Molecular characterization of *Streptococcus uberis* strains, isolated in a longitudinal study from milk of a commercial South African dairy herd.

by

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Table of contents

Table of contents i
Declaration vi
Ethics statement vii
Dedication viii
Acknowledgements ix
List of tables x
List of figures x
List of abbreviations xi
Abstract xv
CHAPTER 1 : INTRODUCTION AND BACKGROUND 1
1.1 Background 1
1.1.1 *Streptococcus uberis* 3
1.1.2 Strain identification and virulence factors 3
1.2 Justification 5
1.3 Aim of the study 6
1.4 Objectives of the study 6
CHAPTER 2 : LITERATURE REVIEW 7
2.1 Introduction to mastitis 7
2.1.1 Dairy situation in South Africa 9
2.1.1.1 General 9
2.1.1.2 Herd size 10
2.1.1.3 Milk yield 11
2.1.1.4 Farming systems 11
2.1.1.5 Breeds 12
2.1.1.6 Parlours 12
2.1.2 Global dairy situation 13
2.1.3 Economics of bovine mastitis 17
2.1.4 Clinical mastitis 22
2.1.5 Mastitis pathogen 23
2.1.6 Mastitis-causing bacteria 24
2.1.7 Environmental versus host-adaptive organisms 25

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2.1.8 *Streptococcus uberis* ... 26
2.1.8.1 Mastitis caused by Streptococcus uberis globally ... 27
2.1.8.2 Mastitis caused by Streptococcus uberis in South Africa ... 28
2.1.8.3 Identifying Streptococcus uberis ... 28
2.1.8.4 Diverse strains of Streptococcus uberis ... 29
2.1.8.5 Virulence factors of Streptococcus uberis ... 31
2.1.8.6 Biofilm ... 32
2.1.8.7 Streptococcus uberis adhesion molecule (SUAM) ... 33
2.2 Immune system of the bovine udder ... 34
2.2.1 Udder physiology ... 34
2.2.2 Primary udder defence ... 35
2.2.3 Secondary udder defence: Innate and adaptive immunity ... 35
2.2.4 Vaccination ... 36
2.3 Herd health management ... 38
2.3.1 The environment ... 39
2.3.2 The host (cow) ... 39
2.3.3 Milking equipment and system types ... 40
2.3.4 Milking routine ... 40
2.3.5 Farming systems: Total mixed ration (TMR) versus pasture-based 41
2.3.6 Managing host-adaptive organisms ... 43
2.3.7 Managing environmental organisms ... 45
2.3.8 Proactive management ... 45
2.3.8.1.1 Animal welfare benefits ... 46
2.3.8.1.2 Cow longevity ... 47
2.3.8.1.3 Milk quality ... 47
2.4 Somatic cell count ... 47
2.5 Antibiotics ... 49
2.5.1 Antibiotic resistance ... 53
2.5.2 Mechanisms of Bacteria Developing Antimicrobial Resistance (AMR) ... 55
2.5.3 Horizontal gene hopping (transfer) ... 55
2.5.4 Resistance genes ... 57

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2.5.5 *Streptococcus uberis* resistance 58
2.5.6 South African situation - regulatory 58
2.5.7 Intramammary products available on the South African market and globally 59
2.5.8 One Health 73
2.6 Concluding remarks 74
CHAPTER 3 : MOLECULAR CHARACTERISATION OF *STREPTOCOCCUS UBERIS* STRAINS, ISOLATED IN A LONGITUDINAL STUDY FROM THE MILK OF A COMMERCIAL SOUTH AFRICAN DAIRY HERD 76
3.1 Introduction 77
3.2 Methodology 79
3.2.1 Experimental design 79
3.2.2 Herd selection 79
3.2.3 Historical data from the herd 80
3.2.4 Samples 81
3.2.4.1 Sampling method 81
3.2.4.2 Sample selection 81
3.2.5 Microbiology and cytology 82
3.2.5.1 Identifying Streptococcus uberis 82
3.2.5.2 Freezing bacterial isolates 82
3.2.5.3 Verifying bacteria with the MALDI-TOF 82
3.2.5.4 Multilocus-sequence typing 83
3.2.5.5 Method of submission of genes (PubMLST Gene bank) 84
3.2.6 Statistical analysis 85
3.2.7 Biosecurity and ethics consideration 85
3.3 Results 86
3.3.1 Retrospective data: Routine isolates and clinical mastitis cases 86
3.3.1.1 Multilocus-sequence type (MLST) data for cows from which Streptococcus uberis were isolated on consecutive samplings 104
3.3.1.2 Multilocus-sequence type (MLST) data on identifying novel alleles 107

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3.3.1.3 Multilocus-sequence type (MLST) data and existing clonal complexes 107

3.3.2 Multilocus-sequence type (MLST) database global comparison ... 109

3.4 Discussion ... 113

3.4.1 General ... 113

3.4.2 Retrospective data ... 114

3.4.3 Overall multilocus-sequence type (MLST) data ... 115

3.4.4 Multilocus-sequence type (MLST) data and existing clonal complexes ... 117

3.4.4.1 Differences among ST-CC for Strep uberis ... 117

3.4.5 Multilocus-sequence type (MLST) data for cows from which *Streptococcus uberis* were isolated on consecutive samplings ... 118

3.4.6 Multilocus-sequence type (MLST) data summary ... 122

3.5 Conclusion ... 125

CHAPTER 4 : SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS ... 127

4.1 Introduction ... 127

4.1.1 Advantages of this study ... 128

4.1.2 Recommendations of this study ... 128

4.1.3 Limitations of this research ... 129

4.1.4 Future research ... 129

4.2 General conclusion ... 129

References ... 131

Appendix 1: Permission to conduct research ... 160

Appendix 2: Communication from Agriculture, Land Reform and Rural development ... 162

Appendix 3: Schedule of accreditation ... 163

Appendix 4: Certificate of approval ... 164

Appendix 6: Recommendation report ... 166

Appendix 7: Research Ethics Committee approval ... 168

Appendix 8: Faculty of Veterinary Science: Approval certificate ... 168

Appendix 9: Faculty of Veterinary Science – Animal Ethics Committee approval certificate – annual renewal ... 170

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Appendix 10: Editing certificate 172

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Declaration

I, Dr Grant Kevin van Lelyveld, declare that the dissertation which I hereby submit for the degree MSc (Production Animal Studies) in the Faculty of Veterinary Science at the University of Pretoria is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

29 Janaury 2025

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Ethics statement

The author, whose name appears on the title page of this thesis, has obtained the required ethics approval/exemption for the research described in this work.

The author declares that they have observed the ethical standards required in terms of the University's Code of Ethics for scholarly activities.

Section 20 approval was obtained from Department of Agriculture, Land Reform and Rural Development (DALRRD) ref no. 12/11/1/1/8 (2923 LH) dated 6 April 2023 (Appendix 1) and extended ref no. 12/11/1/1/8 (July 2024) (6562 CMo) (Appendix 2) for the use of the isolates from the SANAS-accredited milk laboratory (Appendix 3) and DALRRD-approved milk laboratory, Faculty of Veterinary Science, University of Pretoria, Onderstepoort (Appendix 4), stored in the Department of Agriculture, Land Reform and Rural Development (DALRDD) approved biobank (Appendix 5). Ethical clearance was obtained from the University of Pretoria, Faculty of Veterinary Science Research Ethics Committee dated 15 May 2023 (Appendix 6) and Animal Ethics Committee No: REC040-23 dated 5 June 2023 before commencement of lab work (Appendix 7) and extended REC040-23 EXT 1 (5 August 2024) (Appendix 8).

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Dedication

I would like to dedicate this to my family, especially my pets, who have often been by my side when studying, working, and writing. I would also like to thank the people who have inspired and guided me in achieving my goals and dreams.

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List of tables

Table 2.1: A comparison of the somatic cell count (SCC) penalty and premium payment scheme from two independent South African milk processors/buyers 199

Table 2.2. Comparing independent milk processors in South Africa, setting up a bonus payment scheme for milk components (fat, protein, and volume) as a rand value per kilogram. 20

Table 2.3: A selection of intramammary antibiotics on the market in countries where *Streptococcus uberis* is a common pathogen 60

Table 3.1: Retrospective laboratory culture results of *Streptococcus uberis* (SUB) from routine herd examinations and clinical mastitis cases in 2021 886

Table 3.2: Percentage of *Streptococcus uberis* (Strep uberis) isolated during 2021 from clinical mastitis cases per cow 88

Table 3.3: Multilocus-sequence typing (MLST), strain typing (ST) data of the 70 *Streptococcus uberis* isolates, including allele numbers, ST numbers and available clonal complexes (CC) 959

Table 3.4: Summary of cows from which *Streptococcus uberis* were isolated on two or more consecutive samplings (31 isolates from 14 cows) 95

Table 3.5: Global multilocus-sequence typing (MLST) data comparison of *Streptococcus uberis* from South Africa, China, Canada, UK, and New Zealand 99

List of figures

Figure 3.1: Dendrogram of MLST data of all *Streptococcus uberis* isolates tested (n= 70) 1004

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List of abbreviations

AB .... active/binding

ACR........ Automatic cluster removal

AEC........ Animal Ethics Committee

AMR .... antimicrobial resistance

APES........ Academic and Professional Editing Services

ARG .... Antibiotic-resistant genes

AWI .... Animal Welfare Indicators

BMSCC .... Bulk milk SCC

BTSCC .... Bulk tank somatic cell count

BVD........ Bovine viral diarrhoea

CC .... Clonal complex

CLSI .... Clinical and Laboratory Standards Institute

CMCT........ California milk mastitis tests

CoA .... Coenzyme A

DALRDD .... Department of Agriculture, Land Reform and Rural Development

DALRRD .... Department of Agriculture, Land Reform and Rural Development

DCT........ Dry cow therapy

DIM........ Days in milk

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DSCC .... Differential Somatic Cell Count

EC .... Electrical conductivity

ELISA .... Enzyme-Linked Immunosorbent Assay

EMA .... European Medicine Agency

ESAC .... European Surveillance of Antimicrobial Consumption

ExoSAP .... Exonuclease I and Shrimp Alkaline Phosphatase

FASTA .... Fast all sequence alignment

FC .... Flow cytometry

FecA .... Ferric citrate transporter A

FepA .... Ferric enterobactin transporter A

GapC .... Capsule formation protein C

HGT .... Horizontal gene transfer

HKY .... Hasegawa-Kishino-Yano

IgD .... Immunoglobulin D

IgG .... immunoglobulin G

IMI .... Intramammary infection

LS .... Linear Score

LSCC .... Low somatic cell count

MDR .... Multi-drug resistance

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MER .... Modulation error ratio

MIC.. Minimal inhibitory concentration

MLST... Multilocus-sequence typing

NCBI... National Center for Biotechnology Information

NMC .... National Mastitis Council

NSAID ....Non-steroidal anti-inflammatory drugs

PauA ....Plasminogen activator A

PauB ....Plasminogen activator B

PCR... Polymerase Chain Reaction

PFGE .... Pulsed-field gel electrophoresis

PMN ....polymorphonuclear

PubMLST .... Public multilocus-sequence typing

QS .... Quorum sensing

REC... Research Ethics Committee

RNA...ribonucleic acid

ROI..Returns on investments

SAAC .... Slime Associated Antigenic Complex

SARA .... Sub-acute ruminal acidosis

SAVC .... South African Veterinary Council

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ST .... Strain typing

SUAM... Streptococcus uberis adhesion molecule

SUB...*Streptococcus uberis*

Strep. Uberis ....*Streptococcus uberis*

TMR ....Total mixed ration

TSB ....Tryptic soy broth

UHT... Ultra-high Temperature

VGT.. Vertical gene transfer

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Abstract

Streptococcus uberis is a significant emerging mastitis pathogen with environmental and host-adaptive properties. Mastitis caused by *Strep. uberis* is increasing in South Africa, particularly in pasture-based herds irrigating with slurry. This field study aimed to identify strain types using MALDI-TOF and MLST and evaluate the Strep's behavioural patterns (host-adaptive versus environmental)*. uberis* isolates over an extended period from a South African dairy herd with a high prevalence of *Strep. uberis* intramammary infection. The study involved 70 (63 retrospective and seven recent) samples, isolated from 53 cows, which were used for MLST sequencing of the seven housekeeping genes (*gki*, *recP*, *ddl*, *tdk*, *arc*, *tpi*, and *yqiL*). The study disclosed a herd prevalence of 7.44% *Strep. uberis* intramammary infection (IMI), responsible for 21.26% of clinical mastitis cases in this herd. Of the *Strep. uberis* positive clinical cases, 25% had repeat occurrences during the same lactation period. The consecutive repeat *Strep. uberis* cases were 34 (2x), 13 (3x), and 7 (4x). The study identified 41 novel *Strep. uberis* strain types from 64/70 isolates, attributable to novel alleles (29) or novel sequences of existing alleles of previously identified alleles. The study revealed that one existing *Strep. uberis strain type (ST) 1613 (6/70) was to be identified and belonged to the* clonal complex (CC) ST-86. All the other strain types of the isolates investigated in this study were novel. They were within the common CC (ST-5, ST-86, and ST-143) or not allocated (owing to the lack of data generated from whole genome sequencing). The longitudinal study 2021 (three-monthly milk samples) n=64/70 and 2024 (specific milk samples) n=7/70 from cows identified as high-interest cows, possibly showing as chronic cases on repeat collections (culture, MALDI-TOF, and Kirby Bauer). The finding was not entirely down to suspected host-adaptive strains of *Strep. uberis* but relatively novel strains creating new infections. The heterogeneity of the *Strep. uberis* isolates in this study agreed with other research identifying the environment as the primary source of strains. This study can aid farmers and veterinarians in managing *Strep. uberis* mastitis is more effective in the field by better classifying what is meant by repeat, non-cure, and new infections amid farming practices favouring higher pathogen loads (environment) and challenges for the cows. Contrary to what was expected, the study identified new infections with diverse Strep caused most suspected chronic intramammary infection (IMI)*. Uberis strains are* dominated by novel environmental strains (high heterogeneity of ST and CC from all 70 isolates).

Keywords: *Streptococcus uberis*, molecular characterisation, slurry, bovine mastitis.

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CHAPTER 1: INTRODUCTION AND BACKGROUND

1.1 Background

Mastitis is the disease that causes the most significant economic losses for the dairy sector in first-world countries (Blosser, 1979; Azooz *et al.*, 2020). This sector is under more pressure from the "green" shift compelled by government policies and public perception associated with climate change, such as greenhouse gases, water pollution, soil erosion, and the prudent use of antibiotics, including selective dry cow therapy. We also need to consider the cost implications, which will result in farmers observing and using alternative farming practices to reduce the ever-increasing costs and prices of feed, fertilisers, and fuels. Farmers need to meet the increased demands required for reproduction performance and to cope with heat stress from their genetically superior dairy animals (Neculai-Valeanu & Ariton, 2022; Youngerman, 2004).

In so doing, the cows are positioned under increasing risk of suffering from clinical and subclinical mastitis. A change in farming practices and management, such as a break in hygiene, which has a shorter time or completely stopping the composting process, is being implemented to save on processing costs. Consequently, pastures are being sprayed with untreated slurry. In contrast, cubicle bedding used in total mixed ration (TMR) intensive dairy farming is replaced with compressed/extruded fresh manure (reduced moisture percentage). This practice will increase IMI risks by increasing and causing higher pathogen loads. This causes dirtier cows with poorer hygiene scores (manure and mud coverage over the body: udder, legs, tail, and legs), increased heat and mud stress, and discomfort of the animals (fermentation and high moisture content of the manure).

Finally, the introduction of environmental and contagious pathogens, such as *Streptococcus uberis* (*Strep uberis*) into areas where highly susceptible groups of animals, such as cows fresh in milk, heifers close to calving, and high-producing cows early in lactation may be housed, will increase the risk of udder infection (Blignaut *et*

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al., 2018). There will be an increased risk of IMIs, which will be costlier to the farmer, decrease herd productivity, and compromise the animals' well-being.

Mastitis is a multifactorial disease influenced by several factors. These include the cow's health, stage of lactation, udder conformation and condition, milk yields and other levels of productivity, and reproductive phase performances. Environmental conditions influence cow comfort, risk of infection and contamination, and causes of injury, illness, and disease. The milking machine, with its elements of animal contact points and damage, calibrations and settings for diverse needs to improve the efficiency of milking time versus milk yield and production stages of dairy cows (over milking) and the risk of infection through transfer from cow to cow or being a source of infection.

The milkers and the milking routine with incorrect handling of cows, improper milk let-down stimulation and timing, and mastitis identification or treatments. Mastitis is also a concern for animal welfare and food safety (sustainability and traceability of antibiotic use, disease transfer, and the supply of safe, sound, wholesome food products). For a farmer to be able to develop and provide a safe, sound, and wholesome product (milk) to the public, they have to ensure the entire system operates efficiently and effectively to limit the risk of IMI in their herd. It is well known that the type of pathogen influences the type, severity, and chronicity of an IMI.

The risk factors influencing this are breaks in hygiene associated with pre-and post-milking routines (use of dips, foremilk stripping, drying of the teats, etc.), higher exposure to environmental hot spots (walkways, waiting areas, etc.), and poor teat end condition (chapped, hyperkeratosis of the teat canals/orifices). These conditions are strongly influenced by the milking routine (time of pre-milking stimulation) and the milking machine calibrations/settings (milking times, automatic cluster removal settings, vacuum stability and settings within the system and at the teat end) an+d service intervals (Phuektes *et al*., 2001). Global trends have indicated shifts from non-aureus staphylococci and *Staphylococcus aureus* (*Staph. aureus*) pathogens that induce IMI to those of *Strep uberis*-induced IMI (Petzer *et al*., 2009).

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1.1.1 *Streptococcus uberis*

Streptococcus has recently been separated into three genera: *Streptococcus*, *Enterococcus*, and *Lactococcus* (Oliver, 2011). *Streptococcus uberis* is a gram-positive cocci bacterium (Watts, 1988).

Local South African IMI data revealed an increased presence of *Strep uberis*. There has been an increase of 10% during 2022 (Personal communication Inge-Marié Petzer, 2023). It has also been known that 25% of cows harbour *Strep. uberis* in their faeces, and 50% of cows harbour the bacteria on their skin, indicating higher risks of environmental contamination with both (environmental > host-adaptive) strains (McDougall *et al*., 2004; Sampimon *et al*., 2009; Srithanasuwan *et al*., 2022; Tomita *et al*., 2008; Zadoks *et al*., 2001; Zadoks, 2007).

It is known that *Strep. uberis is challenging to treat successfully because the bacteria is intracellular and has various virulence factors, such as Strep. uberis* adhesion molecule (SUAM), prevalent resistance genes (tetM, blaZ, and ermB) (Zhang *et al*., 2020), biofilm formation (virulence factor) (Magagula, 2023), and capsule formation (has active/binding (AB) gene) (Srithanasuwan *et al*., 2022). Recent South African data from 30 dairy farms has revealed that all *Strep. uberis* strains isolated in the trial produced biofilms of varying strength (Magagula *et al*., 2023). Sixty per cent of *Strep. uberis* IMI lasts <30 days, and 18% could become chronic and last >100 days (Srithanasuwan *et al*., 2022

1.1.2 Strain identification and virulence factors

With the misuse of antibiotics through the incorrect use of treatments, prophylactic dry cow therapy protocols, and drug selection, we have observed a decrease in the efficacy of antibiotics, such as erythromycin (0.2%) and tetracycline (36.7%) (de Jong *et al*., 2018). This may have led to possible mutations, increased resistance or virulence factors (development of characteristics to avoid antibiotics), and the expression and sharing of bacteria, e.g., biofilm production observed in all *Strep. uberis* strains (Magagula, 2023; Sipka *et al*., 2021).

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Recent research from China has revealed that more than 59.3% of analysed *Strep. uberis* strains carried four to seven virulence-related genes (encoding antimicrobial resistance and invasiveness factors). Given the diverse genetic ecology, it is essential to understand the similarities and differences among strains (Tomita *et al*., 2008; Davies *et al*., 2016; Reyes *et al*., 2019) to ensure that the correct control (preventative and treatment) measures and actions are implemented.

This literature revealed various virulence gene patterns within and between the same and diverse *Strep. uberis* strains (Zhang *et al*., 2020). The virulence factors established in *Strep. uberis* bacteria comprise plasminogen activator/streptokinase, surface dehydrogenase proteins, CAMP factor, lactoferrin-binding proteins, surface adhesion molecules, hyaluronic acid capsules, and several others (Calonzi *et al*., 2020; Zhang *et al*., 2020). All contribute to a high level of antibiotic resistance across a wide range of diverse antibiotics because prophylactic use of antibiotics is a common practice. This becomes even more important when there is a drive for the prudent use of antibiotics.

The reported prevalence rate of *Strep. uberis* varies among studies. Still, it is increasing, and the data collected in South Africa (single dairy herd) has similarities to studies in China (4.9%), Finland (1.5%) (Pitkala *et al*., 2008), Canada (2.0%) (Cameron *et al*., 2016), and Argentina (3.3%) (Reinoso *et al*., 2011; Zhang *et al*., 2020). The prevalence of *Strep. uberis* in 303,895 milk samples examined from South African herds was reported to be 8.03% from 2018 to 2020 (Personal communication Inge-Marié Petzer, 2022).

The process of identification of virulence factors includes the isolation and correct identification of *Strep. uberis* bacteria (phenotypic appearances and responses to agar plates with blood media), the confirmation by using the MALDI-TOF (Van Dyk *et al*., 2016) technique (96% accuracy), and the molecular characterisation of virulence factors (using gene similarities) through the comparison of genomic Deoxyribonucleic acid (DNA) using seven housekeeping genes: Glucose kinase (*gki*), Transketolase (*recP*), D-ala-D-ala ligase (*ddl*), Thymidine kinase (*tdk*), Carbamate kinase (*arcC*),

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Triosephosphate isomerase (*tpi*), and Acetyl Coenzyme A (CoA) acetyl-transferase (*yqiL*) or full genomes (Coffey *et al*., 2006; Tomita *et al*., 2008; Reyes *et al*., 2019).

Confirmation could also be conducted using the multilocus sequence typing (MLST) technique (http://pubmlst.org/suberis) (Jolley *et al*., 2018; Coffey *et al*., 2006; Vezina *et al*., 2021) and PCR profiles (Srithanasuwan *et al*., 2022).

1.2 Justification

With local South African IMI data following global trends and showing increased *Strep. uberis* from 2.36% (TMR) and 2.63% (pasture-based) (Blignaut *et al*., 2018) to 8.03% (Personal communication 2023, Inge-Marié Petzer, Milk laboratory, University of Pretoria) over a period from 2008 to 2020, this increases the importance of determining the correct identification of the strains present (Reyes *et al*., 2019) and differentiating between environmental or host-adaptive strains. This could be conducted by evaluating the genomic data to identify new, similar, or diverse strains.

This type of molecular characterisation of *Strep. uberis* has not been conducted on *Strep. uberis* isolates from South African dairy farms. Understanding the dynamics on or among farms will make implementing prevention and treatment strategies easier for farmers and veterinarians. This includes the selection of antibiotics and separation/isolation of cows with host-adaptive *Strep. uberis* IMI and the incorporation of global practices, such as vaccinating against mastitis-causing pathogens with monoclonal or multivalent vaccines.

Knowing the characterisation of the virulence factors, strain, and type (environmental vs. host-adaptive) could reduce the losses associated with clinical and subclinical *Strep. uberis* IMI. Money can be saved through decreased culls of highly productive cows, reduced use of medications and treatment costs, reduced milk losses owing to erosion of potential or milk being discarded, improved somatic cell counts (SCC), and fewer penalties due to improved milk quality.

More importantly, it will allow for the correct and prudent use of antibiotics, improve animal welfare parameters by limiting and reducing clinical mastitis, and finally allow

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for sustainable farming and the production of safe, sound, and wholesome milk products for a developing economy where cheap protein sources are needed.

1.3 Aim of the study

This longitudinal study was conducted to use the molecular characterisation of Strep. uberis to identify diverse strains and virulence profiles and improve the understanding of the diverse behaviour associated with environmental and host-adaptive *Strep. uberis* strains.

The aim is to evaluate the influence of farming practices, such as pasture fertilisation with untreated slurry on strain pathogenesis and see if a predicted outcome can be implemented into udder health and herd health management systems.

1.4 Objectives of the study

1. To use molecular characterisation (housekeeping genes) to identify new, duplicate, or similar *Strep. uberis* strains (ST numbers and clonal complexes (CC)) from isolates sourced from a large commercial dairy farm in South Africa (stored samples in the biobank or fresh milk samples). Compare the genomic data and strains to other countries with similar farming practices and IMI trends.
2. To evaluate the genomic differences between the strains (ST number and CC) occurring within an individual cow over time (repeat or chronic IMI) and/or among cows at the same time in the same herd (same groupings or production stages) to determine the pathogenesis of *Strep. uberis* strain as that of environmental or host-adaptive origin.
3. To observe if a farming practice favours developing a host-adaptive strain over an environmental strain.

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CHAPTER 2: LITERATURE REVIEW

Multiple literature sources were used to develop a complete understanding of mastitis. These sources include its pathogenesis, adaptability, and consequences associated with one particular mastitis pathogen—*Strep uberis.*

2.1 Introduction to mastitis

Mastitis is an inflammatory response involving polymorphonuclear (PMN) leucocytes, lymphocytes, macrophages, and antibodies in the udder that follows a classification scale system which includes subclinical and clinical mastitis (Ruegg, 2012a; Morin, 2015). The National Mastitis Council (NMC) defines subclinical mastitis as an increase of the SCC equal to or greater than 200,000 cells/mL of milk with no clinical signs being recorded in the milk or animal (National Mastitis Council Guidelines, 2001) due to an IMI.

Clinical mastitis is defined as an increase in the SCC equal to or greater than 200,000 cells/mL of milk with clinical signs present in both milk, such as floccules, blood, watery changes, and discolouration, and the animal showing symptoms of an oedematous udder, pain, fever, lethargy, and anorexia (Fogsgaard *et al.*, 2012; Morin, 2015; Adkins & Middleton, 2018; Hsieh *et al.*, 2019). Clinical mastitis can be classified as acute to chronic in duration and mild to severe (Ruegg, 2012a; Oliveira & Ruegg, 2014; Argaw, 2016; Ruegg, 2017).

Most clinical IMIs are mild and are difficult to detect unless foremilk (first milk of a milking), composed of residual milk and cells (PMNs and epithelial) that occur in the teat cistern and canal between milkings, is examined. A study involving over 50 Wisconsin dairy farms evaluated farm data focusing on the classification, type, and occurrence of IMIs and revealed that around 50% of IMI cases had only abnormal milk. Conversely, 35% of IMI cases had abnormal milk and udder involvement (swelling of the quarters), and only 15% of the IMI cases had abnormal milk, udder involvement, and systemic symptoms (Apparao, 2009; Oliveira & Ruegg, 2014; Van Lelyveld, 2022b).

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The costly disease in dairy-producing animals (cows, water buffalo, sheep, etc.) is due to a multitude of factors, including environmental factors (i.e., the milking machine and routine), cow immunity, and the microorganisms involved. An imbalance in one or more of these areas will result in mastitis. It boils down to a break in hygiene, such as dirty cows, dirty environment, and higher pathogen loads (De Vliegher *et al.*, 2012; Blignaut *et al.*, 2018) and damage to the udder, including physical defence mechanisms – teat orifices that are cracked and have hyperkeratosis, everted sphincters, oedematous and congested teat and udder parenchyma (Neijenhuis, 2000). Some pathogens are strictly or lean towards contagious/host-adapted or environmental organisms, causing transient or chronic infections and species with varying pathogenicity (du Preez, 2000).

In addition to the challenges creating mastitis, problems arising from mismanagement of udder health include failure to identify pathogens and deficient farming practices. These include the lack of training staff and milker motivation, incorrect services or calibration of milking machinery, and antibiotic resistance (Van Lelyveld, 2022b). Pathogen mutations may occur owing to incorrect treatment through the inappropriate or lack of medical stewardship of drugs and identification methods (De Vliegher *et al.*, 2012). This is a global, regional, farm, and animal problem that needs to be studied to create innovative solutions and reduce animal welfare issues and financial losses (Viguier *et al.*, 2009).

There are ample reasons for creating mastitis management programmes and having veterinarian involvement. This will ensure records are kept and actions are implemented, such as frequent parlour audits, ensuring the correct identification of mastitis pathogens (sample collection for culture or PCR) and recording animal identification correctly to ensure that the type and severity of mastitis (foremilk stripping, California milk mastitis tests (CMCT), and electroconductivity)) is appropriately recorded. This develops a history of all the cows (number, cure rate versus non-cures), which is essential to evaluate and implement actions effectively. This can reduce the adverse effects of mastitis on cows and farms (dropped milk production and decreased milk quality, such as pH, protein structures, and butterfat percentages). It may also reduce large volumes of milk discarded due to defects (unfit

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for human consumption) and antibiotic residues. It will also reduce the loss of animals and genetic potential through premature culling and/or death. Finally, udder health programmes can save farmers from incurring increased costs associated with treatment, diagnostics, and losses from returns on investments (ROI) (Ruegg, 2012b

2.1.1 Dairy situation in South Africa

2.1.1.1 General

The past 17 to 20 years, the South African dairy farming industry has experienced several changes and developments. Some of these changes have involved management practices, types of farming, and herd sizes (Blignaut *et al.*, 2018). The general situation led to increased yields from fewer dairy herds (Lactodata, 2018; Lactodata, 2022). Some of these advances or changes can be attributed to the worsening economic situation of the country and the constant low milk price for farmers, therefore the need to achieve enhanced ROI or reduce losses experienced owing to inefficiencies and the high costs of mastitis. A perfect example is the limitations of constant electrial supplies due to poor infirstructure and governmental operations, affecteing different parts of the dairy farm from the irrigation systems to running the parlour. Which includes feeding, the fluctuations in the milking system of the machine and the interruptions of the milking routines all compiling into increase IMIs. This is further compounded by the higher levels of stress due to the Temperature Humidity index challenges specific to South African dairy farms (du Preez, 1990).

Herd sizes have increased substantially (Lactodata, 2018; Lactodata, 2022). Several trends have followed that of the global market, pursuing improved genetics (Pryce *et al.*, 2012). Herd health programmes have focused on fertility and the introduction of the concept of vaccinating against mastitis to reduce antibiotic use. Other areas of focus have been the efficiency of the milking parlour (size, milking capacity per stall) and the milking machine (correct settings and servicing) (Blignaut *et al.*, 2018; Vermaak *et al.*, 2022; Van Lelyveld, 2022c; Van Lelyveld, 2022d

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2.1.1.2 Herd size

The most significant change has been the transition from smaller milking herds, averaging 110 lactating cows, to herds with over 1,000 cows in milk, up to 5,000 cows. This created the need for more byproducts (animal feeds, irrigation, fertilisation of pastures, etc.) to produce more cost-effective milk. The South African dairy industry is a serious contender, with average herd sizes of 453 cows (lactating and dry) (Lactodata, 2023) and 48% of herds with a headcount of over 300 cows (Lactodata, 2018). This places the country in the class of large dairies when compared to Saudi Arabia (6,924 cows per herd), New Mexico (2,752 cows per herd), New Zealand (419 cows per herd), and the United States of America (USA) (316 cows per herd) (Lactodata, 2018; Hanson, 2022).

Due to government and economic pressures, the trend in South Africa has followed that of several international countries (USA, New Zealand, Canada, and Saudi Arabia). The number of South African dairy farms has decreased by 65% from 2006 to 2020. The number of registered dairy herds (Milk SA data) has decreased from 4,184 herds in January 2006 to around 984 herds in January 2020 (Lactodata, 2013; Lactodata, 2018; Lactodata, 2022). There has not been a decrease in the national animal numbers for commercial dairy cows.

The increase in herd size is attributed to organic growth on the farm and the purchasing of “new” animals. Organic growth on the farm involves retaining more replacement stock by using sexed semen and improved rearing practices and retaining multiparous cows for longer (reduced culling restrictions, milking cows for longer as non-return to breeding animals or having more lactations per animal) (Nielsen *et al.*, 2010). Growth by purchase involves acquiring whole herds that are transported after a selection/culling programme is used to remove cows with a history of mastitis, especially *Staph. aureus* positive animals. Both methods have led to a rapid growth in milk yield. Negative consequences of not keeping closed herds include elevated bulk tank SCC (BTSCC) levels and the spread or introduction of diseases, such as bovine leukosis, bovine viral diarrhoea (BVD), and *Streptococcus agalactiae*

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and *Staph. aureus* mastitis outbreaks due to deficient biosecurity practices (Haxhiaj, 2022).

2.1.1.3 Milk yield

The milk yield for South Africa has also increased, with the average daily cow yield rising from 20.1 kg in 2012 (Lactodata, 2013) to 21.0 kg in 2018 (Lactodata, 2018). These higher-yielding animals have resulted in a net increase in the milk yield for South African dairy herds of 31% over the same period, equating to a 273% increase in milk production per producer (Lactodata, 2018).

The dairy industry in South Africa has observed a shift in animals over 20 years (1997 to 2017). This involved moving animals from the central region (Gauteng, North West, and the Free State) to the coastal regions. Notably, 85.4% of milk production originates from coastal regions (Lactodata, 2022). The milk production increases were recorded for each region: Western Cape [percentage of milk yield change from 22.9% (1997) to 30.6% (2021)]; KwaZulu-Natal [percentage of milk yield change from 15.7% (1997) to 28.2% (2017)]; and the Eastern Cape [percentage of milk yield change from 13.8% (1997) to 29.7% (2017)] (Lactodata, 2018; Lactodata, 2022).

The South African dairy industry has generally observed an increase in milk production. In 2004, the annual milk yield was 2.3 million tonnes, which increased to 3.245 million in 2017 (Lactodata, 2018), with expectations for it to continue on this upward trajectory. The yields for 2021 were down from previous years (0.7% drop) (Lactodata, 2022), and this could be owing to a variety of factors, including the importation of milk from Europe, Covid-19, drops in milk prices, and an increase in production costs (inflation, exchange rates, feed, and fertiliser).

2.1.1.4 Farming systems

South Africa has two dairy farming types that developed over the same period: TMR, where cows are housed in camps or intensive housing comprising free stall barns with or without cubicles (sand to wet manure bedding) (Schingoethe, 2017), and pasture-based farms with large pastures of mono or multi-cultivar grazing systems and

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supplemental feeding in parlour/post feeding troughs (Blignaut *et al.*, 2018; Lactodata, 2018). The TMR farming systems have generally been in the central regions (Gauteng, North West, Free State provinces), with the pasture-based farming systems occurring more along the coastal areas (Western Cape, Eastern Cape, and KwaZulu-Natal).

Over the same period, when the number of milk producers decreased, there was an artificial increase in pasture-based farming systems. The pasture-based farming systems tend to mimic the farming practices of New Zealand to obtain low input costs and smaller mixed breed (Theron & Mostert, 2010) cows. This system has contributed the most towards the growth in cow numbers in the coastal regions. The growth in the coastal regions has been attributed to a lower attrition rate of herds (Eastern Cape: -31% versus Free State: -50%) (Lactodata, 2013) and the increase (organic and purchasing of cows) in the average number of cows per herd [Eastern Cape: (536 cows in 2012 to 865 cows in 2022), KwaZulu-Natal: (425 cows in 2012 to 766 cows in 2022), and Western Cape: (246 cows per herd in 2012 to 446 cows in 2022) (Lactodata, 2013; Lactodata, 2018; Lactodata, 2022)].

2.1.1.5 Breeds

The dairy herds in South Africa predominantly comprised Holstein cows, followed by Jersey cows and mixed-breed animals (Theron & Mostert, 2010). The economic benefits of more energy-efficient animals, enhanced adaptation to environmental stressors (heat efficiency and adaptability), and disease resistance (Dezetter, 2017) motivate the deviation from purebred herds.

2.1.1.6 Parlours

Over this period, the dairy parlours have had a slight lag in development, with several herds outgrowing the older parlours. Several South African dairy parlours are designed to be a Highline swing-over system (milking/system vacuum levels from 45 to 48 kPa or higher in some cases) (Van Lelyveld, 2022c). The milk line runs above the heads of the milkers in the milking pit. This style of milking parlour allows for a maximum number of cows to be milked per milking unit and will, from field experience during parlour audits, have the cows stacked in a herringbone manner down both sides of the

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milking pit. Some modernisations have included automatic cluster removal systems (ACR) (Petzer & Swan, 2019; Vermaak *et al.*, 2022; Prendergast *et al.*, 2024). Another trend observed in the past 15 years is an increase in the number of sizeable rotary turntables (with 60 to 65 units per table but increasing to 100 units) used by pasture-based and TMR systems (KwaZulu-Natal, Western Cape, and Eastern Cape) (Personal communication: Afimilk & Delaval SA).

2.1.2 Global dairy situation

The dairy industry globally comprises subsistence farming (predominantly observed in many parts of Asia and Africa, and South Africa, where a household will have one to five cows for their consumption of milk and the possibility of pooling milk and selling to milk processors), family-owned (predominantly observed in Europe where a family farm can have up to 300 cows), and commercial dairy farms (predominantly observed in the USA, Saudi Arabia, New Zealand, and South Africa) (Lactodata, 2022). Although these are the norms, there are exceptions to the rule where there are farms in China that house cows under intensive settings and have around 10,000 cows at one site (Dairy Global, 2021). The global trend is the same as in South Africa, where the dairy sector is expanding and consolidating simultaneously (Sewell, 2021; Lactodata, 2013; Lactodata, 2017; Lactodata, 2022).

Registered herd numbers have decreased (Lam *et al.*, 2013) globally (currently at a rate of 1.4%) (Lactodata, 2022). There is still an increase in the global milk yield to meet the increased demands. The attrition rate of milk producers globally varies, with some areas seeing over 10% annually (Sewell, 2021). Nearly 80% of the global population consumes milk-derived products – fresh milk, Ultra-high Temperature (UHT) pasteurised long-life milk, yoghurts, cheese, and milk powders (Bojovic, 2023). There is a desire in developing countries for a high-quality protein that is more affordable than other products (meats).

The world commercial milk yield for 2020 was 910 million tonnes (Lactodata, 2022), an increase from 826 million tonnes in 2017 (Lactodata, 2018). South Africa only contributes 0.4% to the global milk production, whereas other countries, such as India (24.8%), USA (10.7%), Pakistan (5.4%), Brazil (3.8%), and Germany (3.7%) contribute

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larger volumes of milk annually (Lactodata, 2022). Cultures, traditions, and living costs strongly influence milk production and use. Although India may not be as developed as the USA or Europe, it produces more milk at a lower cost per cow or cost per litre of milk; however, no matter the system or size of the farm, the breed or species of animal milked, and the diverse milking techniques, from hand to automatic or robot milking machines, mastitis occurs in all of them and will have varied levels of effect on or for the people (loss of social ranking, loss of draught power, loss of income, and loss of pets).

Some interesting trends are occurring in other countries to capture a market or fit into new socio-ecological challenges and legislation set out by governments or governing bodies, such as veterinary councils and the World Health Organization (Bojovic, 2023). They include the following:

- **Organic milk**: The idea to meet consumers' needs of producing safe, sound, and wholesome products that are sourced from dairy animals fed organic foods, raised on organic pastures (no artificial fertilisers), no use of chemicals, hormones, and antibiotics in animals (prophylactic use), and meet "higher" welfare standards for milk production (Lombotte *et al.*, 2023; Pol & Ruegg, 2007). This is a very small market in South Africa and could be considered irrelevant due to priorities to increase production yeilds and overcome economical and political difficulties faced by the farmers.
- **One Health**: Promoted to regard the collaboration of governments (legislation), nonprofit organisations, public health sectors, and the veterinary professions. This will help ensure general improvement and management in all animal, environmental, and human health sectors. This is because of a significant overlap, and animals are close to humans (humans, ecosystems, and animals). This has an accumulative effect that can result in adverse consequences within each aspect and at the interfaces (e.g., water quality due to contamination from animal or human byproducts, accumulation of medications and hormones in water resources that favour resistance, and pollution, which goes full circle destabilising food safety, food production efficiencies, and general population dynamics) (Mazet *et al.*, 2009; Verraes *et al.*, 2013; Schmidt *et al.*, 2017; Loo *et al.*, 2019). There has been a

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motivation to promote antibiotic stewardship, which involves the prudent and reduced use of antibiotics in the food-producing sector, as it is being blamed for developing antibiotic resistance (Essack *et al.*, 2018). The latter is now challenged by new research indicating that it is invalid (Health for Animals, 2023). This equates to the involvement of veterinary services to ensure that antibiotics are available to treat diseases and, more importantly, use antibiotics to enhance udder health management.This is a growing practice in South Africa, with the DALRRD and the SAVC highlighting this practice at professional development conferences, although limited legeslation at present many producers and milk processing plants regularly perferm quality control check points for antibiotic residues.

- **Selective dry cow therapy**: Fine-tuning of antibiotic selection through the "culture to treat" approach to managing mastitis cases. An individual drug selection (according to antibiogram results) can be made for lactation intramammary application and treatment. Early IMI pathogen classification for selecting antibiotics and early detection of animals with subclinical or clinical mastitis (inline milk labs, electroconductivity readings) is essential to ensuring promising results (Van Lelyveld, 2022b). While this is being implemented, there are varying degrees of implementation, with some countries, such as the Netherlands, having stricter systems versus other countries where it is optional or lacking completely (Haenni *et al.*, 2018; Van Werven, 2018). Not commonly practiced in South Africa and is still limited due to the free access of antibiotics for parasite control.
- **Vaccination against IMI-causing pathogens**: This strategy is used to reduce using antibiotics by immunising the animals and empowering them to better respond to IMI by using the lower virulence factors or properties of the bacterial microbiome (Hoque *et al.*, 2020). Currently, vaccines are available to protect against IMI pathogens, such as *Staph. aureus*, *Escherichia coli*, *Mannheimia* spp., and *Strep uberis*. Countries that practice vaccination against mastitis include Zambia, Canada, New Zealand, the majority of Europe, the United Kingdom, small parts of China, and parts of South America and Mexico (private communication with Hipra representative). South Africa currently has commercial vaccines (Two Bovine mastitis vaccines – one polyvalent and one monovalent, and One Ovine vaccine) and one autogenous mastitis vaccine available on the market.

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- **Selective dry cow therapy under the veil of prudent use of antibiotics (stewardship)**: Driven by regulatory parties; however, many will debate that the dry cow period is the ideal time for treating and preventing an IMI. Treating during this time allows for extended periods of exposure while the udder recuperates through tissue replenishment and involution of the glandular tissue (Van Werven, 2018).
- **Sustainability and reduction of greenhouse gases**: (Megatrend – implies the complex relationship between human, non-human, environment, and economy) (Bojovic, 2023). These trends are promoting the decrease of greenhouse gases by reducing cow numbers, increasing the longevity of animals, implementing fines for pasture use and methane production, and promoting the development and use of biofuel energy plants (Bojovic, 2023; Pieper *et al*., 2020; De Vries, 2017). These are important to identify and understand as the dairy industry (production, processing, transportation, and storage) is a high-energy user (Bojovic, 2023; Ladha-Sabur *et al*., 2019). Not a current feature or practice in South African Dairy herds.

According to studies, such as that of Willett *et al.* (2019), which emphasise the 2006 report from the United Nations Food and Agriculture Organisation, there are serious climate-related concerns. In addition to the high-energy usage, there are also other adverse side effects of intensified agricultural systems, as observed in the dairy sector, such as land degradation (erosion, deforestation), loss of biodiversity (soil, plant, and animal/insect), and water contamination (surface and underground sources), which were emphasised in a study by Saari *et al.* (2021). Combined with plant-based alternative milk sources, this is a significant challenge that the dairy sector must overcome (Bojovic, 2023).

These trends will be compelled by animal rights activists, climate change campaigners, and consumer perceptions and propaganda (vegetarians, vegans, age-related changes) as they seem to think that this is a better solution to resolve the socio-ecological issues associated with the dairy sector, which finds itself needing to commercialise and intensify (automation and robotic milking systems) (Van Lelyveld, 2022d; Van Lelyveld, 2023; Prendergast *et al*., 2024). There are trends in Europe

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similar to this, and they are expected to become the new social norms and put the global and local dairy sector's profit margins (most often felt by the producer) under pressure.

These trends threaten or challenge the dairy sector, which means that the veterinary, agriculture, and food safety sectors need to use any opportunities to ensure the profitability of cows and farming systems. The best way is to reduce the losses owing to mastitis.

2.1.3 Economics of bovine mastitis

The monetary value of mastitis is measured as losses (direct losses and indirect losses) and costs (direct costs and indirect costs) (Shim *et al.*, 2004). This makes mastitis the costliest disease in the dairy sector and has been so for many years (Blosser, 1979; Philpot, 1984; Azooz *et al.*, 2020). It has been remarked to cost billions of dollars, with literature showing that mastitis costs the USA $1 billion annually and North America $2 billion annually (NMC, 1996; Ruegg, 2012b).

South Africa is not exempt from such losses and costs, but additional factors, such as exchange rates and politics, play a noticeable role. This can influence the importation and costs of fuels, grains, and electricity. Such economic strains force farmers to shift practices and possibly use less than desired farming practices, such as using chicken litter as a source of urea or following less favourable farming trends from other countries (pasture slurry irrigation and wet manure bedding) (Lactodata, 2022). The cost of mastitis has been evaluated in several ways, observing direct and indirect factors. It varies according to the severity of the mastitis cases (subclinical and clinical IMI) (Halasa *et al.*, 2007), milk quality regulations (SCC, bacterial counts, milk/butter fat, etc.) (Allore & Erb, 1998), and changes in the market (demands, economics, etc.) (Hogeveen & Østerås, 2005). A South African study (Banga, 2014) estimated that mastitis costs would average R919.96 per cow annually with an incidence rate of 0.9, but the cost per clinical IMI was R1,079.51 per incident.

When calculating the value of mastitis, the herd dynamics need to be evaluated. These parameters and factors will influence the BTSCC levels and the milk value achieved

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per litre (Baucells, 2016). The SCC influences and indicates the quality and quantity of milk produced by a cow. Several factors influence the SCC of a cow, such as heat/environmental stresses; housing and grouping practices; age and parity of the cow; presence, incidence, rate and history of IMIs; and condition of the cow's udder and overall hygiene of the environment, cow, and udder through any stage of lactation or even in the dry cow period (Bonestroo *et al.*, 2023). A negative correlation exists between the SCC and the milk price achieved per litre (Miller *et al.*, 1993; Schukken *et al.*, 2003).

This is because the SCC lowers the milk/butter fat, protein quality, lactose, and milk volume. It is thought that milk yields will be reduced when the SCC exceeds 100,000 cells/mL of milk (Schukken *et al.*, 2003). The milk buyer also implements penalties on the milk price as the SCC levels increase, and at certain thresholds, the milk buyer will refuse to take the milk, which is country-dependent. These regulatory upper limits are a part of the milk marketing agreements to improve the quality and safety of milk entering the food sector, and there could be variations between countries (Hand *et al.*, 2012; Magagula, 2023).

This process (quality payment scheme) motivates general improvements in the supply and production of high-quality milk. It controls all milk producers/farmers through an effective standard means of communication, ensuring no double standards or nepotism (Hurst, 1993). Each milk processor/buyer will vary the agreement to set up a premium, bonuses, and penalties system (Hurst, 1993; Banga, 2014), as depicted by modified tables from Banga's (2014) evaluation of economics and SCC in dairy cattle.

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Table 2.1: A comparison of the somatic cell count (SCC) penalty and premium payment scheme from two independent South African milk processors/buyers

Company A – SCC payment scheme	
SCC levels ($x10^3$ cells/mL)	Penalty/Premium (cents/L)
<400	+0.4 for every 10,000 reductions in SCC up to a maximum of +4
400–500	0.00
>500	−0.1 for every 10,000 increases in SCC up to a maximum of −4
Company B – SCC payment scheme	
<350	+0.4 for every 10,000 reductions in SCC up to a maximum of +4
350–400	0.00
>400	−0.4 for every 10,000 increases in SCC up to a maximum of −10

Adapted from Banga *et al.* 2014.

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Table 2.2. Comparing independent milk processors in South Africa, setting up a bonus payment scheme for milk components (fat, protein, and volume) as a rand value per kilogram.

Payment system (PS)				
Component	Company A	Company B	Company C	Company D
Fat (ZAR/kg)	16.00	20.60	17.26	19.00
Protein (ZAR/kg)	16.00	30.26	28.26	28.50
Volume (ZAR/L)	0.77	0.00	0.00	0.77

Adapted from Banga *et al.* 2014.

The herd size influenced the BTSCC (as less than 20% of a herd can adversely affect the entire bulk tank reading). This is observed with heifers producing smaller volumes and lower SCC levels versus multiparous animals producing larger volumes with higher SCC levels. If a herd is out of balance (herd size, lactating versus dry cow percentages, and the uneven split between parity and production groups), the overall BTSCC can be pushed up or down. A perfect example is when cows are kept in milk as non-rebreeders due to their high milk yields but having low fertility.

These cows can be in milk for more than 400 days, and as this time increases, so does the SCC level. An individual cow with a high milk yield contributes more to the BTSCC level than an average cow with an average SCC, which is explained by the differences in the average, median, and mean of a herd SCC. Similarly, a cow with an elevated SCC can be hidden by the rest of the herd, and trying to manage the problem could result in significant losses to the producer/farmer (Bonestroo *et al.*, 2023).

The demand versus supply influences the milk price. Often, with seasonal fluctuations in the milk price per litre, there is a drop in the summer seasons versus implementing winter premiums. Similarly, the milk price changes according to the quality of milk produced. This can be influenced by breed, quality and quantity of feed, feed and

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water intake volume, milk/butter fat percentages, milk protein type and quantity, bacterial count, and milk contaminants present. The milk price is generally set for a period and can be altered with bonuses or penalties implemented by the buyer. There are external factors that are influenced by the country's economic pressures. A herd with a good milk/butter fat %, within their quotas, low BTSCC will receive a bonus for each factor, meaning that a single farm could earn above the general milk price per litre (Personal communication with milk buyers, 2014).

The most significant income loss is the loss of production from subclinical mastitis, chronic mastitis, clinical mastitis, and then milk being discarded (Ruegg, 2012b) due to antibiotics and not being fit for human consumption. Approximately 66% of losses are due to milk not being produced due to the erosive effects of subclinical mastitis (Miller *et al.*, 1993), while 6% of milk is genuinely discarded due to milk clots, antibiotics, and blood staining. Knowing the breakdown of a herd's clinical and subclinical mastitis cases is crucial while comparing the data to performance indexes and benchmarks (Personal Communication E. Keane, 2014).

There are also perceived and known costs involved in managing and preventing mastitis (DeGraves & Fetrow, 1993). These are direct and indirect costs to the farmer to reduce, treat, and replace animals suffering from mastitis. Direct costs include diagnostics, which accounts for 15% of costs for mastitis management (Bonestroo *et al.*, 2023), treatments (supportive, antibiotics, etc.), labour (increased overtime), and additional treatment by a veterinarian, which accounts for 5% of treatment costs (Lam *et al.*, 2013; Petrovski *et al.*, 2006; Wolfová *et al.*, 2007; Bonestroo *et al.*, 2023). Indirect or hidden costs include involuntary culling, forced culling, replacement stock, and reproductive loss due to increased service intervals (Rajala-Schultz *et al.*, 1999; Rollin *et al.*, 2015; Magagula *et al.*, 2023).

Using the direct and indirect factors in a relatively simple calculation, one can determine the costs of mastitis and even further break it down into specific categories in a scenario where a farm with 120 cows in milk (100 multiparous animals and 20 first lactation animals) and 30 dry cows, with a moderate concern associated with environmental pathogens (Mastitis: 12 cases monthly of which the breakdown is 55%

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mild/low severity, 25% moderate severity, and 15% high severity). The calculation then uses data that needs to be input, such as the cost for treatments for the varying severities of mastitis (€150, €200, and €400, respectively), milk price (€0.40/L of milk), and the penalty or bonus (-€0.01/L of milk when the BTSCC level is higher than the cut-off for the company). The volume of milk per age/production group (first lactation animals 20L/day and multiparous animals 30L/day), milk volume discarded (2,000L), and deaths/culls associated with mastitis (10 cows).

This sort of situation would result in losses amounting to €79,244.00 [including milk loss due to subclinical mastitis €16,015.00 (first lactation animals €1,737.00 and multiparous animals €14,287.00), discarded mastitis milk (€8,640.00), losses due to milk penalties (€15,330.00), cost of mastitis treatment (€29,520.00), and replacement stock/lost stock (€12,000.00)] (Van Lelyveld, 2021a; Van Lelyveld, 2021b). This simple calculation mimics the typical small family farm in Europe. Studies, such as Bonestroo (2023) and other supporting literature, have illustrated that the total cost (median) of mastitis is €230.00 per IMI (ranging from €210 per clinical case and €53 to €72 for a subclinical case). This demonstrates an accumulative effect of the costs and losses of mastitis in all its forms.

2.1.4 Clinical mastitis

Clinical mastitis is a multifactorial disease of the mammary tissue mainly caused by infectious agents but can also be created by toxins, trauma, and chemicals. Mastitis will result in chemical and physical alterations to components in the milk and an abnormal appearance of mammary tissue. The disease can significantly affect the health, welfare, and longevity of dairy animals and, at the same time, decrease the dairy farmer’s profitability. Given the high level of incidence of mastitis (clinical and subclinical), there have been developments in mastitis management, prevention, and diagnostics to reduce the harmful effect of mastitis, which saves animals and improves genetics (Narayana *et al.*, 2021), increases production and longevity [IMI consistently affects milk yield (Heikkilä *et al.*, 2018)], improves profitability, and improves milk quality (De Vries, 2017; Haxhiaj, 2022).

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Two main areas for the occurrence, reoccurrence, and non-cure of IMIs are determined by the interaction of the pathogen and the animal's immune system (innate and adaptive) (Youngerman, 2004; Piepers *et al.*, 2017; Piepers, 2022) and the type and degree of the reaction are influenced by the type of pathogen and specific cow factors (Heikkilä *et al.*, 2018). By being able to understand and identify the pathogen (biomarkers, resistance, variance, and type) and know when an IMI occurs in the time of lactation or age of an animal, as the prevalence of an IMI is higher during late gestation, at calving, and in the earlier periods (<30 days in milk) of a heifer's lactation (Piepers *et al.*, 2017; Narayana *et al.*, 2021), one can be better equipped to manage and reduce IMIs.

2.1.5 Mastitis pathogen

Mastitis-causing pathogens can be categorised into diverse types based on their physiological characteristics: gram-positive and gram-negative bacteria, aerobic and anaerobic bacteria, rods and cocci, and environmental, host-adapted, and opportunistic bacteria. They can also be grouped according to pathogenicity as major or minor pathogens. The primary pathogen group includes *Staph. Aureus*, a highly contagious, often host-adaptive pathogen, is difficult to eliminate due to its intermittent shedding behaviour due to it being intracellular and encased in biofilms at times. *Streptococcus agalactiae* (host-adaptive) and *Strep. uberis* (primarily environmental) are growing groups, as is the Enterobacteriaceae group, such as *E. coli*, *Klebsiella*, and *Serratia* species, and a minor pathogen group including non-aureus staphylococci.

Udders of cows infected with major pathogens often result in severe clinical IMIs exhibiting milk, udder, and cow signs, meaning that the animals require intensive care, such as non-steroidal anti-inflammatory drugs (NSAID), antibiotics, and additional supportive care to control the pathogen in the udder. An additional level of udder health management and herd management is often needed, including biosecurity, isolation, and culling to be implemented to control these pathogens (Narayana *et al.*, 2021; Haxhiaj, 2022).

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The prevalence rate of IMI, resulting in clinical mastitis, ranges from 13.0% to 50.0% in various countries and cattle breeds (Hoque *et al.*, 2020). The percentage of IMI-causing pathogens: gram-negative (35.5%), no growth (27.2%), gram-positive (27.6%), and other (9.7%) and high contamination rates during collection or identifying multiple bacteria being cultured from a single milk sample (Oliveira & Ruegg, 2014). South African data (2021) of bacteria isolates from 1,806 clinical mastitis cases revealed that 11.5% were gram-negative bacteria, 21.9% *Staph. aureus*, 23.5% non-aureus staphylococci, and 31.9% *Strep. uberis* bacteria (Submitted July 2024, Karzis & Petzer, 2024).

2.1.6 Mastitis-causing bacteria

Pathogenesis is the process in which bacteria invade, colonise, and replicate in the teat and/or udder, causing an IMI. The pathogen generally originates from the environment, cow (udder parenchyma), and/or existing IMIs (chronic udder infections) through direct contact or mechanical transfer. The bacteria override the udder's physical defence barriers and establish themselves in the mammary gland, where they interact with the innate and adaptive immune system (Oviedo-Boyso *et al.*, 2007).

The bacteria enter the teat canal, overcoming the keratin plug with the antibacterial and the lactoferrin in the milk. The bacteria then move into the udder (alveoli and epithelial tissues), eliciting the local immune reaction to start, and a clinical or subclinical mastitis case ensues (Oviedo-Boyso *et al.*, 2007; Piepers *et al.*, 2017; Piepers, 2022). Cows are susceptible to mastitis throughout their lactation; however, the periods of highest risk are at the beginning and end (30 to 100 days in milk) of the dry cow period and at points when the cows are under additional stress (immunosuppression), for example, parturition, metritis, sub-acute ruminal acidosis (SARA) (Haxhiaj, 2022).

It has been remarked that the distribution of the causative pathogens differs between IMI and clinical mastitis cases and that a series of milk samples need to be tested to classify the status of an IMI and, therefore, a new, repeat, or non-cure of a clinical mastitis case (McDougall *et al.*, 2001). The accuracy of the test is influenced by the dilution factor of the milk sample (quarter level versus cow composite level) (Narayana

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et al., 2021). Pathogens involved in IMIs also elicit an immune response from the cow. The microbial communities can influence the pathophysiology of mastitis due to microbiome dynamics (genomic features, such as the diverse metabolic pathways, functional genes, and gene expression associated with mastitis pathogenesis and dysbiosis with an increase in opportunistic pathogenic bacteria reducing the healthy milk commensal bacteria).

The bovine mastitis microbiome is comprised of bacteria (99.58%), archaea (0.24%), viruses (0.18%), and other microorganisms, with a focus on bacteria. Understanding the bacteria's avoidance tactics (antimicrobial resistance (AMR), biofilm production, adhesion to host cell uptake, bacterial communication, etc.) to override the host's immune system is also essential (Hoque *et al.*, 2020).

In the case of *Strep uberis*, the bacteria are environmental and host-adaptive in their properties and infection routes. *Streptococcus uberis* has a critical role to play in the development of an IMI due to the genomic properties of the bacteria. The phenotypic properties of the bacteria result in rapid changes to the pathogen load and genetic variation of the population in the environment due to the development, sharing, and expression of virulence factors or adaptive mechanisms to enhance viability and infection rates. The role of the pathogen in an IMI in a herd also depends on the dynamics of the other pathogens (competitiveness, coinfections, and competitiveness for resources in the udder and environment). We often see dominant strains or types of pathogens in a herd or a group of animals and how a farming practice can influence this, such as the spread of *Staph. Aureus*, misdiagnosis of *Mycoplasma*, and the misidentification of pathogen *Strep uberis*.

2.1.7 Environmental versus host-adaptive organisms

The microbiome comprises mainly host-adaptive udder pathogens, including *Staph. Aureus*, *Strep. agalactiae*, *Strep. dysgalactiae*, *Mycoplasma* spp., and *Corynebacterium bovis*, and mainly environmental pathogens, including *E. coli*, *Klebsiella pneumonia, and Strep. dysgalactiae*, and *Strep. uberis* (Oliver, 2011; Hoque *et al.*, 2020).

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Environmental IMI-causing pathogens account for a significant proportion of subclinical and clinical mastitis cases in lactating and dry cows. The general farming practices aimed at reducing new IMI have initially focused on host-adaptive pathogens and indirectly selected environmental pathogens in opting for this approach (Oliver, 2011).

Host-adaptive pathogens account for pathogens that colonise the udder of persistently infected dairy animals. This means pathogens can be shed in varying degrees when animals are under stress or udder damage occurs. The transmission or infection routes are generally associated with direct contact during milking with milk impaction and indirect contact, such as milkers using contaminated communal drying clothes. This spreads the IMI-causative pathogen from infected to uninfected quarters and cows (Nickerson, 2011).

The spread of contagious pathogens is generally rapid and associated with incomplete diagnostics (missed by intermittent shedding). This includes the high virulence factors that help build biofilms and adhesion molecules, promoting bacteria entering epithelial cells or macrophages and avoiding defence mechanisms associated with the cellular response in the udder defence system. Management systems have focused on identification and aggressive treatment with extended courses of antibiotics due to very low cure rates (<30%) in the case of *Staph. Aureus* cows during lactation, culling out of the herd, extra precautions in the milking routine, such as gloved hands routinely disinfected and dried, and backflushing of milking machine claw pieces/clusters with disinfectants (Nickerson, 2011).

2.1.8 *Streptococcus uberis*

Streptococcus has been recently separated into three genera: *Streptococcus*, *Enterococcus*, and *Lactococcus* (Oliver, 2011). *Streptococcus uberis* is a gram-positive cocci bacterium (Watts, 1988) commonly established in cow manure (25%) and on the skin of the cow (50%), emphasising the risk factor associated with contaminated environments (bedding and pastures) (Lopez-Benavides *et al.*, 2007). This confirms the behaviour (environmental and some host-adaptive) of the diverse strains (Davies *et al.*, 2016; McDougall *et al.*, 2004; Oliver *et al.*, 2011; Reyes *et al.*,

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2019; Sampimon *et al.*, 2009; Srithanasuwan *et al.*, 2022; Tomita *et al.*, 2008; Werner *et al.*, 2018; Zadoks *et al.*, 2001; Zadoks, 2007).

Streptococcus uberis has been known since the late 60s and early 70s to be responsible for IMIs (Watts, 1988) and is a common environmental pathogen. It is in the top three of the family *Streptococcaceae* involved in bovine mastitis (Watts, 1988); however, more recently, *Strep. uberis* has also been revealed to be a host-adaptive pathogen responsible for recording moderate clinical signs. These are IMI traits associated with milk loss throughout the lactation, often causing visible udder and milk sign changes, but have no specific correlation to SCC, duration of IMI (persistent or transient infections), severity, and recovery of clinical or subclinical mastitis (Heikkilä *et al.*, 2018). An interesting point identified was that *Strep. uberis* has a more significant adverse effect on milk yield in multiparous animals due to a double effect of having higher yields than healthy animals before diagnosis and lower yields than healthy cows post-diagnosis (Gröhn *et al.*, 2004).

In more recent studies in Thailand, *Strep. uberis* was detected to have a prevalence of 8.4% of the cows isolated from milk samples from around 20,000 cows over two years and accounted for 27% of the clinical cases in the study. The incidence of clinical IMIs is equally distributed between early and late lactation periods (Heikkilä *et al.*, 2018). Sixty per cent of *Strep. uberis* IMI lasts <30 days, and 18% could become chronic and last >100 days (Srithanasuwan *et al.*, 2022).

2.1.8.1 Mastitis caused by Streptococcus uberis globally

Streptococcus uberis is a mastitis pathogen that is increasing globally (Loures *et al.*, 2017). It is a common pathogen associated with IMIs and clinical mastitis cases in areas with temperate climates, for example, New Zealand, South-Eastern Australia (Charman *et al.*, 2012), United Kingdom (33% of all clinical IMIs) (Hillerton *et al.*, 1993; Bradley, 2002; Green & Bradley, 2013), and France (18% - 37% clinical and 6% - 27% subclinical cases) (Poutrel *et al.*, 2018), which also happens to be shared in pasture-based farming systems.

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This trend is most likely compelled by the changes in farming practices (Lopez-Benavides *et al.*, 2007) and the adaptations of virulent factors and resistance developing in the *Strep. uberis* strains. It is also a growing problem in herds with managed contagious IMIs, which have opened up a new area of udder health microbiome dysfunction (Denis *et al.*, 2009; Petrovski *et al.*, 2011; Green & Bradley, 2013; Keane *et al.*, 2013; Poutrel *et al.*, 2018; Rainard *et al.*, 2021). The reported prevalence rates of *Strep. uberis* in various countries are all on the rise, with China (4.9%) (Zang *et al.*, 2020), Finland (1.5%) (Pitkala *et al.*, 2008), Canada (2.0%) (Cameron *et al.*, 2016), and Argentina (3.3%) (Reinoso *et al.*, 2011).

2.1.8.2 Mastitis caused by Streptococcus uberis in South Africa

Local South African IMI data revealed increased prevalence and identification of *Strep—uberis* on TMR and pasture-based dairy farms. Over five years, from 2008 to 2013, the increase was remarked as (2.36% to 3.10%) in TMR systems and (2.63% to 3.64%) in pasture-based systems (Blignaut *et al.*, 2018). There has been a further increase in 2022, which was recorded to have increased to 8.03% (Personal communication 2023, Inge-Marié Petzer, Milk laboratory, University of Pretoria). Recent South African data from 30 dairy farms has revealed that all *Strep. uberis* strains isolated in the trial produced biofilms of varying strength (Magagula *et al.*, 2023).

2.1.8.3 Identifying *Streptococcus uberis*

Initially, *Strep. uberis* isolates can be excluded using the Lancefield grouping test (Lancefield Streptococcal Grouping Kit, Thermo Fisher Scientific). A series of culturing kits are used to select for *Strep. uberis* (Werner *et al.*, 2018). Following the identification step, laboratories often run antibiograms for culture and sensitivity. This may aid in identifying virulence expression and the potential development of AMR or multi-drug resistance (MDR). These isolates are then confirmed through the multilocus-sequence typing (MLST) (Coffey *et al.*, 2006) and/or the older, more time-consuming technique of pulsed-field gel electrophoresis (PFGE) with the macro-restriction analysis of the bacterial chromosomal DNA (Werner *et al.*, 2018).

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Through these techniques, the isolates from various studies have been ranked to evaluate evolution or genetic similarities, with literature revealing a 75% to 100% similarity (Werner *et al.*, 2018). The similarities or differences were observed within a herd and between diverse herds. This phenomenon can be explained by the fact that *Strep. uberis* is an environmental pathogen (Wang *et al.*, 2013; Werner *et al.*, 2018; McDougall *et al.*, 2004) and an opportunistic adaptive host-pathogen (Loures *et al.*, 2017). This means that *Strep. uberis* can result in multiple resources from which it can be introduced. These similarities and/or differences between strains are essential to understanding and explaining the probable reason behind reinfection, non-cure, or persistent infections (McDougall *et al.*, 2004; Werner *et al.*, 2018; Zadoks *et al.*, 2001).

Jolley *et al.* (2004) indicate that MLST is a means of sharing data on the Public multilocus-sequence typing (PubMLST) platform for research and identifying pathogens using molecular characterisation using fragments (housekeeping genes) and complete genomes. The sequence for housekeeping genes comprises 500 base pairs (Coffey *et al.*, 2006) and usually comprises between six and eight loci. These are identified based on the sequence and combination of alleles, forming a profile allocated a specific sequence-type (ST) number, which forms part of a specific clonal complex (CC) number.

This is a classification system used to analyse comparative data on a gene-by-gene basis to evaluate the phenotypic appearances, behaviours, and dynamics of the pathogen's genome over a time and place spectrum (Jolley *et al.*, 2004; Jolley *et al.*, 2018). In so doing, a grouping of similar or common ancestor strains and identifying shared genetic material (plasmids) generating GrapeTree - minimum - spanning trees (Khan *et al.*, 2003; Werner *et al.*, 2018; Jolley *et al.*, 2004; Jolley *et al.*, 2018).

2.1.8.4 Diverse strains of *Streptococcus uberis*

The diverse *Strep. uberis* strains contain a wide array of genetic diversity. Studies in Germany (Werner, 2018), Canada (Reyes et al., 2019), China (Zhang et al., 2020), and India (Srithanasuwan et al., 2022) indicate that various strains have been identified from field isolates. Over 500 diverse Strep. uberis strains are currently known (Sherwin *et al.*, 2021).

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It has been revealed that strains have similarities and differences within and between herds, host species, and temporal and geographical settings (Khan *et al.*, 2003; Tomita *et al.*, 2008; Werner *et al.*, 2018; Gilchrist *et al.*, 2013). This means that it is vital to identify diverse strains of specific organisms correctly, using the *Strep. uberis* MLST scheme, using housekeeping genes and the complete sequence of the genome (Coffey *et al.*, 2006; Jolley *et al.*, 2004; Tomita *et al.*, 2007; Davies *et al.*, 2016; Jolley *et al.*, 2018). There are seven housekeeping genes. These genes are required and expressed by all single cells and cells of an organism under normal and pathophysiological conditions (Butte *et al.*, 2001; Coffey *et al.*, 2006). The evolution of housekeeping genes is relatively stable, resulting in uniform expression, with CC favouring infectious patterns or origins, e.g., ST-5 CC causing clinical mastitis, ST-86 CC causing latent IMI, and ST-143 CC causing subclinical IMI (Tomita *et al.*, 2008; Davies *et al.*, 2016; Reyes *et al.*, 2019).

Using these housekeeping genes and complete genomes, the virulence genes can be studied when confronted with farming management practices that could be associated with the emergence of *Strep uberis*. Further benefits also include evaluating virulence factors, developing and sharing resistance factors between strains, plotting and identifying strains contributing to the biodiversity of the pathogen population, and evaluating the properties between environmental and host-adaptive strains.

The literature emphasises the high level of heterogeneity between *Strep. uberis* strains (Coffey *et al.*, 2006; Gilchrist *et al.*, 2013; Loures *et al.*, 2017) and how this is influenced by its environmental origins (Tomita *et al.*, 2008; Reyes *et al.*, 2019).

It has even been suggested that there are *Strep. uberis* subtypes I and II (*Strep. para-uberis*) (Khan *et al.*, 2003). Certain traits dominate in specific countries; for example, bovine *Strep. uberis* had common CC isolated from milk samples per region: United Kingdom (GCC5), New Zealand (GCC143) (Pullinger *et al.*, 2006; Gilchrist *et al.*, 2013), and Canada (ST-5 CC, ST-86 CC, and ST-143 CC) (Reyes *et al.*, 2019). Although within the bovine host species, the clonal complexes (GCC) can be present in multiple countries, they do not cross into ovine host species. Ovine and bovine *Strep. uberis* strains do not share global CC 5 or 143. Similarly, there are differences

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in the presence of plasminogen activator genes (Plasminogen activator A (PauA) in bovines and plasminogen activator B (PauB) in ovines) and the lack of yqil and capsule formation protein C (GapC) genes (Gilchrist *et al.*, 2013). With vaccination becoming a game changer in managing several IMI-causative pathogens, knowing the similarities and differences could aid in controlling IMI and the efficiencies of vaccines.

2.1.8.5 Virulence factors of *Streptococcus uberis*

It is known that *Strep. uberis* is challenging to treat successfully because the bacteria is intracellular and has various virulence factors. These virulence factors include the *SUAM*, prevalent resistance genes, such as tetracycline resistance (tetM), beta-lactamase enzyme production (blaZ), and ribosomal methylase enzyme (ermB) (Hoque *et al.*, 2020; Zhang *et al.*, 2020), biofilm formation (Magagula, 2023), and capsule formation (has AB gene) (Srithanasuwan *et al.*, 2022).

Virulence factors are products, such as enzymes, that are byproducts of ribonucleic acid (RNA) or DNA sequences within virulence factor genes (Hoque et al., 2020; Zhang et al., 2020; Srithanasuwan et al., 2022). They promote resistance or evading techniques of the microorganism from the host's immune system (Almeida *et al.*, 2006) and antibiotic treatments (de Jong *et al.*, 2018).

Streptococcus uberis strains carry four to seven virulence-related genes (Zhang *et al.*, 2020; Srithanasuwan *et al.*, 2022). These genes comprise:

- Biofilms (Magagula, 2023).
- Surface adhesion molecules (SUAM) (Almeida *et al.*, 2006).
- Hyaluronic acid capsules (*has AB* gene) (Srithanasuwan *et al.*, 2022). Hyaluronic acid is a biomaterial with multiple properties which can support microbial life. These include moisture retention (humectant) and resistance to mechanical damage (reducing the effects of the immune system and/or toxins/drugs) (Liu *et al.*, 2011).
- Plasminogen activator and receptors. These components of the bacterial surface turn bacteria into proteolytic organisms that use the host's fibrin and non-collagenous proteins from the extracellular matrices. This allows the bacteria to

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invade tissue and cells and potentially avoid the immune system and antibiotic treatments (Lähteenmäki *et al.*, 2001).

- CAMP factor (*CFA). The CAMP protein associated with Strep. uberis and other bacteria is a cohemolytic agent and is thought to be involved in cell physiology and pathogenicity (immune suppression*—binding to immunoglobulins) (Gase *et al.*, 1999).
- Lactoferrin-binding proteins. These proteins are secreted in milk and play a role in the immune system by depriving microorganisms of this nutrient, causing a bacteriostatic effect. Bacteria that can overcome this can survive in the udder, causing severe mastitis cases (García-Montoya *et al.*, 2012).
- Antibiotic deactivator/resistance: tetracycline resistance (*tetM*), beta-lactamase enzyme production (*blaZ*), and ribosomal methylase enzyme (*ermB*) (Hoque *et al.*, 2020; Zhang *et al.*, 2020).

2.1.8.6 Biofilm

A biofilm is a complex matrix of microorganisms that interact cooperatively, providing support and nutrients within an extracellular matrix comprising bacteria, polysaccharides, proteins, lipids, and DNA (Magagula *et al.*, 2023). Biofilms may form on living surfaces, such as mammary glandular epithelium or non-living surfaces like water pipes. The bacteria that produce biofilms have a virulence gene and can choose to express it when under threat. This is to avoid exposure to antibiotics and avoid cell recognition by the host's immune system. A biofilm develops as a result of the communication of the colonising bacteria with each other using quorum sensing (QS) to transfer products, such as N-acyl homoserine lactone, and it will grow as the colony grows by cell division and recruitment (Magagula *et al.*, 2023).

It is known that diverse strains of bacteria may have or lack the gene for biofilm production (Magagula *et al.*, 2023). There has been adequate research in the dairy sector, as several IMI-causative agents are known to produce biofilms. According to Moore (2009), *Strep. uberis* strains produce biofilms to varying degrees. There are several genes associated with biofilm production, bacterial proliferation, uptake, and binding, such as *sua*, plasminogen activator (*pauA*), Streptokinase gene (*skc*) –

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activation of multiplication/replication, *gapC*, *cfu*, Lipopolysaccharide Binding Protein (*lbp*), Hyaluronic acid production or support for biofilms (*hasA*, *hasB*, *hasC*).

Other studies have revealed that the most frequently identified genes from biofilm-producing *Strep. uberis* isolates from IMI and clinical cases were *gapC* (98%), *sua* (96%), and *pauA/skc* (94%), while *lbp* was not identified (Melchior *et al.*, 2006). To produce the biofilm, a need exists for the expression of competence genes (*comX*, *comEa*, and *comEC*) (Suntharalingam & Cvitkovitch, 2005), the sortase gene (*srtA*), and the QS genes (*luxS* and *gapC*). Additionally, *Strep. uberis* has been demonstrated by Reinoso (2011) to express plasminogen activator, lactoferrin-binding protein, or CAMP factor. These defence mechanisms allow for the development of antibiotic resistance, host-adaptive, and chronic/repeat IMIs (Magagula *et al.*, 2023).

The literature has revealed that *Strep. uberis* bacteria isolated from IMIs associated with clinical and subclinical cases were stimulated to produce biofilms (Moore, 2009; Magagula *et al.*, 2023). Lactose in cow's milk is a good source of carbohydrates supporting bacterial growth and biofilm expression by most *Strep. uberis* (Moore, 2009; Magagula *et al.*, 2023; Shree, 2023).

2.1.8.7 Streptococcus uberis adhesion molecule (SUAM)

The *Streptococcus uberis* adhesion molecule (SUAM) is a protein (comprising a 17 amino acid long peptide chain with the N-terminal sequence M T T A D Q S P K L Q G E E A C A) that plays a role in *Strep. uberis* bacteria attachment and uptake into bovine mammary epithelial cells during colonisation of the mammary parenchyma (Almeida *et al.*, 2006). The pathogenesis mimics that of a contagious bacterium, and the mechanism is similar to that of the lactoferrin-binding protein that elicits the uptake of bacteria. This process is a mechanism used by *Strep. uberis* to avoid the host's defence system, allowing the pathogen to maintain a state of persistence within the host and be classified as a virulence factor (Almeida *et al.*, 2006).

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2.2 Immune system of the bovine udder

Pathogens involved in IMIs need to interact and initiate a response once they have overcome the physical barriers, such as the anatomical structures or micro-environments of the teat end. Udder health management practices (pre- and post-milking routines, milking machine calibrations/services, etc.) and strategies (routine testing, teat dip actives, teat sealants, antibiotic selection, culling selection criteria, etc.) need to be implemented on dairy farms. The immunological process starts when the bacteria come into contact with the epithelial tissue of the mammary gland, activating the innate response (unprimed PMN cells) and then the adaptive immune system (humoral antibodies and primed cells) (Wellnitz, 2012).

2.2.1 Udder physiology

Mastitis is an inflammatory reaction in the udder parenchyma and supportive tissues making up the udder, such as suspensory ligaments and glandular and cistern tissue of the udder body and teat canals. The immune response involves the body being triggered by the physical udder barriers and epithelial tissue being breached, which activates the innate response (cellular and cytokines) and adaptive response (cell-mediated and humoral antibodies) immune system (Chase, 2022). The response is often rapid, and the degree of response varies due to the pathogen type, severity of the pathogen load, and cow parameters influenced or determined by animal husbandry and milking routines (Oviedo-Boyso *et al.*, 2007; Wellnitz, 2012; Piepers *et al.*, 2017; Chase, 2022; Haxhiaj, 2022; Piepers, 2022).

There is an increase in the number of somatic cells (PMNs) in the milk, compelled by the migration of the PMNs across the blood-udder barrier into the mammary gland and surrounding tissues (Piepers, 2022). This responds to pro-inflammatory cytokines triggered by stimuli, such as protein binders, biofilm recognition, and other common antigens (Oviedo-Boyso *et al.*, 2007). Factors that could adversely affect the innate and adaptive immunity of dairy animals include age (PMN cells of older cows have lower activity for reactive oxidative types); diet deficiencies (vitamin E, vitamin A, selenium, zinc, copper, manganese, and magnesium); a negative energy state (increase in B-hydroxybutyrate, which lowers the efficacy of chemotaxis, lower

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oxidative burst, and leukocyte phagocytosis); other infections or diseases; and the stage of lactation (high-risk areas) (Piepers, 2022). Other factors include the breed of cow and genetic selection for resistance (Narayana *et al.*, 2021).

2.2.2 Primary udder defence

The epithelial cells lining the teat canal, cistern, and alveolar glandular tissue act as the first line of defence. They serve as a mechanical barrier and a focal point for mucosal immunity. The physical barriers often have additional properties, such as keratin and the wax plug, which not only block the opening of the teat canal to potential IMI-causing pathogens but also contain fatty acids with antibacterial properties, bactericidal and bacteriostatic (Wellnitz, 2012).

2.2.3 Secondary udder defence: Innate and adaptive immunity

The innate immunity comprises cells and chemical triggers that elicit indiscriminate death to everything foreign to the udder and, in turn, prime the adaptive immunity. Adaptive immunity comprises antibodies (B lymphocytes and memory cells) against the antigens developed to complement the innate system. The other part of adaptive immunity involves the development of target-specific T helper cells, cytotoxic T cells, and regulatory T cells (T lymphocytes). This process often takes two to four weeks (Chase, 2022).

Antibodies (immunoglobulin G (IgG), immunoglobulin A (IgA), immunoglobulin M (IgM), immunoglobulin E (IgE), and immunoglobulin G (IgG)) can comprise polyclonal or monoclonal antibodies that are artificially produced. This ensures that the multitude of surface proteins for a particular pathogen can be incorporated into the adaptive immune response, aiding the animal in overcoming the IMI. Antibodies do not kill the pathogen directly but emphasise the antigen, targeting it for destruction by phagocytic or cytotoxic cells. Antibodies also function by blocking the attachment to and/or uptake of the pathogen into the host cells. They can reduce the severity of the infection and the adverse effects of toxins released from pathogens by neutralising them through agglutinating the pathogen or chelating and inactivating the toxin, respectively (Chase, 2022).

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In the case of an IMI-causative agent, such as *Strep uberis*, the exposed lipopolysaccharides stimulate innate immunity. This can initiate a cascade of pro-inflammatory cytokines, causing an acute phase response resulting in phagocytosis of the intruding bacteria and curing the IMI. The T lymphocytes are then activated, facilitating the development of cytotoxic cells and the activation of B lymphocytes to produce antibodies, aiding in the elimination and further memory development. Depending on the type and scale of the pathogen invasion, the cow will experience clinical or subclinical mastitis (Oviedo-Boyso, 2007; Wellnitz, 2012; Piepers *et al.*, 2017; Chase, 2022; Haxhiaj, 2022; Piepers, 2022).

2.2.4 Vaccination

Mastitis vaccines have been available for some time, but their efficacy has been a limiting factor. More than ever, there is a need to develop safe, effective, and cost-efficient vaccines. The vaccines aim to reduce and/or alleviate the financial burdens associated with mastitis (reducing the losses associated with decreased milk yield) and animal welfare (less severe and long-lasting clinical cases) (Bradley, 2015). The mastitis vaccines aim to reduce the severity and duration of the symptoms (milk, udder, and cow signs) experienced by cows suffering from IMIs (clinical and subclinical cases) (Rainard *et al.*, 2021). These vaccines must overcome the udder's unique anatomy, such as the blood-udder barrier and the short-lived local immunity of polymorphonuclear cells (Piepers *et al.*, 2017).

Mastitis vaccines aim to act as an immune modulator and improve the response and robustness of the immune system when challenged by an IMI. They provide protection at cow and farm levels, improving cure rates. Vaccination allows for the reduction in SCC or linear scores because there are fewer PMN cells. Research reviews, such as that of Rainard et al. (2021), emphasise the specifics (differences and similarities) of vaccines and the immunity associated with the major mastitis pathogens E. coli and *Staph. aureus*, and *Strep uberis*.

Currently, the vaccines on the market use alternative strategies to initiate protection by developing pathogen/antigen-specific antibodies in high serum levels. These include, for example, the slime-associated antigenic complex (SAAC) associated with

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staph*. aureus* vaccines and *E. coli* J5 with their corresponding IgG1 and IgG2 subtypes and the antigen-specific IFN-γ, IL-4, and IL-17 production by blood lymphocytes (opsonisation for phagocytosis or even the neutralisation of endotoxins) (Piepers *et al.*, 2017; Piepers, 2022). There is also the thought of improved T helper memory cells, which promote the cell-mediated immune response and accelerate and/or amplify the influx of neutrophils at the onset of IMI (Rainard *et al.*, 2021). There is the killed bacterin method and the concept of subunit vaccines, which target the siderophore receptor involved in the uptake of iron to support the growth and replication of coliforms by interrupting the nutrient supply to the pathogen, such as vaccinating with ferric enterobactin transporter A FepA and/or ferric citrate transporter A (FecA) antigens (Rainard *et al.*, 2021).

The commercial *E. coli* vaccines use bacterins made up of killed rough *E. coli* (J5 strain) and adjuvants. There has been cross-protection against all identified *E. coli* strains. This is due to the lack of the oligosaccharide side chains of the lipopolysaccharide chains that expose the common core or to membrane-associated proteins and antigens, identical to those of most other gram-negative bacteria (Rainard *et al.*, 2021). Tyler (1993) reported that vaccination improved protection against IMI-causative pathogens, such as *E. coli*. This was depicted in dairy cows not vaccinated having lower Enzyme-Linked Immunosorbent Assay (ELISA) titres of IgG1 for *E. coli* J5, which were associated with five times the rate of clinical coliform mastitis cases compared with animals vaccinated and had higher titres of IgG for *E. coli* J5 (Rainard *et al.*, 2021).

In the case of *Strep. uberis* vaccines, a live bacterial vaccine approach, was initially used. This resulted in the leucocyte response that only protected against the homologous strain for *Strep. uberis* and was less effective against a heterologous strain of *Strep uberis*. This had little to no effect on the opsonisation (neutrophils and macrophages) of bacteria in the mammary gland, rendering the vaccination less effective.

Another approach would be to target the nutrient uptake mechanism PauA or binding mechanisms, such as glyceraldehyde-3-phosphate dehydrogenase (GapC), Christie–

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Atkins–Munch-Petersen factor (CAMP), and *SUAM* (Rainard *et al.*, 2021). These all reduced the severity of the clinical cases to some degree but did not stop the shedding or initiation of the IMIs.

Finally, it is a means of protecting the udder and cow against *Strep. uberis* would control biofilm production using a lipoteichoic acid extracted from the biofilm. A biofilm comprises a complex matrix of exopolysaccharides with a dense aggregate of surface-adherent microorganisms. It is vital to use an antigen and building component to prevent biofilm development, as the majority of *Strep. uberis* strains are associated with the ability to produce biofilm. They can then survive in biofilm, avoiding exposure to the animal's immune system and contact with intramammary or systemic antimicrobial agents (Rainard *et al.*, 2021; Magagula *et al.*, 2023; Shree, 2023). Similarly, *Staph. aureus* has had a series of vaccines comprising killed whole-cell bacteria adhesins or leukotoxins focusing on inhibiting biofilm production (Piepers *et al.*, 2017; Rainard *et al.*, 2021; Piepers, 2022).

The general practice of vaccination aims at covering the most vulnerable times of lactation (dry-off and early lactation < 30 days in milk), ensuring individual protection is obtained. It can also be used with off-label vaccination protocols involving vaccinating an entire herd at set time intervals throughout the year (block vaccinations). This approach aims at herd immunity to control seasonal challenges or contagious pathogens. In the case of *E. coli* and *Strep. uberis* commercial vaccines, the vaccination protocol involves vaccinating a cow around the time of dry-off, at steam-up, and 15 to 30 days in milk (DIM). In summary, it is essential for a mastitis vaccine to have both an effect on the cellular and humoral response to reduce the severity, chronicity, and number of IMIs (clinical, subclinical, chronic non-cure) (Rainard *et al.*, 2021; Hipra correspondence).

2.3 Herd health management

The branch of herd health that mainly focuses on effective mastitis management is udder health. The latter focuses on the various pillars: environment, host (cow), and microorganism (Van Lelyveld, 2021a; Van Lelyveld, 2021b).

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Herd health management mainly focuses on general herd information and aids in the necessary changes or implementation of farm practices. This includes grouping animals based on production stages (daily milk yield), mastitis status (SCC levels, clinical, chronic cases), and fresh and dry cow status (parity). This is to meet the nutritional and performance needs with the correct ration mixes, milking frequency (twice to thrice daily), and sequence (fresh cows, low milk yield producers, high milk yield producers, dry-off animals, and the hospital group). This will require the processing of large data sets from various interested parties, including feed suppliers, nutritionists, milk buyers, veterinarians, and suppliers of teat dip and chemicals for cleaning and disinfection (Ruegg, 2017).

2.3.1 The environment

Housing (pasture, camps, or free stall systems), bedding (organic, sand, mattresses, moisture percentage), and managing both (stocking density, cleaning routines, cubicle dimensions) are essential for cow comfort. Cow comfort affects the animal's immunity. By ensuring that cow comfort is maintained, there will be a reduction in stressors and challenges. This may include reduced heat stress (temperature humidity index), mastitis pathogen load (environmental contamination), population stress and hierarchy, and the expression of natural behaviours, such as resting and eating (personal correspondence and training with Oriol Franquesca, 2014).

2.3.2 The host (cow)

Cow signs are cues used during parlour and farm audits. These include scores, such as cow hygiene scores (flank, leg, and udder), body condition scores, teat end scores, and lameness scores. Parlour efficiency (animal throughput rates, noise levels, cleanliness of parlour and walkways) and effectiveness of milkers (correct routine, hygiene, noise level, etc.) are evaluated (Van Lelyveld, 2022b; Van Lelyveld, 2022c; Van Lelyveld, 2023; Neave *et al.*, 1969; Marnet, 2013; Ruegg, 2017). These evaluations generally relate to animal welfare and can be extended to animal health. Other areas would then extend to vaccinations for diseases, such as BVD, Mastitis (*Staph. aureus*, *Strep uberis*, *E. coli*), and the general vaccine protocol.

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2.3.3 Milking equipment and system types

Milking equipment and system types (highline swing over, lowline rotary tables, and others) (Prendergast *et al.*, 2024) and maintenance timing to inspect the condition and replace parts, such as liners, filters and vacuum pump regulators, play an important role (Teagasc/IMQCS). The importance of equipment cleaning protocol and frequency should not be underestimated. Cleaning also extends to parts of the machine not directly in contact with the cow, such as airline filters, vacuum release valves, pulsator diaphragms, and sanitary traps. Finally, the evaluation of the system and teat end vacuum level and stability is crucial (Vermaak *et al.*, 2022; Van Lelyveld, 2022c; Van Lelyveld, 2022d; Van Lelyveld, 2023; Schwarz *et al.*, 2023).

2.3.4 Milking routine

Factors affecting the milking routine start with the number of daily milkings, seasonality, the number of staff in the parlour, and the cluster removal method (automatic or by hand) (Prendergast *et al.*, 2024). Parlour design influences temporal or sequential patterns and the movement or restriction of staff. The milking routine is a structured sequence of events performed to prepare the udder for milking, achieve optimum milk flow during milking, and end milking at the optimum time while practising high levels of hygiene, which can reduce IMIs during milking by 44% (Ruegg, 2017; Sun *et al.*, 2022).

Incorrect timing or sequence of steps in the milking routine could adversely affect udder health and increase the number of IMIs experienced on the farm to the extent that the type of dominating pathogen can be influenced. The selection of a teat dip is essential to prevent new IMIs. There are vital actions during milk harvesting to protect the udder that should be adhered to (Neave *et al.*, 1969; Marnet, 2013; Ruegg, 2017). These include gentle milking, which efficiently (reducing the milking time) and effectively (rapid milk let-down with limited over or under-milking) milks the animal with minimum milking irregularities (kick-offs, liner slips, power outages, cluster reattachments, or premature removals) (Van Lelyveld, 2022b; Van Lelyveld, 2022c; Van Lelyveld, 2023). Milking routine steps/actions include the following:

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- Disinfection with the pre-milking teat dip (10 to 30 sec) (Ruegg, 2017)
- Fore milk stripping with gloved hands that are to be disinfected and cleaned between each animal (hand versus machine) (Dzidic *et al.*, 2019)
- Dry teats (individual paper or cloth towels)
- Lag time is 60 to 120 sec in dairy cows, with the optimal time being 90 sec (Vermaak *et al.*, 2022) and 30 sec in dairy goats (Dzidic *et al.*, 2019). There are some thoughts of 40 sec for rotary tables of 50 milking points (personal communication I. M. Petzer, 2023)
- Cluster attachment
- Cluster detachment (manual, automated, cut-off volumes and arm retraction vacuum cut-off settings) (Vermaak *et al.*, 2022; Van Lelyveld, 2022c; Van Lelyveld, 2022d; Van Lelyveld, 2023; Dzidic *et al.*, 2019)
- Post-milking teat dip
- Backflushing of cluster

The animal husbandry around each pillar influences the management of mastitis, and there are several crossover benefits of having the balance right to optimise the health, production, and longevity of animals in dairy systems, irrespective of country, farming system, and breed (host-adapted pathogenic bacteria versus environmental pathogenic bacteria). The main objective for the preventative and control measures to work is consistency in their application, and the cost to implement them should be less than the losses caused by mastitis (Ruegg, 2017).

2.3.5 Farming systems: Total mixed ration (TMR) versus pasture-based

A TMR farming system feeds cows according to the production stage and milk yield performance (Schingoethe, 2017) while housing the animals in groups according to the stage of production, parity, and lactation stages. The TMR system uses a housing or camp type, opting for a high density of animals in a small area. They comprise housing (free-standing deep litter camps, cubicles, or non-cubicles). These systems attempt to manage the environment of the cow. Often, the resting areas use bedding material to provide cow comfort and maintain animal welfare. Still, at the same time, it is a high-risk factor for having dirty animals with high pathogen loads. It is a science

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on its own to manage these systems properly. This requires daily to twice-daily cleaning of walkways, refreshing or replacing bedding (correct depth, moisture percentage, etc.), treating bedding to decrease pathogen load, and aiding in keeping the bedding comfortable (raking or tilling).

This is to ensure that the pH and temperature of the bedding are managed to lower the pathogen load and simultaneously reduce its moisture percentages. Wet bedding or extruded manure with composting has been a new bedding used by farmers to reduce costs of buying new bedding, such as sand, straw, or mattresses (composting time varies from zero days to two weeks; dry matter percentage 34–42%) (Jeppsson *et al.*, 2024). Intensive housing allows the farmer to reduce environmental stressors (temperature regulation, feeding multiple times), manage nutritional deficits to limit deficiencies occurring, and promote correct udder health (Schingoethe, 2017).

Pasture-based farming combines mixed or monoculture pastures with complementary feeding to meet the needs of the dairy animals depending on their production stage and yields. The pathogen loads tend to be highest in spring and summer systems (Harmon *et al.*, 1992) in summer rainfall areas and areas with higher density of animals like calving pens (Harmon *et al.*, 1992).

There are alternative degrees of infection rates and pathogen loads between the host-adaptive and environmental pathogens experienced between the TMR and pasture-based dairy farming systems (Blignaut *et al.*, 2018). In the case of *Strep uberis*, both TMR and pasture systems have high faecal matter levels that form the largest reservoir. Due to the indiscriminate defecation pattern of cows, the environment is seeded with the pathogen in varying degrees (Zadoks *et al.*, 2001; Zadoks *et al.*, 2007; Lopez-Benavides *et al.*, 2007). The risk for faecal bacteria increases when raw slurry is used for irrigation and fertilising pastures and when wet/green manure bedding is used (Jeppsson *et al.*, 2024).

Both practices are growing and becoming a standard in South African dairy farms. This is similar to what is done in New Zealand and parts of Europe, where the pathogen loads are recorded as rising. *Streptococcus uberis* is on the rise in both farming systems because of increases in herd numbers and competition with

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management due to cost-cutting farming practices (Jeppsson *et al.*, 2024) and the change resulting in the over-representation of the dominant pathogens of the udder. *Streptococcus uberis* pathogen load from the environment (soil and faeces) ranged from 10.2% on a New Zealand farm during the October 2014 grazing season to 33.2% during the housed season, which mimics the possible differences between pasture and TMR farming systems (Sherwin *et al.*, 2021).

In summary, the involvement of farming type, barn design, bedding material, environmental conditions, and overall cow comfort will affect the pathogen load and exposure risk of *Strep uberis*. This, in turn, will require diverse methods to manage the IMI risk and reduce the incidence and prevalence of subclinical and clinical mastitis cases (Blignaut *et al.*, 2018; Sherwin *et al.*, 2021).

2.3.6 Managing host-adaptive organisms

Udder health management systems accepted by the NMC were generally based on the Five Point Plan and have now been adapted to be based on the ten-point plan (Petzer *et al.*, 2009):

1 Milking routine practices: This is important to control host-adapted mastitis as the basis involves having individual towels to dry the teats during pre-milking, reducing cross-contamination from quarter to quarter and cow to cow, and an effective post-milking teat dip.

2 Antibiotic use: It is recommended that blanket antibiotic dry cow therapy be implemented for all infected and healthy cows with a long-acting and effective antibiotic over a long therapeutic period. This practice prophylactically protects uninfected cows. Implementing this practice is an appropriate treatment plan for all clinical and chronic IMIs.

3 Identification and recordkeeping: An effective and easily applicable process of identifying and isolating or segregating bacterial-positive IMI animals from the rest of the herd with a culling programme that removes chronically affected cows. This now includes the vaccination of cows (both infected and non-infected animals) as this reduces the shedding of pathogens into the environment and protects naïve animals by having a more effective immune system (Ruegg, 2017).

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4 Culling programme: To ensure that cows infected with host-adapted bacteria that do not cure are removed from the herd as a source of infection.

5 Milking machine efficacy: Maintaining milk equipment in good condition and using correct settings to ensure an optimal and stable teat end vacuum, reducing milk impaction between quarters to preserve the integrity of the teat canals. Additionally, maintaining hygiene by using backflush systems with chemical disinfectant to reduce cross-contamination from the milking equipment (Ruegg, 2017). In so doing, this will reduce the risk of new IMIs from occurring in the parlour.

There are areas of focus which equate to the newer ten-point plan. This focuses on using selective dry cow therapy and the selection of an appropriate antibiotic through the prudent use of antibiotics:

1. Establish goals for Udder Health (current situation, current target/goal, how close we are to achieving the set goal, revise and adapt protocols).
2. Maintenance of a clean, dry, and comfortable environment (cow comfort and reduced risk factors).
3. Proper milking procedures (timing, hygiene, stimulation, and lag periods) (Rasmussen *et al.,* 1992).
4. Proper maintenance and use of milking equipment (services of parts, replacement of parts and calibrations of settings).
5. Good recordkeeping.
6. Appropriate management of clinical mastitis during lactation (identification, quick treatment, alternative treatments, and separation in hospital pen) (Laven, 2010a; Laven, 2010b).
7. Have an effective dry cow management programme (selective versus blanket).
8. Maintenance of the herd and parlour biosecurity for host-adapted pathogens and marketing (easily visible) of chronically infected cows. This includes the control of youngstock and avoiding or reducing cross-suckling between calves. An effective and efficient fly control system should be used.
9. Regular monitoring of the herd’s udder health status.
10. Periodic reviews of the mastitis control programme.

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Each of the above points can be expanded to cover various aspects of udder health, focusing on the longevity, productivity, and welfare of the animal while maintaining economic goals. This can be achieved through simple and more detailed steps.

2.3.7 Managing environmental organisms

The best ways to manage environmental pathogens involve reducing the pathogen load at the source. This includes reducing exposure to high-risk areas, such as straw and manure bedding or contaminated pastures (Crompton *et al.*, 2007), where cows are housed in higher densities or high-throughflow areas, such as calving pens or pasture camps (Sherwin *et al.*, 2021). The best means to protect dairy animals is to use pre-milking teat dips and ensure that teat dips and teat seals are used hygienically at steam-up cows before calving down (Petzer *et al.*, 2009). This helps reduce colonisation of the skin and teat orifice and canal from environmental contaminants like *Strep. uberis* bacteria.

Managing housing design and bedding, such as the frequency of changes, treatment with chemicals, such as soda lime, and aeration or composting of organic-based bedding (Jeppsson *et al.*, 2024), is associated with reducing the risk of *Strep. uberis* pathogens. Mastitis vaccines against bacteria, such as *E. coli* and *Strep. uberis* are now an additional part of controlling and managing environmental mastitis, with vaccination at set intervals or at high-risk times to maximise protection when dairy animals are most susceptible (Berry & Hillerton, 2003). Elements of the five- and ten-point control plans also work for environmental pathogens, but pre-milking teat dips are more critical than post-milking teat dips.

2.3.8 Proactive management

Many farming practices have become standardised (ten-point system) and are often implemented at alternative frequencies or intervals depending on the management level and the degree of involvement of a veterinarian. The more proactive, adaptive, and innovative a farmer and veterinarian are, the better they will be able to manage mastitis by using newer solutions (Petzer *et al.*, 2009; Ruegg, 2017). The most proactive step is to monitor and interpret farm data through routine and regular

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sampling of the herd (healthy and hospital animals) to identify the common pathogens involved in clinical and subclinical IMIs. This will aid in selecting intramammary antibiotics, elucidate whether to use blanket or selective dry cow therapy, segregate and cull chronic mastitis animals, review the milking routine, and monitor the cow environment for high-risk areas.

2.3.8.1.1 Animal welfare benefits

All animals have the right to be free of pain, suffering, and illness. Ensuring cow comfort, housing, and nutrition are part of achieving this, ensuring that the animal is in a good state of health and able to display regular activity and behaviour. The Animal Protection Act No. 71 of 1962 and the Veterinary and Para-veterinary Professions Act, Act 19 of 1982, are legislative controls ensuring all production animals are appropriately cared for (Schellack, 2017). Animal Welfare Indicators (AWI) are used at farm and herd levels to assess and quantify the degree of animal welfare achieved (Barry *et al.*, 2024). This type of strategy can also aid in correcting areas of concern. Dairy animals are monitored on the subsequent AWIs (Barry *et al.*, 2024):

1 Calf (deaths, abortions, and number of treated calves)
2 Cattle claw health (conditions, number of animals needing claw trimming, additional veterinary care)
3 Dehorning practices (age, pain control, polled breeds)
4 Fertility (inseminations to conception, calving interval, culls due to poor fertility)
5 Longevity (culled within first 14 DIM, culled pregnant animals between 84–290 days after last insemination, culled non-pregnant animals between 84–290 days after last insemination)
6 Metabolic diseases (number of milk fever cases, ketosis and rumen acidosis cases, body condition scores, carcase scores and classifications)
7 Milk yield
8 Mortality (number of deaths on farm, number of emergency slaughters, number of animals euthanised)
9 Udder health (number of animals with an SCC >200,000 cells/ml, number of clinical mastitis cases, number of culls due to deficient udder health)

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10 Youngstock (births, stillbirths, growth rates, morbidity, and mortality)

Animal welfare includes managing udder health through preventative measures and by early and effective detection and treatment (antibiotics, anti-inflammatories, etc.) of IMIs.

2.3.8.1.2 Cow longevity

The term 'longevity' refers to the period during which dairy cows are considered productive and economical, concerning efficiency, profitability, and average milk yield. There are subjective (social acceptance of the industry) and objective parameters linked to the environmental effect of dairy production (Adamie *et al.*, 2023).

2.3.8.1.3 Milk quality

Milk quality refers to the components of milk, including butter/milk fat, protein types and percentages, volume, colour, and appearance of floccules, SCC levels, and the level of physical and bacterial contaminants. The milk buyer will often set standards (total bacterial counts) to evaluate and determine a price per litre of milk and if the milk is fit for human consumption (safe, sound, and wholesome) (Martin *et al.*, 2023).

2.4 Somatic cell count

The somatic cells comprise epithelial cells and PMN cells, forming part of the immune system. SCC values indicate the cow's udder health (individual cow SCC) and the herd dynamics. Bulk milk SCC (BMSCC) increases due to environmental and management stressors, and incorrect milking machine settings (Haxhiaj *et al.*, 2022; Rienesl *et al.*, 2022). The interpretation of the SCC and BMSCC could be better represented as a Linear Score (LS), which accounts for the "hidden" individual SCC data that causes an erroneous interpretation of herd SCC data (mean SCC, median SCC, and bulk milk SCC).

The LS is a base 2 log of the SCC of a cow/herd and is calculated as LS = log2 (SCC/100) + 3 (Schukken *et al.*, 2003; Van Lelyveld, 2022a; Van Lelyveld, 2021a; Van Lelyveld, 2021b). By analysing the trends observed in the current and historical SCC

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or LS data (recorded annually, quarterly, or monthly), the veterinarian, farmer, and animal interface can be improved as problems, such as an increase in new IMIs, are identified. Corrective measures could be implemented, and progress can be assessed (cure or non-cure) and benchmarked (Schukken *et al.*, 2003; Van Lelyveld, 2022a; Van Lelyveld, 2021a; Van Lelyveld, 2021b).

There are slight variations in the diverse milk regulatory standards determining if an animal has mastitis. An average healthy dairy animal would realistically have an SCC of 70,000 cells/ml (Haxhiaj *et al.*, 2022) to 150,000 cells/ml (Vissio *et al.*, 2014). The cut-off SCC level that most entities have accepted is 200,000 cells/ml (Koeck *et al.*, 2012). Nevertheless, milk buyers and authorities are more interested in the BMSCC. The BMSCC provides information on what is happening in the whole herd. The milking yield of an animal influences it, also used to generate a weighted SCC (milk yield (ℓ) x SCC).

The latter influences the milk price and determines the fitness of the milk for human consumption or for processing (yoghurt, milk, etc.). In the USA, the BMSCC cut-off for human consumption is 750,000 cells/ml (Berry & Hillerton, 2002); in Canada, 400,000 cells/ml; in Europe, 400,000 cells/ml (Beli, 2016); and in South Africa, 500,000 cells/ml. In South Africa, there are similarities, but the milk buyer implements penalties, and after a series of poor results without corrective actions from the farmer, the milk buyer might not accept any further milk from the offending farm. The BMSCC is recorded for each delivery taken by the milk buyer. This data would be valuable to the veterinarian and the farmer to initiate an action plan to manage the different pillars of udder health (Rhoda & Pantoja, 2012).

The monitoring process involves sampling the herd and/or mastitis animals with cow-side tests, such as CMCT or electroconductivity or by laboratory tests using cytometry and fluorescence (electro-optical Fossomatic counter) (Gunasekera, 2003). The CMCT test is a helpful cow-side test, cost-effective, and provides a visual test result subjective due to the varying degrees of viscosity changes but can be receptive to interpretation for grading the level of severity of an IMI. The viscosity changes then grade the SCC levels and determine the risk of mastitis (0 - negative; 1 - weak positive;

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2 - distinct positive; and 3 - strong positive). The degree of positivity comes down to the chemical reaction of the milk (level of PMN cells) with the reagent (detergent and bromocresol purple), meaning the more nuclei present, the more viscous the mixture becomes (Pyorala, 2003; Van Lelyveld, 2022a).

The permeability of the blood-udder barrier changes in the presence of inflammation, and the increase in the SCC can be indicated by electrical conductivity (EC), which measures the electrical resistance of the milk. The resistance is due to the changes in the intra- and extracellular concentrations of sodium and chloride, the changes in the pH, and the fat/protein composition (decrease) of the milk. The classification of an IMI using EC: green light representing a healthy udder (>30 mΩ/cm), an orange light representing a suspect IMI (25-30 mΩ/cm), and a red light representing a positive IMI (<25 mΩ/cm). The CMCT strategy (sensitivity 94.5% and specificity 77.7%) is more accurate than the EC reading (sensitivity 66.7% and specificity 54.8% (Petzer *et al.*, 2013) of modulation error ratio (MER) at a cut-off point of >25 mΩ/cm) (Van Lelyveld, 2022a; Fosgate *et al.*, 2013). The correct classification of the varying classification levels of an animal regarding IMI or health status varies with EC strategies. It has been indicated to be correctly identified: subclinical (45% correct diagnosis), clinical (80% correct diagnosis), and healthy (74% correct diagnosis) (Norberg *et al.*, 2004).

As farming advances, so must the monitoring strategies. Differential somatic cell count (DSCC) measures the total SCC and expresses the percentage of a cow's SCC comprising PMN cells (leukocytes and lymphocytes). The DSCC is an early indicator of an IMI due to its high sensitivity and correlation to parity and not being influenced by DIM or milk yield (Dal Prà, *et al.*, 2022).

2.5 Antibiotics

Antibiotics form a part of the antimicrobial group, which are bacteriostatic or bactericidal. Their mechanisms of action interrupt the bacteria's ability to function and replicate. Antibiotics assist the immune system in eliminating bacterial infections (Berti *et al.*, 2020). Antibiotics move through the body in a particular manner (pharmacokinetics) due to their chemical composition (lipophilic or hydrophilic properties), resulting in organ-specific accumulation, which determines the route of

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administration (oral, systemic injection, or intramammary). The antibiotic intends to effectively treat an infection using a desired minimal inhibitory concentration (MIC) to inhibit the bacterium.

The pharmacodynamics of an antibiotic include how the active ingredient interacts with the body, therefore influencing the MIC. The MIC is the level or concentration of the active ingredient at the desired location/site that needs to be reached or maintained for a set period to ensure cure or prevention of infection. This varies according to the antibiotic, as some actives are time-dependent (≥ 50% of the dosing interval). In contrast, others are concentration-dependent (required to be 10 times above the MIC at the site of infection), and some actives are both concentration- and time-dependent. As a combination, the active will need to exceed the concentration and the period to be effective (Levison, 2000).

Another aspect is how the cidal or static effects are generated, such as the point of interaction. Antibiotics target diverse sites of action: cell wall or membrane, disruption of chemical processes across the cell wall/membrane, or the synthesis of proteins (Ribosomal 50 or 80), which will block a step in the replication of the bacteria and/or the maintenance of homeostasis within the bacteria. Finally, antibiotics are specific to the pathogen type and effective against only gram-positive or gram-negative bacteria, or are broad-spectrum, being effective against gram-positive and gram-negative bacteria simultaneously (Van Lelyveld, 2022b).

Antibiotics are registered with regulatory bodies, such as the European Medicine Agency (EMA) and the Clinical and Laboratory Standards Institute (CLSI) or governmental departments (DALRRD) according to the registration guidelines, which use the mode of action, class of active, and treatment protocol based on the pharmacodynamics/kinetics of the antibiotic. The product needs to be packaged to emphasise the treatment protocol and meet the legal and ethical aspects of the product claims. The treatment protocols (duration of treatment, treatment interval, antibiotic concentration, withdrawal periods, etc.) guide the user to ensure that the IMI is appropriately managed to promote cure (lactating and dry cow periods) and prevention (dry cow periods) (Karzis *et al.*, 2007; Stevens *et al.*, 2016; Van Werven,

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2018). The main aim of mastitis treatment is to cure and prevent an animal from suffering an IMI:

1 Clinical cure: Reducing and returning the symptoms (milk floccules/clots, blood, oedema, fever, etc.) observed in the milk, udder, and cow back to normal.
2 SCC cure: <200,000 cells/ml.
3 Bacterial cure: Decreasing or removing all bacteria or colony-forming units (cfu) from the milk, udder, and teat.

To achieve a clinical and bacterial cure of an IMI, an intramammary antibiotic should be used to achieve an optimal dosing schedule (correct dosing interval and number of treatments for an appropriate treatment duration). The optimal dosing includes the following: Single daily application (time-dependent active), twice-daily application (concentration-dependent active), off-label/extra-label use, and duration of treatment varies from three days, seven days, or extended treatment (cure vs non-cure).

The various combinations of antibiotic properties and the anatomy of the cow’s udder influence the drug's efficacy in treating the IMI. The blood-udder barrier separates the udder’s immune system and tissue from the blood that circulates in the rest of the body and, as a result, affects the penetration of the antibiotics into the glandular and parenchymal tissue of the udder. It is vital to select the correct treatment type (active and application method/route) to optimise the antibiotic reaching the target site and achieving the appropriate concentration to be most effective in curing or preventing the IMI (Owens *et al.*, 1988; Van Lelyveld, 2022b); however, in severe cases, a combination of intramammary and systemic antibiotics seems to be beneficial.

Overall, the best results in all scenarios will be achieved by treating cases appropriately through the quick detection and correct classification of IMI cases (Wilson *et al.*, 1999; Vasquez, 2017; Yang *et al.*, 2019). It does not stop there, as the method in which the intramammary antibiotic is applied (Aly *et al.,* 2022) to the teat/udder will also affect the outcome (cure and prevention) of an IMI. The treatment method can constitute a risk of creating further IMI cases with poor application hygiene and technique, causing damage to the physical defences of the teat canal.

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The steps for intramammary antibiotics and/or internal teat sealants are as follows: All staff conducting IMI treatments using intramammary tubes/applicators should be familiar with the correct procedure (clean gloved hands, clean equipment, correct techniques, good recordkeeping, and correct animal).

Step 1: After identifying (fore milk stripping and/or CMCT) and recording the animal suffering from mastitis (cow number, lactation, stage of lactation, quarter/s affected, and marking the cow according to a system, for example, leg or tail bands, separate cow group), the milker will need to milk out the infected quarter/s of the cow. Similarly, cows would have to be managed and prepared for dry cow therapy (DCT).

Step 2: The udder (DCT) or affected quarter/s (cow with an IMI) would need to be prepared to ensure that a good level of hygiene (cleaned and disinfected) is maintained and achieved at the body, tip, and orifice of the teat/s. The sequence involves the milker (with clean and gloved hands) starting with an appropriate teat dip with a good detergent (cleaning ability) with a high level of disinfection (active agent) to reduce pathogen load at the teat orifice (Van Lelyveld, 2020). In the case of DCT, the milker must start cleaning from the furthest teat and move towards the closest teat. Once the milker has cleaned the teat/s, they would need to dry the teat/s (using an individual paper towel per teat) similarly from the furthest to the nearest teat. Then, the milker would have to disinfect the teat end/s with an alcohol or methylated spirit swab, working in the same direction from the furthest to the nearest teat.

Step 3: The milker would need to place the recently opened antibiotic applicator (taking care to avoid sharp edges) into the teat orifice to infuse each quarter or the affected quarter/s with an antibiotic. In the case of DCT, the milker would start with the nearest teat and move away from themselves towards the furthest teat.

- Insert only the tip (<6mm) of the recently opened clean antibiotic tube (ensure that there are no sharp edges) into the teat canal/orifice using a slight rotating movement as the tip is inserted. Lightly compress the tip of the teat around the tip of the applicator using the thumb and forefinger.

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- Slowly administer the entire content of the dry cow or lactating cow intramammary tube into the teat canal. Remove the tube while maintaining the orifice of the teat closed between the thumb and forefinger.
- The milker would use the other hand to massage the administered treatment (antibiotic) up into each quarter.
- In the case of DCT, repeat steps a, b, and c per quarter.
- When teat sealants are used as a part of DCT, the milker should follow the same basic principles; however, when administering the tube content, the milker must stop applying pressure to the plunger of the applicator once resistance is felt. There should be no upward massaging of the product into the udder to avoid permanent damage (teat sealant entering the udder).

Step 4**:** Immediately following applying the intramammary antibiotic/teat sealant, the teats are dipped with an appropriate teat dip (Paduch, *et al.*, 2020; Van Lelyveld, 2020). The treated cows should stand in a clean area for 30 minutes (attempt to avoid letting cows walk long distances in pasture-based systems) to ensure proper coverage is maintained.

Step 5**:** As part of the DCT, each animal needs to be monitored for 36 hours, and the udder needs to be checked daily for signs (udder and animal) of mastitis, as the rapid or gradual drying-off methods have varying risk factors. In treating IMI (clinical and subclinical cases), the entire process needs to be repeated according to the registered protocol until a cure is achieved.

2.5.1 Antibiotic resistance

Antimicrobial resistance (AMR) is defined by Loo *et al.* (2019) as the capability of a microorganism to evolve and develop mechanisms to withstand or avoid the inhibitory activities (static or cidal) of an antimicrobial agent at the MIC to which the microorganism is normally susceptible. Repeated exposure to antibiotics or maintaining a constant low concentration selects the more resilient strains of bacteria and promotes evolution. Evolution can occur by adaptation and acquisition of genes through various methods: horizontal gene transfers from another microorganism,

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transformation, transduction, conjugation, or gene mutation, which can increase the risk of AMR (Verraes *et al.*, 2013) and MDR. MDR occurs when a microorganism is resistant to three or more antibiotic groups, forming the well-defined criteria of MDR (Magiorakos *et al.*, 2012).

Both AMR and MDR constitute a risk to the health of animals and the environment and are considered difficult to eradicate once developed (Van Duin & Peterson, 2016). This is a common occurrence in the food production sector, where high population densities of animals from mixed origins are often housed under high stress levels (environmental, disease, etc.). Antimicrobials are used as in-feed growth promoters or blanket prophylactic treatments to promote good health and lower costs when obtaining high performance in production, growth, and replication (Loo *et al.*, 2019).

One area in food production is the dairy industry, with as much as 80% of all antibiotics used in the dairy industry being solely for managing and treating mastitis (DCT and in-lactation treatments) (Pol & Ruegg, 2007). About 60% of the antibiotics used on farms are for mastitis management in the form of intramammary antibiotics (lactation and dry cow tubes). Forty per cent of the intramammary tubes are in the form of dry cow tubes, and 22% of intramammary tubes are lactation cow tubes applied to treat subclinical and clinical IMI.

Approximately 5% of antibiotics used for mastitis treatment are provided as systemic antibiotic treatment, primarily for severe or repeat IMI cases, often involving resistant pathogens (Vasquez *et al.*, 2017; Burke, 2021). Intramammary administration, being the first line of treatment for most cases of mastitis, is often applied by untrained workers or workers with lower levels of education. It is understandable why there could be errors in using and selecting intramammary antibiotics. In most cases, the selection is based on cost, availability in stock, and the presence of symptoms (Laven, 2010a; Laven, 2010b); therefore, treatments are often provided regardless of aetiology.

With the misuse or overuse of antibiotics being common practice, AMR/MDR will remain a high concern for regulatory authorities and consumers, especially with studies showing changes in responses and susceptibility of bacteria, such as *Strep uberis*. This organism initially responded well to β-lactam (*Strep. uberis* susceptibility

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ranges from 23% to 99%, with a median of 82%) (Cameron *et al.*, 2016) and third- or fourth-generation cephalosporin antibiotics (Haenni *et al.*, 2018). Now, both antibiotics are shown to be less effective due to lower sensitivity and higher resistance (McDougall *et al.*, 2020; Haenni *et al.*, 2018).

It is observed that through the prudent use of antibiotics, the risk of AMR/MDR is noticeably reduced (Oliver & Murinda, 2012). Several pathogens develop AMR/MDR, and it has been remarked in research studies that 9.9% of *Strep. uberis* isolates are thought to be resistant (Boireau *et al.*, 2018). The antibiotic classes with the highest level of resistance are: beta-lactams (11 genes identified), macrolides (10 genes), tetracyclines (five genes identified), quinolones (five genes identified), and trimethoprim (three genes identified) (Hoque *et al.*, 2020).

In the presence of AMR and the prudent use of antibiotics, there has been more emphasis on antibiograms and sensitivity tests (Klinker *et al.*, 2021). The overall procedure involves isolation of the pathogen from the milk sample and antibiotic susceptibility testing: disc method (Kirby Bauer) or MIC testing (Bauer *et al.*, 1966; de Jong *et al.*, 2018; Gurjar, 2012; Bagul & Sivkuma, 2016; Thermo Fisher Scientific, 2021).

2.5.2 Mechanisms of Bacteria Developing Antimicrobial Resistance (AMR)

Antimicrobial resistance (AMR) is developed through intrinsic (natural) resistance or acquired (bacteria obtained the ability) resistance. These comprise diverse mechanisms or strategies within bacterial phylum and/or between species of bacteria (Zhu *et al.*, 2023). The development of resistomes or antibiotic-resistant genes (ARG) or the acquisition and sharing of ARG can involve DNA mutation, vertical gene transfer (VGT), and horizontal gene transfer (HGT) (Hoque *et al.*, 2020; Zhu *et al.*, 2023). This process can occur in vivo or in vitro, such as in the udder or other environmental hot spots, such as slurry dams or waterways (Lopez-Benavides *et al.*, 2007; Blignaut *et al.*, 2018).

2.5.3 Horizontal gene hopping (transfer)

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Deoxyribonucleic acid (DNA) or RNA mutations may lead to several changes in the original structure of the specific target of an antibiotic or immune system, decreasing the affinity between drugs and targets. This can be due to errors in the replication phases of cells, bacteria, splicing of genes through deletion or addition of sequences, sometimes through horizontal or vertical transfer, and plasmids.

Examples of this include ribosome methylation of ribosomal RNA, which accounts for the development of resistance to macrolide antibiotics (Bagul & Sivkuma, 2016), or the mutation within the *gyrA* gene, which accounts for the alteration of a part of the DNA gyrase and the development of resistance to fluoroquinolone antibiotics (Zhu *et al.*, 2023). The transfer of genes can occur vertically through the sexual or asexual replication of bacteria (Zhu *et al.*, 2023), and horizontal transfer can occur between diverse strains of bacteria by using mobile genetic material, such as plasmids, transposons, and integrons (Zhu *et al.*, 2023). Interestingly, antibiotics at low levels and various non-antibiotic factors (temperature, pollutants, etc.) can contribute to the process (Zhu *et al.*, 2023). The method of horizontal transfer involves four main mechanisms:

1 Conjugation—requiring cell-to-cell contact, and DNA is transported through cell membranes from donor to recipient through pil-T-regulated trichome retraction common in gram-negative bacteria, e.g., *E. coli* and *Escherichia faecalis* (Zhu *et al.*, 2023).
2 Transformation—occurs through the uptake of cell-free (exogenous) DNA that can exist in the environment due to the release from dead or damaged cells/bacteria. The recipient bacteria can now alter its phenotype by expressing the obtained DNA (independently or when incorporated into the DNA), e.g., *Streptococcus pneumoniae* (Nnadozie & Odume, 2019; Zhu *et al.*, 2023).
3 Transduction—when an “infected” bacteriophage transfers the donor bacteria’s DNA, which it has incorporated into its own genome, and releases it into the recipient cell. Then the transduction process will occur (Zhu *et al.*, 2023).
4 Vesiduction—when vesicles containing DNA, metabolites, and proteins are secreted from the surface of the donor bacterium and transported to the recipient

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bacterium, where they fuse with the cell membrane and enter the cytoplasm (Zhu *et al.*, 2023).

All these mechanisms are supported or even promoted due to pollutants, non-antibiotic drugs (bioaccumulation or reverting to an active form of the drug from the metabolites), disinfectants, and water treatment processes, emphasising the concern of slurry being used for irrigation of pastures presenting a high risk of environmental bacteria, such as *E. coli* and *Strep. uberis* (Tiedje *et al.*, 2023).

Another aspect of AMR is the ability to express virulence factors like being impermeable to antibiotics, e.g., biofilms or impenetrable barriers produced by *Strep. uberis* strains (Sipka *et al.*, 2021; Magagula *et al.*, 2023). Several gram-negative bacteria develop resistance to beta-lactam antibiotics by releasing enzymes, such as beta-lactamase, which hydrolyse any beta-lactam antibiotics. Other bacteria have efflux pumps that expel antibiotics from the cell or specific metabolic pathways that alter the activity of an antibiotic to become ineffective before the target (ribosomes, etc.) is reached and the bacteria is inactivated or killed (Bagul & Sivkuma, 2016).

Several bacteria can even have several resistance mechanisms; for example, over 59.3% of analysed *Strep. uberis* strains carried four to seven virulence-related genes (Zhang *et al.*, 2020). These virulence factors comprise plasminogen activator/streptokinase, surface dehydrogenase proteins, CAMP factor, lactoferrin-binding proteins, SUAM, hyaluronic acid capsules, and several others (Calonzi *et al.*, 2020; Zhang *et al.*, 2020; Srithanasuwan *et al.*, 2022).

2.5.4 Resistance genes

Mastitis pathogens use microbial traits (pathogenicity, virulence factors, ARGs, and metabolic potentials) to function as potential opportunists, creating a monoclonal population that causes dysbiosis by interfering with metabolism. This includes promoting the growth/replication of the pathogen, uptake of nutrients for and by the pathogen, and overcoming the host defence and immune systems by developing avoidance tactics (adhesion and motility structures, biofilms, secreting and

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manufacturing of enzymes to deactivate oxidative substances and improve intracellular survivability, efflux pumps to avoid antibiotics).

2.5.5 *Streptococcus uberis* resistance

Antimicrobial susceptibility research studies have varied reports of drug efficiency for *Strep. uberis* in vivo and in vitro scenarios, and different drug selection criteria. For several years, there have been concerns regarding the development and worsening of AMR and MDR (Hoque *et al.*, 2020). An example of beta-lactamase resistance in *Strep. uberis* species has been remarked globally in South America (Gianneechini *et al.*, 2002), New Zealand (Petrovski *et al.*, 2011), Canada (Cameron *et al.*, 2016; Reyes *et al.*, 2019), and North Africa (Saed & Ibrahim, 2020). *Streptococcus uberis* also resists tetracyclines (Cameron *et al.*, 2016; Gurjar, 2012).

2.5.6 South African situation - regulatory

The agricultural and veterinary sectors are being regulated to control using antimicrobials in South Africa. The controlling bodies are the National Department of Health, the Department of Agriculture, Forestry and Fisheries, and the South African Veterinary Council. These bodies have set up and used the Stock Remedies Act 36 of 1947 and The Medicines and Related Substances Act 101 of 1965 to regulate at various levels (veterinary, farmer, etc.) which products are available over the counter and require prescriptions (Henton, 2011). Currently, South Africa uses antibiotics to treat or prevent external and internal parasites (mainly tetracycline antibiotics) and in-feed growth promotion.

The South African Veterinary Council has also started implementing One Health aspects to reduce using antibiotics in the livestock sector (Schellack, 2017). This means that the access and use of antibiotics have mainly been freely available, complicating the regulation of the prudent use of antibiotics. The European Surveillance of Antimicrobial Consumption (ESAC) project developed quality indicators to measure appropriate outpatient antibiotic use in Europe and is much stricter, meaning that using antibiotics requires proof of need for use, analysis of the

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number of sales (average use, sales, and recorded prescriptions), and implementing penalties (Coenen *et al.*, 2007).

2.5.7 Intramammary products available on the South African market and globally

Intramammary antibiotics have become harder to obtain over the past few years. The reasons include the discontinuation of some products and the shift of pharmaceutical companies' focus to developing alternative products, such as immunomodulators, vaccines, or teat sealants. Currently, the products available in distinct parts of the world are summarised in Table 2.3. An interesting point observed from the products available on the global market of intramammary antibiotics, such as dry cow or lactating cow therapies, is that the common actives used are those of penicillin and cephalosporins.

Several countries observe alternative treatments with early use of NSAIDs, oxygenated oils, and glucose/dextrose infusions. Several of these alternatives still require further research to support their assertions of efficacy and safety.

Table 2.3 summarises the product characteristics: the active ingredient, mechanism of action, target site of the pathogen, milk withdrawal periods, and pricing in respective countries (South Africa, United Kingdom, and New Zealand).

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Table 2.3: A selection of intramammary antibiotics on the market in countries where *Streptococcus uberis* is a common pathogen

A: United Kingdom

Antibiotics - Intramammary application						
United Kingdom						
Product name	Use (dry cow or lactating cow)	Manufacturer	Active ingredient	Method of action	Price per tube	Additional information
Bovaclox DC Xtra	DC	Norbrook	Cloxacillin and ampicillin	Broad-Spectrum (Gram + and Gram -), cell wall synthesis and cidal.	**£2.15** per tube	Milk withdrawal period 156hr after calving
Cepravin DC	DC	MSD	Cephalonium	Broad-Spectrum (Gram + and Gram -), first-generation	**£3.17** per tube	Milk withdrawal period 96 hours after calving if the dry period is longer than 54 days

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Antibiotics - Intramammary application						
United Kingdom						
				Cephalosporin, cell wall synthesis, and cidal		58 days following treatment if the dry period is less than or equal to 54 days
Cepritect DC	DC	Norbrook	Cephalonium	Broad-Spectrum (Gram + and Gram -), first-generation Cephalosporin, cell wall synthesis, and cidal	**£2.19** per tube	Milk withdrawal period 96 hours after calving if the dry period is longer than 54 days 58 days following treatment if the dry period is less than or equal to 54 days
Multisheild DC	DC	Duggan	Neomycin sulphate, Penethamate Hydriodide and Procaine Benzylpenicillin	Broad-Spectrum with various actives targeting the cell membranes, cell wall and B-lactamase enzyme, and cidal	**£2.68** per tube	Milk withdrawal period 96 hours post calving in cows with a dry period of more than 50 days. 50 days plus 96 hours after treatment from

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Antibiotics - Intramammary application						
United Kingdom						
						cows with a dry period of 50 days or less
Noroclox	DC	Norbrook	Cloxacillin Benzathine	Broad-Spectrum (gram + and gram -), cell wall synthesis and cidal	**£2.26** per tube	Milk withdrawal period 108 hours after calving. Should a cow calve earlier than 35 days after the last treatment, milk for human consumption may only be taken from 35 days plus 108 hours after the last treatment
Orbinin	DC	Zoetis	Cloxacillin Benzathine	Broad-spectrum (gram + and gram -), cell wall synthesis and cidal	**£2.69** per tube	Milk withdrawal period 204 hours after calving. If calving occurs before 30 days after last treatment, milk for human

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Antibiotics - Intramammary application						
United Kingdom						
						consumption may only be taken after 30 days plus 204 hours after the last treatment
Orbinin Extra	DC	Zoetis	Cloxacillin Benzathine	Broad-spectrum (gram + and gram -), cell wall synthesis and cidal	**£3.13** per tube	Milk withdrawal period 96 hours after calving. If calving occurs before 45 days after the last treatment, milk for human consumption may only be taken after 45 days plus 96 hours after the last treatment
Ubrostar Red	DC	Boehringer Ingelheim		Broad-spectrum with various actives targeting the Cell membranes, cell	**£2.89** per tube	Milk withdrawal period 36 hours after calving if calving occurs after 35 days after the last

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Antibiotics - Intramammary application						
United Kingdom						
			Penethamate hydriodide, Benethamine penicillin and Framycetin sulphate	wall and B-lactamase enzyme and cidal		treatment. Milk for human consumption may only be taken after 37 days if calving occurs before 35 days after the last treatment
Albiotic 330mg/100 mg	LC	Huvepharma	Lincomycin, neomycin	Gram +, Cell membrane and Cell wall synthesis, and both Static and cidal.	**£3.48** per tube	Milk withdrawal period 84 hours
Mastiplan LC	LC	Intervet	Cephapirin, Prednisolone	Broad-Spectrum (Gram + and Gram -), cell wall synthesis and cidal.	**£3.29** per tube	Milk withdrawal period 132 hours
Orbenin LA	LC	Zoetis	Cloxacillin sodium	Broad-Spectrum (Gram + and Gram -), cell wall synthesis and cidal.	**£3.84** per tube	Milk withdrawal period 96 hours

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Antibiotics - Intramammary application						
United Kingdom						
Pathocef	LC	Zoetis	Cefoperazone (sodium salt)	Broad-Spectrum (Gram + and Gram -), cell wall synthesis and cidal.	**£15.07** per tube	Milk withdrawal period 72 hours
Ubropen	LC	Boehringer Ingelheim	Benzylpenicillin procaine monohydrate	Broad-Spectrum (Gram + and Gram -), cell wall synthesis and cidal.	**£3.02** per tube	Milk withdrawal period six days.
Ubrolexin	LC	Boehringer Ingelheim	Cefalexin as monohydrate and Kanamycin as monosulphate	First-generation cephalosporin (Cell wall) and Kanamycn (Ribosomal inhibituion of protein) synthesis)	**£3.76** per tube	Milk withdrawal period 10 days
Synulox	LC	Zoetis	Amoxicillin Trihydrate, Clavulanic Acid, Prednisolone	Broad-Spectrum (Gram + and Gram -), cell wall synthesis and cidal	**£3.33** per tube	Milk withdrawal period 84 hours, i.e. seven milkings with two times a day milking or 11

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Antibiotics - Intramammary application						
United Kingdom						
						milkings with three times a day milking.

B: South Africa

South Africa						
Cephudder	LC/DC	MSD	Cephapirin	First-generation cephalosporin, Broad-spectrum (Gram + and Gram -), cell wall synthesis, and cidal	R per tube	Milk withdrawal period is 96 hours after calving if calving occurs after 54days.
Cobactan	LC	MSD	Cefquinome	Broad-spectrum (Gram + and Gram -), cell wall systhesis and cidal	**R58.77** per tube	Milk withdrawal period is five days or 120 hours following the last infusion.
Mastiject Forte	LC	MSD	Tetracycline, Neomycin, Bacitracin and Prednisolone	Broad-spectrum with various actives targeting the Inhibition of protein synthesis (RB30s) and Cell wall synthesis, and both Static and cidal	**R60.67** per tube	Milk withdrawal period for eight milkings following the last infusion.

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South Africa						
Mastiplan	LC	MSD	Cefapirin and Prednisolone	First-generation cephalosporin, Broad-spectrum (Gram + and Gram -), cell wall synthesis, and cidal	R per tube	Milk withdrawal period for 5.5 days or 132hours following the last infusion.
Rilexine 200/500	LC/DC	Virbac	Cephalexin, Neomycin sulphate and Prednisolone	Broad-spectrum with various actives targeting the inhibition cell wall synthesis, and cidal	**R45.52** per tube/ **R43.36** per tube	Milk withdrawal period for 96 hours after the last infusion
Spectramast	LC/DC	Zoetis	Ceftiofur	Broad-spectrum (Gram + and Gram -), cell wall systhesis and cidal	**R54.78** per tube/ **R58.81** per tube	Milk withdrawal period for 72hours after the last infusion in lactating animals and no withdrawal period if the dry cow period is 30 days or longer.
Spectrazole	LC	MSD	Cefuroxime	Broad-spectrum (Gram + and Gram -), cell wall systhesis and cidal	R per tube	Milk withdrawal period of 48 hours if treatments are three infusions and 72hours if treatmetns are four to eight infusions.
Ubrolexin	LC	Boehringer	Cefalexin as monohydrate and Kanamycin as monosulphate	First-generation cephalosporin (Cell wall) and Kanamycn (Ribosomal inhibituion of	**R68.15** per tube	Milk withdrawal for five days post the last infusion.

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South Africa						
		Ingelheim		protein) synthesis)		
Cepravin	DC	MSD	Cephalosporins	Broad-Spectrum (Gram + and Gram -), cell wall synthesis and cidal	**R51.91** per tube	Milk withdrawal period for 96 hours after the last infusion
Orbenin extra	DC	Zoetis	Cloxacillin Benzathine	Broad-Spectrum (Gram + and Gram -), cell wall synthesis and cidal	**R23.06** per tube	Milk withdrawal period 96 hours after calving if the dry period is longer than 35 days 58 days following treatment if the dry period is less than or equal to 35 days.

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C: New Zealand

New Zealand						
Amoxi-Mast	LC	Merck	Amoxicillin	Broad-Spectrum (Gram + and Gram -), cell wall synthesis and cidal	NZ$ 3.75 per tube	Milk withdrawal period 60 hours
Albiotic	LC	Agrihealth	330mg Lincomycin/100mg Neomycin	Broad-spectrum (Gram + and Gran -), Cell membrane and Cell wall systhesis, and both Static and cidal	NZ$ 15.89 per tube	Milk withdrawal 60 hours or five milkings when infused three times in a two milking per day system and 96hours or four milkings in a one milking per day system
Dari-Clox	LC	Merck	Sodium Cloxacillin (Semisynthetic penicillin)	Broad-Spectrum (Gram + and Gram -), cell wall synthesis and cidal.	NZ$ 3.75 per tube	Milk withdrawal period 48 hours

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C: New Zealand

New Zealand						
Masti-clear	LC	Hanford's U.S. Vet	Penicillin G Procaine	Broad-Spectrum (Gram + and Gram -), cell wall synthesis and cidal.	NZ$ 2.05 per tube	Milk withdrawal period
PolyMast	LC	Boehringer Ingelheim	Hetacillin Potassium	Broad-Spectrum (Gram + and Gram -), cell wall synthesis and cidal.	NZ$ 3.66 per tube	Milk withdrawal period
ToDay	LC	Boehringer Ingelheim	Cephapirin Sodium	Broad-Spectrum (Gram + and Gram -), cell wall synthesis and cidal.	NZ$ 3.75 per tube	Milk withdrawal period

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C: New Zealand

New Zealand						
Dry-Clox	DC	Boehringer Ingelheim	Cloxacillin Benzathine	Broad-Spectrum (Gram + and Gram -), cell wall synthesis and cidal	NZ$ 2.92 per tube	Milk withdrawal period
Go-Dry	DC	Hanford's U.S. Vet	Penicillin G Procaine	Broad-Spectrum (Gram + and Gram -), cell wall synthesis and cidal.	NZ$ 2.31 per tube	Milk withdrawal period
Orbinin -DC	DC	Merck	Benzathine Cloxacillin	Broad-Spectrum (Gram + and Gram -), cell wall synthesis and cidal.	NZ$ 2.91 per tube	Milk withdrawal period

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C: New Zealand

New Zealand						
Spectramast DC	DC	Pfizer/Zoetis	Ceftiofur	Broad-Spectrum (Gram + and Gram -), cell wall synthesis and cidal.	NZ$ 5.41 per tube	Milk withdrawal period
ToMorrow	DC	Boehringer Ingelheim	Cephapirin Sodium	Broad-Spectrum (Gram + and Gram -), cell wall synthesis and cidal.	NZ$ 3.11 per tube	Milk withdrawal period

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2.5.8 One Health

The One Health concept studies the interaction between animals, humans, and the environment (Essack, 2018). This is a legislative and active approach to protect (effective, ethical, and practical) the diverse arms of the ecosystem, including human health, animal health (domestic and wildlife, insects), and environmental health (plant, air, and water). Understanding this interaction lowers the risk of biodiversity loss, damage or function degradation, and the emergence or outbreak of diseases (Hayman *et al.*, 2023). This is achieved through the optimisation of sustainable practices, utilisation of surveillance systems regulating exposure and/or bioaccumulation of detrimental substances through the prudent use or replacement, and reduction theory (Hayman *et al.*, 2023; Cattell, 1996).

The perception is to strengthen cross-sectoral coordination and collaboration and share the responsibility, for example, reducing using AMR or MDR through reducing the use of a confirmed selection-based approach to substantiate using an antibiotic. There has now been the development of regulatory bodies and legislation that place financial, structural, and procedural steps and strategies into play to ensure One Health is implemented across all fields (consumer awareness, professional awareness and protocols, and recording systems and reports) (Hayman *et al.*, 2023).

The importance of identifying and understanding the effects (standalone or accumulative) of the molecular characteristics of IMI-causative pathogens, such as *Strep. uberis* and managing slurry from dairy farms falls well within the One Health practice. By being able to interpret farm data in the context of knowing the behaviours of the species and subspecies of *Strep uberis*, we are able to observe the effects of imbalances and interactions at the human, environment, and dairy animal interface that will promote or demote the development of IMIs. Knowing the molecular characteristics of a pathogen could aid in the reduction of risk, improvement of treatments and cure, and the reduction of pathogenicity, all while promoting the health of other sectors that could contribute to a healthier ecosystem (farm, region, country).

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2.6 Concluding remarks

Several studies aim to control and reduce the occurrence and severity of IMIs in dairy animals. The use of molecular characterisation to evaluate the housekeeping genes and full genomes of emerging pathogens, such as *Strep. uberis* will enable veterinarians and farmers to understand better the evolution, adaptability, and predictability of IMIs. By correctly identifying an IMI pathogen, the human, animal, and environment interface can be efficiently and effectively managed, facilitating monitoring, corrective steps, and setting standards for the various pillars of udder health management plans.

In the encounter of climate or environmental health pressures and the associated consumer perception and regulatory authorities, the relevance of molecular characterisation will increase in the identification and treatment of IMIs. Farmers and veterinarians should focus on and use what they learn from this research to overcome the challenges of IMI pathogens. The information generated using the molecular characteristics of pathogens (resistance factors, knowing and evaluating the biodynamics of the IMI pathogen in the cow and in the environment, etc.) can and should be used to implement changes in protocols and farming practices. The practice of correct selection and prudent use of antibiotics will increase and be the responsibility of veterinarians (regulators and implementers of current and future strategies for udder health management). Molecular characterisation could aid in developing and further improving immunomodulation techniques to overcome the virulence factors and infectious rates of pathogens.

Molecular characterisation of pathogens needs to focus on the IMI pathogens that are occurring at higher rates, especially when farming practices are promoting this despite the geographical areas (same or similar trends observed globally: New Zealand versus South Africa), housing and animal husbandry practices (TMR versus pastures, dry cow period lengths, DCT practices). The breeds (mixed versus pure breeds) and milking systems (parlour designs and operations, machines and their calibrations and/or settings, etc.) will also influence the pathogen load, exposure risk, and damage or overcoming the defence systems of the udder (physical and immune system).

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Streptococcus uberis is a major environmental IMI-causative pathogen with an increasing prevalence in dairy herds locally in South Africa and globally. This is because of changes in the microbiomes and the ever-growing number of virulence factors, which lead to the concern of this evolving into a host-adaptive pathogen. More needs to be learnt about this pathogen locally in South Africa (identifying the current strains, identifying existing or new molecular characteristics that could be associated with resistance or virulence factors associated with IMI, and the treatment efficacy in the encounter of increasing cost factors due to poor economic performance and challenges putting pressure on the dairy industry) and globally.

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CHAPTER 3: MOLECULAR CHARACTERISATION OF *STREPTOCOCCUS UBERIS* STRAINS, ISOLATED IN A LONGITUDINAL STUDY FROM THE MILK OF A COMMERCIAL SOUTH AFRICAN DAIRY HERD

Abstract

Streptococcus uberis is a significant emerging mastitis pathogen increasing in South Africa, particularly in pasture-based herds irrigating with slurry. This study aimed to identify strain types of *Strep. uberis* isolates and to evaluate their behavioural patterns over an extended period (2021 and 2024) from a South African dairy herd with a high prevalence of *Strep. uberis* intramammary infection (IMI).

A retrospective, longitudinal study was performed on *Strep. uberis* isolated from milk samples of a large commercial dairy herd in South Africa using MLST (seven housekeeping genes) for strain type identification.

The study indicated a herd prevalence of 7.44% *Strep uberis, responsible* for 21.26% of all clinical mastitis cases. Of the *Strep. uberis* positive clinical cases, 25% had repeat occurrences during the same lactation. The MLST study identified (64/70) novel *Strep. uberis* strain types owing to novel alleles (29) or sequences of previously identified alleles. The MLST study revealed one existing strain type, *Strep. uberis* ST 1613 (6/70) CC ST-86; all other strain types of the isolates investigated in this study were novel. The repeat cases indicate that consecutive infection was caused by a diverse strain type, which unexpectedly revealed primarily new strains creating new infections, possibly owing to high environmental risk (pathogen overload). This agreed with additional research, identifying the environment as the primary source of strains. This information will correct the assumption that *Strep. uberis* causes chronic infections while enabling veterinarians and farmers to manage *Strep. uberis* mastitis effectively.

Keywords: *Streptococcus uberis*, molecular characterisation, slurry, bovine mastitis.

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3.1 Introduction

Mastitis is a disease with the most significant economic effect on the dairy sector in first-world countries (Blosser, 1979; Azooz *et al.*, 2020). Owing to the recent increase in socioeconomic pressures from the “green” shift (climate change and prudent use of antibiotics) (Van Werven, 2018), the dairy sector is being scrutinised and restricted by government policies and public perception (Hayman *et al.*, 2023).

These pressures and additional economic constraints (ever-increasing costs of feed, fertilisers, and fuels) have forced farmers to adopt alternative farming practices to reduce costs while aiming to meet the increased demands of modern-day dairy animals (high-energy feed, reproduction performance, and heat stress) (Neculai-Valeanu & Ariton, 2022; Youngerman, 2004). Such cows are expected to produce larger volumes of milk consumers need, such as growing populations and nutritional changes within populations. This increases the risk of dairy animals suffering from clinical and subclinical mastitis.

One such change in farming and management practices involves shortening the time or completely stopping the composting process for manure-based bedding in TMR systems and pasture fertilisation with untreated slurry (Jeppsson *et al.*, 2024). This has increased the risk of IMI owing to higher pathogen loads (Sherwin *et al.*, 2021) and dirtier dairy animals (poorer hygiene scores). The growing herd sizes and higher stocking densities have further contributed to increased stressors and discomfort (Lactodata, 2023). Finally, there could be a change in the biome of the udder, with dysbiosis changing the challenges from environmental and contagious pathogens, such as *Strep. uberis* (Blignaut *et al.*, 2018).

Mastitis is a multifactorial disease with multiple concerns for animal welfare (Schellack, 2017; Berry & Hillerton, 2022), environmental effects, food safety, and sustainability (Essack, 2018). Farmers and veterinarians need to implement, review, and manage efficient and effective practices to develop and supply a safe, sound, and wholesome product (milk) to the public while protecting their herds from the increased risk factors associated with IMIs. These factors include breaks in parlour practices and hygiene, higher exposure to and contamination of environmental hot spots (Crompton *et al.*,

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2007; Reyes *et al*., 2019), and poor teat end condition and udder health (Van Lelyveld, 2020; Van Lelyveld, 2022d).

Global IMI trends have revealed a shift from non-aureus staphylococci and *Staph. aureus* pathogens to *Strep uberis*-induced IMI (Petzer *et al*., 2009). Local South African IMI data has followed this trend, with the presence of *Strep. uberis* increasing by 10% from 2016 to 2022 (Karzis & Petzer, unpublished). Twenty-five per cent of cows harbour *Strep. uberis* in their faeces, and 50% harbour the bacteria on their skin. There are higher risks of environmental contamination with environmental and host-adaptive *Strep. uberis* strains (McDougall *et al*., 2004; Tomita *et al*., 2008; Sampimon *et al*., 2009; Reyes *et al*., 2019; Srithanasuwan *et al*., 2022; Zadoks *et al*., 2001; Zadoks *et al*., 2007).

Some reasons could be stocking density and cutting corners in standard practices of the ten-point mastitis management plan (Petzer *et al*., 2009). This can change from proactive to reactive farming. Several practices promote the selection for virulence and resistance within the *Strep. uberis* pathogens (Hoque *et al*., 2020; Zhang *et al*., 2020). Drug resistance (Zhang *et al*., 2020) in *Strep. uberis* species has been observed globally: in South America (Gianneechini *et al*., 2002), New Zealand (McDougall *et al*., 2020; Petrovski *et al*., 2011), Canada (Cameron *et al*., 2016), and North Africa (Saed & Ibrahim, 2020). Successful treatment of *Strep uberis*, an intracellular bacterium in udder parenchyma, is complex. This is owing to virulence properties, such as *SUAM* (Almeida *et al*., 2006), expression of biofilms (Magagula, 2023; Krömker, 2014), and its antibiotic resistance genes (Reyes *et al*., 2019; Srithanasuwan *et al*., 2022).

By identifying current or new strains using molecular characterisation in farms with known IMIs, we can improve our understanding of the dynamic evolution of the biomes (udder, bedding, pasture) and emerging causative agents that *Strep. uberis* has become (Lopez-Benavides *et al*., 2007). With this pathogen having host-adaptive and environmental properties (Jeppsson *et al*., 2024), it is ever more critical to correctly identify the pathogen to the strain level and know the virulence profiles.

The process of investigating the genomic characterisation to strain type level for South Africa using multilocus-sequence typing (MLST) (7-gene) (Butte *et al*., 2001) will form

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the basis for further studies developing the entire genome or identification of virulence factors within the *Strep. uberis* species–lacking in South Africa. This will become a future strategy for udder health management programmes.

The study aimed to provide patterns of prevalence, probable chronic IMI, and clinical mastitis cases caused by *Strep uberis,* identify the South African strains in one sizeable commercial herd, and compare the genomic data to the existing database (PubMLST Genbank). Through a longitudinal study of milk samples, it is possible to study the behaviours of *Strep. uberis* IMI to predict whether the strains are host-adaptive or environmental. This is an essential application for herds with practices that may favour or inhibit the incidence, chronicity, and severity of *Strep. uberis* IMI. Veterinarians and farmers will be better able to understand and evaluate the risk versus reward scenarios for diverse farming practices, such as green manure bedding and untreated slurry pasture fertilisation to improve dairy herd management in South Africa in the future.

3.2 Methodology

3.2.1 Experimental design

A retrospective, longitudinal study performed on *Strep. uberis* isolated from milk samples from a large commercial dairy herd in South Africa.

3.2.2 Herd selection

The herd selected for the study is a Jersey herd with nearly 1,000 cows in milk that calves year-round. It is a closed herd located in the Eastern Cape, one of South Africa's three major dairy areas. The herd has been using untreated slurry to fertilise its pastures for approximately five years. Before that, it had been using dry manure on its pastures for more than 20 years.

The management of the herd is significant (biosecurity, genetic selection, and nutrition), recordkeeping (Delpro, DeLaval programme), parlour hygiene, and milking machine maintenance of the herd are good, and there have been no changes in

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management over the years and during this study. The herd was known to have a prevalence of *Strep. uberis* IMIs of 7.0% to 9.7% at the time of the study. The herd has participated in a three-monthly milk herd sampling of all lactating cows for more than 15 years. Milk samples of all clinical mastitis cases are frozen and sent monthly for analysis by a milk laboratory.

3.2.3 Historical data from the herd

Composite cow milk samples were collected from each lactating cow in this herd every three months as a proactive udder health management system for the past 15 years. Microbiology and cytology analyses were conducted on all the samples. Milk samples were taken from all clinical mastitis cases diagnosed and analysed monthly for microbiology for the same period. These results were analysed from January 2021 until December 2021 for individual dairy cows for this study (

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Table 3.1 and Table 3.2).

3.2.4 Samples

3.2.4.1 Sampling method

The milk samples were collected aseptically. Cleaning, drying, and disinfecting the teats from furthest to nearest, stripping the fore milk, and collecting the quarter milk and cow milk samples from nearest to furthest teat. The milk samples were stored on ice and transported to the Milk Laboratory, Faculty of Veterinary Science, Onderstepoort (SANAS-accredited, DALRRD and South African Veterinary Council (SAVC) approved) within 48 hours post sampling for microbiology and cytology analysis. Quarter milk samples were collected from all clinical mastitis cases, frozen on the farm, and sent to the milk laboratory monthly.

3.2.4.2 Sample selection

Streptococcus uberis isolates from two routine herd examinations performed during July and October 2021 and the clinical mastitis cases caused by *Strep. uberis* during the same period were frozen. The bacteria examined in this study comprised 70 *Strep. uberis* isolates from 53 cows. The 70 isolates were isolated once from 48 cows, twice from 12 cows, three times from one cow, and four times from one cow.

Of these, 63 *Strep. uberis* isolates were selected from two consecutive routine herd tests (low SCC animals) and clinical mastitis cases during July (33 isolates) and October 2021 (30 isolates). The selection was based on available isolates frozen in preparation for future studies. It was selected based on positive confirmation with a MALDI-TOF using a score exceeding 1.9 and antibiograms (Kirby Bauer disc method), which lowered the number from 143 samples.

Seven *Strep. uberis* isolates were added during 2024, originating from the February and May 2024 routine herd tests and isolates from clinical mastitis cases from June 2024. The authors specifically selected *Strep. uberis* isolates during 2024 (n=7) isolated more than once from the same cow to increase the samples for the

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longitudinal investigation. The 2024 isolates were overseen similarly to the initial isolates.

The sample size (n=70) was checked for statistical relevance using OpenEpi, which indicated that the minimum sample size required was 48 to 68.

3.2.5 Microbiology and cytology

3.2.5.1 Identifying Streptococcus uberis

Initial bacterial identification was conducted phenotypically using traditional microbiological methods. These methods included assessing colony morphology, haemolysis, pigmentation, catalase, staphylase/coagulase, maltose, and potassium hydroxide tests. This was conducted following the guidelines delineated by the NMC, 2017). Somatic cell count (SCC) analysis was performed through fluoro-opto-electronic means using the fossomatic flow cytometry (FC) (Rhine Ruhr, Wendywood, South Africa).

3.2.5.2 Freezing bacterial isolates

The *Strep. uberis* isolates were transferred to cryotubes containing tryptic soy broth (TSB) with 10% glucose. They were then frozen at -80°C and stored in a DALRRD-approved biobank.

3.2.5.3 Verifying bacteria with the MALDI-TOF

The bacteriological identification was verified using Matrix-assisted laser desorption/ionisation—time of flight mass spectrometry (MALDI-TOF) (Bruker Daltronics, Bremen, Germany) at the Department of Plant and Soil Sciences, University of Pretoria, South Africa (Van Dyk *et al*., 2015). *Streptococcus uberis* isolates with a MALDI-TOF score greater than 1.9 were available for the study.

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3.2.5.4 Multilocus-sequence typing

Genetic characterisation was performed by Inqaba Biotec™ (Pretoria, South Africa) on the 70 isolates using MLST. The MLST analysis was performed using well-established methods (Coffey *et al.*, 2006; Jolley *et al.*, 2018) also available in the National Center for Biotechnology Information (NCBI) database, *Strep. uberis* isolates collection (http://pubmlst.org/suberis).

Genomic DNA was extracted from the 70 isolates using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005) according to the manufacturer's instructions (https://files.zymoresearch.com/protocols/_d6005_quick-dna_fungal-bacterial_miniprep_kit.pdf) and according to the study by Coffey *et al.* (2006).

In this study, the MLST target regions comprised the seven housekeeping genes: Glucose kinase (*gki*), Transketolase (*recP*), D-ala-D-ala ligase (*ddl*), Thymidine kinase (*tdk*), Carbamate kinase (*arcC*), Triosephosphate isomerase (*tpi*), and Acetyl CoA acetyl-transferase (*yqiL*), which constituted 3338bp per isolate. The DNA was then amplified and purified for sequencing. The isolates' DNA was prepared using the Exonuclease I and Shrimp Alkaline Phosphatase (ExoSAP) protocol (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050). The integrity of the genomic DNA was checked using the 1% agarose gel (CSL-AG500, Cleaver Scientific Ltd.) stained with EZ-vision® Bluelight DNA dye. The genomic DNA was sequenced in a forward direction using the Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1 (BRD3-100/1000) and the ABI 3730xl Genetic Analyser (Applied Biosystems, Thermo Fisher Scientific).

The method chosen followed other studies where MLST (Reyes *et al.*, 2019; Vezina *et al.*, 2021) and PCR virulence profiles (Monistero *et al.*, 2021; Srithanasuwan *et al.*, 2022) were used to evaluate the behaviour of *Strep. uberis*. Data from the 70 isolates was first used to clarify an IMI's behaviour and better understand the biodynamics and characteristics associated with the diverse strains of *Strep. uberis*. The fast all sequence alignment (FASTA) data of all novel alleles were tested and novel

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combinations were submitted to the PubMLST database, curated, allocated ST numbers and grouped into available CC where possible.

A phylogenetic tree (dendrogram) was drawn to display the relative similarity of the isolates tested. The dendrogram was constructed with CLC Main Workbench 24.0, involving the neighbour joining method. This was generated without an existing tree, using the Hasegawa-Kishino-Yano (HKY) nucleotide and WAG protein substitution models. Other parameters used included: Transition/transversion ratio = 2.0; Gamma distribution parameter = 1.0; Replicates of 100; number of substitution rate categories of four, which included rate of variations, no gamma distribution, all with a bootstrap analysis strategy.

The allocated ST numbers were used with the farm data (cow and longitudinal sequence of IMIs and isolated strains) to track the strain types of *Strep. uberis* over time in the same cow. This made it possible to differentiate between *Strep uberis, responsible for causing chronic udder infections,* and those that only remained in the udder for a short period. This is important as it edifies the bacteriological findings on farm-level data, confirming an IMI as a non-cure, chronic, or new IMI.

3.2.5.5 Method of submission of genes (PubMLST Gene bank)

The MLST tests generated FASTA files summarising the genomic characteristics for the seven housekeeping genes associated with the 70 *Strep. uberis* isolates. The FASTA data was submitted to PubMLST to be curated, and allele numbers for existing and novel alleles and ST numbers for novel MLST combinations of alleles were allocated. This was species-specific and identifiable to the genetic allele level, observing variation in genetic similarity and/or pattern of IMI, which can later be dedicated to virulence factors emphasising behaviour, resistance, and adaptability. The MLST data were submitted according to the stipulations from the PubMLST webpage (https://bigsdb.readthedocs.io/en/latest/submissions.html).

1. All the alleles were checked against the existing database: (individual or batches)

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https://pubmlst.org/bigsdb?db=pubmlst_suberis_seqdef&l=1&page=batchSequenceQuery

2. Novel alleles were submitted to the allele database and allocated new allele numbers: https://pubmlst.org/bigsdb?db=pubmlst_suberis_seqdef&page=submit&alleles=1
3. Once all alleles had allele numbers allocated, the isolates were submitted, https://pubmlst.org/bigsdb?db=pubmlst_suberis_seqdef&page=submit&profiles=1&scheme_id=1

After curation and acceptance of all the isolates by the database curator, the novel MLST sequence-type profiles were submitted for the allocation of ST numbers for each isolate, https://pubmlst.org/bigsdb?db=pubmlst_suberis_isolates&page=submit&isolates=1

This study identified isolates up to strain type level. Characterisation of novel ST-CC was outside the scope of this study.

3.2.6 Statistical analysis

The software CLC Main Workbench 24.0 was used to construct the phylogenetic tree (dendrogram). It employed bootstrap analysis to calculate the relevant similarities among the isolates tested.

3.2.7 Biosecurity and ethics consideration

Ethics approval (REC 040-23) was obtained from the Animal Ethics Committee (AEC) (Appendix 7 and 8) and the Research Ethics Committee (REC) of the University of Pretoria (Appendix 6). An application for the DALRRD approved the research under Section 20 of the Animal Disease Act (ACT NO 35 of 1984) to occur from March 2023 until December 2027 (Appendix 1 and 2). Samples were stored in a DALRRD-approved biobank (Appendix 5).

Laboratory work was performed in a SANAS-accredited (Appendix 3) and DALRRD-approved (Appendix 4) laboratory. Turnitin was used to ensure that the constructed

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report does not infringe any plagiarism rules established by the University of Pretoria and meets the ethical writing requirements for publication.

3.3 Results

3.3.1 Retrospective data: Routine isolates and clinical mastitis cases

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Table 3.1 presents the retrospective laboratory culture results from the study herd, indicating *Streptococcus uberis* (SUB) isolated during four (three-monthly) routine herd examinations and clinical mastitis cases in 2021.

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Table 3.1: Retrospective laboratory culture results of *Streptococcus uberis* (SUB) from routine herd examinations and clinical mastitis cases in 2021

	Jan to March 2021	Apr to Jun 2021	Jul to Sept 2021	Oct to Dec 2021	Averages for 2021
Number of lactating cows tested (n)	1061	988	960	1011	1005
Number and (%) of SUB isolated	78 (7.35%)	80 (8.09%)	71 (7.18%)	72 (7.12%)	75 (7.44%)
Number of SUB isolated from clinical mastitis cases (as a % of clinical cases)	27 (34.61%)	17 (21.25%)	9 (12.67%)	11 (15.27%)	16 (21.26%)
Number and (%) of cows from which SUB was isolated once during 2021	142 cows (47.2% of SUB isolates)				
Number and (%) of cows where SUB was isolated 2x in consecutive tests during 2021	35 cows (23.2% of SUB isolates)				
Number and (%) of cows where SUB was isolated 3x in consecutive tests during 2021	13 cows (13.0% of SUB isolates)				

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	Jan to March 2021	Apr to Jun 2021	Jul to Sept 2021	Oct to Dec 2021	Averages for 2021
Number and (%) of cows where SUB was isolated 4x in consecutive tests during 2021	7 cows (9.3% of SUB isolates)				
Number and (%) of cows where SUB was isolated 2x (not consecutive tests) during 2021	8 cows (5.3% of SUB isolates)				
Number and (%) of cows where SUB was isolated 3x (not consecutive tests) during 2021	2 cows (2.0% of SUB isolates)				

The herd prevalence for *Strep. uberis* was 7.44% (

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Table 3.1), based on data generated from the routine three-monthly herd sampling as part of the udder health herd milk samples collected during 2021. The prevalence for the two sample dates used in this study were 7.18% (June 2024) and 7.12% (October 2024) respectively (

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Table 3.1). Of all the routine milk samples taken for the 2021 period, 207 cows tested positive for *Strep uberis*. Among these cases, the animals with multiple positives (137 cows) were broken down as follows: 35 cows tested positive twice, 13 thrice, and seven four times positive in consecutive test/collection batches. Meanwhile, the cows that tested positive multiple times but not for consecutive sample dates were broken down as follows: eight cows tested positive twice, and two tested positive thrice (

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Table 3.1). Data from the routine sampling from 2021 to 2024 emphasise that several cows were identified to have had multiple cases of *Strep. uberis* IMIs.

The number of *Strep. uberis* clinical mastitis cases varied during 2021, from 34.61% of all clinical mastitis cases from January - March 2021 to 12.67% of clinical cases from July - September of that same year (

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Table 3.1). There was no known reason for January to March having a significantly higher prevalence of clinical mastitis owing to *Strep uberis*. During 2021, there were seven cows, of which *Strep. uberis* was isolated in all four consecutive routine herd examinations (

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Table 3.1).

Table 3.2: Percentage of *Streptococcus uberis* (*Strep. uberis*) isolated during 2021 from clinical mastitis cases per cow

Strep. uberis isolations from clinical mastitis per cow	As a percentage of *Strep. uberis* clinical mastitis cases during 2021 (total n=64)
Strep. uberis isolated once from the same cow	48 (75.00%)
Strep. uberis isolated 2x from the same cow	14 (21.88%)
Strep. uberis isolated 3 or 4x from the same cow	2 (3.12%)

Observations indicate that 25% of clinical *Strep. uberis* mastitis cases repeated during 2021, and of these, 3.12% repeated more than twice (Table 3.2).

3.3.2 Overall multilocus-sequence type (MLST) data.

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Table 3.3: Multilocus-sequence typing (MLST), strain typing (ST) data of the 70 *Streptococcus uberis* isolates, including allele numbers, ST numbers and available clonal complexes (CC)

Isolate ID	Cow ID	*Strep. uberis* isolated from routine sampling (2021 - 2024)	Sample date	Somatic cell count level	*arcC	*ddl	*gki	*recP	*tdk	*tpi	*yqiL	ST number	Clonal complex
ZZ100	990	5 (random with first 3 clustering 3 months apart)	27/07/2021	SC	1	10	3	2	28	4	3	2103	**
ZZ119	1102	3 (random)	27/07/2021	SC	9	10	5	1	2	1	114	2102	**
ZZ86	1216	6 (3 months apart)	27/07/2021	SC	9	1	111	2	7	1	3	2105	**
ZZ73	1228	2 (3 months apart)	27/07/2021	SC	1	10	5	1	17	67	7	2112	**
ZZ99	1234	5 (random with first 3 clustered)	27/07/2021	SC	122	94	109	2	29	4	3	2106	**
ZZ13	1234	5 (random with first 3 clustered)	18/10/2021	SC	122	94	109	2	29	4	3	2106	**
ZZ74	1243	3 (3 months apart)	27/07/2021	SC	1	1	2	2	2	2	3	2138	ST-5
ZZ18	1316	3 (random)	18/10/2021	LSCC	2	10	5	1	17	1	7	2155	
ZZ112	1318	7 (random)	27/07/2021	LSCC	1	2	3	2	2	2	3	2127	Nearest match: 5 loci. ST-86
ZZ19	1318	7 (random)	18/10/2021	SC	1	2	3	2	2	2	3	2127	**
ZZ121	1327	6 (random with last 3 clustered and seasonal)	27/07/2021	SC	1	1	2	1	175	1	3	2096	ST-5
ZZ63	1327	6 (random with last 3 clustered and seasonal)	18/10/2021	CM	1	10	111	2	2	2	111	2113	**
ZZ64	1327	6 (random with last 3 clustered and seasonal)	18/10/2021	CM	9	49	2	1	4	1	3	2137	Nearest match: 5 loci. ST-5
ZZ24	1342	4 (random)	18/10/2021	LSCC	123	2	3	42	5	2	3	2119	**
ZZ29	1489	1	18/10/2021	LSCC	3	2	3	12	28	2	3	2152	ST-86
ZZ33	1520	2 (random)	18/10/2021	SC	3	2	6	2	5	4	3	2136	**
ZZ38	1559	seasonal/random	18/10/2021	SC	1	1	5	1	2	3	3	2148	ST-5

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Table 3.3: Multilocus-sequence typing (MLST), strain typing (ST) data of the 70 *Streptococcus uberis* isolates, including allele numbers, ST numbers and available clonal complexes (CC)

Isolate ID	Cow ID	*Strep. uberis* isolated from routine sampling (2021 - 2024)	Sample date	Somatic cell count level	*arcC	*ddl	*gki	*recP	*tdk	*tpi	*yqiL	ST number	Clonal complex
ZZ88	1640	4 (random)	27/07/2021	SC	1	2	3	2	2	2	3	2127	Nearest match: 5 loci. ST-86
ZZ42	1693	3 (random)	18/10/2021	LSCC	5	2	109	1	173	4	3	2118	**
ZZ44	1714	8 (every 3 months then random)	18/10/2021	LSCC	124	95	6	60	3	1	3	2117	**
11_24/324	1788	4 (random)	20/05/2024	CM	3	1	3	2	5	1	3	2159	ST-86
10_24/301	1788	4 (random)	20/02/2024	CM	1	49	111	60	4	1	6	2123	ST-5
10_24/324	1788	4 (random)	05/06/2024	CM	1	92	6	2	5	4	3	2162	**
ZZ53	1788	4 (random)	18/10/2021	CM	1	49	111	1	2	1	6	2115	ST-5
ZZ69	1861	1	27/07/2021	CM	9	10	5	42	7	3	3	2129	**
ZZ57	1895	1	18/10/2021	LSCC	1	10	2	1	2	3	6	2146	Nearest match: 5 loci. ST-5
ZZ68	1910	1	18/10/2021	CM	1	49	2	2	2	1	3	2142	ST-5
ZZ87	1928	5 (random)	27/07/2021	CM	1	49	5	1	2	1	6	2135	ST-5
ZZ60	1943	1	18/10/2021	LSCC	1	49	2	2	2	1	3	2142	ST-5
ZZ71	1954	3 (different quarters)	27/07/2021	LSCC	1	10	5	1	2	3	7	2124	Nearest match: 5 loci. ST-5
205_24/292	1981	2	20/02/2024	SC	1	49	111	60	4	1	6	2123	**
11_24/301	1981	2	05/06/2024	CM	1	91	2	2	28	4	3	2164	ST-143
ZZ75	8175	1	27/07/2021	SC	9	49	111	2	7	3	7	2111	**
ZZ111	10242	1	27/07/2021	LSCC	56	1	3	2	2	2	3	2128	Nearest match: 6 loci. ST815
ZZ94	11232	2 (3 months apart)	27/07/2021	SC	1	10	2	1	2	3	7	2132	ST-5

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Table 3.3: Multilocus-sequence typing (MLST), strain typing (ST) data of the 70 *Streptococcus uberis* isolates, including allele numbers, ST numbers and available clonal complexes (CC)

Isolate ID	Cow ID	*Strep. uberis* isolated from routine sampling (2021 - 2024)	Sample date	Somatic cell count level	*arcC	*ddl	*gki	*recP	*tdk	*tpi	*yqiL	ST number	Clonal complex
ZZ2	11232	2 (3 months apart)	18/10/2021	SC	1	10	2	1	2	3	7	2132	ST-5
ZZ117	12179	1	27/07/2021	SC	9	10	5	1	2	1	114	2102	**
ZZ120	11251	3 (every 3 months)	27/07/2021	SC	3	2	3	3	5	4	3	1613	ST-86
ZZ3	11251	3 (every 3 months)	18/10/2021	SC	3	2	3	3	5	4	3	1613	ST-86
ZZ9	12236	1	18/10/2021	SC	1	49	108	1	7	67	3	2122	**
ZZ10	12264	2 (3 months apart)	18/10/2021	SC	56	1	3	2	2	2	3	2128	**
ZZ83	12264	2 (3 months apart)	27/07/2021	SC	1	10	111	1	2	2	3	2108	**
ZZ92	13117	3 (every 3 months)	27/07/2021	SC	3	2	3	3	5	4	3	1613	ST-86
ZZ113	13204	4 (every 3 months)	27/07/2021	LSCC	9	1	111	2	7	1	3	2105	**
ZZ20	13204	4 (every 3 months)	18/10/2021	SC	9	1	111	2	7	1	3	2105	**
ZZ21	13249	2 (random)	18/10/2021	SC	5	49	3	2	17	3	3	2147	**
ZZ118	13294	1	27/07/2021	LSCC	9	10	5	1	2	1	114	2102	**
ZZ125	14226	3 (every 3 months)	27/07/2021	LSCC	1	10	5	1	2	3	7	2124	Nearest match: 5 loci. ST-5
ZZ76	15218	1	27/07/2021	SC	9	49	2	1	4	1	3	2137	Nearest match: 5 loci. ST-5
ZZ77	15310	4 (every 3 months)	27/07/2021	SC	3	2	6	2	5	4	3	2136	**
ZZ35	15310	4 (every 3 months)	18/10/2021	SC	1	4	3	12	13	6	3	2150	**
ZZ34	19392	1	18/10/2021	LSCC	1	4	3	12	13	6	3	2150	**
ZZ90	15302	4 (3 month and then random)	27/07/2021	SC	1	4	3	12	13	6	3	2150	**
ZZ37	15353	1	18/10/2021	SC	3	2	3	3	5	4	3	1613	ST-86
ZZ66	16195	1	18/10/2021	CM	1	10	111	2	2	2	111	2113	**

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Isolate ID	Cow ID	*Strep. uberis* isolated from routine sampling (2021 - 2024)	Sample date	Somatic cell count level	*arcC	*ddl	*gki	*recP	*tdk	*tpi	*yqiL	ST number	Clonal complex
ZZ70	16410	1	27/07/2021	LSCC	1	10	2	1	2	3	7	2132	ST-5
ZZ45	17144	2 (random)	18/10/2021	SC	5	1	5	2	174	1	7	2116	**
ZZ79	17151	3 (random)	27/07/2021	SC	123	1	3	2	5	4	3	2110	ST-143
ZZ14	17151	3 (random)	18/10/2021	SC	1	1	2	2	2	2	3	2138	ST-5
ZZ101	17266	5	27/07/2021	SC	9	10	5	42	7	3	3	2129	**
ZZ107	17175	2 (3 months apart)	27/07/2021	SC	9	10	5	42	7	3	3	2129	**
ZZ47	17175	2 (3months apart)	18/10/2021	SC	5	49	3	2	17	3	3	2147	**
ZZ116	17255	1	27/07/2021	SC	4	49	2	2	2	2	3	2126	Nearest match: 6 loci. ST-143
369_24/292	17280	3 (random)	20/02/2024	SC	1	10	5	2	28	2	7	2160	**
5_24/301	17280	3 (random)	28/05/2024	CM	1	10	5	2	28	2	7	2160	**
ZZ81	17324	3 (every 3 months)	27/07/2021	LSCC	3	2	3	4	5	1	113	2109	ST-86
ZZ124	17352	4 (every 3 months)	27/07/2021	LSCC	3	2	3	3	5	4	3	1613	ST-86
ZZ50	17352	4 (every 3 months)	18/10/2021	LSCC	3	2	3	3	5	4	3	1613	ST-86
ZZ123	18136	3 (every 3 months)	27/07/2021	SC	9	10	5	2	17	1	3	2125	Nearest match: 5 loci. ST-143
ZZ59	19103	2 (random)	18/10/2021	SC	1	10	3	2	13	3	3	2145	**

Cluster:3-month max interval; random: different years or timing intervals irregular; SC: Subclinical mastitis cases; C: clinical mastitis cases; low somatic cell count (LSCC): cases (< 300 000cells/ml milk)

* arcC, ddl, gki, recP, tdk, tpi, ygit are the seven housekeeping genes investigated

** Clonal complexes were not available on PubMLST for all ST numbers

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Key for colour codes:

Colour	Description
Orange	Different ST strains from the same cow
Dark blue	Similar ST strains from the same cow
Light blue	Isolates during the year 2024
Yellow	Novel allele numbers
Purple	Existing ST number in PubMLST database
Light grey	Clonal complex ST-5
Grey	Clonal complex ST-86
Green	Clonal complex ST-143

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369_24/292_ST2160_02/2024
5_24/301_ST2160_05/2024
11_24/301_ST2164_06/2024
ZZ100_ST2103_07/2021
ZZ90_ST2150_07/2021
ZZ35_ST2150_10/2021
ZZ34_ST2150_10/2021
ZZ29_ST2152_10/2021
ZZ59_ST2145_10/2021
11_24/324_ST2159_05/2024
ZZ81_ST2109_07/2021
ZZ124_ST1613_07/2021
ZZ120_ST1613_07/2021
ZZ92_ST1613_07/2021
ZZ50_ST1613_10/2021
ZZ37_ST1613_10/2021
ZZ3_ST1613_10/2021
ZZ24_ST2119_10/2021
ZZ79_ST2110_07/2021
ZZ77_ST2136_07/2021
ZZ33_ST2136_10/2021
10_24/324_ST2162_06/2024
ZZ44_ST2117_10/2021
ZZ99_ST2106_07/2021
ZZ13_ST2106_10/2021
ZZ42_ST2118_10/2021
ZZ73_ST2112_07/2021
ZZ18_ST2155_10/2021
ZZ123_ST2125_07/2021
ZZ47_ST2147_10/2021
ZZ21_ST2147_10/2021
ZZ121_ST2096_07/2021
205_24/292_ST2123_02/2024
10_24/301_ST2161_02/2024
ZZ53_ST2115_10/2021
ZZ9_ST2122_10/2021
ZZ68_ST2142_10/2021
ZZ60_ST2142_10/2021
ZZ87_ST2135_07/2021
ZZ75_ST2111_07/2021
ZZ113_ST2105_07/2021
ZZ86_ST2105_07/2021
ZZ20_ST2105_10/2021
ZZ107_ST2129_07/2021
ZZ101_ST2129_07/2021
ZZ69_ST2129_07/2021
ZZ119_ST2102_07/2021
ZZ118_ST2102_07/2021
ZZ117_ST2102_07/2021
ZZ76_ST2137_07/2021
ZZ64_ST2137_10/2021
ZZ116_ST2126_07/2021
ZZ66_ST2113_10/2021
ZZ63_ST2113_10/2021
ZZ83_ST2108_07/2021
ZZ74_ST2138_07/2021
ZZ14_ST2138_10/2021
ZZ112_ST2127_07/2021
ZZ88_ST2127_07/2021
ZZ19_ST2127_10/2021
ZZ111_ST2128_07/2021
ZZ10_ST2128_10/2021
ZZ94_ST2132_07/2021
ZZ70_ST2132_07/2021
ZZ2_ST2132_10/2021
ZZ71_ST2124_07/2021
ZZ125_ST2124_07/2021
ZZ57_ST2146_10/2021
ZZ45_ST2116_10/2021
ZZ38_ST2148_10/2021

0,002

Figure 3.1: Dendrogram of MLST data of all *Streptococcus uberis* isolates tested (n= 70)

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Multilocus-sequence typing (MLST) was performed on 70 *Strep. uberis* isolates. Of these isolates, 12 originated from clinical mastitis cases, 40 from subclinical mastitis, and 18 from milk with low SCC (<300,000 cells/ml milk); 42 *Strep. uberis* strain types (ST) were identified (Table 3.3). Of these, 41 were novel strains (including combinations of novel and existing alleles) isolated from 64 of the 70 samples (Table 3.3 and

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Figure 3.1). Only one strain, ST 1613, already existed in the PubMLST data bank (previously isolated from bovine milk in Australia). The six ST 1613 isolates were isolated once from four cows and twice from two cows (Table 3.3 and

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Figure 3.1). All four of these cows had subclinical mastitis or low SCC. No set patterns were identified in the 64 isolates from which novel strains were isolated (Table 3.3).

All seven isolates from 2024 were only isolated during 2024 and not in 2021. Two strains were isolated twice: ST 2160 from the same cow and ST 2,123 from different cows (Table 3.3 and

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Figure 3.1). Of the 14 cows with multiple isolations of *Strep. uberis*, 12 had the bacterium isolated twice. Among these, seven cows had similar strains, while five had different strains isolated on the two sampling occasions. Both cows with three and four *Strep. uberis* isolations had different strains isolated at each sampling (Table 3.3).

3.3.1.1 Multilocus-sequence type (MLST) data for cows from which Streptococcus uberis were isolated on consecutive samplings

Table 3.4: Summary of cows from which *Streptococcus uberis* were isolated on two or more consecutive samplings (31 isolates from 14 cows)

Cow identification	Quantity and (dates) of isolations	*PubMLST ST numbers	Clonal complex	Number of isolated from clinical mastitis	Number of isolated form subclinical mastitis
1234	5 (Jul21 & Oct21)	2106	**	0	2
1318	7 (Jul21 & Oct21)	2122127	**	0	2
1327	6 (Jul 21, Oct21 & Oct^)	2096, 2113 & 2137	ST-5CC	2	1

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Cow identification	Quantity and (dates) of isolations	*PubMLST ST numbers	Clonal complex	Number of isolated from clinical mastitis	Number of isolated form subclinical mastitis
1788	4 (Oct21, Feb24, May24 & June24)	2115, 2123, 2159 & 2162	ST-5CC & ST-86	3	1
1981	2 (Feb24 & May24)	2123 & 2164	ST-143	1	1
11232	2 (Jul21 & Oct21)	2132	ST-5	0	2
11251	2 (Jul21 & Oct21)	1613	ST-83	0	2
12264	2 (Jul21 & Oct21)	2128 & 2198	**	0	2
13204	4 (Jul21 & Oct21)	2105	**	0	2
15310	4 (Jul21 and Oct21)	2136 & 2150	**	0	2
17151	3 (Jul21 and Oct21)	2110 & 2138	ST-143 & ST-5	0	2
17175	2 (Jul21 and Oct21)	2129 & 2147	**	0	2
17280	3 (Feb24 and May24)	2160	**	1	1
17352	4 (Jul21 and Oct21)	1613	ST-86	0	2

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*PubMLST data (*Streptococcus uberis* isolates (pubmlst.org)), ** Clonal complexes were not provided by PubMLST for all ST numbers.

There were 14 cows with *Strep. uberis* isolated on two 3-monthly consecutive tests. Of the 14 cows recorded as having consecutive positive isolates for *Strep uberis,* two cows (cow 11251 and cow 17352) had a pre-existing ST - 1613 and ST-86 CC. Of these 14 cows, seven had a different strain identified in the second test, indicating new IMI, while seven had the same strain that indicated a possible chronic IMI. The strains similar and isolated on consecutive tests from the same cow were ST- 2106 (cow 1234); ST- 2127 (cow 1318), ST – 2132 (cow 11232), ST – 2105 (cow 13204), ST-1613 (cow 11251), ST-1613 (cow 17352) and ST-2160 (cow 17280). Only one of these cows had clinical mastitis, namely cow 17352, with the first isolation of the existing ST - 1613 strain. From four of these cows, *Strep. uberis* was isolated more than twice during 2021 in the routine tests (Table 3.3 and Table 3.4). One of the most typical novel *Strep. uberis* strains isolated from the herd was ST 2129, isolated 3 times from three different cows. Novel ST strains 2102, 2105, 2127, 2132, and 2150 were isolated 3 times, Novel ST strains 2106, 2113, 2124, 2128, 2136, 2137, 2138, 2142, 2147, and 2160 twice, while the remaining 24 strains were isolated only once (Table 3.3 and

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Figure 3.1).

3.3.1.2 Multilocus-sequence type (MLST) data on identifying novel alleles

The study identified new alleles for the seven housekeeping genes. There were three novel alleles for *arcC* (122, 123, and 124). Of these, *arcC* 122 repeated in the same cow once, and *arcC* 123 occurred once in two separate cows (Table 3.3). There were two novel alleles (94 and 95) for *ddl*, with *ddl* 94 repeating in the same cow once (Table 3.3). There were two novel alleles (109 and 111) for *gki*, with *gki* 109 occurring in two cows and repeating in the same cow once and *gki* 111 occurring in eight cows and repeating in two cows once (Table 3.3). There was one novel allele (60) for *recP*, with *recP* 60 occurring in three separate cows (Table 3.3). There were three new alleles (173, 174, and 175) for *tdk*, each occurring once in a distinct cow (Table 3.3). There were no new alleles for *tpi* (Table 3.3). There were three new alleles (111, 113, and 114) for *yqiL*, with *yqiL* 111 occurring once in two cows, *yqiL* 113 occurring in one cow, and *yqiL* 114 occurring in three cows (Table 3.3). Some of these alleles had a one-base difference, with the maximum number of differences being 17 base differences (Table 3.3).

3.3.1.3 Multilocus-sequence type (MLST) data and existing clonal complexes

The herd had three common CCs: ST-5 CC (12/70 isolates), ST-86 CC (9/70 isolates), and ST-143 CC (2/70 isolates) (Table 3.3). As part of the preliminary research, some isolates were identified as similar to an existing CC but have not been confirmed by the PubMLST database.

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Whole genome sequencing is necessary to identify the CC level and confirm the suspicions (isolates ZZ116 and ZZ123 as ST-143, isolates ZZ112 and ZZ88 as ST-86, and isolates ZZ64, ZZ57, ZZ71, ZZ125, and ZZ76 as ST-5) (Table 3.3, Table 3.4, and

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Figure 3.1).

3.3.2 Multilocus-sequence type (MLST) database global comparison

Table 3.5 signifies global multilocus-sequence typing (MLST) data patterns, comparing available results from South Africa to other countries (China, Canada, United Kingdom, and New Zealand).

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Table 3.5: Global multilocus-sequence typing (MLST) data comparison of *Streptococcus uberis* from South Africa, China, Canada, UK, and New Zealand

Country	*ST- clonal complex									No. of isolates tested (% isolates from the 3 ST-clonal Complexes in the table)
	ST-5			ST-86			ST-143			
	No.	Clinical mastitis	Subclinical Mastitis	No.	Clinical mastitis	Subclinical mastitis	No.	Clinical mastitis	Subclinical mastitis	
New Zealand	15	0%	100%	23	13.04%	86.96%	113	10.61%	89.39%	266 (56.77%)
United Kingdom	671	N/A	N/A	18	N/A	N/A	16	N/A	N/A	1166 (60.46%)

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Country	*ST- clonal complex									No. of isolates tested (% isolates from the 3 ST-clonal Complexes in the table)
	ST-5			ST-86			ST-143			
	No.	Clinical mastitis	Subclinical Mastitis	No.	Clinical mastitis	Subclinical mastitis	No.	Clinical mastitis	Subclinical mastitis	
China	15	67%	33%	1	100%	0%	2	50%	50%	81 (22.22%)
Canada	4	0%	100%	4	0%	100%	5	0%	100%	63 (20.65%)
South Africa	11	9.09%% 1	90.9% 10	9	11.11% 1	88.89% 8	2	50% 1	50% 1	70 (37.14%)

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*PubMLST data (*Streptococcus uberis* isolates (pubmlst.org)).

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In Table 3.5, the results of the multilocus-sequence typing (MLST) from the *Strep. uberis* isolated from the South African herd are compared to those of other large dairy-producing countries (New Zealand, China, Canada, and the United Kingdom). Of the samples tested, most belonged to the three CC ST-5, ST-143, and ST-86 in New Zealand (56.77%) and the United Kingdom (60.46%), while the isolates tested from China, Canada, and South Africa accounted for 22.22%, 20.65%, and 37.14% respectively.

3.4 Discussion

3.4.1 General

Mastitis is a costly multifactorial disease that affects the animal's health and well-being, the farm's profitability and sustainability, and the quality of the desired product (food safety and decreased processing to develop higher value products) (Blosser, 1979; Azooz *et al*., 2020). Mastitis is caused by pathogens when there is an imbalance favouring higher contamination, udder damage, and deficient parlour hygiene (Blignaut *et al*., 2018; Phuektes *et al*., 2001). Knowing the causative pathogen aids in managing and reducing the number, severity, and chronicity of mastitis cases.

Streptococcus uberis is a pathogen identified as a growing concern in the dairy sector in multiple countries (Petzer *et al*., 2009). This is important to understand and identify, as perception and factual data (seven housekeeping genes and virulence genes) can be used to manage mastitis better. In South Africa, this data for *Strep. uberis* was missing but had been recorded for other pathogens. The reason was most likely being a combination of the pathogen numbers only recently starting to increase, making this an emerging mastitis pathogen, and the level of diagnostics and laboratory tests (MLST, PCR, and MALDI-TOF) (Jolley *et al*., 2018; Wang *et al*., 2013; Werner *et al*., 2018) being costly. In other countries, such practices have been conducted for several years,; therefore, more research is available on this mastitogenic organism (Jolley *et al*., 2018; Zhang *et al*., 2020; Srithanasuwan *et al*., 2022). Another contributing factor to the continued problem with mastitis has been the limited focus on udder health in practice, as several veterinarians are predominantly focused on reproduction.

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3.4.2 Retrospective data

A previous South African study by Blignaut *et al*. (2018) found *Strep. uberis* IMI to vary from 2.36% to 3.10% in TMR systems and 2.63% to 3.64% in pasture-based systems before the initiation of slurry use on pastures. These figures have grown to 8.03% (Karzis *et al*., 2024 unpublished) and have been supported by the results from

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Table 3.1, where the percentage of IMI cases caused by *Strep. uberis* varied between 7.12% and 8.09% during 2021. The number of isolates in milk from clinical mastitis cases varied from 12.67% to 34.61% during the collection period, with an average of 21.6% of *Strep. uberis* isolates causing clinical mastitis (Table 3.2). These variations could be attributed to seasonal influences of heat stress and higher density of calving down but could not be set to a particular cause or influence; however, these levels recorded agree with the environmental origin theory.

3.4.3 Overall multilocus-sequence type (MLST) data

Cows with more than two *Strep. uberis* IMI did not reveal the same ST number, possibly correlating with an environmental source where the pathogen population had high biodiversity. Twenty-four of the STs had only been isolated once, further strengthening the likelihood of being of environmental origin. This is further compelled by the increasing number (>59.3%) of *Strep. uberis* strains identified in a Chinese study, which indicated strains carry between four to seven virulence-related genes (Zhang *et al*., 2020).

Table 3.3, Table 3.4, and

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Figure 3.1 emphasise the host-adaptive *Strep. uberis* strains versus environmental *Strep. uberis* strains and origin focal points. The herd's infection pattern generally followed that of an environmental origin, with several novel STs being isolated. Sixty-four of the 70 isolates, including repeat cases, belonged to 41 ST numbers, while there was one pre-existing ST number (ST 1613) (6/70 isolates, including repeat cases) (Table 3.3).

Half of the cows where *Strep. uberis* was isolated on more than one occasion did not remain in the udder parenchyma for longer than six months (Table 3.4), indicating that some of these infections, although chronic, are not lifelong. The study results indicated that the heterogeneity of the ST numbers and the divide of the CC were essential in understanding the biodynamics and diversity in the herd. An interesting observation was the similarities and differences among the isolates of the South African herd and different countries (Table 3.5).

Generally, the CC pattern/distribution available in this study was similar to but not the same as that of other countries (Table 3.5). The ST-5 CC was prominent in the United Kingdom, China, and South Africa. In New Zealand, the ST-143 CC was the most

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dominant and ST-5 CC the least. This is of interest, as several pasture-based dairy farms in South Africa copy dairy practices from farms in New Zealand (seasonal milking, pasture with untreated slurry fertilisation, cross-breed farming practices) (Theron & Mostert, 2010) and low input, high output practices. Both could be attributed to the climates, stocking density of camps/pastures (a focal point of infection if environmental or host-adaptive) and breed. In this study, Jersey cows were used versus predominantly cross-breeds in New Zealand (Theron & Mostert, 2010).

3.4.4 Multilocus-sequence type (MLST) data and existing clonal complexes

3.4.4.1 Differences among ST-CC for Strep uberis

Differences among the ST-CC for *Strep. uberis* have been reported in other studies (Reyes *et al.*, 2019) to be associated with pathogenicity potential and varying infectious behaviours and patterns. The ST-5 CC was reported to cause clinical cases (Tomita *et al.*, 2008), and in this study, the pattern did not follow what was expected, with one out of 11 being clinical mastitis cases (Table 3.3, Table 3.4, and

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Figure 3.1). The ST-86 CC and ST-143 CC were responsible for most clinical mastitis cases in New Zealand, while there was a more even distribution among all three CCs in China and South Africa (Table 3.5).

Strain type 1613 (ST-86 CC) was isolated in South Africa (six isolates) and Australia (Table 3.5). The ST-86 CC was reported to cause subclinical cases (Tomita *et al.*, 2008), and in this study, the pattern followed what was expected, with eight subclinical cases and one clinical mastitis case. The ST-143 CC was reported to induce latent infections with some host-adaptive traits (Tomita *et al.*, 2008), which disagreed with the results from this study, where the pattern was neutral with one subclinical case and one clinical mastitis case (Table 3.3, Table 3.4, and Table 3.5). These variations could depend on the selection criteria of the samples, but in this study, the selection was from a mixed grouping of animals during routine and clinical IMI samplings and could be considered a true representative of the CC split.

3.4.5 Multilocus-sequence type (MLST) data for cows from which *Streptococcus uberis* were isolated on consecutive samplings

According to Srithanasuwan *et al.* (2022), 66% of *Strep. uberis* IMI remains in the udder parenchyma for less than 30 days, while 18% could become chronic, lasting

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more than 100 days. This generates the question of why some *Strep. uberis* induce a chronic IMI, and others do not. Is this owing to the microbiome change or the ever-growing number of virulence factors, which lead to the concern of this evolving into a host-adaptive pathogen? The time interval of sampling in the current study was 85 days and did, therefore, not exceed 100 days which could confirm the chronicity of an IMI. Table 3.4 evaluates the 14 cows with consecutive *Strep. uberis* IMI at the two sampling dates. From seven of these 14 cows, the same ST strain was isolated from the same cow at the second sampling (Table 3.4), lowering the likelihood of host-adaptive *Strep. uberis* strains being the primary source of the IMI.

The most common isolated *Strep. uberis* strain, ST-1613, was the only one of 42 strains isolated in this South African study that already existed in the PubMLST database (October 2024), while 41 were novel strains, further indicating the biodiversity and unique strains (Table 3.3 and

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Figure 3.1). The ST 1613 strain was previously isolated from Australia in 2021 (Table 3.5). Six ST-1613 strains were isolated from four different cows; two were isolated from the same cow at both samplings, while two were isolated once only from different cows (Table 3.3 and Table 3.4). The ST-1613 was identified as belonging to the ST-86 CC, and none of the cows infected with this strain had clinical mastitis (Table 3.3).

One of the second most common novel *Strep. uberis* ST isolated from the herd was the ST 2129 strain (Table 3.3 and

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Figure 3.1). This strain was isolated only during the first test in July 2021 and did not follow a set clinical or subclinical pattern. This could raise the question of whether it is a point infection. The fact that we did not isolate this strain again at the follow-up test in October of the same year lowers the likelihood that it was a host-adaptive strain and instead points in the direction of an environmental source of infection with lower virulence, as it could not establish itself.

The *Strep. uberis* isolates from the study were dominated by environmental strains (heterogeneity), favouring the source having a higher pathogen load, which could account for the higher rates of new strains being identified. This could be achieved through mutation or sharing genes (such as virulence factors) (Zhu *et al*., 2023). This was beyond the scope of this study but would be of interest, particularly to compare with other countries in future work. Another point emphasised by the high level of heterogeneity was a rapid eradication of the IMI, possibly attributed to rapid identification, treatment, or self-cure owing to low-level infection and significant immune response from the animal.

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Except for seven cows with consecutive samples harbouring the same strain: 1,234 (two subclinical), 1,318 (two subclinical), 11,232 (two subclinical), 13204 (two subclinical), 17,280 (one clinical and one subclinical), and all were novel strains (Table 3.4). Except for cows 11,251 (two subclinical) and 17352 (two subclinical), both being pre-existing strains (ST 1613 and CC ST-86 CC) (Table 3.5). The ST and CC homogeneity of the two cows, 11,251 and 17,352 (generally at a three-month interval), could allude to a possible contagious transmission and host-adaptive *Strep. uberis* strain (ST and CC if available). Strain type 1613 was also isolated six times in South Africa and once in Australia (subclinical: ST-86 CC) in 2021 (PubMLST database) (Table 3.3, Table 3.4, and Table 3.5).

This is interesting as it has been involved in only subclinical mastitis cases, raising the question of its ability to avoid the immune system of the cow and whether this could be a host-adaptive strain. Whereas, cows 1,327 and 1,788, which have had several identifications of *Strep. uberis* (in this study identified to be different strains for all isolates, Table 3.3 and Table 3.4) over the four-year period (2021 to 2024), could at the surface appear to be chronic IMI cows, but rather the results indicated new infections as opposed to chronic infections. This could be an aspect of value to emphasise the importance of MLST and diagnosis of strain types for the farmer and the veterinarian to manage mastitis in dairy herds better.

3.4.6 Multilocus-sequence type (MLST) data summary

The dendrogram showing the genetic relatedness of the 70 South African *Strep. uberis* isolates confirms the heterogeneity of the strains. The results suggest a grouping of cases with the same strain during the same period rather than being the same strain as a repeat in the same cow. The distance between the different strains in the consecutive infections in the same cow tends to be related, indicating randomised infections from an environmental population (

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Figure 3.1).

The question remaining unanswered is—why these cows? The persistence of *Strep. uberis* (subclinical and clinical cases) in an udder is largely unknown when testing is not conducted to the strain type level and at a higher frequency. This follows the findings (Table 3.3 and Table 3.4) and aligns with the theory of *Strep. uberis* being a short, low-level infection quickly eradicated from the udder. Currently, there were no

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distinct patterns associated with the cows that had high or low SCC levels. Limited patterns were observed with seasonal influences (two cows, 1327 and 1559), and the underlying predisposing cow factors were unclear in this study (no teat end and hygiene scores conducted).

These results will enable veterinarians and farmers to adapt to, correct, or prevent problems caused by *Strep. uberis* by knowing which strain types are mostly present (environmental versus host-adaptive strains) and with which predominant virulence factors to avoid further development of the risk. More frequent sampling would indicate an enhanced distinction between the environmental and host-adapted strains. It could result from high environmental risk (pathogen overload) and new infections with rapidly changing microbiomes or mutating strains of a pathogen, such as *Strep uberis*. This becomes economically pressing when the cost of mastitis in South Africa can be broken down to an average cost of R919.96 per cow annually (Banga, 2014) and could constitute an interesting point for using selective DCT (Essack *et al*., 2018) in *Staph. aureus*-free herds.

Another question remaining unanswered is whether the *Strep. uberis* mastitis vaccines manufactured in European countries based on their *Strep. uberis* strains will be effective in all countries across the globe; therefore, knowledge of strain types of *Strep. uberis* in South Africa can assist farmers and veterinarians in the field to manage mastitis caused by these particular primarily novel *Strep. uberis* strains in herds, which would have several benefits, including economic, animal welfare, and providing an improved product.

To improve the study and possibly provide an improved representation of the dynamics of the IMIs, more samples could have been collected from the same cows more frequently to evaluate cure and reinfection, and aid in avoiding possible bias. More evaluation of the cows with several consecutive cases of *Strep. uberis* isolates could have been included to anticipate possible explanations regarding susceptibility (teat end scores, udder and cow hygiene scores, parity, and stage for interpreting reoccurring positive *Strep. uberis* cases with the same strain type and CC). Similarly the use of data and isolates sourced from a TMR herd that contained a high prevalence

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of *Strep. uberis* and a second pasture based farm not using the slurry system of pasture fertalisation. This could have been used to cross refference (source point IMIs dominated by environmental infections) and to anticipate possible explanations regarding farming practices and dominating strain types (ST and CC).

This is an area for future study and the whole genome development for *Strep. uberis* profiles specific to Southern Africa (dynamics of infection and duration of infection, developing geographical data, farm data, and identifying the virulence factors) that could be used to develop vaccines or aid in the correct use of exisiing *Strep. uberis* vaccines available on the market (selection for preventation knowing the strain, influence of environmental and farming factors specific to Southern Africa that could reduce the efficacy of the vaccines).

3.5 Conclusion

Contrary to what was expected, the study identified that most suspected chronic IMI were caused by new infections with different *Strep. uberis* strains, dominated by 41 novel environmental strains (high heterogeneity of ST and CC from all 70 isolates). From the results of this study, the *Strep. uberis* strains isolated from the milk of the South African herd are diverse and unique compared to those of other countries. The *Strep. uberis* strains identified appear to have been dominated by environmental strains (high heterogeneity of ST and CC from all 70 isolates).

The consecutive repeat cases had a 50:50 ratio (seven cows with identical strains (ST and CC) for the first and second IMI and seven cows with different strains (ST and CC) for the first, second, third, and even fourth repeat IMI) and do not conclusively confirm the presence or absence of host-adaptive strains within the herd. Even though two strains occurred multiple times (ST 2129 and 1613), only ST 1613 was isolated more than once from the same cow. None of the strains isolated during 2024 were isolated during 2021, also tipping the scale towards a single-source point environmental origin. Most IMI cases appeared to be short-lived and are most likely caused by the farming practice where untreated slurry is used to fertilise pastures. It is, therefore, more likely that most strains were environmental rather than host-adapted.

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It has been established, that certain animals are more susceptible to *Strep. uberis* IMIs. This study showed that half of the reoccurring *Strep. uberis* infections were actually new infections. Since this study only took two samplings into consideration, additional studies are needed over a longer period of time with more samplings to provide a better indication on the duration (chronicity) of *Strep. uberis* infection in the udder. This will provide a better indication of the relationship of host adapted and environmental strains, which would have an affect on the management. This will also show the importance of carrier animals, and cow to cow transfer, including the role of parlour hygiene and the necessity of further research into reducing the shedding via slurry. A large amount of host adapted *Strep. uberis* IMI strains can have an impact on decision making on selective dry cow therapy.

The current study can aid farmers and veterinarians in classifying what is meant by chronic, non-cure, and new infections more effectively in contempt of farming practices that favour higher pathogen loads (environment) and challenges for the cows to manage mastitis caused by *Strep. uberis* better in such herds, with positive economic, animal welfare, and high-quality products being some of the benefits.

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CHAPTER 4: SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

4.1 Introduction

The study used a retrospective approach, employing herd history and baseline data, comprising three-monthly investigations of microbiology and cytology, which emphasised the high incidence of *Strep uberis*, and a longitudinal study evaluating the genomic characteristics using MLST (Jolley *et al*., 2018). The seven housekeeping genes (*gki*, *recP*, *ddl*, *tdk*, *arcC*, *tpi*, and *yqiL*) were used to identify strains of *Strep. uberis* (novel or existing) and evaluate the biodynamics (environmental or host-adaptive properties) (Coffey *et al*., 2006; Tomita *et al*., 2008; Reyes *et al*., 2019) of the pathogen in the context of a South African pasture-based dairy herd using untreated slurry as a fertiliser. Southern African data was compared to global data, judging the similarities and differences to genomic traits observed in other dairy-dominant countries experiencing higher numbers of IMI caused by *Strep uberis*.

Sixty per cent of *Strep. uberis* IMIs last less than 30 days, and 18% could become chronic and last more than 100 days (Srithanasuwan *et al*., 2022). Knowing this, in conjunction with the high percentage of major gram-positive bacteria being isolated from milk samples in South Africa comprising *Strep. uberis* (47.4% recorded in 2021 by the Milk Laboratory, Onderstepoort), farming and veterinary care practices need to be identified and managed better to curb the effects of mastitis and allow for adapted management of environmental or host-adapted IMI pathogens, such as *Strep uberis*.

The results indicated that the IMI, although possibly resembling a chronic IMI pattern when observing basic bacteriological identifications, was primarily a new IMI caused by diverse strains. These strains are more likely of environmental origin attributable to the higher heterogeneity of the pathogen population (Davies *et al*., 2016) and the high number of novel strains (41 from 42) identified from the isolates (64/70) versus six isolates of an identical pre-existing strain (ST 1613); however, the possibility of an original infection with a host-adapted strain and a subsequent co-infection with an environmental strain exists. The results generally followed trends observed in

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additional studies identifying the subsequent CC: ST-5 CC n=11 (clinical 9.09%; subclinical 90.91%), ST-86 CC n=9 (clinical 11.11%; subclinical 88.89%), and ST-143 CC n=2 (clinical 50%; subclinical 50%) (Table 3.3), but differed from data from New Zealand. The ST-143 CC (2/70 isolates versus 113/266 isolates) > ST-5 CC (13/70 isolates versus 15/266 isolates) respectively (Table 3.5). This is interesting as several farming practices are routinely similar and commonly shared between Southern Africa and New Zealand.

Differences among the CC for *Strep. uberis* have been reported in other studies (Reyes *et al*., 2019) to be associated with pathogenicity potential and varying infectious behaviours and patterns. The ST-5 CC was reported to cause clinical cases (Tomita *et al*., 2008), and in the study, the pattern did not follow what was expected, with 10 subclinical cases and one clinical mastitis case. The ST-86 CC was reported to cause subclinical cases (Tomita *et al*., 2008), and in the study, the pattern followed what was expected, with eight subclinical cases and one clinical mastitis case. The ST-143 CC was reported to induce latent infections (host-adaptive traits) (Tomita *et al*., 2008). The pattern was neutral in the study, with one subclinical case and one clinical mastitis case (Table 3.3 and Table 3.4).

The scope of this study omitted sequencing for identification to the CC level. Further genomic data (whole genome sequencing) are needed for identification at the CC level.

4.1.1 Advantages of this study

The comprehensive background history and baseline dataset of the farm, the three-monthly microbiology assessments, and clinical mastitis records over 15 years were significant advantages of this study. The study focused solely on the molecular characterisation (MLST using the seven housekeeping genes) of known *Strep. uberis* positive cases (identified through phenotypic identification and MALDI-TOF).

4.1.2 Recommendations of this study

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If this study were to be repeated, the subsequent recommendations could potentially enhance the results, therefore, strengthening the observations and outcomes:

- Store more samples from consecutive sampling (random and repeat cases). This would allow for a more complete longitudinal evaluation of the herd.
- Repeat the sampling, culture, and MLST evaluation process of known clinical IMIs caused by *Strep. uberis* at the end of intramammary treatment to ensure the successful treatment (milk cure and bacterial cure). This would aid in the identification and confirmation of host-adaptive and environmental strains.

4.1.3 Limitations of this research

The study was conducted mainly on retrospective samples collected from routine samples. Not all routine samples are stored in the biobank, attributable to the high total sample numbers and storage space constraints. Cost constraints were the leading cause of limiting cases being tested with the MLST, a test still costly in South Africa. Knowing this, an enhanced selection plan could have been implemented for subsequent samples to be tested.

4.1.4 Future research

It would be more valuable to sequence the whole genomes of the tested isolates and collect additional samples for cross-referencing. This would include environmental samples (faecal, cow, pasture/untreated slurry) and all clinical mastitis samples identified as *Strep uberis*. Increasing the sample size by storing more isolates identified and confirmed would also be beneficial.

4.2 General conclusion

Contrary to what was expected, the study identified that most suspected chronic IMIs were caused by new infections with diverse *Strep. uberis* strains, dominated by 41 novel environmental strains (high heterogeneity of ST and CC from all 70 isolates). The Southern African genomic profile mimics that of several countries, and no set isolates indicate a chronic repeat pattern among cows, except for ST 2129 and 1613;

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however, the IMI appeared to be short-lived and were unlikely to be influenced by developing host-adaptive strains from the farming practice of untreated slurry fertilisation of pastures. These results differed from New Zealand despite having the most similarities in farming practices (seasonal pasture-based systems, low input versus max output, untreated slurry management and fertilisation of pastures, mixed-breed cows with small frames, etc.).

This research can help farmers and veterinarians classify repeat, non-cure, and new infections more effectively. It focuses on farming practices favouring higher pathogen loads (environment) and challenges for the cows. This will improve the management of mastitis caused by *Strep. uberis* in such herds. The benefits include positive economic outcomes, better animal welfare, and high-quality products.

It has been established, that certain animals are more susceptible to *Strep. uberis* IMIs. This study showed that half of the reoccurring *Strep. uberis* infections were actually new infections. Since this study only took two samplings into consideration, additional studies are needed over a longer period of time with more samplings to provide a better indication on the duration (chronicity) of *Strep. uberis* infection in the udder. This will provide a better indication of the relationship of host adapted and environmental strains, which would have an affect on the management. This will also show the importance of carrier animals, and cow to cow transfer, including the role of parlour hygiene and the necessity of further research into reducing the shedding via slurry. A large amount of host adapted *Strep. uberis* IMI strains can have an impact on decision making on selective dry cow therapy.

The findings also contain a word of caution: products that work in other countries with diverse *Strep. uberis* strains need to be tested in South Africa, preferably under field conditions, to ensure they are effective against the unique South African *Strep. uberis* strains.

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References

Adamie, B. A., Owusu-Sekyere, E., Lindberg, M., Agenäs, S., Nyman, A.-K., & Hansson, H. 2023. Dairy cow longevity and farm economic performance: Evidence from Swedish dairy farms. *Journal of Dairy Science, 106*(12), 8926-8941, ISSN 0022-0302, https://doi.org/10.3168/jds.2023-23436.

Adkins, P. R. & Middleton, J. R. 2018. Methods for diagnosing mastitis. *Veterinary Clinics: Food Animal Practice, 34*(3), 479-491.

Allore, H. G. & Erb, H. N. 1998. Partial budget of the discounted annual benefit of mastitis control strategies. *Journal of Dairy Science, 81*(8), 2280-2292.

Almeida, R. A., Luther, D. A., Park, H. M., & Oliver, S. P. 2006. Identification, isolation, and partial characterization of a novel Streptococcus uberis adhesion molecule (SUAM). *Veterinary Microbiology, 115*, 183–191.

Aly, S. S., Okello, E., ElAshmawy, W. R., Williams, D. R., Anderson, R. J., Rossitto, P., Tonooka, K., Glenn, K., Karle, B., & Lehenbauer, T. W. 2022. Effectiveness of Intramammary Antibiotics, Internal Teat Sealants, or Both at Dry-Off in Dairy Cows: Clinical Mastitis and Culling Outcomes. *Antibiotics (Basel, Switzerland)*, *11*(7), 954. https://doi.org/10.3390/antibiotics11070954

Apparao, M. D., Ruegg, P. L., Lago, A., Godden, S., Bey, R., & Leslie, K. 2009. Relationship between in vitro susceptibility test results and treatment outcomes for gram-positive mastitis pathogens following treatment with cephapirin sodium. *Journal of Dairy Science, 92*(6), 2589-2597. <https://www.journalofdairyscience.org/action/showPdf?pii=S0022-0302%2809%2970574-0> [Viewed 18/12/2021]

© University of Pretoria

Argaw, A. 2016. Review on epidemiology of clinical and subclinical mastitis on dairy cows. *Food Science Quality Managment, 52*(6), 56-65.

Azooz, M. F., El-Wakeel, S. A., & Yousef, H. M. 2020. Financial and Economic Analyses of the Impact of Cattle Mastitis on the Profitability of Egyptian Dairy Farms. *Veterinary World, 13*, 1750–1759.

Bagul, U. S. & Sivakuma, S. M. 2016. Antibiotic susceptibility testing: A review on current practices. *International Journal of Pharmacy, 6*(3), 11-17. ISSN 2249-1848

Banga, C. B., Neser, F. W. C., & Garrick, D. J. 2014. Breeding objectives for Holstein cattle in South Africa. *South African Journal of Animal Science, 44*(3), 199-214.

Barry, C., Ellingsen-Dalskau, K., Winckler, C., & Kielland, C. 2024. Exploring uses for an algorithmically generated “Animal Welfare Indicator” for welfare assessment of dairy herds. *Journal of Dairy Science, 107*(6), 3941-3958. https://doi.org/10.3168/jds.2023-24158.

Baucells, J. 2016. A simple method to calculate the cost of mastitis under Spanish field conditions. *Published at the 29th World Buiatrics Congress*, Dublin, 3rd to 8th July 2016. <https://www.hipra.com/portal/en/hipra/knowledge/pubdetail/A-simple-method-to-calculate-the-cost-of-mastitis-under-Spanish-field-conditions> [Viewed on the 03/05/2022].

Bauer, A. W., Kirby, W. M., Sherris, J. C., & Turck, M. 1966. Kirby Bauer method antimicrobial susceptibility testing by a standardized single disk method. American *Journal of Clinical Pathology, 45*(4), 493–496. doi:10/1093/ajcp/45.4 ts.493

Behind the Bench staff. 2021b. The Importance of Sensitivity and Specificity in Diagnosing Bovine Mastitis. Thermo Fisher Scientific.

© University of Pretoria

<https://www.thermofisher.com/blog/behindthebench/the-importance-of-sensitivity-and-specificity-in-diagnosing-bovine-mastitis/> [Viewed 25/01/2022]

Beli, E. 2016. Evaluation of Albanian Raw Milk Quality Situation by Using Somatic Cell Counts. *Journal of Multidisciplinary Engineering Science and Technololgy, 3*, 5971–5974.

Berry, E. A. & Hillerton, J. E. 2002. The Effect of Selective Dry Cow Treatment on New Intramammary Infections. *Journal Dairy Science, 85*, 112–121.

Berti, A., Rose, W., Nizet, V. &., & Sakoulas, G. 2020. Antibiotics and Innate Immunity: A Cooperative Effort Toward the Successful Treatment of Infections. *Open Forum Infectious Diseases, 7*, (8), 302. doi: 10.1093/ofid/ofaa302. PMID: 32818143; PMCID: PMC7423293.

Blignaut, D., Thompson, P., & Petzer, I. M. 2018. Prevalence of mastitis pathogens in South African pasture-based and total mixed ration-based dairies during 2008 and 2013. *The Onderstepoort Journal of Veterinary Research, 85*(1), 1–7. https://doi.org/10.4102/ojvr.v85i1.1482.

Blosser, T. H. 1979. Economic losses from and the National Research Program on Mastitis in the United States. *Journal of Dairy Science. 62*, 119.

Boireau, C., Cazeau, G., Jarrige, N., Calavas, D., Madec, J., Leblond, A., Haenni, M., & Gay, É. 2018. Antimicrobial resistance in bacteria isolated from mastitis in dairy cattle in France, 2006–2016. *Journal of Dairy Scienc,. 101*(10), 9451-9462. doi: 10.3168/jds.2018-14835

Bojovic, M. & McGregor, A. 2023. A review of megatrends in the global dairy sector: what are the socioecological implications? *Agricultural and Human Values*, 40 , 373–394 .<https://doi.org/10.1007/s10460-022-10338-x> [Viewed 16 June 2023]

© University of Pretoria

Bonestroo, J., Fall, N., Hogeveen, H., Emanuelson, U., Klaas, I. C., & Van der Voort, M. 2023. The costs of chronic mastitis: A simulation study of an automatic milking system farm. *Preventive Veterinary Medicine*. 210, 105799. https://doi.org/10.1016/j.prevetmed.2022.105799

Bradley, A. J. 2002. Bovine Mastitis: An Evolving Disease. *The Veterinary Journal, 164*(2), 116-128.

Bradley, A. J., Breen, J. E., Payne, B., White, V., & Green, M. J. 2015. An investigation of the efficacy of a polyvalent mastitis vaccine using different vaccination regimens under field conditions in the United Kingdom. *Journal of Dairy Science, 98*, 1706-1720.

Burke, N. & Adley, C. C. 2021. Prevalence of intramammary antibiotic usage in dairy farming. *Journal of Dairy Research, 88*, 176–178. https://doi.org/10.1017/S0022029921000431> and <https://www.cambridge.org/core/services/aop-cambridge-core/content/view/803A0C153C0BFFDC5DA2DB28D120AFEF/S0022029921000431a.pdf/prevalence-of-intramammary-antibiotic-usage-in-dairy-farming.pdf> [Viewed 03/02/2022]

Butte, A. J., Dzau, V. J., & Glueck, S. B. 2001. Further defining housekeeping, or maintenance, genes Focus on A compendium of gene expression in normal human tissues. Physiological Genomics. 7 (2), 95–96. doi:10.1152/physiolgenomics.2001.7.2.95. PMID 11773595.

Calonzi, D., Romano, A., Monistero, V., Moroni, P., Luini, M. V., Biscarini, F., Castiglioni, F. B., & Cremonesi, P. 2020. Technical note: Development of multiplex PCR assays for the molecular characterization of *Streptococcus uberis* strains isolated from bovine mastitis. *Journal of Dairy Science, 103*(1), 915-921. https://doi.org/10.3168/jds.2019-16823

© University of Pretoria

Cameron, M., Saab, M., Heider, L., Mcclure, J. T., Rodriguez-Lecompte, J. C., & Sanchez, J. 2016. Antimicrobial susceptibility patterns of environmental Streptococci recovered from bovine milk samples in the Maritime Provinces of Canada. *Frontiers in Veterinary Science*, 3, 79.

Cattell, M. B. 1996. An outbreak of *Streptococcus uberis* as a consequence of adopting a protocol of no antibiotic therapy for clinical mastitis. Proceedings of the 35th National Mastitis Council at Nashville (TN). Madison (WI): *National Mastitis Council*; . 123 - 130.

Charman, N., Dyson, R., Hodge, A., Robertson, N., & Chaplin, S. 2012. A survey of mastitis pathogens in the South-Eastern Australian dairy industry, Proceedings of the Countdown Symposium 2012, Melbourne, Victoria, Australia, 19 July, . 18–22.

Chase,C.C.L. 2022. The Essentials The Who, What and Where of the Bovine Immune System. Hipra, S.A., Acda., La Selva 135 17170 Amer (Girona) Spain, .3-29.

Coenen, S., Ferech, M, Haaijer-Ruskamp, F. M., Butler, C. C., Vander Stichele, R. H., Verheij, T.J. M., Monnet, D. L., Little, P., & Goossens, H. 2007. European Surveillance of Antimicrobial Consumption (ESAC): quality indicators for outpatient antibiotic use in Europe. *Qualality Safe Health Care*, 16, 440-445. http://qualitysafety.bmj.com.uplib.idm.oclc.org/content/16/6/440.short. [Accessed 6 April 2024]

Coffey, T. J., Pullinger, G. D., Urwin, R., Jolley, K. A., Wilson, S. M., Maiden, M. C., & Leigh, J. A. 2006. First Insights into the Evolution of *Streptococcus uberis*: a Multilocus Sequence Typing Scheme That Enables Investigation of Its Population Biology. *Applied and Environmental Microbiology*, 72(2), 1420–1428. http://doi:10.1128/AEM.72.2.1420–1428.2006.

© University of Pretoria

Crompton, C. W. R., Heuer, C., Parker, K., & McDougall, S. 2007. Epidemiology of Mastitis in Pasture-Grazed Peripartum Dairy Heifers and Its Effects on Productivity. *Journal of Dairy Science, 90*, 4157–4170. doi:10.3168/jds.2006-880.

Dal Prà, A., Biscarini, F., Cavani, G. L., Bacchelli, S., Iotti, A., Borghi, S., Nocetti, M., & Moroni, P. 2022. Relationship between total and differential quarter somatic cell counts at dry-off and early lactation. *PloS one*, *17*(10), e0275755. https://doi.org/10.1371/journal.pone.0275755.

Davies, P. L., Leigh, J. A., Bradley, A. J., Archer, S. C., Emes, R. D., & Green, M. J. 2016. Molecular Epidemiology of *Streptococcus uberis* Clinical Mastitis in Dairy Herds: Strain Heterogeneity and Transmission. *Journal of Clinical Microbiology 54*(1):68-74. doi: 10.1128/JCM.01583-15. Epub 2015 Oct 21. PMID: 26491180; PMCID: PMC4702729.

De Jong, A., El Garch, F., Simjee, S., Moyaert, H., Rose, M., Youala, M., Siegwart, E., & VetPath Study Group, 2018. Monitoring of antimicrobial susceptibility of udder pathogens recovered from cases of clinical mastitis in dairy cows across Europe: VetPath results. *Veterinary Microbiology*, 213, 73-81. doi: 10.1016/j.vetmic.2017.11.021. Epub 2017 Nov 21. PMID: 29292007.

De Vliegher, S., Fox, L. K., Piepers, S., McDougall, S., & Barkema, H. W. 2012. Invited review: Mastitis in dairy heifers: Nature of the disease, potential impact, prevention and control. *Journal of Dairy Science, 95*(3),1025-1040. doi:10.3168/jds.2010-4074.

De Vries, A. 2017. Economic Trade-Offs between Genetic Improvement and Longevity in Dairy Cattle. *Journal of Dairy Scienc,. 100*, 4184–4192.

DeGraves, F. J. & Fetrow, J. 1993. Economics of mastitis and mastitis control., *Veterinary Clinics of North America: Food Animal Practice*, 9(3), 421–443. https://doi.org/10.1016/s0749-0720(15)30611-3.

© University of Pretoria

Denis, M., Wedlock, D. N., Lacy-Hulbert, S. J., Hillerton, J. E., & Buddle, B. M. 2009. Vaccines against bovine mastitis in the New Zealand context: What is the best way forward? *New Zealand Veterinary Journal*,. 57, 132-140.

Dezetter, C., Bareille, N., Billon, D., Côrtes, C., Lechartier, C., & Seegers, H. 2017. Changes in Animal Performance and Profitability of Holstein Dairy Operations after Introduction of Crossbreeding with Montbéliarde, Normande, and Scandinavian Red. *Journal of Dairy Science, 100*, 8239–8264.

Du Preez, J.H., Hattingh, P.J., Giesecke, W.H. & Eisenberg, B.E. 1990. Heat stress in dairy cattle and other livestock under southern African conditions. III. Monthly temperature – humidity index mean values and their significance in the performance of dairy cattle. *Onderstepoort Journal of Veterinary Research*, 57(4), 243-248

Du Preez, J. H. 2000. Bovine mastitis therapy and why it fails: continuing education. *Journal of the South African Veterinary Association, 71*(3), 201-208.

Dzidic, A., Rovai, M., Poulet, J. L., Leclerc, M., & Marnet, P. G. 2019. Review: Milking routines and cluster detachment levels in small ruminants, *Animal,* 13(1), 86-93. ISSN 1751-7311, https://doi.org/10.1017/S1751731118003488.

Essack, S. Y. 2018. Antibiotic resistance and One Health: a mapping project. The *Lancet* 6 (Special Issue), S27. doi:10.1016/S2214-109X (18)30156-6

Fogsgaard, K. K., Røntved, C. M., Sørensen, P., & Herskin, M. S. 2012. Sickness behaviour in dairy cows during Escherichia coli mastitis. *Journal of Dairy Science, 95*(2), 630-638. doi:10.3168/jds.2011-4350.

Fosgate, G. T., Petzer, I. M., & Karzis, J. 2013. Sensitivity and specificity of a hand-held milk electrical conductivity meter compared to the California Mastitis Test for

© University of Pretoria

mastitis in dairy cattle, *Veterinary Journal, 196*(1), 98-102. doi: 10.1016/j.tvjl.2012.07.026.

García-Montoya, I. A., Cendón, T. S., Arévalo-Gallegos, S., & Rascón-Cruz, Q. 2012. Lactoferrin a multiple bioactive protein: an overview*. Biochim Biophys Acta*. 1820(3), 226-236. http://doi: 10.1016/j.bbagen.2011.06.018. Epub 2011 Jun 25. PMID: 21726601; PMCID: PMC7127262.

Gase, K., Ferretti, J. J., Primeaux, C., & McShan, W. M. 1999. Identification, cloning, and expression of the CAMP factor gene (cfa) of group A streptococci. *Infection and Immunity.* 67(9), 4725-4731. https://doi: 10.1128/IAI.67.9.4725-4731.1999. PMID: 10456923; PMCID: PMC96801.

Gianneechini1, R. E., Concha, C., & Franklin, A. 2002. Antimicrobial susceptibility of udder pathogens isolated from dairy herds in the Western Littoral Region of Uruguay. *Acta Veterinaria Scandinavica*, 43, 31-41.

Gilchrist, T. L., Smith, D. G. E., Fitzpatrick, J. L., Zadoks, R. N., & Fontaine, M. C. 2013. Comparative molecular analysis of ovine and bovine *Streptococcus uberis* isolates. *Journal of Dairy Science, 96*(2), 962-970. ISSN 0022-0302, https://doi.org/10.3168/jds.2012-5705.

Green, M. & Bradley, A. J. 2013. The changing face of mastitis control. *Veterinary Record*, 173, 517-521.

Gröhn, Y. T., Wilson, D. J., González, R. N., Hertl, J. A., Schulte, H., Bennett, G., & Schukken, Y. H. 2004. Effect of Pathogen-Specific Clinical Mastitis on Milk Yield in Dairy Cows. *Journal of Dairy Science, 87*(10), 3358-3374. https://doi.org/10.3168/jds.S0022-0302(04)73472-4.

© University of Pretoria

Gunasekera, T. 2003. Potential for Broad Applications of Flow Cytometry and Fluorescence Techniques in Microbiological and Somatic Cell Analyses of Milk. International *Journal of Food Microbiology, 85*, 269–279.

Gurjar, A., Gioia, G., Schukken, Y., Welcome, F., Zadoks, R., & Moron, P. 2012. Molecular Diagnostics Applied to Mastitis Problems on Dairy Farms. *Veterinary Clinics of North America: Food Animal Practice*, 28, 565–576. http://dx.doi.org/10.1016/j.cvfa.2012.07.011

Haenni, M., Lupo, A., & Madec, J. Y. 2018. Antimicrobial resistance in Streptococcus spp. *Microbiology spectrum*, 6(2), 6-12. doi: 10.1128/microbiolspec.arba-0008-2017

Halasa, T., Huijps, K., Østerås, O., & Hogeveen, H. 2007. Economic effects of bovine mastitis and mastitis management: A review. *Veterinary quarterly*, 29(1), 18-31.

Hand, K. J., Godkin, M. A., & Kelton, D. F. 2012. Bulk milk somatic cell penalties in herds enrolled in Dairy Herd Improvement programs. *Journal of Dairy Science, 95*(1), 240-242.

Hanson, M. 2022. Decline in U.S. Dairy Herd Numbers Slows. *Dairy Herd Management* <https://www.dairyherd.com/news/dairy-production/decline-us-dairy-herd-numbers-slows> [Viewed 14 June 2023]

Harmon, R. J., Clark, T., Ramesh, T., Crist, W. L., Langlois, B. E., Akers, K., & Smith, B. 1992. Environmental pathogen numbers in pastures and bedding of dairy cattle. *Journal of Dairy Science, 75*(1), 256.

Haxhiaj, K., Wishart, D. S., & Ametaj, B. N. 2022. Mastitis: What It Is, Current Diagnostics, and the Potential of Metabolomics to Identify New Predictive Biomarkers. *Dairy,* 3(4), 722-746. https://doi.org/10.3390/dairy3040050

© University of Pretoria

Hayman, D.T.S., Adisasmito, W.A., Almuhairi, S., Behravesh, C.B., Bilivogui, P., Bukachi, S.A., Casas, N., Becerra, N.C., Charron, D.F., Chaudhary, A., Janice R. Ciacci Zanella, Cunningham, A.A., Dar, O., Debnath, N., Dungu, B., Farag, E., Gao, G.F., Khaitsa, M., Machalaba, C., Mackenzie, J.S., Wanda Markotter, W., Mettenleiter, T.C., Morand, S., Smolenskiy, V., Zhou, L. & Koopmans, M. 2023. Developing One Health surveillance systems, One Health, 17.

Health for Animals, Global animal Association 2023. Animal Health and AMR: Livestock Data Analysis. https://healthforanimals.org/animal-health-in-data/antimicrobial-resistance/animal-health-and-amr/ cited July 2024.

Heikkilä, A. M., Liski, E., Pyörälä, S., & Taponen, S. 2018. Pathogen-specific production losses in bovine mastitis. *Journal of Dairy Science, 101*, 9493–9504. https://doi.org/10.3168/jds.2018-14824

Henton, M. M., Eager, H. A., Swan, G. E., & Van Vuuren, M. 2011. Part VI. Antibiotic management and resistance in livestock production. *The South African Medical Journal. 101* (8) http://www.samj.org.za/index.php/samj/article/view/5063/3369

Hillerton, J. E. & Berry, E. A. 2003. The management and treatment of environmental streptococcal mastitis, Veterinary Clinics of North America: Food Animal Practice, 19(1), 157-169. ISSN 0749-0720, https://doi.org/10.1016/S0749-0720(02)00069-5.

Hillerton, J. E., Shearn, M. F. S., Teverson, R. M., Landridge, S., & Booth, J. M. 1993. Effect of pre-milk teat dipping on clinical mastitis on dairy farms in England. *Journal of Dairy Research, 60,* 31-41.

Hogeveen, H. & Østerås, O. 2005. Mastitis management in an economic framework. In Mastitis in Dairy Production. Proceedings of the 4th IDF International Conference, Edited by, Hogeveen, H, pp. 41-52.

© University of Pretoria

Hoque, M. N., Istiaq, A., Rahman, M. S., Islam, M. R., Anwar, A., Siddiki, A. M. A. M. Z., Sultana, M., Crandall, K. A., & Hossain, M. A. 2020. Microbiome dynamics and genomic determinants of bovine mastitis. *Genomics*, 6(112), 5188-5203.

Hsieh, J. C., Yen, Y. S., & Chuang, S. T. 2019. Identification of Streptococcus spp. isolated from bovine milk and characterization of their antimicrobial susceptibility profiles in Taiwan. *The Thai Journal of Veterinary Medicine, 49*(1), 57-63. Available at: https://he01.tci-thaijo.org/index.php/tjvm/article/view/193694

Hurst, M. 1993. Mastitis, milk quality and milk price*. British Mastitis Conference* 1993, Cambridge, United Kingdom, Stratagene Ltd., 1993, 77-82.

Jeppsson, K.-H., Magnusson, M., Bergström Nilsson, S., Ekman, L., Winblad von Walter, W., Jansson, L. E., Landin, H., Rosander, A. & Bergsten, C. 2024. Comparisons of recycled manure solids and wood shavings/sawdust as bedding material -implications for animal welfare, herd health, milk quality and bedding costs in Swedish dairy herds. *Journal of Dairy Science, 107*(8), 5779- 5793. ISSN 0022-0302, https://doi.org/10.3168/jds.2023-24192.

Jolley, K. A., Chan, M. S., & Maiden, M. C. 2004. mlstdbNet – distributed multi-locus sequence typing (MLST) databases. *BMC Bioinformatics*, 5, 86. https://doi.org/10.1186/1471-2105-5-86

Jolley, K., Bray, J., & Maiden, M. 2018. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. Welcome Open Res. https://doi.org/10.12688/wellcomeopenres.14826.1

Karzis, J. & Petzer, I. M. 2024. A preview of antibiotic resistance and mastitis pathogen trends in South African dairy herds from 2010 to 2021. *Journal of Dairy Research* (Submitted 5 Aug 2024).

© University of Pretoria

Karzis, J., Donkin, E. F., & Petzer, I. M. 2007. The influence of intramammary antibiotic treatment, presence of bacteria, stage of lactation and parity in dairy goats as measured by the California Milk Cell Test and somatic cell counts. *Journal of Veterinary Research, 74*(2), 161-167. https://repository.up.ac.za/bitstream/handle/2263/5123/Karzis_Influence(2007).pdf?sequence=1> [Viewed 18/12/2022]

Keane, O. M., Budd, K. E., Flynn, J., & McCoy, F. 2013. Pathogen profile of clinical mastitis in Irish milk-recording herds reveals a complex aetiology. *Veterinary Record*, 173, 17.

Khan, I. U., Hassan, A. A., Abdulmawjood, A., Lammler, C., Wolter, W., & Zschock, M. 2003. Identification and epidemiological characterization of *Streptococcus uberis* isolated from bovine mastitis using conventional and molecular methods. *Journal of Veterinary Science, 4*(3), 213-223. https://doi.org/10.4142/jvs.2003.4.3.213

Klinker, K. P., Hidayat, L. K., DeRyke, C. A., DePestel, D. D., Motyl, M., & Bauer, K. A. 2021. Antimicrobial stewardship and antibiograms: importance of moving beyond traditional antibiograms. *Therapeutic Advances in Infections Disease*. Jan-Dec (8): 20499361211011373. Doi: 10.1177/20499361211011373

Koeck, A., Miglior, F., Kelton, D. F., & Schenkel, F. S. 2012. Alternative Somatic Cell Count Traits to Improve Mastitis Resistance in Canadian Holsteins. *Journal of Dairy Science, 95*, 432–439.

Kromker, V., Reinecke, F., Paduch, J. H., & Grabowski, N. 2014. Bovine *Streptococcus uberis* Intramammary Infections and Mastitis. *Clinical Microbiology*, 3, 157. doi:10.4172/2327-5073.1000157.

Lactodata Milk SA, 2013. *MPO* 16(2) <09/BIPLAC017%20LACTO%20DATA%20November%202013.pdf> [Viewed 15 June 2023]

© University of Pretoria

Lactodata Milk SA, 2018. *MPO* 21(1) <https://milksa.co.za/sites/default/files/2018-09/LAC005%20May%202018.pdf> [Viewed 15 June 2023]

Lactodata Milk SA, 2022*. MPO* 25 <https://www.mpo.co.za/wp-content/uploads/2022/05/TDM0522-Lacto-Data-Vol-25-May-2022-Final.pdf> [Viewed 15 June 2023]

Lactodata Milk SA, 2023*. MPO* 26 https://milksa.co.za/sites/default/files/2023-06/Lacto%20Data%20June%202023.pdf> [Viewed 21 June 2023]

Ladha-Sabur, A., Bakalis, S., Fryer, P. J., & Lopez-Quiroga, E. 2019. Mapping energy consumption in food manufacturing. *Trends in Food Science and Technology*, 86, 270–280. https://doi.org/10.1016/j.tifs.2019.02.034

Lähteenmäki, K., Kuusela, P., & Korhonen, T. K. 2001. Bacterial plasminogen activators and receptors. *FEMS microbiology reviews*, 25(5), 531–552. https://doi.org/10.1111/j.1574-6976.2001.tb00590.x

Lam, T., Van Den Borne, B. H. P., Jansen, J., Huijps, K., Van Veersen, J. C. L., Van Schaik, G. H., & Hogeveen, H. 2013. Improving bovine udder health: a national mastitis control program in the Netherlands. *Journal of Dairy Science, 96*, 1301-1311.

Laven, R. 2010a. Mastitis Part 4 - Detecting and Treating Clinical Mastitis*. National Animal Disease Information Service (© NADIS).* <https://nadis.org.uk/disease-a-z/cattle/mastitis/mastitis-part-4-detecting-and-treating-clinical-mastitis/> [Viewed 18/12/2022]

Laven, R. 2010b. Mastitis Part 8 - Dry Cow Therapy. *National Animal Disease Information Service (© NADIS).* <https://nadis.org.uk/disease-a-z/cattle/mastitis/mastitis-part-8-dry-cow-therapy/> [Viewed 18/12/2022]

© University of Pretoria

Levison, M. E. 2000. Pharmacodynamics of antibacterial drugs*. Infectious disease clinics of North America,* 14(2), 281. https://doi.org/10.1016/s0891-5520(05)70248-8

Liu Y., Li J., Du G., & Chen J. 2011. Microbial production of hyaluronic acid: Current state, challenges, and perspectives*. Microbial. Cell Factories*, 10, 99. https://doi: 10.1186/1475-2859-10-99.

Loo, E., Lai, K. S., & Mansor, R. 2019. Antimicrobial usage and resistance in dairy cattle production. *Veterinary Medicine and Pharmaceuticals*, 1-15.

Lopez-Benavides, M. G., Williamson, J. H., Pullinger, G. D., Lacy-Hulbert, S. J., Cursons, R. T., & Leigh, J. A. 2007. Field observations on the variation of *Streptococcus uberis* populations in a pasture-based dairy farm. *Journal of Dairy Science, 90*, 5558–5566.

Loures, R., de Pádua Pereira, U., de Carvalho Castro, G., Mian, G., da Costa Custódio, D. & da Silva, J. 2017. Genetic diversity and virulence genes in *Streptococcus uberis* strains isolated from bovine mastitis. *Semina: Ciênc Agrár,* 38, 2595-2606.

Magagula, S., Petzer, I. M., Famuyide, I. M., & Karzis, J. 2023. Biofilm expression and antimicrobial resistance patterns of *Streptococcus uberis* isolated from milk samples of dairy cows in South Africa. *Journal of Dairy Research, 8*, 1-5. https://doi.org/10.1017/ S0022029923000158 Rec (Cambridge University Press, CUP).

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

© University of Pretoria

resistance. *Clinical Microbiology and Infection*, 18(3), 268-281. doi: 10.1111/j.1469-0691.2011.03570.x.

Marnet, P. 2013. Milking Procedures and Facilities. Milk and Dairy Products in Human Nutrition: Production, Composition and Health. Editors: Young W Pak and Georges F.W. Haenlein John Wiley and sons, Ltd. pp. 46-64 10.1002/9781118534168.ch3.

Martin, N. H., Vanowski, R. L., & Wiedmann, M. 2023. Invited review: Redefining raw milk quality—Evaluation of raw milk microbiological parameters to ensure high-quality processed dairy products. *Journal of Dairy Science, 106*(3), 1502-1517. ISSN 0022-0302, https://doi.org/10.3168/jds.2022-22416.

Mazet, J. A., Clifford, D. L., Coppolillo, P. B., Deolalikar, A. B., Erickson, J. D., & Kazwala, R. R. 2009. A “one health” approach to address emerging zoonoses: the HALI project in Tanzania. *PLoS medicine*, 6(12), 1000190.

McDougall, S., Parkinson, T. J., Leyland, M., Anniss, F. M., & Fenwick, S. G. 2004. Duration of infection and strain variation in *Streptococcus uberis* isolated from cows' milk. *Journal of Dairy Science, 87*(7), 2062-2072.

Melchior, M. & Vaarkamp, H., & Fink-Gremmels, J.. 2006. Biofilms: A role in recurrent mastitis infections?. *Veterinary journal* (London, England : 1997). 171. 398-407. 10.1016/j.tvjl.2005.01.006.

Miller, R. H., Paape, M. J., Fulton, L. A., & Schutz, M. M. 1993. The relationship of milk somatic cell count to milk yields for Holstein heifers after first calving. *Journal of Dairy Science, 76(*3), 728-733.

Monistero, V., Barberio, A., Cremonesi, P., Castiglioni, B., Morandi, S., Lassen, D. C. K., Astrup, L. B., Locatelli, C., Piccinini, R., Addis, F., Bronzo, V., & Moroni1, P.

© University of Pretoria

2021. Genotyping and Antimicrobial Susceptibility Profiling of *Streptococcus uberis* Isolated from a Clinical Bovine Mastitis Outbreak in a Dairy Farm. *Antibiotics* (Basel). 10(6), 644. http://doi: 10.3390/antibiotics10060644.

Moore, G.E. 2009. Biofilm Production by Streptococcus uberis Associated with Intramammary Infections. Chancellor's Honors Program Projects. https://trace.tennessee.edu/utk_chanhonoproj/1299

Morin, D. E. 2015. Mammary gland health, in B.P Smith, P.L. Ruegg & R.L. Erskine(eds.), *Large animal internal medicine*, 5th Ed, Chapter 36, pp 1015-1043. Mosby Elsevier, St. Louis, Missouri.

Narayana, S.G, Schenkel, F., Miglior, F., Chud, T., Emhimad A. Abdalla, Naqvi, S.A., Malchiodi, F. & Barkema, H.W. 2021. Genetic analysis of pathogen-specific intramammary infections in dairy cows. *Journal of Dairy Science, 104* (2), 1982-1992. https://doi.org/10.3168/jds.2020-19062.

National Mastitis Council. Laboratory Handbook on Bovine Mastitis, 3rd ed.; National Mastitis Council: Verona, WI, USA, 2017.

Nielsen, L.R., Jørgensen, E., Kristensen, A.R., & Østergaard, S. 2010. Optimal replacement policies for dairy cows based on daily yield measurements. *Journal of Dairy Science*. 93 :75–92. http://doi.org/10.3168/jds.2009-2209

Neave, F. K., Dodd, F. H., Kingwill, R. G., & Westgarth, D. R. 1969. Control of mastitis in the dairy herd by hygiene and management. *Journal of Dairy Science, 52*, 696-707.

Neculai-Valeanu, A. S. &. & &Ariton, A. M. 2022. Udder Health Monitoring for Prevention of Bovine Mastitis and Improvement of Milk Quality. *Bioengineering*, 9(11), 608. https://doi.org/10.3390/bioengineering9110608.

© University of Pretoria

Neijenhuis, F., Barkema, H. W., Hogeveen, H., & Noordhuizen, J. P. T. M. 2000. Classification and longitudinal examination of callused teat ends in dairy cows. *Journal of Dairy Science, 83*, 2795-2804.

Nickerson, S. C. 2011. Mastitis Pathogens, Contagious Pathogens, Editor(s): John W. Fuquay, *Encyclopaedia of Dairy Sciences*, Second Edition), Academic Press. pp. 408-414.

Norberg, E., Hogeveen, H., Korsgaard, I. R., Sloth, K. H. M. N., & Lovendahl, P. 2004. Electrical conductivity of milk: Ability to Predict Mastitis Status. *Journal of Dairy Science, 87*(4), 1099-1107.

Oliveira, L. & Ruegg, P. L. 2014. Treatments of clinical mastitis occurring in cows on 51 large dairy herds in Wisconsin. *Journal of Dairy Science, 97*(9), 5426-5436. doi:10.3168/jds.2013-7756.

Oliver, S. P. & Murinda, S. E. 2012. Antimicrobial resistance of mastitis pathogens. *Veterinary Clinical North American Food Animal Practice*, 28(2), 165-85. doi: 10.1016/j.cvfa.2012.03.005. Epub 2012 Apr 28. PMID: 22664201.

Oliver, S. P., Pighetti, G. M., & Almeida, R. A. 2011. Mastitis Pathogens. Environmental Pathogens. Editor(s): John W. Fuquay, J.W. *Encyclopaedia of Dairy Sciences*, Second Edition, Academic Press. pp. 415-421.

Oviedo-Boyso, J., Valdez-Alarcón, J. J., Cajero-Juárez, M., Ochoa-Zarzosa, A., López-Meza, J. E., Bravo-Patiño, A., & Baizabal-Aguirre, V. M. 2007. Innate immune response of bovine mammary gland to pathogenic bacteria responsible for mastitis. *Journal of Infection, 54*(4), 399-409. ISSN 0163-4453, https://doi.org/10.1016/j.jinf.2006.06.010.

© University of Pretoria

Owens, W. E., Watts, J. L., Boddie, R. L., & Nickerson, S. C. 1988. Antibiotic Treatment of Mastitis: Comparison of Intramammary and Intramammary Plus Intramuscular Therapies. *Journal of Dairy Science, 71*(11), 3143-3147. <https://www.journalofdairyscience.org/action/showPdf?pii=S0022-0302%2888%2979915-4> [Viewed 18/12/2022]

Paduch, J.H., Lücking, J., Vries, E., Zinke, C., Wente, N., & Krömker, V. 2020. Prevention of Intramammary Infections by Prepartum External Application of a Teat Dip Containing Lactic Acid Bacteria with Antimicrobial Properties in Dairy Heifers. *Pathogens*. 9. 10.3390/pathogens9040288.

Petrovski, K. R., Trajcev, M., & Buneski, G. 2006. A review of the factors affecting the costs of bovine mastitis. *Journal of the South African Veterinary Association*, 77(2), 52-60.

Petrovski, K. R., Williamson, N. B., Lopez-Villalobos, N., Parkinson, T. P., & Tucker, I. G. 2011. Culture results from milk samples submitted to veterinary diagnostic laboratories from August 2003 to December 2006 in New Zealand. *New Zealand Veterinary Journal, 59*, 317- 322.

Petzer, I. M. & Swan, A. C. 2019. The importance of teat liners in udder health. *SAVA Southern Cape Congress*, Hyatt Hotel, Heroldsbay. 12 April 2019.

Petzer, I. M., Karzis, J., Meyer, I. A., & Van der Schans, T. J. 2013. A cost-benefit model comparing California Milk Cell Test and Milk Electrical Resistance Test. *Onderstepoort Journal of Veterinary Research, 80*(1), 538. doi: 10.4102/ojvr.v80i1.538. PMID: 23718179.

Petzer, I. M., Karzis, J., Watermeyer, J. C., Van der Schans, T. J., & Van Reenen, R. 2009. Trends in udder health and emerging mastitogenic pathogens in South African dairy herds. *Journal of the South African Veterinary Association, 80*(1), 17-22. doi: 10.4102/jsava. v80i1.163. PMID: 19653514.

© University of Pretoria

Petzer, I., Karzis, J., Donkin, E. F., Webb, E. C., & Etter, E. M. 2017. Somatic cell count thresholds in composite and quarter milk samples as indicator of bovine intramammary infection status. *The Onderstepoort Journal of Veterinary Research*, 84(1), 1-12.

Philpot, W. N. 1984. Economics of Mastitis Control. *Veterinary Clinics of North America: Large Animal Practice*, 6(2), 233-245.

Phuektes, P., Mansell, P. D., Dyson, R. S., Hooper, N. D., Dick, J. S., & Browning, G. F. 2001. Molecular epidemiology of *Streptococcus uberis* isolates from dairy cows with mastitis. *Journal of Clinical Microbiology, 39*(4), 1460-1466. doi: 10.1128/JCM.39.4.1460-1466.2001. PMID: 11283072; PMCID: PMC87955.

Pieper, M., Michalke, A., & Gaigler, T. 2020. Calculation of external climate costs for food highlights inadequate pricing of animal products. *Nature Communications*, 11(1), 6117.

Piepers, S. 2022. Dealing with the major culprit of the dairy cow: The mammary gland and the immune response. Hipra, S.A., Acda., La Selva 135 17170 Amer (Girona) Spain, pp.108-129.

Piepers, S., Prenafeta, A., Verbeke, J., De Visscher, A., March, R., & De Vliegher, S. 2017. Immune response after an experimental intramammary challenge with killed Staphalococcus aureu in cows and heifers vaccinated and not vaccinated with Startvac, a polyvalent mastitis vaccine. *Journal of Dairy Science, 100*, 769–782. https://doi.org/10.3168/jds.2016-11269.

Pitkala, A., Koort, J., & Bjorkroth, J. 2008. Identification and antimicrobial resistance of *Streptococcus uberis* and *Streptococcus parauberis* isolated from bovine milk samples. *Journal of Dairy Science, 91*, 4075–4081.

© University of Pretoria

Pol, M. & Ruegg, P. L. 2007. Treatment practices and quantification of antimicrobial drug usage in conventional and organic dairy farms in Wisconsin. *Journal of Dairy Science, 90*(1), 249 -261. <https://www.journalofdairyscience.org/action/showPdf?pii=S0022-0302%2807%2972626-7> [Viewed 19/12/2022]

Poutrel, B., Bareille, S., Lequeux, G., & Leboeuf, F. 2018. Prevalence of mastitis pathogens in France: antimicrobial susceptibility of *Staphylococcus aureus*, *Streptococcus uberis* and *Escherichia coli. Journal of Veterinary Science and Technology, 9*, 2.

Prendergast, R., Murphy, M. D., Buckley, F., & Upton, J. 2024. The effects of seasonality, management, infrastructure, and automation on the milking efficiency of herringbone and rotary milking parlors in Ireland. *Journal of Dairy Science, 107*(2), 917-932. ISSN 0022-0302, https://doi.org/10.3168/jds.2023-23540.

Pryce, J. & Daetwyler, H. 2012. Designing dairy cattle breeding schemes under genomic selection: a review of international research. *Animal Production Science* 52(3):107-114. https://doi.org/10.1071/AN11098.

Pullinger, G. D., Coffey, T. J., Maiden, M. C., & Leigh, J. A. 2006. Multilocus-sequence typing analysis reveals similar populations of *Streptococcus uberis* are responsible for bovine intramammary infections of short and long duration. *Veterinary Microbiology*, 119, 194–204. https://doi.org/10.1016/j.vetmic.2006.08.015.

Pyorala, S. 2003. Indicators of inflammation in the diagnosis of mastitis. *Veterinary Research*, 330(34), 565-578. https://www.vetres.org/articles/vetres/pdf/2003/05/V3507.pdf [Viewed 4/12/2021]

Rainard, P. & Riollet, C. 2006. Innate immunity of the bovine mammary gland. *Veterinary Research, 37*, 369-400.

© University of Pretoria

Rainard, P., Gilbert, F. B., Germon, P., & Foucras, G. 2021. Invited review: A critical appraisal of mastitis vaccines for dairy cows, *Journal of Dairy Science, 104*(10), 10427-10448.

Rajala-Schultz, P. J., Gröhn, Y. T., McCulloch, C. E., & Guard, C. L. 1999. Effects of clinical mastitis on milk yield in dairy cows. *Journal of dairy science, 82*(6), 1213-1220.

Rasmussen, M. D., Frimer, E. S., Galton, D. M., & Petersson, L. G. 1992. The influence of pre-milking teat preparation and attachment delay on milk yield and milking performance. *Journal of Dairy Science, 75*(8), 2131-2141. ISSN 0022-0302, https://doi.org/10.3168/jds.S0022-0302(92)77973-9.

Reinoso, E. B., Lasagno, M. C., Dieser, S. A. &., & Odierno, L. M. 2011. Distribution of virulence-associated genes in *Streptococcus uberis* isolated from bovine mastitis. *FEMS Microbiology Letters*, 318, 183–188.

Reyes, J., Rodriguez-Lecompte, J. C., Blanchard, A., McClure, J. T., & Sánchez, J. 2019. Molecular variability of *Streptococcus uberis* isolates from intramammary infections in Canadian dairy farms from the Maritime region. *Canadian Journal of Veterinary Research. 83*(3):168-176. PMID: 31308588; PMCID: PMC6587884.

Rhoda, D. A. & Pantoja, J. C. F. 2012. Using Mastitis Records and Somatic Cell Count Data*. Veterinary Clinics of North America: Food Animal Practice*, 28(2), 347-361. ISSN 0749-0720, https://doi.org/10.1016/j.cvfa.2012.03.012.

Rienesl, L., Marginter, M., Stückler, P., Köck, A., Egger-Danner, C., & Sölkner, J. 2022. Use of differential somatic cell count, somatic cell score, and milk mid-infrared spectral analysis for monitoring mastitis in dairy cows during routine milk recording. *Livestock Science,* 264, 105050. ISSN 1871-1413, https://doi.org/10.1016/j.livsci.2022.105050.

© University of Pretoria

Rollin, E., Dhuyvetter, K. C., & Overton, M. W. 2015. The cost of clinical mastitis in the first 30 days of lactation: An economic modelling tool. *Preventive veterinary medicine,* 122(3), 257-264. doi: 10.1016/j.prevetmed.2015.11.006.

Ruegg, P. L. 2012a. New perspectives in udder health management. *Veterinary Clinics: Food Animal Practice,* 28(2), 149-163.

Ruegg, P. L. 2012b. Premiums, Production and Pails of Discarded Milk How Much Money Does Mastitis Cost You? *Resources Milk Money*, 3, 50-56. University of Wisconsin, Madison.

Ruegg, P. L. 2017. A 100-Year Review: Mastitis detection, management, and prevention. *Journal of dairy science, 100*(12), 10381-10397.

Saari, U.A., Damberg, S., Frömbling, L., & Ringle, C.M. 2021. Sustainable consumption behavior of Europeans: The influence of environmental knowledge and risk perception on environmental concern and behavioral intention, *Ecological Economics*, 189. https://doi.org/10.1016/j.ecolecon.2021.107155.

Saed, H. A. E. R. & Ibrahim, H. M. M. 2020. Antimicrobial profile of multidrug-resistant Streptococcus spp. isolated from dairy cows with clinical mastitis. *Journal of Advanced Veterinary and Animal Research, 7*(2), 186–197. https://doi.org/10.5455/javar.2020.g409.

Sampimon, O., Barkema, H. W., Berends, I., Sol, J., & Lam, T. 2009. Prevalence of intramammary infection in Dutch dairy herds. *Journal of Dairy Research, 76*(2), 129-136.

Schellack, N., Benjamin, D., Brink, A., Duse, A., Faure, K., Goff, D., Mendelson, M., Meyer, J., Miot, J., Perovic, O., Pople, T., Suleman, F., Van Vuuren, M., & Essack, S. 2017. A situational analysis of current antimicrobial governance, regulation, and

© University of Pretoria

utilization in South Africa. *International Journal of Infectious Diseases, 64*, 100-106. ISSN 1201-9712, https://doi.org/10.1016/j.ijid.2017.09.002.

Schingoethe, D. J. 2017. A 100-Year Review: Total mixed ration feeding of dairy cows. *Journal of Dairy Science,* 100(12), 10143-10150. https://doi.org/10.3168/jds.2017-12967.

Schmidt, T., Kock, M. M., & Ehlers, M. M. 2017. Molecular characterization of *Staphylococcus aureus* isolated from bovine mastitis and close human contacts in South African dairy herds: genetic diversity and inter-species host transmission. *Frontiers in microbiology,* 8, 511. doi:10.3389/fmicb.2017.00511.

Schukken, Y. H., Wilson, D. J., Welcome, F., Garrison-Tikofsky, L., & Gonzalez, R. N. 2003. Monitoring udder health and milk quality using somatic cell counts. *Veterinary Research*, 2(34), 579 - 596.

Schwarz, D., Kleinhans, S., Witzel, G., Stückler, P., Reith, R., & Danø, S. 2023. Usefulness of the total and differential somatic cell count based udder health group concept for evaluating herd management practices and udder health in dairy herds. *Preventive Veterinary Medicine*, 218, 105977. ISSN 0167-5877, https://doi.org/10.1016/j.prevetmed.2023.105977.

Sewell, S. 2021. Small farms vanish every day in America’s dairy land: There ain’t no future in dairy. *The Guardian*. <https://www.theguardian.com/environment/2021/jul/21/small-farms-vanish-every-day-in-americas-dairyland-there-aint-no-future-in-dairy> [Viewed 15 June 2023].

Sherwin, V. E., Egan, S. A., Green, M. J., & Leigh, J. A. 2021. Survival of *Streptococcus uberis* on bedding substrates. *The Veterinary Journal, 276*, 105731. ISSN 1090-0233, https://doi.org/10.1016/j.tvjl.2021.105731.

© University of Pretoria

Shim, E. H., Shanks, R. D., & Morin, D. E. 2004. Milk loss and treatment costs associated with two treatment protocols for clinical mastitis in dairy cows. *Journal of Dairy Science, 87*(8), 2702–2708. https://doi.org/10.3168/jds.S0022-0302(04)73397-4

Shree, P., Singh, C. K., Sodhi, K. K., Surya, J. N., & Singh, D. K. 2023. Biofilms: Understanding the structure and contribution towards bacterial resistance in antibiotics. *Medicine in Microecology*, 16. https://doi.org/10.1016/j.medmic.2023.100084

Sipka, A., Wieland, M., Biscarini, F., Rossi, R. M., Roman, N., Santisteban, C., Moroni, P., & Nydam, D. V. 2021. Short Communication: Comparative performance of 3 on-farm culture systems for detection of mastitis pathogens interpreted by trained and untrained observers. *Journal of Dairy Science, 104*, 4936–4941.

Srithanasuwan, A., Pangprasit, N., & Suriyasathaporn, W. 2022. Comparison of virulence patterns between *Streptococcus uberis* causing transient and persistent intramammary infection. *Frontiers in Veterinary Science, 9*, 806674.

Stevens, M., Piepers, S., & De Vliegher, S. 2016. Mastitis prevention and control practices and mastitis treatment strategies associated with the consumption of (critically important) antimicrobials on dairy herds in Flanders, Belgium. *Journal of Dairy Science,* 99(4), 2896-2903. <https://www.journalofdairyscience.org/action/showPdf?pii=S0022-0302%2816%2900148-X> [Viewed 18/12/2021]

Sun, L., Lundh, Å., Höjer, A., Bernes, G., Nilsson, D., Johansson, M., Hetta, M., Gustafsson, A. H., Saedén, K. H., & Dicksved, J. 2022. Milking system and premilking routines have a strong effect on the microbial community in bulk tank milk. *Journal of Dairy Science*, 105(1), 123-139. ISSN 0022-0302, https://doi.org/10.3168/jds.2021-20661.

© University of Pretoria

Suntharalingam, P., & Cvitkovitch, D. G. 2005. Quorum sensing in streptococcal biofilm formation. *Trends in microbiology*, *13*(1), 3–6. https://doi.org/10.1016/j.tim.2004.11.009

Teagasc/IMQCS Recommendations for the installation and testing of milking machines, Editor Egan, M.

Theron, H. E. & Mostert, B. E. 2010. Production and breeding performance of South African dairy herds. *South African Journal of Animal Science.* 39. 10.4314/sajas.v39i1.61324.

Tiedje, J. M., Fu, Y., Mei, Z., Schäffer, A., Dou, Q., Amelung, W., Elsner, M., Adu-Gyamfi, J., Heng, L., Virta, M., Jiang, X., Smidt, H., Topp, E., & Wang, F. 2023. Antibiotic resistance genes in food production systems support One Health opinions. *Current Opinion in Environmental Science & Health,* 34. https://doi.org/10.1016/j.coesh.2023.100492.

Tomita, T., Meehan, B., Wongkattiya, N., Malmo, J., Pullinger, G., Leigh, J., & Deighton, M. 2008. Identification of *Streptococcus uberis* multilocus sequence types highly associated with mastitis. *Applied and Environmental Microbiology*. 74(1):114-24. doi: 10.1128/AEM.01373-07. Epub 2007 Nov 16. PMID: 18024686; PMCID: PMC2223209.

Tyler, J.W., Cullor, J.S., & Ruffin. D.C. 1993. Immunization and Immunotherapy for Mastitis, *Veterinary Clinics of North America: Food Animal Practice*, Volume 9, Issue 3, 537-549, https://doi.org/10.1016/S0749-0720(15)30620-4.

Van Dyk, B. N., De Bruin, W., Du Plessis, E. M., & Korsten, L. 2015. Microbiological Food Safety Status of Commercially Produced Tomatoes from Production to *Marketing. Journal of Food Production ,79*(3), 392-406. https://doi:10.4315/0362-028X.JFP-15-300.

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Van Dyk, B. N., De Bruin, W., Du Plessis, E. M., & Korsten, L. 2016. Microbiological food safety status of commercially produced tomatoes from production to marketing. *Journal of Food Protein*, 79, 392-406. https://doi.org/10.4315/0362-028X.JFP-15-300

Van Lelyveld, G. 2020. The Chemistry behind teat dips and the art in using them properly. *The Review/Oorsig,* 24(5), 7-17. <https://online.fliphtml5.com/icpyl/nzsa/#p=7> [Viewed 19/12/2021]

Van Lelyveld, G. 2022a. Problem solving using somatic cell counts in dairy animals. *The review/Oorsig*, 26(1), 10–21.

Van Lelyveld, G. 2022b. Intramammary Antibiotics: Are we using them correctly? *The Review/Oorsig*, 26(2), 9-22 <https://online.fliphtml5.com/icpyl/iggk/#p=9>

Van Lelyveld, G. 2022c. Milking Machine - The history and functionality (Harvesting Milk). *The Review/Oorsig,* 26(4), 9–15. <https://online.fliphtml5.com/icpyl/rkwk/#p=9>

Van Lelyveld, G. 2022d. Milking Machine - Problem Solving (Parlour Assessment). *The Review/Oorsig*, 26(5), 10–17. <https://online.fliphtml5.com/icpyl/cqrg/#p=10>

Van Lelyveld, G. 2023. The Milking Machine -Problem Solving (Listen to the cow). *The Review/Oorsig,* 27(1), 4–12. <https://online.fliphtml5.com/icpyl/qknv/>

Van Lelyveld, G. K. 2021a. An “analysis” of mastitis Part 1 Understanding Data. *The Review/Oorsig,* 25(4), 6-8. <https://online.fliptml5.com/icpyl/sdot/#p=6> [Viewed 19/04/2022].

Van Lelyveld, G. K. 2021b. An “analysis” of mastitis Part 2 Management tool to analyse udder health. *The Review/Oorsig*, 25(5), 6-17. <https://online.fliptml5.com/icpyl/hwry/#p=6> [Viewed 19/04/2023]

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Van Werven, T. 2018. Managing Dairy Cows with Less Antibiotics! *WCDS Advances in Dairy Technology*, 30, 37-47. <https://wcds.ualberta.ca/wcds/wp-content/uploads/sites/57/2018/05/p-037-050-van-Werven-WCDS-2018-Managing-Dairy-Cows-with-Less-Antibiotics.pdf> [Viewed 27/06/2022].

Vasquez, A. K., Nydam, D. N., Capel, M. B., Eicker, S., & Virkler, P. D. 2017. Clinical outcome comparison of immediate blanket treatment versus a delayed pathogen-based treatment protocol for clinical mastitis in a New York dairy herd. *Journal of Dairy Science, 100*(4), 2992–3003. [Viewed 28/12/2022].

Vermaak, P., Petzer, I. M., & Karzis, J. 2022. Effects of milking machine settings and teat liners on bovine udder health. *South African Journal of Animal Science, 52*(4), 421-432. <http://dx.doi.org/10.4314/sajas.v53i4.02>

Verraes, C., Van Boxstael, S., Van Meervenne, E., Van Coillie, E., Butaye, P., Catry, B., De Schaetzen, M. A., Van Huffel, X., Imberechts, H., Dierick, K., & Daube, G. 2013. Antimicrobial resistance in the food chain: a review. *International journal of environmental research and public health, 10*(7), 2643-2669. doi: 10.3390/ ijerph10072643.

Vezina, B., Al-harbi, H., Ramay, H. R., Soust, M., Moore, R. J., & Olchowy, T. W. J. 2021. Sequence characterisation and novel insights into bovine mastitis-associated *Streptococcus uberis* in dairy herds. *Science Report,* 11, pp. 3046. https://doi.org/10.1038/s41598-021-82357-3.

Viguier, C., Arora, S., Gilmartin, N., Welbeck, K., & O'Kennedy, R. 2009. Mastitis detection: current trends and future perspectives. *Trends in biotechnology*, 27(8), 486-493.

Vissio, C., Dieser, S. A., Agnelli, H. L., Odierno, L. M., & Larriestra, A. J. 2014. Accuracy of the composite somatic cell count to detect intra-mammary infection in

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dairy cows using latent class analysis. *Preventative Veterinary Medicine*, 113, 547–555.

Wang, L., Chen, W., Zhang, L., & Zhu, Y. 2013. Genetic diversity of *Streptococcus uberis* isolates from dairy cows with subclinical mastitis in Southern Xinjiang Province, China. *Journal of General Applied Microbiology*, 59, 287-293.

Watts, J. L. 1988. Etiological Agents of Bovine Mastitis. *Veterinary Microbiology*, 16, 41-66.

Wellnitz, O. & Bruckmaier, R. M. . 2012. The innate immune response of the bovine mammary gland to bacterial infection. *The Veterinary Journal, 192*(2), 148-152.

Werner, C., Sauerwald, C., Sundrum, A., El-Sayed, A., & Zschöck, M. 2018. Genotyping of *Streptococcus uberis* isolates in healing process of bovine clinical mastitis. *International Journal of Veterinary Science and Medicine, 6*(2), 274-278. doi: 10.1016/j.ijvsm.2018.09.005. PMID: 30564609; PMCID: PMC6286412.

Willett, W., Rockström, J., Loken, B., Springmann, M., Lang, T., Vermeulen, S., Garnett, T., Tilman, D., DeClerck, F., Wood, A., Jonell, M., Clark, M., Gordon, L. J., Fanzo, J., Hawkes, C., Zurayk, R., Rivera, J. A., De Vries, W., Majele Sibanda, L., Afshin, A., … Murray, C. J. L. 2019. Food in the Anthropocene: the EAT-Lancet Commission on healthy diets from sustainable food systems. *Lancet* (London, England), 393(10170), 447–492. https://doi.org/10.1016/S0140-6736(18)31788-4

Wilson, D. J., Gonzalez, R. N., Case, K. L., Garrison, L. L., & Gröhn, Y. T. 1999. Comparison of seven antibiotic treatments with no treatment for bacteriological efficacy against bovine mastitis pathogens. *Journal of Dairy Science, 82*(8), 1664–1670. [Viewed 29/12/2022].

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Wolfová, M., Wolf, J., Kvapilík, J., & Kica, J. 2007. Selection for profit in cattle: I. Economic weights for purebred dairy cattle in the Czech Republic. *Journal of Dairy Science, 90*(5), pp. 2442-2455.

Yang, W.-T., Ke, C.-Y., Wu, W.-T., Lee, R.-P. 1., & Tseng, Y.-H. 2019. Effective treatment of bovine mastitis with intramammary infusion of Angelica dahurica and Rheum officinale extracts. Hindawi, Evidence-Based Complementary and Alternative Medicine, Article ID 7242705, 8 pages. <https://doi.org/10.1155/2019/7242705> or <https://www.hindawi.com/journals/ecam/2019/7242705/> [Viewed 18/12/2021].

Youngerman, S. M., Saxton, A. M., Oliver, S. P., & Pighetti, G. M. 2004. Association of CXCR2 polymorphisms with subclinical and clinical mastitis in dairy cattle. *Journal of Dairy Science, 87*(8), 2442-2448.

Zadoks, R. N. 2007. Sources and epidemiology of *Streptococcus uberis*, with special emphasis on mastitis in dairy cattle. CAB Reviews: Perspectives in agriculture. *Veterinary Science*, Nutrition and Natural Resources, 2, pp. 30.

Zadoks, R. N., Allore, H. G., Barkema, H. W., Sampimon, O. C., Gröhn, Y. T., & Schukken, Y. H. 2001. Analysis of an outbreak of *Streptococcus uberis* mastitis. *Journal of Dairy Science, 84*(3), 590-599.

Zhang, H., Feng, Y., Xin-pu, L., Jin-yin, L., Ling, W., Yu-long, Z., Yong, Y., Xu-rong, W., & Hong-sheng, L. 2020. Detection of antimicrobial resistance and virulence-related genes in *Streptococcus uberis* and *Streptococcus parauberis* isolated from clinical bovine mastitis cases in north western China. *Journal of Integrative Agriculture, 19*(11), 2784–2791. doi: 10.1016/S2095-3119(20)63185-9.

Zhu, A., Yang, B., Wang, Z., & Liu, Y. 2023. Augmented dissemination of antibiotic resistance elicited by non-antibiotic factors. *Ecotoxicology and Environmental Safety*, 262.

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Appendix 1: Permission to conduct research

Directorate Animal Health, Department of Agriculture, Land Reform & Rural Development
Private Bag X138, Pretoria 0001

Enquiries: Ms Mama Laing • Tel: +27 12 319 7632 • Fax: +27 12 319 7470 • E-mail: MarnaL@dalrrd.gov.za
Reference: 12/11/1/1/6 (2023 LH)

Dr Grant Kevin van Lelyveld
Department of Production Animal Studies
Faculty of Veterinary Sciences
University of Pretoria
Onderstepoort
Tel:
E-mail: grantvanlelyveld@gmail.com

Dear Dr van Lelyveld,

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Your undated application, received by us on 7 March 2023, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. This permission is given upon finding the biosecurity of the research project as described to be acceptable to DALRRD;
3. The research project is approved as per the application form received on 7 March 2023 and any correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this research project under this Section 20 permit. Please apply in writing to MarnaL@dalrrd.gov.za;
4. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 permit. Please apply in writing to MarnaL@dalrrd.gov.za;
5. No part of this research project may begin until the valid ethical approval has been obtained in writing from the relevant South African authority;

SUBJECT: S20 PERMISSION FOR: Gene analysis of *Streptococcus uberis* strains, isolated in a longitudinal study from milk samples of a South African dairy herd

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6. Only *Streptococcus uberis* isolates (isolated from bovine milk samples) currently stored at the DALRRD approved Milk Laboratory biobank of the University of Pretoria Faculty of Veterinary Sciences may be used in this study;
7. The research project may only be done at the DALRRD approved Milk Laboratory of the University of Pretoria Faculty of Veterinary Sciences, as per the application form;
8. Samples for molecular characterisation may be sent to Inqaba Biotec;
9. Samples must be packaged and transported in accordance with International Air Transport Association (IATA) requirements and/or the National *Road Traffic Act*, 1996 (*Act* No. 93 of 1996);
10. All potentially infectious material utilised or generated during or by the research project, is to be destroyed at completion of the study. No samples may be stored after the completion of the study;
11. Only a waste disposal company registered for the disposal of biohazardous waste may be used for the removal of all potentially infectious waste from the research project; Records must be kept for five years for auditing purposes;
12. Samples may not be outsourced for research without prior written approval from the Director: Animal Health.

Title of research/study: Gene analysis of Streptococcus uberis strains, isolated in a longitudinal study from milk samples of a South African dairy herd
Researcher: Dr Grant Kevin van Lelyveld
Institutions: University of Pretoria, Faculty of Veterinary Sciences, Production Animal Studies
Permit Expiry date: 31 December 2023
Our ref Number: 12/11/1/1/8 (2823 LH)
Your ref:

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2023 -04- 06

SUBJECT: S20 PERMISSION FOR: Gene analysis of Streptococcus uberis strains, isolated in a longitudinal study from milk samples of a South African dairy herd

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Appendix 2: Communication from Agriculture, Land Reform and Rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development
Private Bag X138, Pretoria 0001

Enquiries: Ms Marna Laing • Tel: +27 12 319 7442 • Fax: +27 12 319 7470 • E-mail: MarnaL@Dalrrd.gov.za
Reference: 12/11/1/1/8 (5562 CMo)

Responsible person: Dr Grant Kevin van Lelyveld
Institution: Department of Anatomy and Physiology
Faculty of Veterinary Science
University of Pretoria
Old Soutpan Road, Onderstepoort, 0110
Tel: 012 529 8405
Email: grantvanlelyveld@gmail.com; joanne.karzis@up.ac.za

Dear Dr Grant Kevin van Lelyveld,

RE: AMENDMENT OF SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) – EXTENSION OF THE EXPIRY DATE

Title of research project / study: "Gene analysis of *Streptococcus uberis* strains, isolated in a longitudinal study from milk samples of a South African dairy herd.

An amendment is hereby granted on the Section 20 approval with reference number 12/11/1/1/8 (2923 LH) that was issued for the above-mentioned study on 2023-04-06.

i) The validity of the section 20 approval is extended to 31 December 2027;

All other conditions as specified in the Section 20 approval with reference number 12/11/1/1/8 (2923 LH) of 2023-04-06 remain in full effect. This includes the validity of laboratory approvals in terms of SANAS and DALRRD.

Kind regards,

DIRECTOR: ANIMAL HEALTH
Date: 2024 -07- 1 8

– 1 –

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Appendix 3: Schedule of accreditation

Facility Number: V0030

ANNEXURE A

SCHEDULE OF ACCREDITATION

Facility Number: **V0030**

Permanent Address of Laboratory:
University of Pretoria
Milk Laboratory, Faculty of Veterinary Science
Pathology Building
Old Soutpan Road, Onderstepoort
0110

Technical Signatories:
Ms R Badenhorst
Ms D Nchabo
Dr R Magadu
Ms N Mmamo

Postal Address:
Private Bag X04
Onderstepoort
0110

Nominated Representative:
Mr Nazeem Cassim

Tel: (012) 529 8368
Fax: (012) 529 8410
E-mail: nazeem.cassim@up.ac.za

Issue No.: 07
Date of Issue: 23 September 2024
Expiry Date: 19 June 2026

Material or Products Tested	Type of Tests / Properties measured, Range of measurement	Standard Specifications, Techniques / Equipment Used
CYTOLOGY		
Raw Milk	Somatic cell count	PAS/MET 001
MICROBIOLOGY		
Raw Milk	Brucella Milk Ring Test	PAS/MET 015

Original Date of Accreditation: 20 June 2016

ISSUED BY THE SOUTH AFRICAN NATIONAL ACCREDITATION SYSTEM

Accreditation Manager

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Appendix 4: Certificate of approval

CERTIFICATE OF APPROVAL

This is to certify that:

University of Pretoria - Faculty of Veterinary Science

Milk Laboratory

DAH Approval Number:

DAH-24

is a DAH Approved Veterinary Laboratory
provided that all DAH conditions and requirements are complied with.
This certificate is valid as per scope on the accompanying schedule of approval,
bearing the above approval number, for the following sections:

Microbiology

The Laboratory complies with the requirements of PM 2018.

Director: Animal Health

Initial DAH Approval Date: 2010-10-11
Certificate Commences: 2023-02-27
Certificate Expires: 2025-02-27

This certificate is only valid when accompanied by its schedule of approval.

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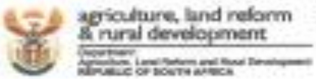
Appendix 5: Schedule of approval



DIRECTORATE ANIMAL HEALTH

CERTIFICATE OF APPROVAL
FOR DAH APPROVED VETERINARY
LABORATORIES

FORM: S 2018

SCHEDULE OF APPROVAL

DAH Approval Number: DAH-24 **Date: 2023-02-27**

DETAILS OF LABORATORY		
Physical Address of Laboratory:	University of Pretoria - Faculty of Veterinary Science Milk Laboratory Department of Production Animal Studies Milk Laboratory Faculty of Veterinary Science University of Pretoria Onderstepoort 0110	
Postal Address:	Private Bag X04 Onderstepoort 0110	
Tel:	0125298461	
Fax:	0125298410	
E mail:	inge-marie.petzer@up.ac.za / joanne.karzis@up.ac.za	
Head of Laboratory:	Dr Inge-Marie Petzer	
Contact Person:	Joanne Karzis	
MATERIALS AND/OR PRODUCTS TESTED	**TYPES OF TESTS**	**METHODS USED**
Microbiology Raw Milk	Milk Ring Test	PAS/MET 015

DAH Auditor

Revised by: J Koch; K Raseleka and R Theron	Authorised by: Director: DAH	Authorisation Date: March 2018	Controlled Document Page 1 of 1

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Appendix 6: Recommendation report

DIRECTORATE ANIMAL HEALTH

RECOMMENDATION REPORT

FORM:
RR 2018

RECOMMENDATION REPORT	**DAFF NO:** N/A
THIS REPORT COVERS DAFF APPROVAL / COMPLIANCE	
Date of Visit:	24 February 2022
Laboratory:	University of Pretoria (UP) Milk Laboratory Biobank
Location:	Onderstepoort Pretoria, Gauteng Province
DAFF Auditors:	Riette Theron, Keneilwe Raseleka, Zandile Mbonxa
OUTCOME OF THIS VISIT	

A Biobank Compliance inspection was carried out since the Recommendation Report expired September 2020. An extension of the DAH Biobank Compliance status was granted in response to the Covid-19 pandemic and travel restrictions put in place by the government to prevent the spread of the SARS-coV-2. This made it possible to continue using samples from the Biobank for research purposes.

The Checklist: Biobank (FORM: DAFF BB) was utilised.

Important matters to be addressed: (addressed on 19 May 2023, *italics* & **bold**)

- The laboratory's documented procedures on equipment service and decontamination must be updated to include the decontamination procedure for pipettes before sending them for service. The updated SOP must be supplied as evidence; *Clarification is required on the F10 product range, concentration and contact time for equipment decontamination;*
  Cleared.
- Inspection records of safety audits were not available. The latest safety audit records must be sent to DAH;
  Cleared. Evidence emailed 07 March 2023.

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DIRECTORATE ANIMAL HEALTH FORM: RR 2018

RECOMMENDATION REPORT

Proposed Recommendations:

When all matters have been adequately addressed within three (3) months permission will be given for storage of samples related to Section 20 projects.

Thereafter future Section 20 applications will be considered for the next two years without an audit of the biobank and biosafety / biosecurity.

Please note that a Section 20 application is required for each research project.

	Name:	Signature:	Date:
Recommendation proposed by:	Auditor/s **Riette Theron** **Zandile Mbonxa** **Keneilwe Raseleka**	[signatures]	2023 -05- 19
Recommendation approved by:	Director: DAH **Dr Mpho Maja**		

The Directorate Animal Health would like to appreciate your hard work and dedication. You truly take pride in your work! Please keep up the good work.

Revised by:	Authorised by:	Authorisation Date:	Controlled Document
J Koch; K Raseleka and R Theron	Director: DAH	June 2018	Page 2 of 2

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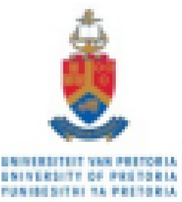
Appendix 7: Research Ethics Committee approval



Faculty of Veterinary Science
Animal Ethics Committee

5 June 2023

Approval Certificate
New Application

AEC Reference No.:	REC040-23
Title:	Molecular characterization of Streptococcus uberis strains, isolated in a longitudinal study from milk samples of a large commercial dairy herd in South Africa
Researcher:	Dr J Karzis
Student's Supervisor:	Prof I Petzer

Dear Dr J Karzis,

The **New Application** as supported by documents received between 2023-04-12 and 2023-05-29 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2023-05-29.

Please note the following about your ethics approval:

1. The use of species is approved:

Samples	Number
- Bacterial culture, isolated from milk samples (Stored- Historic/Retrospective)	74

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2024-06-05.
3. Please remember to use your protocol number (REC040-23) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8434
Fax +27 12 529 8321
Email: marleze.rheeder@up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Disaense tša Bongakadiruiwa

© University of Pretoria

We wish you the best with your research.

Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8434
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Fakulteit Veeartsenykunde
Lefapha la Disaense tša Bongakadiruiwa

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Appendix 9: Faculty of Veterinary Science – Animal Ethics Committee approval certificate – annual renewal

Faculty of Veterinary Science
Animal Ethics Committee

05 August 2024

Approval Certificate
Annual Renewal

AEC Reference No.: REC040-23 Line 1
Title: Molecular characterization of Streptococcus uberis strains, isolated in a longitudinal study from milk samples of a large commercial dairy herd in South Africa
Researcher: Dr J Karzis
Student's Supervisor: Prof I Petzer

Dear Dr J Karzis,

The **Annual Renewal** as supported by documents received between 2024-07-15 and 2024-07-30 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2024-07-30.

Please note the following about your ethics approval:

1. The use of species is approved:

Samples	Approved
Cattle - Bacterial culture, isolated from milk samples - South Afri - Stored	74

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2025-08-05.
3. Please remember to use your protocol number (REC040-23) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. All incidents must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

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Fakulteit Veeartsenykunde
Lefapha la Disaense tša Bongakadiruiwa

© University of Pretoria

We wish you the best with your research.

Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

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Tel +27 12 529 8434
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Fakulteit Veeartsenykunde
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Appendix 10: Editing certificate

Nr: 202916

ACADEMIC AND PROFESSIONAL EDITING SERVICES (PTY) LTD (2024/824663/07)

LANGUAGE EDITING CERTIFICATE

Report title (Article):	MOLECULAR CHARACTERISATION OF STREPTOCOCCUS UBERIS STRAINS, ISOLATED IN A LONGITUDINAL STUDY FROM MILK SAMPLES OF A LARGE COMMERCIAL DAIRY HERD IN SOUTH AFRICA
Author/s:	Grant Kevin van Lelyveld
Institution:	The University of Pretoria
Date Issued:	27 January 2025

This document certifies that the above manuscript was edited for proper English language, grammar, punctuation, spelling, and overall style. Neither the research content nor the author's intentions were altered in any way during the editing process. Documents receiving this certification should be English-ready for publication; however, the author has the ability and choice to accept or reject our suggestions and changes.

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Warm regards

SAFREA regional committee member (2020 & 2023); Represented South Africa in the EFA International Editors' Conference – Chicago: August 2019 https://www.the-efa.org/efas-2019-conference-announcement/

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