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Alternative Treatments for Bovine and Human Mastitis

A Thesis presented to the National University of Ireland for the degree of

Doctor of Philosophy

by

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

Signed: _____ Date: _____

Angeliki Angelopoulou

List of Publications

Chapter 1: Non-antibiotic microbial solutions for bovine mastitis – live biotherapeutics, bacteriophage, and phage lysins. Critical Reviews in Microbiology, DOI: 10.1080/1040841X.2019.1648381.

Chapter 2: The microbiology and treatment of human mastitis. Medical Microbiology and Immunology, 2018;207(2): 83-94. doi: 10.1007/s00430-017-0532-z.

Chapter 3: Bovine mastitis is a polymicrobial disease requiring a polydiagnostic approach. International Dairy Journal, 2019;99: 104539. doi: 10.1016/j.idairyj.2019.104539

Chapter 4: Subclinical mastitis, a frequent but misidentified disease in lactating women. Pediatrics. Submitted

Chapter 5: Vancomycin and Nisin A are effective against biofilms of multi-drug resistant *Staphylococcus aureus* isolates from human milk. PLoS One. Submitted

Chapter 6: Diverse bacteriocins produced by strains from the human milk microbiome. Frontiers in Microbiology. Submitted

Vouloir c’est pouvoir

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Acronym List

AMR – Antimicrobial Resistance

AOI –Area of Interest

APC – APC Microbiome Ireland Culture Collection

ATCC – American Type Culture Collection

BA – Blood agar

Bacteriophage – Phage

BAs – Biogenic Amines

BHI – Brain Heart Infusion

bMEC – bovine Mammary Epithelial Cells

BP – Baird Parker

BSA – Bovine Serum Albumin

BSAC – British Society for Antimicrobial Chemotherapy

BTCEC – Bovine Teat Canal Epithelial Cells

CFS – Cell-Free Supernatant

$CHAP_K$ – Cysteine- and Histidine-dependent Amidohydrolase/Peptidase

CI – Confidence Interval

CLSM – Confocal Laser Scanning Microscopy

CM – Clinical Mastitis

CoNS – Coagulase-Negative Staphylococci

CV – Crystal Violet

DAMPs – Danger-associated Molecular Patterns

Dha – Dehydroalanine

Dhb – Dehydronoutyrine

DMSCC – Direct Microscopic Somatic Cell Count

DPC – Teagasc Culture Collection

DSM – Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures

EAD – Enzymatically Active Domain

ELISA – Enzyme-Linked Immunosorbent Assay

EUCAST – European Committee on Antimicrobial Susceptibility Testing

GBS – Group B Streptococci

GI – Gastrointestinal

GIT – Gastrointestinal Tract

H – Healthy

HIV – Human Immunodeficiency Virus

HPLC – High Performance Liquid Chromatography

HTS – High Throughput Sequencing

IFNγ – Interferon γ

Ig – Immunoglobin

IGM – Idiopathic Granulomatous Mastitis

IL-8 – Interleukin-8

LAB – Lactic Acid Bacteria

Lan – Lanthionine

LMG – Laboratorium voor Microbiologie, Universiteit Gent, Belgium

MALDI-TOF MS – Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry

McC – Mac Conkey

MeLan – β-methyllanthionine

MH – Mueller Hinton

MIC – Minimum Inhibitory Concentration

MLST – Multilocus Sequence Typing

MRD – Maximum Recovery Diluent

MRS – de Man, Rogosa, Sharpe

MRSA – Methicillin Resistant *S. aureus*

MSA – Multiple Sequence Alignment

NAGase – N-acetyl-b-D-glucosaminidase

NCTC – National Collection of Type Cultures, Public Health England

NFκB – Nuclear Factor kappa B

NGS – Next-Generation Sequencing

NIH – National Institute of Health

NRPS – Non-Ribosomal Peptide Synthetases

ORSA – Oxacillin Resistant *S. aureus*

OTU – Operational Taxonomic Unit

PBGC – Potential Bacteriocin Gene Cluster

PBS – Phosphate-Buffered Saline

PCA – Plate Count agar

PFGE – Pulsed-field Gel Electrophoresis

PI – Propidium Iodide

PKS – Polyketides

PMN – Polymorphonuclear Leucocytes

pvl – panton-valentine leucocidin

RANTES – Regulated on Activation Normal T Cell Expressed and Secreted

RiPPs – Ribosomally synthesized and Post-translationally modified Peptides

S – *Staphylococcus*

SAM – S-adenosylmethionine

SCC – Somatic Cell Counts

SLPI – Secretory Leukocyte Protease Inhibitor

SM – Subclinical Mastitis

SMRT – Single-Molecule Real-Time

spa – *S. aureus* staphylococcal protein

SSF – Single-Strip Factor

St – *Streptococcus*

TLR – Toll-like Receptor

TNFα – Tumour Necrosis Factor alpha

TSA – Tryptic Soy Agar

TSB – Tryptic Soy Broth

UPGMA – Unweighted Pair Group Method with Arithmetic Average

VISA – Vancomycin-Intermediate *S. aureus*

WGS – Whole Genome Sequencing

WHO – World Health Organization

MOI – Multiplicity of Infection

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Thesis Abstract

Mastitis, an inflammation of the mammary gland normally caused by infection, is a painful condition with welfare implications recorded both in cows and lactating women. The main treatment for mastitis is the administration of antibiotics for both species. However, there is a compelling need for novel alternative therapies, considering the antibiotic resistance crisis and concomitant problems in the treatment of human and animal infections. Bacteriocins, are a heterogeneous group of small, peptide-based bacterium antimicrobials that have either broad or narrow range inhibition spectra and as such are a viable alternative to antibiotics in some cases.

This thesis presents the current strategies to treat mastitis in cows and women. Chapter 1 provides an overview of alternative microbial treatments for bovine mastitis, with focus on probiotics, bacteriocins, phages, and phage endolysins. Chapter 2 examines the microbiology and treatment of human mastitis with *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Corynebacterium* sp. being identified as the major etiological agents in acute, subacute, and granulomatous mastitis, respectively. Moving forward, Chapter 3 sought to identify the major pathogenic species detected in fifty bovine mastitic milk samples by using cultivation and high-throughput sequencing (HTS). A combination of the two approaches illuminated the polymicrobial complexity of the disease and implied potential for multimicrobial origins of the symptoms. Chapter 4 investigated the milk microbiomes in healthy, subclinical and clinical lactating mothers and evaluated the immune status from these three groups with 37.8% of the asymptomatic women being subclinical. In Chapter 5 insights were provided into using alternative treatments, i.e. nisin A and vancomycin, on *S. aureus* for biofilm inhibition and eradication. None of the applied treatments

were able to eradicate preformed biofilms while the combination treatment significantly inhibited biofilm formation compared with single treatments. Finally, in Chapter 6, 80 strains with antimicrobial activity were isolated from 37 asymptomatic human milk samples. Genome sequencing and *in silico* analysis of those isolates led to identification of sixteen novel bacteriocins representing all known bacteriocin sub-classes. This study suggests that the milk microbiome is a rich source of strains with antimicrobial potential.

Overall, the results of this work expand the large body of research that exists in the field of mastitis by exploring the microbiota composition in both mastitic cows and humans and by investigating the pathogens involved and relating it to local immune responses. Furthermore, the efficacy of a bacteriocin as a co-therapy against a mastitis pathogen along with the discovery of novel antimicrobial peptides within the human milk microbiome, suggests that bacteriocins could provide novel therapies for disease treatment and their deployment in the medical sector in the future is a likely prospect.

Chapter 1

Non-antibiotic Microbial Solutions for Bovine Mastitis-Live Biotherapeutics, Bacteriophage, and Phage Lysins

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Authors Contributions

AA, AKW and RPR have drafted the manuscript. AKW, CH and RPR critically reviewed the manuscript. The final manuscript was read and approved by all authors.

Abstract

Bovine mastitis is a disease with a multi-etiological nature, defined as an inflammation of the udder. The main treatment for mastitis is the administration of antibiotics – usually directly to the udder. There is an urgent need for novel therapies to treat and prevent the disease, given the widespread emergence of antibiotic resistance and concomitant problems in the treatment of human and animal infections. We provide an overview of treatments for bovine mastitis, with emphasis on probiotics, bacteriocins, bacteriophages (phages), and phage endolysins. Probiotics have in recent years proved to be particularly efficacious in bovine mastitis treatment and prevention. In this case, the mode of action is most likely to be due to stimulation of the host immune response which clears the mastitis pathogen. Bacteriocins have the potential to be incorporated into teat washes and wipes, thus preventing pathogen spread on the farm. Phage therapy is limited by the inability of some phages to replicate in raw milk, as reported for some staphylococcal phages, and by their narrow host specificity. The use of phage endolysins is more promising, by enabling the development of broad host range potent antimicrobials, but additional research is required in terms of efficacy, safety, and production.

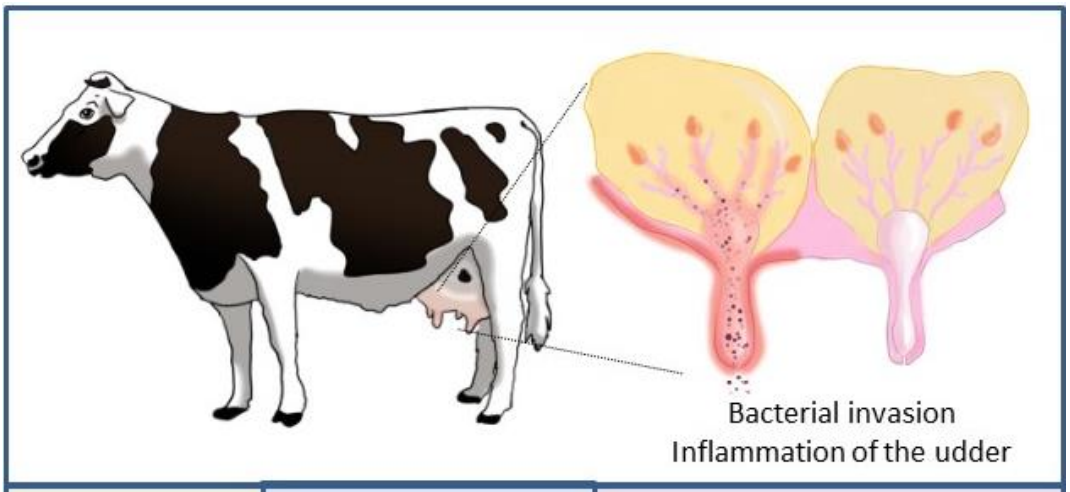
Introduction

Bovine mastitis, an inflammation of the udder, is a disease with a high prevalence which results in economic losses due to depleted milk production, discarded milk, premature culling, and treatment costs [Halasa et al., 2007; Bar et al., 2008; Hertl et al., 2010]. In 2015, an average case of clinical mastitis in the first 30 days of lactation was estimated to cost the US dairy producer $444 [Rollin et al., 2015]. Bovine mastitis can be classified into subgroups based on the clinical appearance (clinical or subclinical), with both being characterized by an increase in somatic cell count (SCC) [Vanderhaeghen et al., 2015]. A cow with a composite milk SCC $\geq$ 200,000 cells mL^{-1} is classed as suffering from mastitis. Milk of cows with clinical mastitis generally has obvious alterations such as flakes, clots, and a watery appearance and is usually accompanied by systemic symptoms in the animal such as fever, engorgement and frailty, while subclinical mastitis is denoted solely by an increase in SCC which can often be missed, leading to considerable cost ramifications, particularly as a result of increased cell count in bulk milk samples [Bogni et al., 2011]. The disease is the result of bacterial intramammary infection, with staphylococci, streptococci, and coliforms being the most often identified culprits [Bradley et al., 2007; Vanderhaeghen et al., 2015]. Furthermore, mastitis-causing organisms have been categorized as contagious or environmental based on their distribution and interplay with the teat and teat duct [Smith and Hogan, 1993]. In this respect, the mammary gland is the most common source of *Staphylococcus aureus* and *Streptococcus agalactiae* which are the main contagious bacteria. In contrast, the surroundings of the cow are the main source of environmental pathogens such as *Streptococcus uberis*, *Escherichia coli*, and *Klebsiella* sp. [Bogni et al., 2011].

Treatment of bovine mastitis usually involves the use of antibiotics, both for treatment and for prophylaxis at the drying off period. Pirlimycin, methicillin, cloxacillin, amoxicillin, novobiocin, penicillin G, dihydrostreptomycin, cephapirin, and erythromycin are all approved antibiotics for bovine mastitis treatment and prevention [Nader-Macías et al., 2013]. Another major disadvantage of using antibiotics to treat cows with mastitis is that milk has to be withheld for days due to antibiotic residues in milk. Additionally, the emergence of antibiotic resistance due to their indiscriminate overuse has become a pivotal issue [WHO, 2018]. These issues have driven the search for novel antimicrobial agents which can be used in the battle against bovine mastitis pathogens.

The aim of this review is to provide an overview of the latest findings in terms of microbe-based solutions for bovine mastitis, including probiotics, bacteriocins, bacteriophage (phage), and phage enzymes.

An overview of these potential alternative treatments and their mode of delivery is illustrated in Figure 1. The majority of studies performed to date have assessed the antimicrobial agents *in vitro* with only 21 of 58 studies evaluating the antimicrobials *in vivo* (Table 1). Moreover, very few studies have used a wide range of species to evaluate the inhibition spectra of the antimicrobial(s) tested (Table 1).



Bioactive	Mechanism(s)	Delivery method(s)
Probiotic	Immunomodulation Antimicrobial	Dip, Intra-mammary infusion
Bacteriocin	Immunomodulation Antimicrobial	Dip, Wipe, Wash, Intra-mammary infusion
Bacteriophage	Antimicrobial	Dip, Wash, Intra-mammary infusion
Endolysin	Antimicrobial	Dip, Wipe, Wash, Intra-mammary infusion

Figure 1.1. Alternative treatments and their potential modes of delivery for the prevention and treatment of bovine mastitis.

Table 1.1. Alternative treatments to antibiotics for prevention and/or treatment of bovine mastitis.

Category of alternative treatment	Treatment	Outcome	Tested *in vitro*	Number of species (strains) tested against	Tested *in vivo*/ number of animals participating	References
Probiotics	Cocktail of *L. acidophilus* & *L. casei*	↓ recovery compared to cephapirin in subclinical mastitis	na	na	26	Greene et al. (1991)
	L. lactis DPC 3147	= recovery compared to antibiotic in subclinical mastitis	na	na	12	Klostermann et al. (2008)
		= recovery compared to antibiotic in clinical mastitis	na	na	48	
		Live cells stimulated host intramammary immune system	na	na	3	Crispie et al. (2008)
		Live cells stimulated IL-1β and IL-8 in healthy cows	na	na	6	Beecher et al. (2009)
		Compared efficacy to Terrexine^{TM}; Viability of *L. lactis* up to 5 weeks	na	na	up to 19 quarters	Kitching et al. (2019)
	L. plantarum CRL *1716*	Probiotic inhibited 50% of pathogens and co-aggregated with majority of them; 35% adhesion ability to BTEC	+	12 (14)	-	Frola et al. (2012)
	L. perolens CRL *1724*	Probiotic inhibited 85.7% of pathogens and co-aggregated with all of them; 75% adhesion ability to BTEC; colonisation of healthy cows' udder	+	12 (14)	3	

(continued)

Table 1.1. Continued

Category of alternative treatment	Treatment	Outcome	Tested *in vitro*	Number of species (strains) tested against	Tested *in vivo*/ number of animals participating	References
	L. perolens CRL 1724	Probiotic colonised the udders of healthy cows, causing mild inflammation with no clinical signs	+	na	up to 5	Frola et al. (2013)
	Coctail of *L. perolens* CRL 1724 and *L. lactis* subsp. *lactis* CRL 1655	Probiotics stimulated IgG in healthy cows	-	na	12	Pellegrino et al. (2017)
	12 LAB strains	83.3% of LAB inhibited all 15 strains; 83% of LAB co-aggregated with the 15 strains: Distinct PFGE profiles; similar growth curves	+	12 (15)	-	Pellegrino et al. (2018)
	L. casei 667 *L. casei* BL23 *L. casei* 1542	Probiotic reduced pathogen adhesion and internalization in bMEC	+	1 (2)	-	Bouchard et al. (2013)
	L. lactis V7	Probiotic inhibited pathogen internalization but not adhesion to bMEC*;* ↑IL-8 in pluripotent stem cells under *E. coli* challenge	+	2 (4)	-	Assis et al. (2015)

(continued)

Table 1.1. Continued

Category of alternative treatment	Treatment	Outcome	Tested *in vitro*	Number of species (strains) tested against	Tested *in vivo*/ number of animals participating	References
	L. brevis 1595 *L. brevis* 1597 *L. plantarum* 1610	Modulation of immune response; Inhibition of mastitic pathogens invasion	+ + +	3 (3)	- - -	Bouchard et al. (2015)
	L. lactis LMG 7930 (nisin producer)	Probiotic inhibits majority of pathogens; low auto-aggregation; no co-aggregation; no effect on pathogen invasion	+	7 (10)	-	Armas et al. (2017)
	Cocktail of *L. plantarum* IMAU 80065 & *L. plantarum* IMAU 10155	Slight decrease in probiotic group; alterations in the microbiota of both groups	-	na	11	Yu et al. (2017)
	L. casei BL23	↓ Expression of pro-inflammatory cytokines; Defensin induction not drastically affected by *S. aureus*-stimulated bMEC	+	1	-	Souza et al. (2018)
	13 LAB strains	7 strains showed best adhesion and/biofilm formation	+	na	-	Wallis et al. (2018)
	5 LAB strains	All strains formed biofilms; *L. rhamnosus* ATCC 7469 & *L. plantarum* 2/37 replaced pathogenic staphylococcal biofilms	+	3	-	Wallis et al. (2019)

(continued)

Table 1.1 Continued

Category of alternative treatment	Treatment	Outcome	Tested *in vitro*	Number of species (strains) tested against	Tested *in vivo*/ number of animals participating	References
Bacteriocins	Nisin A	↓ of *S. aureus* and *E. coli;* no inflammation	-	5 (5)	up to 20	Sears et al. (1992)
	Nisin Z	↓NAGase activity and ↑recovery compared to no treatment in subclinical mastitis	-	na	90	Wu et al. (2007)
	Lysostaphin	Enzyme detaches biofilm and kills sessile cells	+	1 (up to18)	-	Ceotto-Vigoder et al. (2016)
	Nisin	Bacteriocin reduces cell viability				
	Nisin+lysostaphin	Treatment kills sessile cells				
	Nisin A producing lactococci	Inactivates majority of streptococci and staphylococci; ↓TNFα and ↑ NAGase when tested on bMEC	+	6 (25)	-	Malvisi et al. (2016)
	Lacticin 3147	Inhibition of *S. aureus* & *Streptococcus*	+	4 (24)	-	Ryan et al. (1998)
		↓ development of mastitis or pathogen shedding at *S. dysgalactiae* challenge	-	1 (1)	18	Ryan et al. (1999)
		↓ shedding in *S. aureus* DPC 5246 challenge	+	2 (4)	up to 16	Twomey et al. (2000)

(continued)

Table 1.1. Continued

Category of alternative treatment	Treatment	Outcome	Tested *in vitro*	Number of species (strains) tested against	Tested *in vivo*/ number of animals participating	References
		Inactivation of mastitic pathogens *in vitro* and *in vivo* challenge	+	3 (3)	8	Klostermann et al. (2010)
	Bacteriocin producing *S. aureus* A53	Inhibition of *S. aureus*	+	1 (65)	-	de Oliveira et al. (1998)
	CoNS bacteriocins	Inhibition of *L. monocytogenes* & *S. agalactiae*	+	12 (16)	-	dos Santos Nascimento et al. (2005)
	Aureocin A70, aureocin A53, aureocin 215FN; Pep5, epidermin, epilancin K7 & epicidin 280	Inhibition of *S. aureus* & *S. agalacatiae*	+	2 (239)	-	Varella Coelho et al. (2007)
	12 Staphylococcal bacteriocins	Inhibition of *Staphylococcus*	+	17 (41)	-	Ceotto et al. (2009)
	CoNS bacteriocin: *Staphylococcus chromogens* L217 producing Nukacin L217	Inhibition of majority of CoNS	+	6 (33)	-	Braem et al. (2014)
	CoNS bacteriocins	Inhibition of *S. aureus*	+	2 (2)	-	Carson et al. (2017)
	L. bulgaricus bacteriocin	Bactericidal against *S. aureus* & *S. agalactiae*	+	17 (17)	-	Kim et al. (2004)

(continued)

Table 1.1 Continued

Category of alternative treatment	Treatment	Outcome	Tested *in vitro*	Number of species (strains) tested against	Tested *in vivo*/ number of animals participating	References
	AS-48	*S. aureus* inhibition	+	15 (15)	5	Davidse et al. (2004)
	Macedocin ST91KM	Bactericidal to *S. agalactiae*, *S. uberis*, *S. dysgalactiae* & *S. aureus*	+	16 (28)	-	Pieterse et al. (2010)
	Morricin 269 Kurstacin 287 Kenyacin 404 Entomocin 420 Tolworthcin 524	*S. aureus* inhibition	+	1 (50)	-	Barboza-Corona et al. (2009)
	Bacillus sp. bacteriocin producing strains	*S. aureus* & *E. coli* inhibition	+	2 (2)	-	Maina et al. (2015)
Phages	Bacteriophage K	Anti-staphylococcal action; ↓ pathogen survival on skin	+	9 (53)	3 human fingers	O' Flaherty et al. (2005a)
		Unable to replicate in raw milk	+	1 (2)	-	O' Flaherty et al. (2005b)
		= recovery compared to placebo in subclinical mastitis; Failure to eliminate *S. aureus*	-	na	up to 24	Gill et al. (2006)
	DW2 & CS1	Inhibition of *S. aureus*; no change in SCC in healthy cows	+	1 (8)	10	O' Flaherty et al. (2005c)

(continued)

Table 1.1. Continued

Category of alternative treatment	Treatment	Outcome	Tested *in vitro*	Number of species (strains) tested against	Tested *in vivo*/ number of animals participating	References
	8 temperate phages	Inhibition of *S. aureus*	+	1 (14)	-	Garcia et al. (2009)
	SAP-1; SAP-3	Inhibition of bovine but not human *S. aureus*	+	9 (20)	-	Son et al. (2010)
	MSA6	Lytic against majority *S. aureus*	+	3 (53)	-	Kwiatek et al. (2012)
	10 phages		+	1 (20)	-	Dias et al. (2013)
	SPW		+	2 (5)	-	Li & Zhang. (2014)
	SA		+	4 (12)	-	Hamza et al. (2016)
	SA; SA2; SNAF		+	2 (11)	-	Tahir et al. (2017)
	λSA2 phage & B30 endolysin	Synergy of λSA2 &B30 in killing streptococci	+	3 (3)	up to 20 mice glands	Schmelcher et al. (2015)
	UFV13	Phage reduces *T. pyogenes* biofilm formation	+	1 (1)	-	da Silva Duarte et al. (2018a)
	Cocktail of 4 phages	Inhibition of *E. coli* in raw milk	+	1 (36)	-	Porter et al. (2016)
	Cocktail of EC6, EC9, EC11	Inhibition of *E. coli*	+	1 (18)	-	McLean et al. (2013)
	UFV13	10-fold decrease in *E. coli*	+	1 (1)	6 mice	da Silva Duarte et al. (2018b)
	ΦSA012; ΦSA013	Both phages killed all tested strains; ΦSA012 ↓ proliferation and inflammation in the mammary gland	+	93 *S. aureus* & 6 MRSA	up to 18 mice	Iwano et al. (2018)
	SAJK-IND; MSP	SAJK-IND was 100% lytic against *S. aureus*; MSP lysed 48% of *S. aureus* strains	+	120	-	Ganaie et al. (2018)

(continued)

Table 1.1. Continued

Category of alternative treatment	Treatment	Outcome	Tested *in vitro*	Number of species (strains) tested against	Tested *in vivo*/ number of animals participating	References
Phage endolysins	JX01	Lytic against *S. agalactiae*	+	62	-	Bai et al. (2013)
	phi11 endolysin	Lytic against CoNS	+	6 (6)	-	Donovan et al. (2006)
	λSA2-E-Lyso-SH3b; λSA2-E-LysK-SH3b	Inactivates *S. aureus* in murine mammary glands	+	1 (16)	up to 22 mice glands	Schmelcher et al. (2012b)
	$CHAP_K$	Elimination & prevention of staphylococcal biofilms	+	1 (2)	-	Fenton et al. (2013)
	LysKΔamidase	Inactivation of methicillin-resistant & methicillin-susceptible staphylococci	+	8 (148)	-	Zhou et al. (2017)

Probiotics

The WHO/FAO describes probiotics as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” [FAO/WHO, 2001; Hill et al., 2014]. Probiotic bacteria can be used to control inflammatory processes through antagonistic activities against etiological agents and via immunomodulation [Bermudez-Brito et al., 2012; Rainard and Foucras, 2018]. When used to treat bovine mastitis, the probiotic microorganisms are generally infused into the teat sinus of the mammary gland. However, more recently, a probiotic teat dip has been assessed [Yu et al., 2017].

Greene et al. [1991] evaluated whether intramammary infusions of lacto-bac, a commercial probiotic composed of *Lactobacillus casei* and *Lactobacillus acidophilus* (Lakeland Vet, Eden Prairie, MN, USA), could cure cows with subclinical mastitis. Of a total of 26 cows with high SCC (> 300,000 cells mL^{-1}), 13 received cephapirin treatment and 13 received lacto-bac with overall cure rates of infected quarters recorded as 73.7% and 21.7%, respectively. At the same time an increase in the SCC occurred in the lacto-bac group due primarily to an increase in SCC in uninfected, low SCC quarters. Thus, the probiotic increased SCC levels but had no impact on infection rate. A subsequent study by Klostermann et al. [2008] investigated whether *Lactococcus lactis* DPC 3147 was able to treat bovine recurrent subclinical or clinical mastitis in two separate field trials as compared to conventional antibiotic therapy. In the first trial (n = 11 quarters for each treatment group), focusing on chronic subclinical mastitis, two groups of 11 quarters received probiotic or antibiotic treatment. The antibiotic treatment was a commercial intramammary antibiotic formulation comprised of amoxicillin (200 mg), clavulanic acid (50 mg), and prednisolone (10

mg). Twelve days post application, 64% of quarters treated with *L. lactis* DPC 3147 were cured compared to 45.5% of quarters treated with antibiotics, with no change observed in the SCC regardless of treatment. In a second trial (n = 25 quarters for each treatment group), which focused on clinical mastitic cases, 60% of quarters were cured in the probiotic group after 14 days compared to 72% of quarters in the antibiotic group. The antibiotic treatment used in this trial was a commercial antibiotic formulation consisting of penethamate hydriodide (150 mg), dihydrostreptomycin (150 mg; as sulfate), framycetin sulfate (50 mg) and prednisolone (5 mg). The outcomes of these trials suggest that *L. lactis* DPC 3147 is as efficacious as antibiotic therapy, however, it should be emphasized that no negative (untreated) controls were included in these studies for welfare concerns. Crispie et al. [2008] demonstrated that recovery for this probiotic treatment correlates with stimulation of the host's intramammary immune system, suggesting that this may be the primary mechanism of action. Infusion of the live culture, but not the cell-free supernatant, into the mammary gland resulted in a substantial recruitment of polymorphonucleocytes and lymphocytes to the udder. A year later, Beecher et al. [2009] endeavored to further elucidate the immune response resulting from *L. lactis* DPC 3147 administration to the mammary gland of six healthy lactating cows. All animals developed transient signs of inflammation seven hours post infusion. Interleukin (IL)-1β and IL-8 were distinctly upregulated matching with high SCC suggesting that infusion of *L. lactis* DPC 3147 induces a short-lived yet substantial immune response which presumably is also effective against mastitic pathogens as well. More recently, Kitching and co-workers [2019] developed a formulation of *L. lactis* DPC 3147 in a liquid paraffin-based emulsion (bio-therapeutic) and compared it to the commercial antibiotic formulation Terrexine™ in order to treat mastitic cows. The bio-therapeutic described

in the study showed comparable efficacy to Terrexine™ which contains both cephalexin and kanamycin. Moreover, the investigators showed that the *L. lactis* cells within the above-described formulation, remained viable for up to five weeks, when the biotherapeutic was stored at 4, 22 or 37 ^{o}C.

Frola et al. [2012] studied the *in vitro* ability of *Lactobacillus perolens* CRL 1724 and *Lactobacillus plantarum* CRL 1716, to adhere to bovine teat canal epithelial cells (BTCEC) and to inhibit and co-aggregate with a selection of 14 mastitis pathogens. *L. perolens* CRL 1724 inhibited 85.7% of the tested pathogens while *L. plantarum* CRL 1716 inhibited 50% of the tested pathogens. Interestingly, *L. perolens* CRL 1724 co-aggregated with all of the tested pathogens whereas *L. plantarum* CRL 1716 did not co-aggregate with *Pseudomonas* sp. 224 and *E. coli* 345. *L. perolens* CRL 1724 exhibited higher (75%) efficacy of adhesion to BTCEC than *L. plantarum* CRL 1716 (37%). In a follow up *in vivo* study, *L. perolens* CRL 1724 was recovered from all tested quarters following intramammary infusion of 10^6 cfu mL^{-1} into bovine udders at drying-off, with an absence of clinical signs or teat injury [Frola et al., 2013]. In another study, Pellegrino et al. [2017] studied the immunomodulatory effect of *L. perolens* CRL 1724 and *Lactococcus lactis* subsp. *lactis* CRL 1655 at the drying-off period. Ten healthy cows were intramammarily inoculated with 10^6 cells of each bacterium and two cows were used as controls. The authors demonstrated that the examined strains increased the concentration of immunoglobulin (Ig) G isotopes in blood and milk and these isotopes were capable of recognizing *S. aureus* epitopes. Thus, these strains have potential as a prophylactic measure for bovine mastitis at the drying-off stage where their mechanism of action may be stimulation of local and systemic defence lines. More recently, Pellegrino et al. [2019] performed *in vitro* characterization of 12 lactic acid bacteria (LAB) strains previously isolated from

bovine milk as potential probiotic strains for the prevention of bovine mastitis. Two of the strains, *L. lactis* subsp. *lactis* CRL 1655 and *L. perolens* CRL 1724 were selected for further analysis based on their adhesion pattern to BTCEC, their ability to inhibit all tested pathogens and to co-aggregate with them. The two strains displayed differences in their Pulsed-field Gel Electrophoresis (PFGE) profiles and similar growth kinetics with the remainder of the 12 LAB.

Likewise, Bouchard et al. [2013] tested three *Lactobacillus casei* strains *in vitro*, including *L. casei* CIRM-BIA 1542 which was isolated from the bovine teat canal, and showed that they can hinder adhesion and internalization of *S. aureus* within bovine mammary epithelial cells (bMEC) without having an effect on bMEC physiology. A more recent study conducted by Assis et al. [2015], demonstrated that *Lactococcus lactis* V7 inhibited the internalization of two strains of *E. coli* and two of *S. aureus* into bMEC, whereas it hindered the adhesion of only one of the two *S. aureus* strains tested. When the bMEC immune response was investigated, *L. lactis* V7 was able to induce a slight increase in the production of the chemokine CXCL8 in bMEC and when cells had been challenged with the *E. coli* strains, V7 increased the inflammatory response. These findings suggest that *L. lactis* V7 could be used for the prevention of bovine mastitis.

In another study, Bouchard et al. [2015], isolated ten LAB strains and investigated their surface properties, their ability to inhibit mastitis-associated *S. aureus, S. uberis* and *E. coli*, their colonization capacities of bMEC and their immunomodulation properties. *Lactobacillus brevis* 1595, *L. brevis* 1597 and *Lactobacillus plantarum* 1610 demonstrated high colonization capacities and a medium surface hydrophobicity suggesting them as good candidates to compete with pathogens for mammary gland colonization.

Armas et al. [2017] examined the *in vitro* probiotic potential of *L. lactis* LMG 7930 against *S. aureus, S. epidemidis, S. chromogenes, S. intermedius, S. agalactiae, S. dysgalactiae* and *E. coli*. *L. lactis* LMG 7930 showed antagonistic properties against a plethora of the tested pathogens, low auto-aggregation and an absence of co-aggregation ability towards any of the tested pathogens. Although the strain was one of the most adhesive to bMEC, its internalization was low. Concerning pathogen invasion, it showed a trend to decrease internalization of some pathogens. Additionally, *L. lactis* LMG 7930 was sensitive to 13 antibiotics. However, *in vivo* studies are required to better assess its safety and efficacy.

Yu et al. [2017] assessed a cocktail of *L. plantarum* IMAU 80065 and *L. plantaru*m IMAU 10155 as a teat-dip probiotic in 11 cows over a 12-day period. The cocktail was applied as a teat-dip (5×10^{10} cfu mL^{-1}) to two quarters of each of the 11 cows and the other two quarters were dipped in a commercial disinfectant product. The authors demonstrated a gradual decrease in SCC following the cleaning regime; however, the SCC in the probiotic group was slightly lower than that of the commercial disinfectant. Analysis of raw milk from the treated teats revealed diverse alterations in microbial composition over the course of treatment, suggesting that the probiotic disinfectant could reduce mastitis associated bacteria. The authors suggest that the LAB “disinfectant” could be used as an alternative to chemical pre- and post-milking teat disinfectants.

Souza et al. [2018] showed that *Lactobacillus casei* BL23, a strain isolated from the mammary gland [Mazé et al., 2010], reduced the expression of pro-inflammatory cytokines in *S. aureus*-stimulated bMEC. At the same time, induction of defensins were not drastically affected by *L. casei* BL23 during *S. aureus* infection.

Wallis et al. [2018] assessed the probiotic potential of 13 LAB strains by investigating their biofilm forming ability and their adhesion capacity to bovine mammary glandular cells *in vitro*. Nine out of the 13 strains were isolated from healthy milk quarters, one from a bulk milk sample, one from a bedding sample and the last two were ATCC strains. All strains formed biofilms and adhered to the epithelium but both traits were found to be strain dependent. Seven out of the 13 strains tested demonstrated strongest biofilm formation and/or adhesion, comprising promising candidates for the treatment of mastitis. The same group a year later [Wallis et al., 2019] selected five LAB from the above aforementioned study [Wallis et al., 2018] and assessed their ability to disrupt and displace *in vitro* pathogenic staphylococcal biofilms with their own biofilms. All five strains were able to eliminate the staphylococcal biofilms. Of the five tested strains, only *Lactobacillus rhamnosus* ATCC 7469 and *Lactobacillus plantarum* 2/37 were able to replace the pathogenic biofilms with their own, hence the authors recommending these two strains for further investigations regarding their probiotic potential.

Overall, while the studies outlined above show the potential for probiotic development in this field, there remains much mechanistic and trial work to be done to demonstrate how effective they can be and whether they could substitute for antibiotics as a front-line treatment in acutely infected animals.

Bacteriocins

Bacteriocins are ribosomally-synthesized antimicrobial peptides produced by bacteria, which are active against other bacteria including antibiotic resistant strains [Cotter et al., 2005]. Some bacteriocins such as nisin are used as bio-preservatives in the food

industry [Mills et al., 2017]. Furthermore, bacteriocin production can also be a key probiotic trait as a result of a number of potential mechanisms, including facilitating colonization of the producer-strain, inhibiting colonization of undesirable bacteria, modifying microbiota composition and modulating the host immune system [Dobson et al., 2012]. Bacteriocins have been suggested as potential antibiotic substitutes [Cotter et al., 2013] and owing to their gene-encoded nature are amenable to genetic manipulation and hence novel drug design [Carroll et al., 2010]. Bacteriocins could be used either independently or in combination with other known antimicrobials to target biofilms. Biofilms are structured bacterial consortia found in polymeric matrices [Mathur et al., 2018] and can be attached to both biotic and abiotic substrates [Karatan and Watnick, 2009]. Biofilm formation is considered a selective advantage for mastitis-causing pathogens [Fox et al., 2005] and has been associated with recurrent mastitis infections [Melchior et al., 2006; Elhadidy and Zahran, 2014; Seixas et al., 2014] and increased resistance to antibiotics [Davies, 2003; Høiby et al., 2010]. For comprehensive reviews on the role of biofilm on the pathogenicity of mastitis and the potential of bacteriocins on fighting biofilms, the reader is referred to Gomes et al. [2016] and Mathur et al. [2018], respectively. Bacteriocins mainly belong to two major groups, the lanthionine-containing lantibiotics (class I) and the non-lanthionine containing bacteriocins (class II).

Nisin

Nisin is currently licensed as a food biopreservative in over 50 countries [Alvarez-Sieiro et al., 2016] and was deemed to be safe for use in food in 1969 [Shin et al., 2016]. Nisin is a single peptide bacteriocin with a mass of 3488 Da and is a member

of the Class I lantibiotics. Sears et al. [1992] tested the efficacy of a nisin A-containing germicidal sanitizer called AMBICIN N® (Applied Microbiology Inc., NY, USA) against bovine mastitis-associated *S. aureus, S. agalactiae, S. dysgalactiae, Klebsiella pneumoniae* and *E. coli* and compared it with conventional chemical treatments such as iodine and chlorhexidine. After the first minute of application on teat surfaces AMBICIN N® significantly reduced *S. aureus* (3.9 log reduction) and *E. coli* (4.2 log reduction) which was comparable with the antimicrobial activity of a 1% iodophor teat dip and significantly greater than a 0.5% chlorhexidine teat dip. The nisin-based formulation demonstrated limited potential for skin irritation. Wipe Out® dairy wipes developed by ImmuCell Corporation (Portland, ME) contain nisin A and can be used to clean, sanitize and dry the teat area and milker's hands before and during milking. Wu et al. [2007] tested the efficacy of nisin Z compared to no treatment in 90 cows with subclinical bovine mastitis. Forty-six cows received an intramammary infusion of nisin (2,500,000 IU) once daily for three days while the remaining 44 cows received no treatment. Nisin Z treatment cured 90.1% of *S. agalactiae* cases, 50% of *S. aureus* cases, 58.8% for coagulase-negative staphylococci (CoNS) and 65.2% of all cases. In the control group, only 15.9% of cows recovered. Following nisin treatment, N-acetyl-b-D-glucosaminidase (NAGase) activity (an indicator of bovine mastitis) as well as the amount of quarters with > 500,000 cells mL^{-1} were significantly reduced in the treated group whereas no changes were detected in the control group. Ceotto-Vigoder et al. [2016] tested nisin and lysostaphin *in vitro* against biofilms formed by *S. aureus.* While four-hour treatment with lysostaphin caused biofilm detachment and death of sessile cells, nisin treatment only reduced cell viability. When both lysostaphin and nisin were combined, it resulted in significant death of sessile cells. The use of

lysostaphin in combination with nisin offers an alternative solution for controlling bovine staphylococcal mastitis.

Malvisi et al. [2016] evaluated the antibacterial activity and immunomodulatory effects on bMEC of a cell-free supernatant (CFS) from two nisin A-producing strains, namely *L. lactis* subsp. *lactis* LL11 and *L. lactis* subsp. *lactis* SL153. Overall, streptococci were more sensitive to CFS than staphylococci including MRSA (Methicillin Resistant *S. aureus*) strains. Treatment of bMEC with CFS did not damage epithelial integrity. However, prolonged treatment (15–24 h) of bMEC with either live culture resulted in an inflammatory response. Interestingly, treatment of bMEC with the live culture of *L. lactis* subsp. *lactis* SL153 downregulated TNFα (Tumor Necrosis Factor alpha). The authors suggested that the control of TNFα release could offer an interesting approach towards reducing symptoms of clinical intramammary infections. When bMEC cells were treated with *L. lactis* subsp. *lactis* LL11, lysozyme activity increased. The combination of antimicrobial spectrum with the stimulation of lysosomal activity makes the two tested strains interesting candidates for the development of intramammary treatment of mastitis.

Lacticin 3147

Lacticin 3147 is another lantibiotic produced by *L. lactis* subsp. lactis DPC 3147. The ability to produce lacticin 3147 was first recognized in an isolate acquired from an Irish kefir grain (Rea et al., 1996). Lacticin 3147 successfully inhibits a broad spectrum of Gram-positive bacteria including *S. aureus* and streptococci. However, no activity has been demonstrated against Gram negative bacteria (Ryan et al., 1998). Ryan et al. (1999) tested the efficacy of an intramammary teat seal formulation with

and without lacticin 3147 at drying-off in 68 healthy quarters of 18 cows. Following infusion with either a standard or a lacticin 3147-containing teat seal, each teat was inoculated with 1.5 x 10^4 cfu of *S. agalactiae*. The lacticin 3147 teat seal significantly decreased the incidence of teats shedding *S. agalactiae*, from 61% in control teats, to 6% in the treated quarters. In a subsequent study, Twomey et al. [2000] tested the efficacy of the lacticin 3147 teat seal against *S. aureus* DPC 5246. In accordance with the results obtained by Ryan et al. [1999], teats treated with the lacticin-containing teat seal shed less *S. aureus* DPC 5246 than control teats. Moreover, the number of teats shedding *S. aureus* DPC 5246 decreased in the treated group. Crispie et al. [2005] produced a milk-based lacticin 3147 fermentate which was incorporated into teat seal and used *in vivo* to challenge *S. aureus* DPC 5246. Results comparable with Ryan et al. [1998] were obtained in that *S. aureus* numbers decreased in teats treated with fermentate teat seal. Klostermann et al. [2010] evaluated the bactericidal activity of the lacticin 3147 fermentate *in vitro* against 10^5 cfu mL^{-1} *S. aureus, S. dysgalactiae* and *S. uberis*. After 15 min of incubation, *S. aureus* and *S. dysgalactiae* were not detected while *S. uberis* was 100-fold reduced. *In vivo* trials followed, where teats were coated with the pathogens and then dipped in a lacticin-containing dip. Following 10 min of contact with the teat dip, an 80% decline of staphylococci was noted whereas *S. dysgalactiae* was reduced by 97% and *S. uberis* by 90%, demonstrating that the lacticin-containing teat dip is an efficient measure to diminish mastitis pathogens from cows' teats.

Staphylococcal Bacteriocins

In a study investigating the antimicrobial potential of five *S. aureus* bacteriocin producers, de Oliveira et al. [1998] showed that four of the five tested strains could inhibit a limited number of 65 *S. aureus* indicator strains which had been isolated from milk of subclinical mastitic cows. The fifth strain, *S. aureus* A53 carrying plasmid pRJ9 which encodes two distinct bacteriocins, was active against all 65 indicators tested, thus the bacteriocins encoded on pRJ9 are potential candidates for the prevention and treatment of bovine mastitis. dos Santos Nascimento et al. [2005] isolated 188 CoNS from bovine mastitic cases and evaluated them for antimicrobial production. In total, 6.4% of the isolated CoNS demonstrated antagonistic activity against *Corynebacterium fimi*, a strain particularly sensitive to staphylococcal antimicrobial substances [Nascimento et al., 2006]. Three of those strains were shown to produce an identical or similar bacteriocin to aureocin A70, a bacteriocin produced by an *S. aureus* strain previously isolated from food, whereas the rest seemed to produce novel bacteriocins. Five of the bacteriocin producers were able to inhibit at least 50% of the *S. agalactiae* tested strains. Therefore, CoNS and especially *S. simulans* 3299 which inhibited *S. agalactiae* (78.4% inhibition), have potential as antimicrobial agents for the treatment or prophylaxis of streptococcal mastitis. Varella Coelho et al. [2007] evaluated the inhibitory activity of three bacteriocins (aureocins A70, A53, 215FN) produced by *S. aureus* and four bacteriocins (Pep5, epidermin, epilancin K7, and epicidin 280) produced by *S. epidermidis* against 165 strains of *S. aureus* and 74 strains of *S. agalactiae*, both isolated from mastitic bovine udders. Epidermin inhibited over 85% of the tested strains followed by aureocin A53 (> 67%). In contrast, aureocin A70 inhibited only a single strain. However, strain MB50 which

produces both aureocins A70 and A53 inhibited over 91% of the tested strains, suggesting synergism between the bacteriocins. The administration of epidermin and/or the combination of A53 and A70 could thus be an alternative for successful treatment of infection by both mastitic staphylococci and streptococci. More recently, Ceotto et al. [2009] isolated 257 staphylococcal strains from bovine mastitic cases and 17.9% were active against the indicator strain *C. fimi* NCTC 7547. The successful strains were identified as *S. aureus* and their antimicrobial properties were assigned to bacteriocins based on their sensitivity to proteolytic enzymes. Further testing showed that only 12 of the tested strains were different to already known bacteriocins. Interestingly, the bacteriocin produced by strain 4185 was successful in inhibiting *Bacillus cereus*, *Listeria monocytogenes* and *Staphylococcus* sp. isolated from nosocomial infections, making it a promising candidate against mastitis pathogens. Braem et al. [2014] also explored the antimicrobial activity of 254 CoNS against mastitis-causing pathogens. After the completion of an initial screening, only 38 displayed bacteriocin-like activity. From the 38 selected, seven demonstrated antagonistic activity against at least one of *S. uberis*, *S. dysgalactiae* and *S. aureus*. *Staphylococcus chromogenes* L217 showed the strongest inhibitory effect, being active against all tested mastitis pathogens and most tested CoNS making it the most promising candidate. Carson et al. [2017] evaluated the inhibitory capacity of 441 non-*aureus* bovine staphylococci against mastitis-associated *S. aureus*. Isolates which efficiently impeded *S. aureus* growth, were also tested against human MRSA. *S. aureus* was inhibited by 40 out of 441 isolates, 23 of which inhibited MRSA. Whole genomes of the non-*aureus* staphylococci were bioinformatically mined for bacteriocin clusters, 105 putative bacteriocin gene clusters from 95 of the initial 441

isolates were identified with 25 novel bacteriocin precursors making non-*aureus* staphylococci a nominee for further research.

Other LAB Bacteriocins

Kim et al. [2004] found a 14 kDa bacteriocin produced by a *Lactobacillus bulgaricus* strain which demonstrated antagonism against bovine mastitis pathogens *S. aureus* ATCC 6538 and *S. agalactiae* ATCC 14364. The mode of action of the bacteriocin was bactericidal thus making it a possible candidate for treating mastitis pathogens. Davidse et al. [2004] demonstrated that peptide AS-48 produced by *Enterococcus faecalis* FAIRE 92 inhibited the growth of *S. aureus* which was isolated from bovine mastitic milk. The partially purified peptide was then liposome-encapsulated and infused into five healthy quarters, while another five healthy quarters were infused with saline as control, this was followed by infusion of all quarters with 3.3×10^3 cfu mL^{-1} *S. aureus*. Consequently, stable shedding of *S. aureus* was observed during daily milking from treated teats over seven days of the experiment, while numbers of *S. aureus* in milk from control teats increased 10-fold. When a higher dose of *S. aureus* infusion was used (5×10^5 cfu mL^{-1}), levels of *S. aureus* were decreased in milk from treated teats 100-fold, and initially increased SCC levels were decreased indicating recovery. Another bacteriocin, macedocin ST91KM which is produced by *Streptococcus gallolyticus* subsp. *macedonicus* ST91KM, is bactericidal against *S. agalactiae, S. dysgalactiae, S. uberis* and *S. aureus* [Pieterse et al., 2010]. The efficacy of a teat seal containing macedocin ST91KM was evaluated and demonstrated successful release of the bacteriocin and a decrease in growth of *S. agalactiae*, making macedocin ST91KM a potential candidate for dry-off therapy.

Bacillus sp. Bacteriocins

Barboza-Corona et al. [2009] evaluated the activity of five bacteriocins produced by *Bacillus thuringiensis* against *S. aureus* isolates associated with bovine mastitis. Morricin 269 and kurstacin 287 were the most active against *S. aureus* followed by kenyacin 404, entomocin 420 and tolworthcin 524. Later, León-Galván et al. [2015] demonstrated that those five bacteriocins were able to inhibit multi-resistant antibiotic bacteria such as *Staphylococcus agnetis, Staphylococcus equorum, S. uberis, Brevibacterium stationis* and *Brachybacterium conglomeratum*, but were inactive against the highly antibiotic resistant *Staphylococcus sciuri*. More recently, Maina et al. [2015] identified and isolated bacteriocin-producing *Bacillus* sp. from fish and screened them against bovine mastitic pathogens. Crude bacteriocins from *Bacillus subtilis* and *B. pumilus* successfully inhibited *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 and their antimicrobial peptides were heat stable, sensitive to low pH and fully or partially inhibited by proteolytic enzymes.

Overall, when one looks at all the literature on bacteriocins against mastitic pathogens, it is encouraging to see such antimicrobial action by so many different producers and bacteriocins. However, to be commercially viable, these bacteriocins would need to be produced in a cost-effective manner and would also need to get approved by veterinary agencies. In our experience with lacticin 3147, production of purified bacteriocin in sufficient quantities cheaply became a rate limiting step that blocked commercially realization of the technology.

Bacteriophages

Another alternative treatment for bovine mastitis is bacteriophage (phage) therapy. Phages are viruses that infect and can kill bacterial cells through cell lysis. Phages are either lytic or lysogenic with the last being able to spend part of its life cycle in a quiescent mode [Basdew and Laing, 2014; Vander Elst and Meyer, 2018]. When it comes to the lytic phage, following infection of the bacterial cell, it replicates within the cell and progeny phages are then released upon cell lysis and proceed to infect other cells. Therefore, the antimicrobial agent is a self-replicating entity. The inhibition spectra of phages can be broad but predominantly are narrow, often being strain specific. Phage therapy is practiced in certain regions of the globe to treat human infections but interest in it has been reignited elsewhere as a result of the antibiotic resistance increase [Hill et al., 2018].

Anti-staphylococcal Phages

Phages against *S. aureus* tend to be broad spectrum, perhaps because of the homogenous surface properties of this species and so staphylococcal phage often infect most members of the species [Kutter et al., 2015]. Phage K is an anti-*Staphylococcus* phage with lytic activity against hospital isolates of *S. aureus* [O'Flaherty et al., 2005a]. In the same study, a wash solution enriched with phage K was capable of reducing staphylococcal numbers on human skin 100-fold. Based on these results, it was hypothesized that phage K may have potential for treatment of bovine mastitis. Given that phage replication is essential for lytic activity its ability to replicate in raw milk was assessed [O'Flaherty et al., 2005b]. While phage K was

capable of replicating in heat-treated milk resulting in elimination of the host culture, it was unable to replicate in raw milk, presumably due to ‘clumping’ of the host bacterial cells due to complement thus restricting phage adsorption to the cell surface. In a placebo-controlled, multisite trial, intramammary infusion of phage K also failed to eliminate *S. aureus* from Holstein cows, due to inactivation/degradation in the mammary gland [Gill et al., 2006]. These results are consistent with a study by Lerondelle and Poutrel [1980] who demonstrated the failure of phage K to cure *S. aureus* mastitis.

O’Flaherty et al. [2005c] isolated two anti-staphylococcal lytic phages from farmyard slurry, CS1 and DW. Infusion of a phage cocktail including phages K, CS1, and DW into healthy bovine teats caused no alteration in the SCC demonstrating the nonirritant nature of these phages. While it is unlikely that the phages would prove efficacious in the mammary gland for pathogen elimination, they may have potential in teat dips and washes. Garcia et al. [2009] isolated eight distinct temperate phages based on their host range. Unlike lytic phages, temperate phages can participate in the phage lysogenic lifecycle and thus integrate into the host genome to become prophage. Temperate phages are not generally favored for phage therapy purposes but in this instance a phage mixture (Ug5 and Ua72, 1:1) proved to be more effective than using a single temperate phage for inhibiting *S. aureus*, since the mixture presumably prevented survival of lysogenized cells. While the phages were able to inhibit *S. aureus* in UHT and pasteurized whole-fat milk they were less potent in semi-skimmed raw milk in agreement with previous findings [O’Flaherty et al., 2005b]. Son et al. [2010] isolated two lytic phages from environmental samples capable of infecting *S. aureus* associated with bovine mastitis but not *S. aureus* strains related to human clinical infections. A mastitic cow was a source of phage MSA6, with morphology

similar to phage K and a polyvalent nature in that it was able to infect a wide range of *S. aureus* strains of both human and bovine origin [Kwiatek et al., 2012]. Dias et al. [2013] isolated 10 lytic phages from mastic cows which were able to infect *S. aureus*. The isolated phages demonstrated promising characteristics such as wide inhibition spectra and thermostability, suggesting they could be good candidates for therapeutic use. A year later, Li and Zhang [2014] isolated a new virulent phage called SPW from wastewater of a dairy farm which showed lytic activity against bovine mastitic *S. aureus*. The isolated phage was stable over a wide range of temperatures and pH values, inhibited a wide spectrum of hosts and demonstrated resistance to chloroform and isopropanol. More recently, Hamza et al. [2016] isolated a lytic phage from sewage water, designated SA phage, which was active against bovine mastitis-associated *S. aureus*. SA phage was stable in a wide range of pH and temperature values, with highest lytic activity at pH 7 at 37 ^{o}C. Tahir et al. [2017] examined the infection ability of SA phage and two other lytic phages against 10 strains of *S. aureus* and one *Micrococcus* sp. Phage SA demonstrated a relatively narrow spectrum of inhibition compared to phages SA2 and SANF while phage SA2 showed potential for reduction of *S. aureus* growth in commercial pasteurized milk. Iwano et al. [2018] evaluated the host range of *S. aureus* phages USA012 and USA039 against *S. aureus* strains isolated from mastitic cows showing that the phages killed all *S. aureus* and MRSA strains examined (93 strains from 40 genotypes and six strains from six genotypes, respectively). In an *in vivo* experiment using a mouse mastitis model, USA012 demonstrated reduced proliferation of *S. aureus* and inflammation in the mammary gland. At the same time, Ganaie et al. [2018] reported the isolation of two phages, namely SAJK-IND (sewage) and MSP (bovine mastitic milk). Both phages were inactivated when heated at 60 ^{o}C and were stable in a pH ranging from 4 to 9.

SAJK-IND displayed 100% lytic activity when tested against 120 isolates of *S. aureus* compared to MSP which lysed only 48% of the tested isolates. SAJK-IND and MSP did not demonstrate lytic activity against *E. coli, S. agalactiae, K. pneumoniae* and *Pseudomonas aeruginosa* suggesting genus specificity.

Anti-streptococcus agalactiae Phages

Bai et al. [2013] isolated a novel bacteriophage, from bovine mastitic milk, JX01 which exhibited lytic activity against clinical isolates of *S. agalactiae*, a major bovine mastitis pathogen. JX01 was found to be heat-sensitive and demonstrated acid and alkaline resistance (pH 3–11). To our knowledge, this article is the only one on antistreptococcal phages that could be used in bovine mastitis therapy.

Anti-Trueperella pyogenes Phages

Trueperella pyogenes (formerly known as *Arcanobacterium pyogenes*) [Yassin et al. 2011] forms pyogenic lesions in cows and is one of the major causes of summer mastitis [Hillerton and Bramley, 1989]. Its ability to form biofilms enables it to avoid recognition by the immune system and makes it difficult to eradicate with antibiotics. da Silva Duarte et al [2018a] tested the use of phage UFV13 for the prevention of *T. pyogenes* biofilms. Even though UFV13 infects *Escherichia* but not *T. pyogenes*, the authors demonstrated that a multiplicity of infection (MOI) of 10 managed to reduce biofilm formation by *T. pyogenes*. The exact mechanism of biofilm disruption is not understood but the genome sequence analysis of phage UFV13 revealed the presence of several virion-associated peptidoglycan hydrolases.

Anti-E. coli Phages

E. coli can be responsible for recurring subclinical mastitis cases and therapeutic trials have demonstrated ambivalent results with regards to antibiotic treatments against *E. coli* infections [Suojala et al., 2013]. Porter et al. [2016] assessed the use of a cocktail of four diverse phages (two T4-like phages, a rV5-related phage, and a phi92-related phage) against 36 mastitis-associated *E. coli* isolates. While the cocktail inhibited growth of over half of the *E. coli* strains in laboratory media, a 3.3–5.6 log decrease in *E. coli* P5-AmpR numbers was observed in raw milk after 12 h of incubation. Interestingly, McLean et al. [2013] also reported the ability of an *E. coli* phage to eliminate *E. coli* from raw milk, thus unlike staphylococcal phages, raw milk components do not appear to hinder replication of *E. coli* phages. Pretreatment of bMEC with the phage cocktail of Porter et al [2016] significantly reduced the adhesion and intracellular survival of *E. coli* compared with controls. When the phage cocktail was combined with a teat-sealant and tested against 1.6×10^3 cfu mL^{-1} *E. coli in vitro*, a successful inhibition of *E. coli* was noted. More recently, da Silva Duarte et al. [2018b] characterized the genetic background of phage UVF13 and evaluated the activity against a mammary pathogenic *E. coli* strain in an *E. coli*-induced mastitis mouse model. Intramammary administration of the phage led to a 10-fold decline in bacterial load.

Phage Endolysins

Phage Endolysins against S. aureus

Endolysins are specialized phage enzymes that lyse phage-infected host cells at the end of the lytic cycle, thus freeing progeny virions. Although endolysins target cells from within, by degrading peptidoglycan, they can be applied externally to lyse Gram-positives since such cells lack an outer membrane [Schmelcher et al., 2012a]. Endolysins from phages of Gram-positive bacteria are composed of two distinct domains; the cell wall binding domain which recognizes the substrate, and the enzymatically active domain (EAD) [Schmelcher et al., 2012a]. Endolysins are classified into at least five groups depending on the mode of action of the EAD; N-acetyl-b-d-muramidases, lytic transglycosylases; N-acetyl-b-d-glucosaminidases; N-acetylmuramoyl-l-alanine amidases and endopeptidases [Schmelcher et al., 2012a]. Donovan et al. [2006b] tested purified phi11 endolysin against *S. aureus* and CoNS. Phi11 was able to lyse mastitis staphylococcal pathogens, with lytic activity maintained at pH 6.7 and in the presence of the 'free' calcium concentration (3 mM) of milk. Schmelcher et al. [2012b] evaluated two chimeric endolysins as antimicrobial agents in a *S. aureus*-induced mastitis mouse model. The two chimeric proteins consisted of the streptococcal kSA2 endolysin endopeptidase domain fused to staphylococcal cell wall binding domains from either lysostaphin (kSA2-E-Lyso-SH3b) or the staphylococcal phage K endolysin, LysK (kSA2-E-LysK-SH3b). The two proteins successfully killed 16 mastitic *S. aureus* isolates. A combination of the endolysins tested in a mastitis mouse model reduced numbers of *S. aureus* Newbould 305 by 3.36 logs. Moreover, Fenton et al. [2013] tested phage-derived cysteine- and histidine-dependent amidohydrolase/peptidase ($CHAP_K$), against mastitis-associated *S. aureus*. $CHAP_K$ was effective against biofilms either by inhibiting biofilm

formation or by disrupting established biofilms of mastitis-associated staphylococcal strains. This study suggested the potential use of $CHAP_K$ as a prophylactic and therapeutic measure against mastitis. Zhou et al. [2017] demonstrated that the engineered lytic endolysin LysKDamidase was capable of inhibiting 66 methicillin-resistant staphylococcal strains and 71 methicillin-susceptible staphylococcal strains isolated from bovine mastitic milk and from infected humans in China, making LysKDamidase a potential treatment for methicillin-resistant and methicillin-susceptible staphylococci.

Phage Endolysins against Streptococci

Schmelcher et al. [2015] characterized endolysins produced from phages kSA2 and B30 and evaluated their ability to inhibit bovine mastitic pathogens. When the two enzymes were tested *in vitro* against streptococci, they showed near-optimum lytic activities at ionic strengths, pH, and calcium ion concentration consistent with bovine milk. However, B30 was less successful than kSA2 in killing *S. dysgalactiae*. Consequently, they evaluated the endolysins in a mouse model of bovine mastitis where both enzymes significantly decreased the intramammary levels of *S. uberis* and *S. agalactiae* and the effects on mammary gland wet weights as well as TNFα concentrations corroborated these findings.

Scholte et al. [2018] used PlyC, a peptidoglycan hydrolase derived from the streptococcal C1 bacteriophage which causes lysis of the cell wall of *S. uberis* [Krause 1957], to study its dose effect on cytotoxicity and the oxidative response on bovine blood polymorphonuclear leukocytes (PMN). The authors demonstrated that

increasing concentrations of PlyC are nontoxic and do not hamper the oxidative response of PMN.

Conclusions and Future Prospects

Bovine mastitis is a highly prevalent disease in dairy cattle with serious consequences for animal health and welfare and presents one of the largest economic costs to dairy farmers. The primary etiological agents are bacterial and it is highly contagious and easily spreads through contaminated machinery, hands, bedding, etc. Broad spectrum antimicrobials are used in the prevention and treatment of mastitis. In terms of prevention, local antibiotic treatment (intramammary infusion) is implemented at the beginning of the drying-off period (dry cow therapy), a practice which has resulted in a considerable reduction in bovine mastitis and is generally recommended for all cows (Kromker and Leimbach, 2017). Treatment of the disease itself requires local treatment with antibiotics or in severe cases, parenteral antibiotic administration (Kromker and Leimbach, 2017). However, in 2015 it was reported that 73% of all intramammary antibiotics were used for dry cow therapy while only 27% were used to treat actual clinical mastitis (Kromker and Leimbach, 2017). The rise in antibiotic resistance has called for the prudent use of antibiotics in both human medicine and animal welfare. Furthermore, recent studies reported the isolation of antibiotic resistant microorganisms from bovine mastitic cases (Silva et al., 2014; Bandyopadhyay et al., 2015; Su et al., 2016; Das et al., 2017). Thus, alternative treatments are urgently needed.

In this review, we presented evidence for four potential alternative treatments for the management of bovine mastitis, probiotics, phages (and their enzymes), and

bacteriocins. Probiotic therapy represents perhaps the most complex of alternative treatments given that an abundance of evidence to date suggests that probiotics exert their antimastitic effect through modulation of the host immune response, while some also directly antagonize the offending pathogen. In this regard, probiotic administration could also prove to be a highly effective prophylactic treatment. Probiotics are commonly used in animal husbandry where they have been shown to improve animal growth rate, production, and general health (Chaucheyras-Durand and Durand, 2010) and thus should be readily accepted on farm for mastitis control. Understanding the exact mechanism(s) of action will be essential to the success of probiotic therapy for bovine mastitis. However, probiotics seem primed for next-stage development in the form of robust evidence-based field trials.

Bacteriocins have been advocated as potential antibiotic alternatives and one bacteriocin, nisin, has been commercially developed for bovine mastitis (AMBICIN® and Wipe Out®). Numerous studies have highlighted the efficacy of a plethora of bacteriocins from LAB, staphylococci, and other bacteria against mastitis pathogens in both *in vitro* and *in vivo* studies. In many cases, the killing effect is comparable to antibiotic treatment. Furthermore, bacteriocins are generally nontoxic to eukaryotic cells and owing to their peptidic nature are broken down in the gut, hence bacteriocin residues in milk should not be an issue. However, one of the major drawbacks with using bacteriocins is the prohibitive production costs associated with generating pure peptides. Crispie et al. (2005) overcame this by generating a milk-based fermentate of lacticin 3147 which proved effective against a mastitis pathogen *in vivo* when combined with teat seal. Bacteriocins are also amenable to use in teat and hand washes and teat wipes to prevent pathogen spread, as proven for nisin. As well as developing innovative and feasible production routes, field trials are urgently required to

determine long-term efficacy, safety and the risk of bacteriocin resistance. In the case of the latter, bacteriocin resistance is a justifiable concern and can arise at frequencies of 10^{-2} – 10^{-9} depending on the bacteriocin (Bastos et al., 2015). But combining bacteriocins with other antimicrobial agents has proven to be a successful strategy for mitigating the development of bacteriocin resistance where certain combinations can be additive or synergistic and expand the antimicrobial spectrum of both agents, enabling bacteriocins, for example, to target Gram-negative bacteria when the outer membrane has been destabilized (Prûdencio et al., 2015).

The direct use of phages for bovine mastitis treatment has met with mixed results. While some of the phages are active in raw milk, others can replicate only in heat-treated milk. Based on current literature for effective inactivation of staphylococci and *E. coli*, a three-step processing would be required, namely, first targeting *E. coli* in raw milk, next heat treatment, finally antistaphylococcal treatment in processed milk. Additionally, the majority of application work was performed in milk models that omitted fat components. More specifically, staphylococcal phages are hindered by raw milk components presumably causing the agglutination of bacterial cells which are then impenetrable to phage attachment, the initial step in the phage lytic cycle. In contrast, *E. coli* phages have provided more promising results being capable of replication in raw milk. Apart from staphylococcal phages, most others are highly specific. This can be overcome through the use of phage cocktails containing multiple phages capable of infecting several pathogens. However, direct experience of human phage therapy in clinical settings clearly states that knowledge of the infectious agent(s) is essential for its success (Kutter et al., 2010). Phage resistance is also a concern although it can be easily overcome by updating phage cocktails with new phages as needed (Hill et al., 2018). However, in reality this would require constant

microbiological monitoring of milk samples and matching of phages to offending bacteria by experienced phage biologists to generate mastitis-specific phage cocktails, a feat which is not impossible but perhaps impractical until all other options have been exhausted. Phage endolysins, however, are emerging as promising antimicrobial alternatives with proven potent activity against mastitis pathogens. Their chimeric nature suggests numerous possibilities for designing novel antimicrobial drugs with improved functionality as well as generating antimicrobials with broader specificity (Love et al., 2018). However, endolysins require genetic engineering for both production and design suggesting that these are drugs of the future rather than the immediate present as much research is required to test their efficacy and safety and ease of large-scale production.

In conclusion, the evidence presented in this review has clearly highlighted the antimicrobial potential of probiotics, bacteriocins, phages and their components against the causative agents of bovine mastitis. In the future, we should not only focus on the discovery of alternative antimicrobials against mastitis pathogens but also should focus on deciphering exact modes of action and innovative means for their production and delivery to ensure optimal performance.

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Chapter 2

The Microbiology and Treatment of Human Mastitis

Angelopoulou A, Field D, Ryan CA, Stanton C, Hill C, Ross RP. The microbiology and treatment of human mastitis. Med Microbiol Immunol. 2018;207(2): 83-94.

Author Contributions

AA drafted the manuscript. DF, CAR, CS, CH, RPR critically reviewed the manuscript. The final manuscript was read and approved by all authors.

Abstract

Mastitis, which is generally described as an inflammation of breast tissue, is a common and debilitating disease which frequently results in the cessation of exclusive breastfeeding and affects up to 33% of lactating women. The condition is a primary cause of decreased milk production and results in organoleptic and nutritional alterations in milk quality. Recent studies employing culture-independent techniques, including metagenomic sequencing, have revealed a loss of bacterial diversity in the microbiome of mastitic milk samples compared to healthy milk samples. In those infected, the pathogens *Staphylococcus aureus*, *Staphylococcus epidermidis* and members of corynebacteria have been identified as the predominant etiological agents in acute, subacute and granulomatous mastitis, respectively. The increased incidence of antibiotic resistance in the causative species is also a key cause of concern for treatment of the disease, thus leading to the need to develop novel therapies. In this respect, probiotics and bacteriocins have revealed potential as alternative treatments.

Introduction

Over the last decade, breastfeeding has been the subject of renewed attention in developed countries because of the demonstrated health benefits to the mother-child dyad [Martin et al., 2016; Victora et al., 2016]. Consequently, international and national health organizations encourage exclusive breastfeeding during the first 6 months of life [WHO, 2000; Abou-Dakn et al., 2010; Amir et al., 2011]. However, in certain cases, exclusive breastfeeding may not be an option for mothers owing to many reasons [Li et al., 2008], with mastitis considered as the greatest cause of undesired weaning.

Mastitis, an inflammation of breast tissue, is an acute, devitalizing condition and a potentially serious illness that may lead to breast abscess and septic fever [Osterman and Rahm, 2000]. The main causes of mastitis are milk stasis and infection [WHO, 2000; Betzold, 2007]. Milk stasis is usually the primary cause and occurs when milk is not removed properly from the breast duct due to poor attachment of the infant, fruitless suckling and blockage of the ducts [WHO, 2000; Cullinane et al., 2015]. It is widely accepted that most mastitic cases are related to changes in the microbiome of the mammary gland and that most mastitis-causing bacteria have the ability to form biofilms in the milk ducts which are quite narrow; this results in the impairment of milk flow and the retention of milk [Fernández et al., 2014; Figure 2a, 2b]. The incidence of lactational mastitis varies between 2 and 33% of lactating mothers [Jiménez et al., 2008; Civardi et al., 2013] and most episodes occur in the first 6 weeks postpartum [Foxman et al. 2002]. According to epidemiologic studies, there are many factors which might be implicated in its occurrence [Kinlay et al., 2001; Foxman et al., 2002; Scott et al., 2008; Mediano et al., 2014]. Risk factors include age, with

mothers under 21 and over 35 years having a decreased incidence [Jonsson and Pulkkinen, 1994], mastitis with a previous child [Mediano et al., 2014], cracked or sore nipples, use of ointments, incorrect breastfeeding practices and peripartum antibiotherapy [Betzold, 2007; Amir et al., 2007; Spencer, 2008].

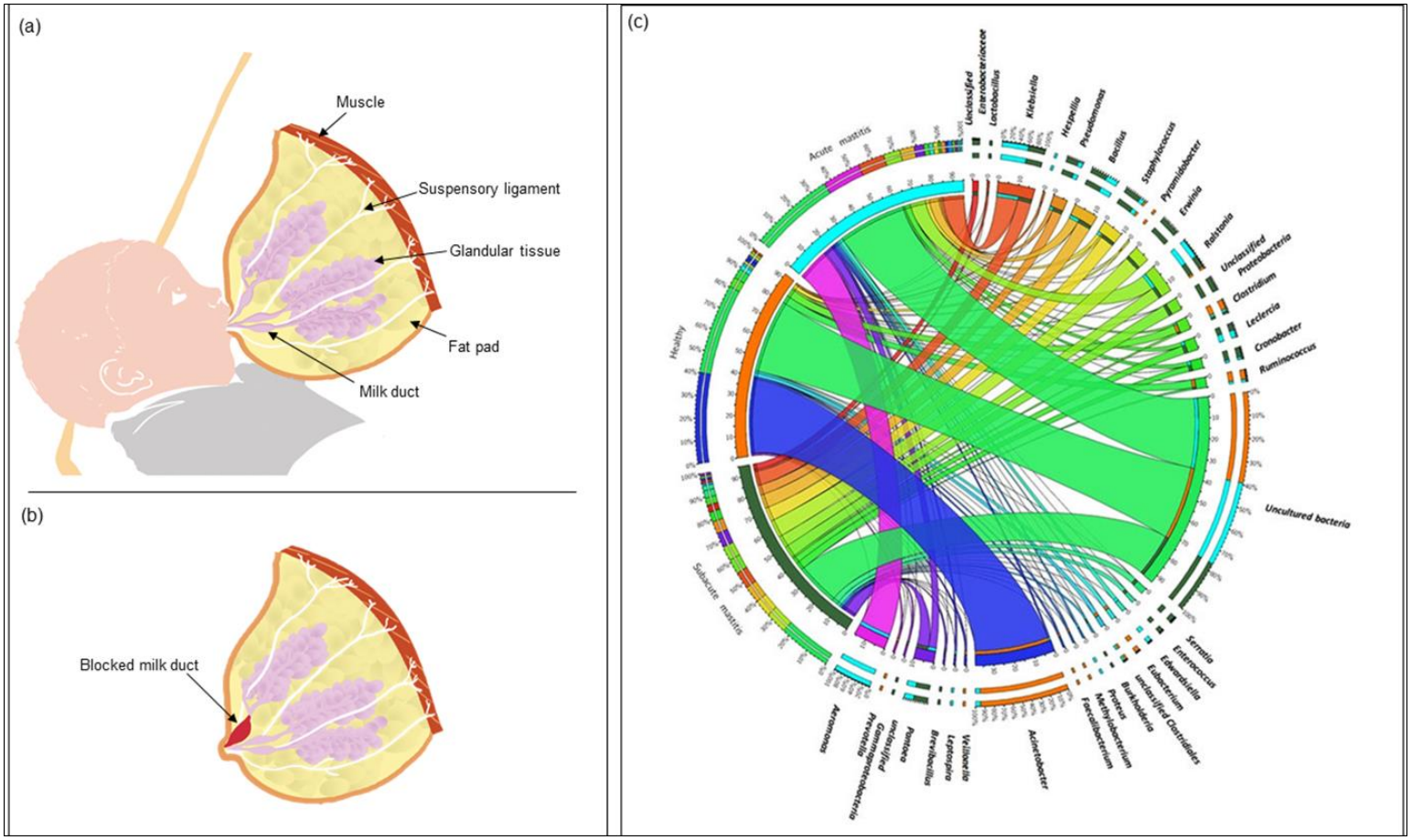

Figure 2.1. Physiological breast anatomy (a) and mastitic enlarged duct. (b) Milk production takes place at the duct system. When mastitis occurs, ducts are blocked due to the formation of bacterial biofilms. **(c) Circos representation of the top most abundant bacterial genera from healthy milk samples and from subacute and acute mastitis milk samples** (Fig 2c is reproduced from Patel et al. [2017] which is licensed under the creative commons license http://creativecommons.org/ licenses/by/4.0/).

The aim of this review is to provide an overview of the latest findings in terms of the microbiological load involved in human mastitis, particularly at a time when the advances in sequencing technologies have provided an excellent platform to study both cultivable and non-cultivable microorganisms, giving a more accurate view of the microbiological dysbiosis which shapes this disease. The review also describes the available therapies to treat mastitis, the most common of which is antibiotics. As antibiotic resistance poses a major challenge to the success of this treatment, we examine alternative therapies, namely probiotics and bacteriocins, for which ongoing studies continue to provide promising results.

Source of Bacteria in Human Milk

Traditionally, human milk was considered to be sterile and any bacteria found within were deemed to be contamination either from the mother's skin or the infant's mouth [West et al., 1979]. This has since been disproven by several studies which demonstrate that human milk contains its own microbiota [Heikkilä and Sarris, 2003; Perez et al., 2007; Collado et al., 2009; Hunt et al., 2011; Jiménez et al., 2015; Mediano et al., 2017; Murphy et al., 2017; Patel et al., 2017]. Despite this, the exact mechanisms by which these bacterial populations reach the mammary gland, and subsequently the milk, have not been fully elucidated.

Recent findings indicated that selected bacteria of the maternal gastrointestinal (GI) microbiota can reach the mammary gland through an entero-mammary pathway [Perez et al., 2007]. The mechanism involves dendritic cells and $CD18^{+}$ cells which have the ability to transfer bacteria from the GI lumen to the lactating mammary gland [Rescigno et al., 2001; Macpherson and Uhr, 2004].

The skin may also be a major source of bacteria for human milk. Indeed, many species of bacteria associated with the skin, namely *Staphylococcus* and *Corynebacterium*, have been isolated from healthy human milk [Hunt et al., 2011]. It is possible that these bacteria are relocated to the milk during ejection, specifically from the nipple, mammary areolas and montgomery glands, although it is worth noting that these species are also associated with mucosal surfaces of the body including that of the GI tract. In addition, retrograde flow of milk from the infant oral cavity to the mammary ducts has the potential to lead to cross-contamination of the milk. Moreover, the partner's bacteria could contribute to bacterial populations in milk as it is known that partners share oral and skin microbiota [Kort et al., 2014; Ross et al., 2017]. Fecal matter can also be a source of contamination in human milk when poor hygienic conditions exist [Wyatt and Mata, 1969; Eidelman and Szilagyi, 1979; Serafini et al., 2003].

All, or some, of these routes of bacterial transfer have the potential to contribute to the microbiota of human milk. As recent studies have indicated, healthy human milk does contain its own populations of bacteria which provide important health benefits not only for the nursing mother but also for the infant, with the suggestion that the microbiota of human milk may aid in establishing the commensal GI microbiota of the infant [Serafini et al., 2003; Jiménez et al., 2008; Martín et al., 2009; Martín et al., 2012; Jost et al., 2014].

How or why the transition from commensal colonization to infection occurs remains to be fully understood, however, several factors have been identified which may predispose women to the development of mastitis. These range from host factors such as genetics, the presence of polymorphisms and even blood grouping, to bacterial factors, e.g., virulence factors of the bacteria present, to medical influences including

the use of antibiotics and the widespread administration of iron to prenatal women [Martín et al., 2012; Fernández et al., 2014].

Microorganisms Detected in Lactational Mastitis

Lactational mastitis is classified into different categories depending on the course (acute, subacute and granulomatous, which is explained later) or the clinical manifestations (clinical or subclinical) [Fernández et al., 2014]. Mastitis is a multifactorial disease caused by a range of different pathogens and is characterized by an alteration in the mammary microbiota [Jost et al., 2014; Mediano et al., 2017; Rodríguez and Fernández, 2017]. Indeed, a metagenomic analysis of 20 milk samples (half from women with mastitis, while the other half were taken from healthy women) showed a noteworthy loss of bacterial diversity in the mastitic milk samples [Jiménez et al., 2015]. The latter is illustrated in Table 2.1. which records the genera detected in healthy human milk and mastitic human milk via culture-dependent and culture-independent analyses. The majority of the available literature is based on culture-independent approaches; however, the scientific community should be handling this data with precaution as there is some argument as to the appropriate controls for such studies. Salter et al. [2014] and Glassing et al. [2016] have both described the potential for incorrect results in low bacterial count samples owing to contamination or other artefacts which could explain the discrepancies between the healthy core microbiome reported by Hunt et al. [2011] and Murphy et al. [2017]. In a more recent study, the healthy core milk microbiome consisted of the genera *Pseudomonas, Staphylococcus, Streptococcus, Elizabethkingia, Variovorax, Bifidobacterium, Flavobacterium, Stenotrophomonas, Brevundimonas, Chryseobacterium, Lactobacillus* and

Enterobacter [Murphy et al., 2017], whereas *Staphylococcus* species dominated the mastitic milk samples with *S. aureus* and *S. epidermidis* being the dominant species in the microbiome of women diagnosed with acute and subacute mastitis, respectively [Jiménez et al., 2015]. In another study, sequencing of 16S rRNA gene of 32 human mastitic milk samples revealed the presence of 17 genera and 30 distinct species [Patel et al., 2016]. Proteobacteria was the predominant phylum accounting for 51.3% of the total followed by Firmicutes (37.8%) and included representatives from the classes Gammaproteobacteria, Alphaproteobacteria and Bacilli. The most frequently isolated genera included *Pseudomonas* (6 species) and *Staphylococcus* (4 species). The following species were frequently isolated; *Staphylococcus* species (87.5% of mastitic samples), *S. aureus* (75.0%), *P. aeruginosa* (53.1%), *Klebsiella pneumoniae* (43.8%) and *Brevundimonas diminuta* (38.0%). Recently, Mediano et al. [2017] identified 5009 isolates from 1849 mastitic milk samples by classical, biochemical and/or molecular methods. Mean total bacterial count was 4.11 log cfu mL^{-1} with *Staphylococcus* being the most commonly isolated bacterial group (97.57%) and *S. epidermidis* being the dominant species (91.56%) followed by *S. aureus* (29.74%). Streptococci and corynebacteria comprised the second (70.20%) and third (16.60%) most dominant groups, respectively. Most recently, a 16S rRNA gene-sequencing method of 50 human milk samples (16 subacute mastitic, 16 acute mastitic and 18 healthy control samples) revealed an enrichment of genera including *Aeromonas, Staphylococcus, Ralstonia, Klebsiella, Serratia, Enterococcus* and *Pseudomonas* in subacute and acute mastitic samples, while *Acinetobacter, Ruminococcus, Clostridium, Faecalibacterium* and *Eubacterium* were consistently depleted [Patel et al. 2017] (Figure 1c). Moreover, dramatic enrichment of aerotolerant bacteria and depletion of obligate anaerobes was observed during the infection. Lower

microbial diversity was also a feature of the mastitic milk samples along with increased abundance of opportunistic pathogens including *S. aureus, S. epidermidis, S. hominis, K. pneumoniae, Ser. marcescens, P. aeruginosa, E. faecalis, Bacillus subtilis, B. cereus* and *Escherichia coli*. Marín et al. [2017] analyzed the cultivable microbial diversity of 647 human mastitic milk samples using Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) technology. The total staphylococcal count was 3.7 log cfu mL^{-1} with *Staphylococcus* genus being the most frequently isolated (94.1%) and *S. epidermidis* being the predominant species (87.6%) followed by *S. aureus* (22.1%), *S. hominis* (5.3%) and *S. lugdunensis* (3.3%). In agreement with the findings of Mediano et al. [2017], streptococci were the second-most abundant group with a mean count of 3.47 log cfu mL^{-1}. *Strep. mitis/or*alis was the most common species (40.8%), while *Strep. salivarius* and *Strep. parasanguinis* were detected with frequencies of 36.8 and 14.4%, respectively. Corynebacteria occurred in 11% of the analyzed samples with the populations of *C. tuberculostearicum* and *C. kropenstedtii* reaching ratios of 3.6 and 2%, respectively. Similarly, Delgado et al. [2008; 2009] identified *Staphylococcus* as the predominant genus in mastitic milk samples using a combination of culture and molecular techniques where *S. epidermidis* was found to be the dominant species. Moreover, antibiotic resistance and the presence of the biofilm-related *icaD* gene were significantly higher in *S. epidermidis* strains isolated from mastitic milk samples [Delgado et al., 2009].

Table 2.1. Genera detected using culture-dependent and culture-independent techniques to study the microbial diversity associated with human milk.

Phylum	Genus	Culture-dependent	Culture-independent	
Firmicutes	*Anaerococcus*		H [27]	
	Bacillus	H [20]		M [28, 42]
	Coprococcus		H [27]	
	Clostridium		H [23, 28, 46]	
	Enterococccus		H [23, 47, 48, 49]	M [28, 50]
	Erysipelotrichaceae incertae *sedis*		H [27]	
	Finegoldia		H [27]	
	Gemella		H [24, 27, 46, 49]	
	Lachnospriraceae incertae sedis		H [27]	
	Lactobacillus	H [20, 51]	H [23, 24, 27, 37, 47-49, 52, 53]	
	Lysinibacillus			M [42]
	Macrococcus			M [42]
	Peptoniphilus		H [27]	
	Peptostreptococcaceae incertae *sedis*		H [27]	
	Planococcus			M [42]
	Staphylococcus	H [20, 51, 54]	H [23-25, 27, 37, 49, 53, 55]	M [25, 26, 42, 45, 56-58]
	Streptococcus	H [20, 51]	H [23, 24, 27, 48, 49, 53, 55]	M [26, 57]
	Veillonella		H [24, 27, 46, 49]	

(continued)

Table 2.1. Continued

Phylum	Genus	Culture-dependent	Culture-independent	
Proteobacteria	*Acinetobacter*	H [20, 51]	H [28]	M [42]
	Alcaligenes	H [20]		M [42]
	Brevundimonas		H [27]	M [42]
	Citrobacter		H [27]	
	Enterobacter		H [27]	
	Erwinia		H [27]	
	Escherichia-Shigella	H [20]	H [27, 46, 49]	
	Hemophilus		H [27]	
	Klebsiella			M [28, 42]
	Moraxella	H [20]		
	Proteus			M [42]
	Providencia			M [42]
	Pseudomonas	H [20]	H [24, 27, 49, 59, 60]	M [28, 42]
	Ralstonia		H [24]	M [28]
	Serratia		H [24, 46, 49]	M [28]
	Sphingomonas		H [24]	
	Stenotrophomonas		H [27]	
	Variovorax		H [27]	
Actinobacteria	*Actinomyces*		H [24, 27, 46]	M [26]
	Atopobium		H [27]	
	Bifidobacterium		H [23, 24, 27, 36, 37, 61]	
	Brevibacterium			M [42, 59]
	Corynebacterium		H [24, 27]	M [26, 43, 56]
	Granulicatella		H [27]	
	Leucobacter			M [42]

(continued)

Table 2.1. Continued

Phylum	Genus	Culture-dependent	Culture-independent	
	Micrococcus	H [20]	M [42]	
	Propionibacterium		H [24, 27, 49]	M [26]
	Rhodococcoccus		H [27]	
	Rothia		H [24, 27]	M [26]
Bacteroidetes	*Bacteroides*		H [23, 27]	
	Chryseobacterium		H [27]	
	Elizabethkingia		H [27]	
	Flavobacterium	H [62]	H [27]	
	Prevotella		H [24, 27, 46]	

H indicates healthy mammary gland while M indicates mastitis.

Acute Mastitis

Acute mastitis is characterized by breast redness, fever, pain, and malaise [Fernández et al., 2014]. Due to the intensity of these symptoms, acute mastitis is typically the sole type of mastitis that is accurately diagnosed despite representing a relatively small proportion of human mastitis cases. A metagenomic study of human milk samples by Jiménez et al. [2015] analyzed the microbiome of 20 milk samples including 10 healthy, 5 acute, and 5 subacute mastitic samples. Variation in the predominant bacterial genus was observed in milk samples obtained from healthy women (*Pseudomonas*, *Bacteroides*), however, *Staphylococcus* was the dominant genus in both acute and subacute mastitis samples. Women who presented with acute mastitis were found to have higher levels of *S. aureus* (approximately 10^6 cfu mL^{-1}) in their milk compared to those with subacute mastitis. The wide range of toxins produced by strains of *S. aureus* have previously been implicated in bovine mastitis and may also be responsible for the symptoms observed in acute mastitis in humans [Delgado et al.,

2011]. Comparatively, *S. epidermidis* was found to be the most abundant staphylococcal species in women with subacute mastitis. Interestingly, Patel et al. [2017] recently reported that acute mastitis samples harbored significantly more *Aeromonas, Klebsiella, Ralstonia, Proteus,* and *Leptospira* at genus level and significantly higher levels of Aeromonadaceae, Burkholderiaceae, Brucellaceae and Streptococcaceae at family level when acute mastitis, subacute mastitis and healthy milk samples were compared (Figure 1c).

Subacute Mastitis

The symptoms of subacute mastitis are distinctly more subtle than acute resulting in a lower rate of diagnosis, whether this is due to misdiagnosis or a lack of understanding by women experiencing these symptoms is not described. Where an official diagnosis has been established, the symptoms of subacute mastitis include a sharp, needling pain and a burning sensation in the breast [Fernández et al., 2014].

As indicated previously, Jiménez et al. [2015] proposed that *S. epidermidis* was the predominant species of *Staphylococcus* present in subacute mastitic milk samples (n=5). An additional, and more extensive study of 20 women with subacute mastitis also found *S. epidermidis* to be the most dominant species (by 85%). Other species of *Staphylococcus* including *S. hominis, S. pasteuri, S. warneri,* and *S. haemolyticus*, were also identified in subacute mastitic samples albeit at much lower levels than *S. epidermidis* [Delgado et al., 2008]. At genus level, *Staphylococcus* was also found to be more enriched in subacute mastitis milk samples along with *Erwinia, Bacillus, Pantoea, Cronobacter* and *Pseudomonas* when compared to acute mastitic and healthy milk samples [Patel et al., 2017].

Collectively, the species of *Staphylococcus* found in subacute mastitic milk samples can be referred to as coagulase negative staphylococci (CoNS). Many species of CoNS are healthy skin commensals; however, they can also be implicated in nosocomial infections, particularly *S. epidermidis* [Otto, 2014].

In addition to CoNS, streptococci, more specifically viridans streptococci, may play a role in mastitis although the strains involved appear to be host dependent. For example, *Strep. uberis* and *Strep. dysgalactiae* have been identified in bovine mastitis [Riffon et al., 2001], yet they are not involved in human mastitis. On the other hand, *Strep. mitis* and *Strep. salivarius* prevail in human mastitis [Delgado et al., 2009]. The combination of viridans streptococci and CoNS have the potential to form thick biofilms leading to confinement of the lumen through which the milk passes. This pressure leads to the symptoms of subacute mastitis described previously [Fernández et al., 2014].

Granulomatous Mastitis

Idiopathic granulomatous mastitis (IGM) is a relatively rare, inflammatory disease which presents as a painful, tender lump or mass in the breast. The presence of a mass and the secondary symptoms of IGM, skin thickness, abscess formation and nipple retraction, often lead to a clinical misdiagnosis of breast malignancy [Sabel, 2009; Dobinson et al., 2015]. IGM is predominantly observed in women of reproductive age, approximately 2–6 years following pregnancy; however, it has also been reported in prepubescent girls, elderly women and men [Korkut et al., 2015].

The causes of IGM may vary and several factors have been proposed that predispose to IGM including diabetes mellitus, the use of the oral contraceptive pill

and undetected organisms amongst others [Kiyak et al., 2014; Korkut et al., 2015]. However, Taylor et al. [2013] suggested that the presence of species of corynebacteria was linked to the development of IGM. A subsequent study by Dobinson et al. [2015] demonstrated the successful isolation of several strains of corynebacteria from breast masses, namely *C. kroppenstedii*, *C. tuberculostearicum*, and *C. freneyi*. Despite the strong suggestion that corynebacteria is a major causative agent of IGM, a successful treatment has not yet been established [Kiyak et al., 2014]. This is principally due to the fact that corynebacteria exist in lipid-filled vacuoles within the granuloma as opposed to the tissue itself thus treatment requires agents that are effective against corynebacteria as well as active in lipid environments. However, most antimicrobials are hydrophilic with weak distribution to lipid environments. A combination of these agents, e.g., clarithromycin or rifampin, with corticosteroids and/or other immunomodulatory modalities has shown some success in treating IGM [Dobinson et al., 2015].

Effects on Milk Quality

Lactational mastitis is a primary cause of decreased milk production and is also related to alterations in the cellular composition of milk. Changes in the metabolic activity of milk-producing cells along with diminished milk synthesis in the mammary gland is normally a direct result of inflammatory mediators [WHO, 2000; Say et al., 2016]. Edema of the interstitial tissues is caused by opening of the paracellular pathways during lactation as a result of protein leakage from blood and milk. Moreover, due to the opening of the paracellular pathways, levels of sodium and chloride increase, while at the same time the levels of potassium and lactose decrease [WHO, 2000]. Due to

milk stasis, white granules may be observed in the milk which are formed from caseins hardened by salts, mainly consisting of calcium [WHO, 2000]. Fatty or fibrous-looking material, sometimes brown or green, is occasionally forced out from blocked ducts accompanied by alleviation of symptoms [WHO, 2000]. Furthermore, during mastitis, a change in the organoleptic properties can occur, in that the milk becomes salty. Generally, the saltiness is provisional and lasts approximately one week [McGregor et al., 1985].

A recent study conducted by Say et al. [2016] focused on the effects of mastitis on the macronutrient content of milk. A total of 30 lactating women were divided into two groups; one diagnosed with mastitis and one consisting of healthy women. Fat, carbohydrate and energy levels were significantly lower in the mastitic milk samples. The authors concluded that the observed differences may have emerged from reduced milk synthesis, compromised permeability of the blood–milk barrier and an increase in enzymatic or proteolytic activity associated with the inflammatory process [Nommsen et al., 1991; Le Roux et al., 2003]. We speculate that the reduced levels of fat, carbohydrates and energy could have a negative effect on the health of the infant with the prerequisite that the mother still breastfeeds.

Perez et al. [2016] concentrated on the impact of mastitis on the biogenic amine (BAs) profile in human milk. BAs are defined as low molecular weight nitrogenous organic compounds with distinct biological activities. In the study, two groups of women participated; one (n=40) healthy group and the other (n=30) diagnosed with mastitis. Putrescine, spermidine and spermine were the predominant BAs identified in both cases, however, the concentrations of BAs in mastitic milk were higher compared to healthy milk. BAs have been shown to contribute to gut maturation and the increased levels seen in mastitic milk may potentially negatively affect the

development of the infants' GI microbiota [Thomas and Thomas, 2001; Chanphai et al., 2016].

Therapies

An overview of current therapies for mastitis treatment are presented in Table 3.

Table 2.2. Overview of mastitis treatment options.

Treatment options	Name of treatment	When to use / Target	Status of the treatment	Duration of therapy
Antibiotics	Dicloxacillin[4, 8, 65]	Staphylococcal mastitis	Standard	7-14 days
	Flucloxacillin[4, 65]	Staphylococcal mastitis	Standard	
	Cephalexin[4, 8, 65]	Penicillin intolerance	Standard	
	Clindamycin[4, 8, 65]	Allergic to penicillin, recurrent mastitis	Standard	
Probiotics	*L. salivarius* CECT5713 and *L. gasseri* CECT5714[11]	Staphylococcal mastitis	Experimental stage	30 days
	L. fermentum CECT5716; *L. salivarius* CECT5713[79]	Infectious mastitis	Experimental stage	21 days
	L. salivarius PS2[80]	Preventive measure against mastitis	Experimental stage	~ 44 days
	L. salivarius PS2[81]	Infectious mastitis	Experimental stage	21 days
Bacteriocins	Nisin[87]	Staphylococcal mastitis	Experimental stage	14 days

Antibiotics

Prior to 1948, the management of lactational mastitis consisted of binding, ice packs, and discontinuance of nursing, in the hope that symptoms would not develop further. Penicillin was considered the most appropriate antibacterial available yet proved to be limited in treatment of the disease [Devereux, 1969]. As stated already, *S. aureus* and *S. epidermidis* are found to be the main causative microorganisms of lactational mastitis with *Strep. mitis* and *Strep. salivarius* being the second and third most reported causative agents, respectively [Mediano et al., 2017]. However, multidrug resistance to antibiotics and/or formation of biofilms is typical among clinical isolates of these two staphylococcal species. Accordingly, it is not surprising that 70–90% of the cases of staphylococcal mastitis in cattle are immune to antibiotherapy [Wall et al., 2005]. Resistance to methicillin and a high ability to form biofilms explains why mastitis tends to be a recurrent or chronic infection. Indeed, it has been reported that 25% of mothers who discontinue breast-feeding as a result of mastitis [WHO, 2000] have already received antibiotherapy (cloxacillin, clindamycin, amoxicillin-clavulanic acid, and/or erythromycin) for 2–4 weeks with little success [Jiménez et al., 2008].

Penicillinase-resistant penicillins such as dicloxacillin or flucloxacillin are the most suitable antibiotic therapies for treatment of the disease (Table 3). First-generation cephalosporins are also approved as a first-line therapy. More specifically, cephalexin can be administered in cases of penicillin intolerance, while in cases of allergy to penicillin, clindamycin is preferred [Schoenfeld and McKay, 2010; Amir et al., 2011]. Dicloxacillin has lesser hepatic inimical effects than flucloxacillin [Jahanfar et al., 2013]. However, dicloxacillin can cause phlebitis when applied intravenously; so, it should be taken orally when symptoms persist [Abou-Dakn et al., 2010]. A later

study demonstrated that chloramphenicol, gentamicin, oflaxacin and ciprofloxacin were the most efficacious antibiotics against mastitis pathogens [Patel et al., 2016]. With regards to duration of antibiotic therapy, there is no consensus with recommendations varying between 7 and 14 days. As outlined by Reddy et al. [2007], where symptoms endure, culturing of the milk and an assessment of the antibiotic resistance of the microbes should be performed due to the escalation of penicillin-resistant, methicillin-resistant (MRSA) and oxacillin-resistant S. *aureus* (ORSA); although it would be of greater benefit if milk cultures were performed as soon as possible to establish a proper diagnosis and to establish the most adequate treatment depending on the main bacterial agent(s) involved. This would greatly help to avoid misuse of antibiotics.

It is broadly acknowledged that antibiotics are the culprit for the disruption in human microbiota which can result in antibiotic-associated diarrhea, urogenital and oral infections [Martín et al., 2012]. Studies have demonstrated an increased vulnerability to infectious, allergic, and inflammatory diseases because of the dysregulation of host immune homeostasis due to altered microbiota. The latter is attributed to the excessive use of antibiotics [Joffe and Simpson, 2009; Willing et al., 2011]. The administration of antibiotics during pre- and post-delivery stages, has been proven to adversely affect breastfed infants as antibiotics can modify the maternal microbiota which is of critical importance to the human GI microbiota in early life [Mueller et al., 2015]. Arvola et al. [2006] demonstrated that infants with deferred colonization or low bifidobacterial counts may be at risk of further gastrointestinal or allergic conditions, while de Weerth et al. [2013] found low *Bifidobacterium* and *Lactobacillus* counts in the stools of infants diagnosed with colic. Moreover, Kummeling et al. [2007] reported that antibiotic exposure via breastfeeding in infants

was associated with a higher risk of recurrent wheeze during the first 2 years of life. Consequently, there is a need to develop strategies to replace antibiotics and, in this context, probiotics may provide a feasible solution.

Probiotics

The WHO/FAO describes probiotics as "live microorganisms that when administered in adequate amounts confer a health benefit on the host" [FAO/WHO, 2001; Hill et al., 2014]. It is now known that the infant gut is colonized by bacteria from human milk [Martín et al., 2003; Albesharat et al., 2011; Jost et al., 2014; Pacheco et al., 2015; Murphy et al., 2017]. Consequently, modulation of maternal gut microbiota during pregnancy and lactation could have a direct impact on infant health [Fernández et al., 2013]. Indeed, recent studies illustrate that the mammary gland accommodates its own microbiota during late pregnancy and lactation [Fernández et al., 2013]. More specifically, strains isolated from breast milk can be good candidates for use as probiotics [Jiménez et al., 2008; Arroyo et al., 2010] as they fulfill several criteria including human origin, adaptation to mucosal substrates and a history of safe extended consumption by sensitive individuals such as infants [Espinosa-Martos et al., 2016].

An initial study by Jiménez et al. [2008] investigated the potential of two lactobacilli strains as an alternative therapy for staphylococcal mastitis, namely *L. salivarius* CECT5713 and *L. gasseri* CECT5714 [Table 3]. Of a total of 20 women presenting with the condition, ten received the two *Lactobacillus* strains (10 log cfu of each) for a 4-week period and ten received the excipient only for the same period. On day 0, mean staphylococcal counts in both groups were similar, but by day 30, the

mean staphylococcal count in the probiotic group was lower when compared to the control group (2.96 log cfu mL^{-1} versus 4.79 log cfu mL^{-1}, respectively). By day 14, clinical signs were no longer observed in the probiotic group although mastitis persisted in the control group. A subsequent study by Arroyo et al. [2010] also investigated the potential of *L. salivarius* CECT5713 and the strain *L. fermentum* CECT5716 in a much larger group of subjects, 352 women presenting with infectious mastitis. Women received either *L. salivarius* CECT5713 (n=127, 9 log cfu), or *L. fermentum* CECT5716 (n=124, 9 log cfu) or antibiotic therapy (n=101) for 3 weeks. By day 21, milk bacterial counts were lower in women receiving probiotics compared to those on antibiotic therapy. The authors state that women in the probiotic groups 'improved more' and had lower recurrence of mastitis than those receiving antibiotic therapy. The outcomes of both these studies are further discussed by Fernández et al. [2014].

A more recent study examined the potential of *L. salivarius* PS2 to hinder infectious mastitis in pregnant women who were previously diagnosed with mastitis [Fernández et al., 2016]—108 women were divided into two groups. Those in the probiotic group ingested 9 log cfu day^{-1} of *L. salivarius* PS2 from approximately week 30 of pregnancy until delivery while those in the control group received a placebo. Following the end of the study, 41% of the participating women were diagnosed with mastitis; although the number of women from the probiotic group suffering from mastitis (25%) was significantly lower than the placebo group (57%). The bacterial counts of women with subacute mastitis from the placebo group were significantly higher when compared to the probiotic group (4.61 and 3.83 log cfu mL^{-1}, respectively). Comparable results were noted in the group of women with acute mastitis where those who belonged in the placebo group had higher bacterial mean

counts (4.51–5.53 log cfu mL^{-1}) than those who belonged in the probiotic group (3.29–4.29 log cfu mL^{-1}). This study demonstrates that *L. salivarius* PS2 could be an effective alternative to forestall infectious mastitis.

Espinosa-Martos et al. [2016] endeavored to correlate microbiological, biochemical or immunological markers in milk, blood or urine with the beneficial outcome observed during probiotic treatment of mastitis. A total of 31 women took part in the study. Among them, 23 women had clinical symptoms of mastitis while the remaining (n=8) were healthy. Over a period of 21 days, both groups received three daily doses (10^9 cfu) of *L. salivarius* PS2. In the mastitic group, the probiotic intake resulted in a notable reduction of mean bacterial (1.1 log cfu mL^{-1}) counts in milk. Moreover, clinical symptoms were alleviated in the probiotic group after day 7 while the somatic cell count significantly declined after the intake of probiotics. The effect of the probiotic strain on the total bacterial counts in human milk is in agreement with previous studies [Jiménez et al., 2008; Arroyo et al., 2010].

Bacteriocins

Bacteriocins are antimicrobial peptides produced by bacteria and exhibit potent activity against other bacteria including antibiotic-resistant strains [Cotter et al., 2005]. They are generally stable, have low toxicity and can exhibit a narrow or broad spectrum of activity. Certain bacteriocins such as nisin have already gained acceptance as antimicrobials in the food industry where they provide protection against pathogen and food spoilage microorganisms. The lantibiotics describe a group of bacteriocins which undergo post-translational modifications and have exhibited a high potency to hinder various multidrug-resistant bacteria combined with a low propensity to

generate resistance. The food-grade lantibiotic, nisin, is produced by certain strains of *Lactococcus lactis*, a common species in the breast milk of healthy women [Martín et al., 2007; Fernández et al., 2014]. Indeed, approximately 30% of isolates of this origin can produce nisin [Beasley and Sarris, 2004]. During the past 2 decades, there has been a revived interest in the use of this lantibiotic as a therapeutic agent in bovine mastitis [Fernández et al., 2014]. Furthermore, another bacteriocin, i.e., lacticin 3147 has generated promising results in the treatment of bovine mastitis [Crispie et al., 2005; Klostermann et al., 2010]. Like nisin, lacticin 3147 is a lantibiotic with a broad spectrum of activity. Indeed, the potential of nisin in treating infectious mastitis in lactating mothers has already been demonstrated (Table 3). In a 2-week study, nisin was investigated as an alternative treatment for staphylococcal mastitis in women who had already received antibiotics for 2–4 weeks but which failed to ameliorate their condition [Fernández et al., 2008]. More specifically, eight women diagnosed with staphylococcal mastitis were divided in two groups. The first group received a solution of nisin which was applied to the nipple and mammary areola, while the second group received a solution lacking nisin. On day 0, the staphylococcal counts were similar in the bacteriocin and placebo groups (5.0 ± 0.2 and 4.9 ± 0.2 log cfu mL^{-1}, respectively), while on day 14, the average counts in the nisin group (3.2 ± 0.4 log cfu mL^{-1}) were significantly lower compared with the placebo group (5.0 ± 0.2 log cfu mL^{-1}). At the end of the study, no clinical signs were observed in the bacteriocin group.

Conclusions and Future Prospects

While the number of studies addressing the microbiological load of human lactational mastitis is limited, those which have been completed provide an insight into the

microbiological dysbiosis associated with the disease and hence shed light on potential new therapies. One study which employed metagenomic analysis of mastitic milk samples clearly identified a loss of bacterial diversity [Jiménez et al., 2015]. Unsurprisingly, *Staphylococcus* is the dominant genus associated with the disease where *S. aureus* is the main etiological agent in acute mastitis and *S. epidermidis* is associated with subacute mastitis. In addition, *Corynebacterium* species are now recognized as the causative agents of human granulomatous mastitis. While the treatment of mastitis with antibiotics has, in the past, been met with mixed results, the looming threat of antibiotic resistance has significantly increased the need to identify alternative therapies. In addition, antibiotics are known to impart deleterious effects on the microbiota of human milk. In this regard, probiotics offer a viable alternative and the limited number of studies available, would seem to indicate that they can be effective against human mastitis both as prophylactics and therapeutics and in the latter case have generated similar or superior results to antibiotics in certain instances. Some of the probiotic strains tested to date have in fact been isolated from breast milk and it is now accepted that the mammary gland has its own microbiota in late pregnancy and during lactation. In this regard, it may be conceivable in the near future to generate 'personalized' probiotics for those at particular risk of mastitis, such as women with a known history of the disease.

The preliminary success of the bacteriocin nisin in the management of human mastitis, as well as the promising results with lacticin 3147 for the treatment of bovine mastitis may pave the way for other bacteriocins or bacteriocin-producing live strains in the treatment of human mastitis in the future. For such alternative therapies to be accepted as preventative agents or for treatment of this condition, randomized controlled trials in healthy and diseased lactating women are required to confirm

efficacy, and furthermore to demonstrate that no negative effects of treatment are imparted on the quality of milk for nutrition of the nursing infant.

Undoubtedly, continued investigation into the microbiology of human mastitis is essential and will help in the identification and development of successful therapies. In this regard, there is an onus on researchers, health agencies and biotechnology companies to work in unison towards reducing the incidence of this devitalizing disease.

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Chapter 3

Bovine Mastitis is a Polymicrobial Disease Requiring a Polydiagnostic Approach

Angelopoulou A, Holohan R, Rea MC, Warda AK, Hill C, Ross RP. Bovine mastitis is a polymicrobial disease requiring a polydiagnostic approach. Int Dairy J. 2019;99: 104539.

Author Contributions

AA performed the experiments and drafted the manuscript. RH performed the bioinformatics analysis and drafted the manuscript. AKW has both drafted and critically reviewed the manuscript. MCR has provided the samples analysed in this study. CH and RPR have critically reviewed the manuscript. The final manuscript was read and approved by all authors.

Abstract

Bovine mastitis, an inflammation of the udder, is associated with increases in milk somatic cell count usually resulting from bacterial infection. We analysed 50 mastitic milk samples via cultivation, 16S rRNA gene sequencing and a combination of the two (culturomics) to define the complete microbial content of the milk. Most samples contained over 10,000 cfu mL^{-1} total bacterial counts including isolates that were haemolysin positive (n = 36). Among colonies isolated from blood agar plates, *Streptococcus uberis* was dominant (11/50) followed by *Streptococcus dysgalactiae* (6/50), *Pseudomonas* (6/50), *Enterococcus faecalis* (6/50), *Escherichia coli* (6/50), *Staphylococcus argenteus* (4/50), *Bacillus* (4/50) and *Staphylococcus aureus* (2/50). 16S rRNA gene profiling revealed that amplicons were dominated by *Rhodococcus*, *Staphylococcus*, *Streptococcus* and *Pseudomonas*. A higher inter-sample diversity was noted in the 16S rRNA readouts, which was not always reflected in the plating results. The combination of the two methods highlights the polymicrobial complexity of bovine mastitis.

Introduction

Mastitis is an inflammation of the cow's udder and is a disease of high frequency and economic significance due to depleted milk production, discarded milk, premature culling, and treatment costs [Halasa et al., 2007; Bar et al., 2008; Hertl et al., 2010]. A large volume of milk is processed to a variety of dairy products and apart from the risk of bacterial contamination, alterations in the composition of mastitic milk can negatively affect the quality of these products [Merin et al., 2008]. For example, it is known that the somatic cell count (SCC) level negatively correlates with cheese yield due to slower coagulation properties of the milk [Le Maréchal et al., 2011].

Mastitis can be classified into clinical or subclinical subgroups, with the latter being indicated by an escalation in SCC in the absence of overt symptoms [Vanderhaeghen et al., 2015]. Milk is classified as being clinical or subclinical based on SCC, with a SCC of 200,000 cells mL^{-1} generally being accepted as an indicator of the presence of mastitis infection [IDF 1997] and the SCC threshold for milk purchasers being 400,000 cells mL^{-1} according to EU regulations (Regulation (EC) No. 853 of 2004). Furthermore, mastitis-causing bacteria have been grouped as contagious or environmental based on their distribution and interplay with the teat and teat duct [Smith and Hogan, 1993]. The disease is normally the result of bacterial intramammary infection, and the most commonly associated causative agents are staphylococci, streptococci and coliforms [Bradley et al., 2007; Vanderhaeghen et al., 2015]. However, up to 200 different microbial species have been documented in mastitic cases. These are primarily bacteria but can include fungi or even monocellular achlorophylic algae [Cvetnić et al., 2016].

Identification of the microbe driving the disease is of critical importance for clinical resolution. The gold standard method used for the characterisation of microorganisms responsible for mastitis is bacterial culture. Nevertheless, restrictions of culture-dependent techniques include a delay of 24–48 h to acquire results and the fact that roughly 25% of milk samples from clinical mastitis cases are culture negative [Taponen et al., 2009]. This highlights the importance of evaluating culture-independent techniques for mastitis diagnosis. It has been suggested that all mastitis treatments should be evidence-based, which primarily requires the identification of the mastitis-causing organism(s) [Milkproduction.com 2007].

Sequencing and analysis of hypervariable regions within the 16S rRNA gene can furnish comparably expeditious and cost-effective methods for appraising bacterial diversity and abundance and has proved an effective tool for pathogen discovery and identification [Oikonomou et al., 2012]. These technologies have enabled the investigation of microbial communities in milk without some of the limitations of culture methods [Oikonomou et al., 2012; Jiménez et al., 2015; Ganda et al., 2016]. It should be noted that the resulting datasets are compositional [Gloor et al., 2017], failing to provide resolution to species/strain level and do not differentiate between living and dead microorganisms.

We employed both culture-dependent and culture-independent methods to identify the major pathogenic species found in milks collected from diseased animals.

Materials and Methods

Sample Collection

Fifty mastitic milk samples were collected from 46 cows which had elevated SCC (≥ 200,000 cells mL^{-1}) during the period of November 2016 to April 2018. Samples were taken after the first streams of milk were discarded and stored below 4 °C, overnight until they were further processed. Aliquots of 1 mL of fresh milk were subject to cultivation within 24 h of donation. Remaining aliquots were immediately frozen at −20 °C for subsequent DNA extraction.

Determination of SCC

Milk samples were analysed for SCC using a Somacount 300 (Bentley Instruments, Inc., Chaska, MN, USA) according to the International Dairy Federation (IDF) guidelines [IDF 1981].

Microbiological Analysis

Clotted samples with high SCC (> 10,000,000 cells mL^{-1}) were homogenised for 30 min with the use of a stomacher machine (IUL Instruments, SA) whereas the remaining samples were directly processed. Aliquots of milk sample, 1 mL, were mixed with 9 mL of maximum recovery diluent (Oxoid, Basingstoke, UK) to make an initial 10^{-1} dilution. Serial dilutions were enumerated by the spread plate method in duplicate onto: (i) de Man, Rogosa, Sharpe (MRS) agar (Oxoid) at 37 °C (pH 5.5) for 3 days in anaerobic jars (gas-pack plus anaerobic system, BBL; BD Diagnostics, USA), which selects for lactobacilli; (ii) blood agar (BA) base (Oxoid) supplemented with 7% (v/v) defibrinated sheep blood (Cruinn Diagnostics, Ireland) at 37 °C for 48 h aerobically, which is a non-selective medium; (iii) Baird Parker (BP) agar (Oxoid)

supplemented with 50 mL egg yolk tellurite emulsion (Oxoid) at 37 °C for 48 h aerobically, which selects for staphylococci; (iv) MacConkey (McC) agar (Oxoid) at 37 °C for 24 h aerobically, which selects for enterobacteria; (v) plate count agar (PCA; Oxoid) at 30 °C for 72 h, aerobically in which total mesophilic bacteria were counted. Plates were assessed for growth and colony morphology characteristics and the blood agar plates were subsequently analysed for haemolytic characteristics.

Species Determination by Sanger Sequencing

Colony PCR was performed on forty isolated colonies from blood agar plates and forty isolated colonies from Baird Parker plates per sample based on different morphology in the analysed samples (Supplementary material, Table S3.1.). Cells were lysed in 10% Igepal 630 (Sigma–Aldrich, Germany) at 95 °C for 10 min. PCR was performed in a total volume of 25 µL using 10 µL Phusion Green Hot Start II High Fidelity PCR master mix (ThermoFisher Scientific, Waltham, MA, USA), 10 µL PCR-grade water, 1.5 µL of the nonspecific primers 27F and 1495R (primer stocks at 0.1 ng μL^{-1}) (Sigma–Aldrich) and 2 µL of DNA template from lysed cells. Amplification was carried out with reaction conditions as follows: initial denaturation at 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s with a final extension step at 72 °C for 10 min. Five microlitres of the resulting amplicons from each reaction were electrophoresed in a 1.5% (w/v) agarose gel. A GeneGenius Imaging System (Syngene, Cambridge, UK) was used for visualisation. The PCR products were purified using the GeneJet Gel Extraction Kit (ThermoFisher Scientific). DNA sequencing of the forward strand was performed by Source BioScience (Tramore, Ireland). The resulting sequences were used for

searching sequences deposited in the GenBank database using NCBI BLAST database (http://www.ncbi.nlm.nih.gov/BLAST/) and the identity of the isolates was determined on the basis of the highest scores (> 98%).

DNA Extraction and MiSeq Sequencing

DNA was purified from milk samples using the PowerFood Microbial DNA Isolation Kit (MoBio Laboratories, Carlsbad, USA) with slight modifications. Four mL of the milk samples were centrifuged twice at 4000 ×*g* for 30 min. The top fat layer was removed with a sterile cotton swab. The pellet was washed twice with sterile phosphate-buffered saline (PBS), re-suspended in 90 µL of 50 mg mL^{-1} lysozyme (Sigma–Aldrich) and 25 µL of 10 KU mL^{-1} mutanolysin (Sigma–Aldrich) and incubated at 55 °C for 15 min. Subsequently, 28 µL of proteinase K (Sigma–Aldrich) was added and the pellet was incubated at 55 °C for 15 min. The supernatant was removed after centrifugation at 13,000 ×*g* at 4 °C. The remaining steps were performed using the PowerFood Microbial DNA Isolation Kit according to manufacturer's instructions with the bead-beating time reduced to 3 min to limit DNA shearing. The microbiota composition of the samples was established by amplicon sequencing of a ~460 base pair (bp) fragment of the V3–V4 hypervariable region of the bacterial 16S rRNA gene following the Illumina 16S Metagenomic Sequencing Library Preparation guide. PCR amplification of V3–V4 region was performed using the forward primer 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′ and reverse primer 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATC

TAAC-3′. Each 30 μL PCR reaction contained up to 5 ng μL^{-1} microbial genomic DNA, 6 μL of each primer (1 μM) and 15 μL Phusion High-Fidelity PCR Master Mix (ThermoFisher Scientific). The PCR conditions were as follows: initial denaturation for 30 s at 98 °C; 25 cycles of 10 s at 98 °C, 15 s at 55 °C and 20 s at 72 °C; and 72 °C for 5 min for final extension. The Agencourt AMPure XP system (Beckman Coulter, UK) was used to purify the amplicons. A subsequent limited-cycle amplification step was performed to add multiplexing indices and Illumina sequencing adapters. Amplicons were quantified, normalised and pooled using the Qubit® dsDNA HS Assay Kit (Life Technologies, Carlsbad, California, USA). Library preparation was carried out by GATC Biotech prior to 2 × 300 bp sequencing on the Illumina MiSeq platform.

Bioinformatic Analysis of HTS Data

Read quality was assessed using FastQC (v0.11.5) [http://www.bioinformatics.babraham.ac.uk/projects/fastqc/] both before and after quality filtering with Trimmomatic (v0.32) [Bolger et al., 2014] where a Phred quality threshold of 20 in a sliding window of size 4 was employed. The leading 15 bases of each read was removed followed by a crop at base 270, all reads greater than or equal to 50 bases in length were retained. Read pairs were merged using FLASH (v1.2.11) [Magoc and Salzberg, 2011] on default settings before being processed using VSEARCH in QIIME2 (v2018.8) [Bolyen et al., 2019]. To do this, reads were dereplicated and clustered *de novo* at 97% forming OTUs. Chimeric reads were removed in two successive steps, both *de novo* and reference based against the ChimeraSlayer Gold database. Taxonomic classification was determined using mothur

(v.1.38.0, bootstrap ≥ 80) [Schloss et al., 2009] and SPINGO [Allard et al., 2015] (v1.3, bootstrap ≥ 0.8, similarity score ≥ 0.5) using the RDP v11.4 database. Analysis was performed using the R programming language (v3.5.1) [Ihaka and Gentleman, 1996] and visualised using ggplot2 (v3.1.0) [Wickham, 2009]. Raw data has been made publicly available in the NCBI's Sequence Read Archive under the accession number: PRJNA509157.

Results and Discussion

The aim of the study was to define the microbial composition of milks from mastitic cows using both cultivation and HTS approaches. Fifty bovine milk samples with elevated SCC were analysed in this study, fluctuating between 221,000 and > 10,000,000 cells mL^{-1} (Table 3.1.). Based on microbiological cultivation, the majority of the samples contained isolates with haemolytic patterns with α-haemolysis being dominant (70% of the samples). β-Haemolytic bacteria were also detected in 40% of the samples, while γ-haemolysis was less common and found in 20% of the samples. Total mesophilic bacteria were enumerated at an average population of 5.92 log cfu mL^{-1} on PCA, with four culture negative samples (M29, M32, M33, and M45). Comparable mesophilic counts were demonstrated by Dobranić et al. [2016], who found up to 5.39 log cfu mL^{-1} total mesophilic counts in bovine milk samples from animals cured of mastitis. The average population of presumptive lactic acid bacteria (LAB) grown on MRS was 4.30 log cfu mL^{-1}, similar to that reported by Qiao et al. [2015] who enumerated lactobacilli using quantitative PCR (qPCR) in 12 mildly subclinical milk samples and 28 severely subclinical milk samples. In the mild subclinical group (SCC < 500,000 cells mL^{-1}), the mean counts were 4.83 log cfu

mL^{-1} whereas in the severely subclinical group (SCC > 500,000 cells mL^{-1}), the mean counts for lactobacilli were 4.74 log cfu mL^{-1}.

Table 3.1. Heat map of the microbial load in fifty bovine mastitic milk samples. [a]

Samples	SCC	Blood agar			McC	BP	PCA	MRS
		α-haemolysis	β-haemolysis	γ-haemolysis				
M1	>10,000,000	4.6			4.6	5.5	6.0	4.4
M2	>10,000,000	5.0				4.9	5.4	5.0
M3	>10,000,000	5.4			4.9	2.0	5.6	5.3
M4	3,343,000	2.8			1.8	1.5	3.7	1.9
M5	324,000	2.5			1.6	1.0	4.2	2.6
M6	3,917,000			2.0	2.0		4.0	3.0
M7	5,707,000		1.7	1.5	2.4		2.5	2.8
M8	346,000	3.5			2.5	3.0	3.8	2.5
M9	700,000		2.0		4.4	1.5	3.5	2.8
M10	9,422,000	5.0			4.2	3.8	4.3	5.7
M11	8,115,000	5.7					5.2	
M12	644,000				5.3	5.0	5.2	7.2
M13	221,000	5.2		1.0	4.6	5.5	6.3	3.9
M14	4,330,000	3.0	1.0	1.2	1.4	1.5	7.4	6.6
M15	2,502.000	3.1		4.5	4.1		4.6	3.5
M16	9,999,000	0.5	2.0	4.4	0.6	5.0	5.0	1.9
M17	809,000				3.1	5.2	6.3	4.0
M18	1,247,000	8.3			8.3	5.5	9.5	1.8
M19	2,607,000	9.0			7.3	4.6	8.3	5.0
M20	>10,000,000	6.5			5.2	4.1	9.5	4.0
M21	>10,000,000	2.8	2.3		1.0	2.4	4.9	
M22	>10,000,000	8.1					7.7	
M23	>10,000,000	4.5			4.5		4.1	
M24	>10,000,000	7.1			4.0		6.8	
M25	>10,000,000	8.9			3.7	6.1	8.9	
M26	>10,000,000	5.6	5.4		5.5	4.5	5.2	
M27	>10,000,000	5.0	4.6		4.4	4.5	5.9	
M28	>10,000,000	6.0		5.1	4.6	3.0	6.1	
M29	592,000			3.0		3.0		
M30	>10,000,000	5.9			4.9		5.7	
M31	>10,000,000			7.2	6.9		7.9	6.4
M32								
M33	8,181,000		6.3			3.0		
M34	>10,000,000		5.8				6.1	3.3
M35	934,000	4.6			4.7	5.3	4.1	3.6
M36	1,061,000		4.1		4.6	4.1	4.3	4.1
M37	>10,000,000				3.1		4.2	4.4
M38	>10,000,000		4.7		4.4		4.5	4.3
M39	>10,000,000	5.6	4.5		6.3	5.4	6.3	4.1
M40	>10,000,000	7.2	6.2		7.5	5.3	6.8	4.6
M41	>10,000,000	6.1		8.2	7.7	5.9	6.9	5.2
M42	>10,000,000	7.1	7.0		7.3	7.3	7.0	5.1

(continued)

Table 3.1. Continued

Samples	SCC	Blood agar			McC	BP	PCA	MRS
		α-haemolysis	β-haemolysis	γ-haemolysis				
M42	>10,000,000	7.1	7.0		7.3	7.3	7.0	5.1
M43	>10,000,000				5.8	6.7	7.5	6.4
M44	>10,000,000	6.4	5.7		7.5	2.0	7.1	3.7
M45	>10,000,000	6.4	6.5		5.8	2.2		5.0
M46	>10,000,000		8.1		2.6		7.3	5.5
M47	>10,000,000	4.6	3.3		4.8	3.9	5.6	
M48	>10,000,000	6.2			5.6	2.2	6.8	3.8
M49	>10,000,000		3.8		4.0	4.2	3.9	
M50	>10,000,000	5.6	6.8			2.0	7.1	6.2

[a] SCC are also shown. Color intensity corresponds to the microbial load on various media. Red represents bacterial numbers ~10 log cfu mL^{-1} and white represents absence of microbial growth in the tested media.

High SCC does not always correlate with a high bacterial load. For example, while samples M7, M21, M37 and M49 had a SCC in excess of 5 million and were clotted in appearance, their total mesophilic counts were only 2.5, 2.4, 4.2 and 3.9 log cfu mL^{-1}, respectively. This could be due to a high load of uncultivable microorganisms in these samples. The identity of microorganisms isolated from blood agar and Baird Parker plates was determined by Sanger sequencing (Figure 3.1., Supplementary Material Table S3.2 and Figure 3.2, Supplementary Material Table S3.3., respectively). Colonies from blood agar plates were dominated by the genus *Streptococcus* (31.6%) and more specifically by *Streptococcus uberis* (18.2%) followed by *Streptococcus dysgalactiae* (11.1%), *Streptococcus agalactiae* (2.2%) and *Streptococcus urinalis* (0.1%). *St. uberis, St. dysgalactiae* and *St. agalactiae* are well-known mastitic pathogens [Klass and Zadoks, 2018], while *St. urinalis* belongs to a subgroup of streptococci which cause urinary tract infections in humans and has not been associated with bovine mastitis until now [Peltroche-Llacsahuanga et al., 2012].

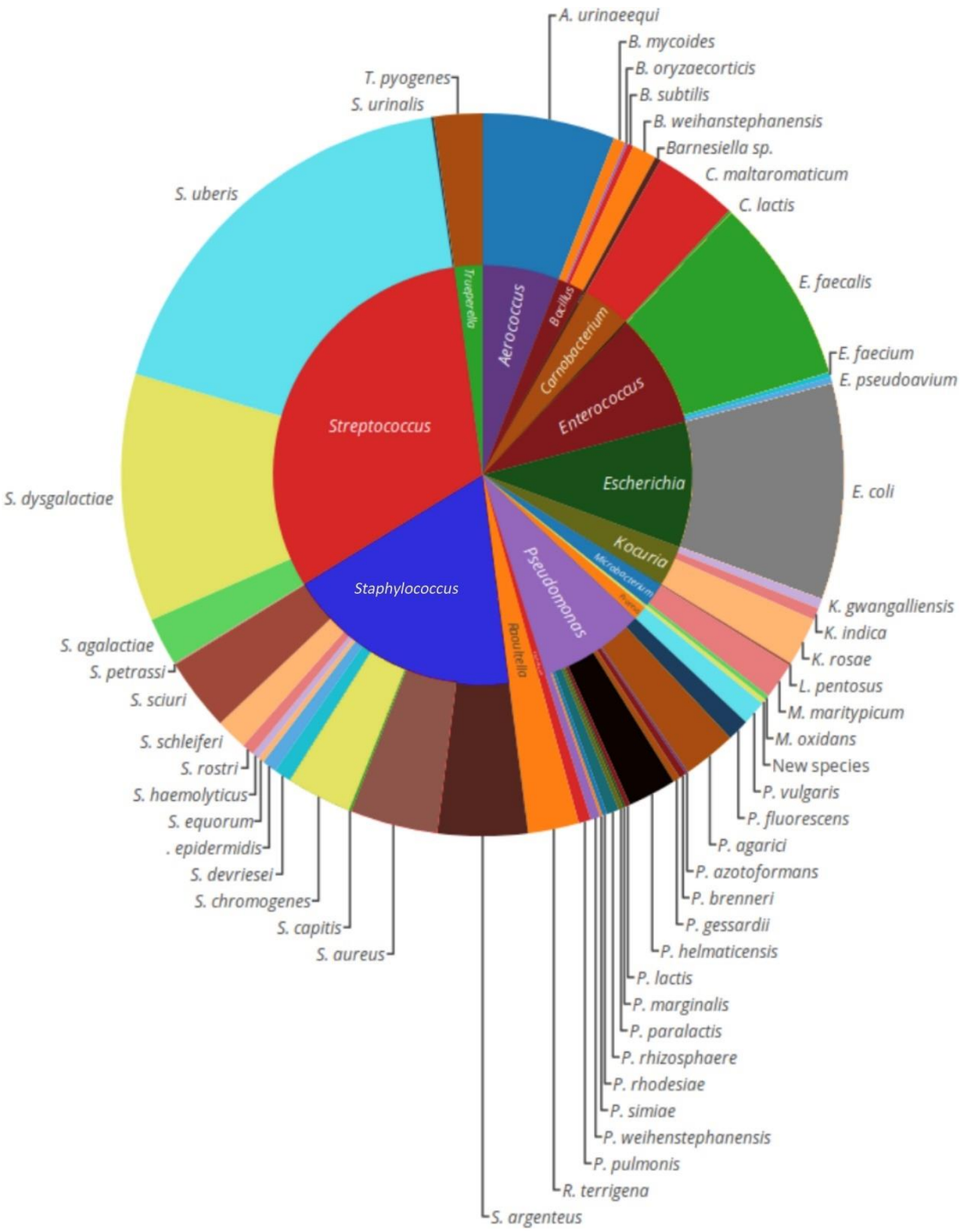

Figure 3.1. Average species identification on blood agar from fifty mastitic milk samples. Inner circle depicts genus and outer circle indicates species. Results depicting *S. argenteus* were inconclusive as to whether it was *S. argenteus* or *S. aureus*.

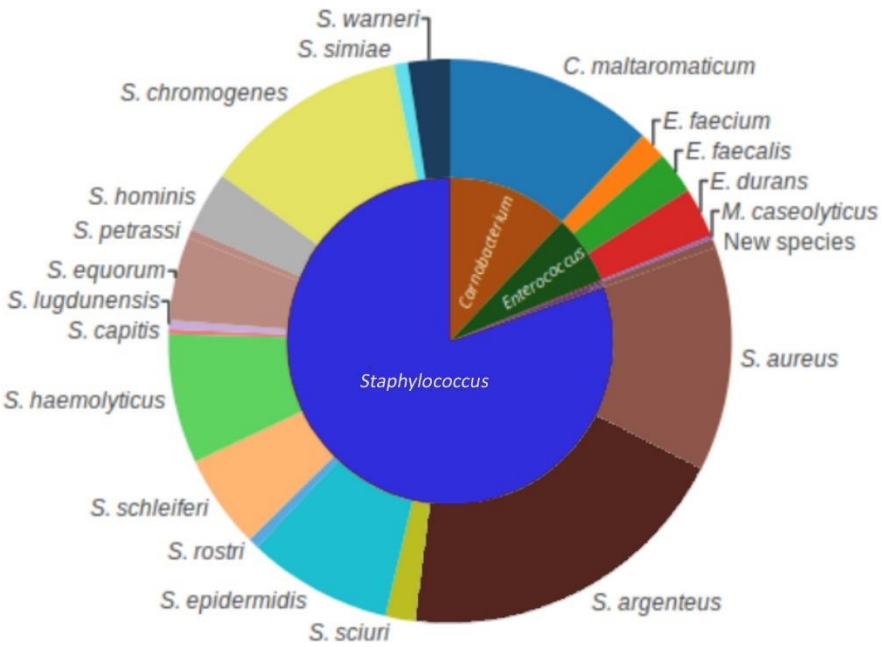

Figure 3.2. Average species identification on Baird Parker agar plates from fifty mastitic milk samples. Inner circle depicts genus and outer circle indicates species. Results depicting *S. argenteus* were inconclusive as to whether it was *S. argenteus* or *S. aureus*.

Of the isolates from blood agar plates, 18.3% were staphylococci with a relatively even distribution of *Staphylococcus aureus* (3.9%), *Staphylococcus argenteus* (4.0%), *Staphylococcus sciuri* (3.3%), and *Staphylococcus chromogenes* (2.9%). *S. aureus* is a well-established mastitis pathogen both in cows and humans while *S. argenteus* is a relatively novel species [Tong et al., 2015] that has been isolated from human infections [Jiang et al., 2018], but not from bovine mastitis until now. The results identifying strains as *S. argenteus* were inconclusive as to whether they were *S. argenteus* or *S. aureus.* *S. sciuri* and *S. chromogenes*, both coagulase-negative staphylococci, have been previously isolated in bovine mastitis studies [Hosseinzadeh and Dastmalchi Saei, 2014; dos Santos et al., 2016]. Members of *Escherichia*, *Enterococcus*, and *Pseudomonas* were identified at comparable

frequencies of 9.7%, 8.9%, and 8.2%, respectively. All *Escherichia* isolates were *Escherichia coli* while almost 98% of the *Enterococcus* belonged to *Enterococcus faecalis*. *E. coli* has been identified as one of the major mastitis-causing pathogens [Luoreng et al., 2018; Vasquez et al., 2019], while enterococci have also been frequently isolated from mastitic cows [Gomes et al., 2016]. Thirteen species of *Pseudomonas* were detected (see Figure 3.1.), but none was identified as *Pseudomonas aeruginosa*, a microbe that is often detected in mastitis [Park et al., 2014]. *Pseudomonas lactis*, *Pseudomonas paralactis* [von Neubeck et al., 2017], and *Pseudomonas weihenstephanensis* [von Neubeck et al., 2016] have been previously isolated from cows' milk. *Kocuria* (3.2%), which is usually found in skin and mucous membranes of humans and animals and is an emerging cause of infection [Kandi et al., 2016], was also detected on blood agar plates together with *Trueperella pyogenes* (2.2%) which has been associated with summer mastitis [Pyörälä et al., 1992].

Due to the semi-selective nature of the media, isolated colonies from Baird Parker agar plates were predominantly identified as *Staphylococcus*, particularly as *S. argenteus* (19.5%), *S. aureus* (19.5%), *S. chromogenes* (11.8%), *Staphylococcus epidermidis* (8.3%), and *Staphylococcus haemolyticus* (7.4%) (Figure 3.2., Supplementary Material Table S3.2.).

It is broadly acknowledged that many bacteria are not cultivable on standard microbiological media under standard conditions [Kamagata and Tamaki, 2005; Sekiguchi, 2006] and so in parallel we applied HTS to characterise uncultivated microbiota [DeLong, 2005].

MiSeq sequencing of 16S rRNA gene amplicons from bovine mastitic milk samples yielded a total of 14,319,524 quality filtered reads, with a median read length

of 234 ± 53 bases. Following quality control, we recorded an average of 286,391 reads per sample. At phylum level, Actinobacteria had the highest relative abundance in 38% of the bovine mastitic milk samples, while Firmicutes which were most abundant in 36% and Proteobacteria in 24% (Figure 3.3).

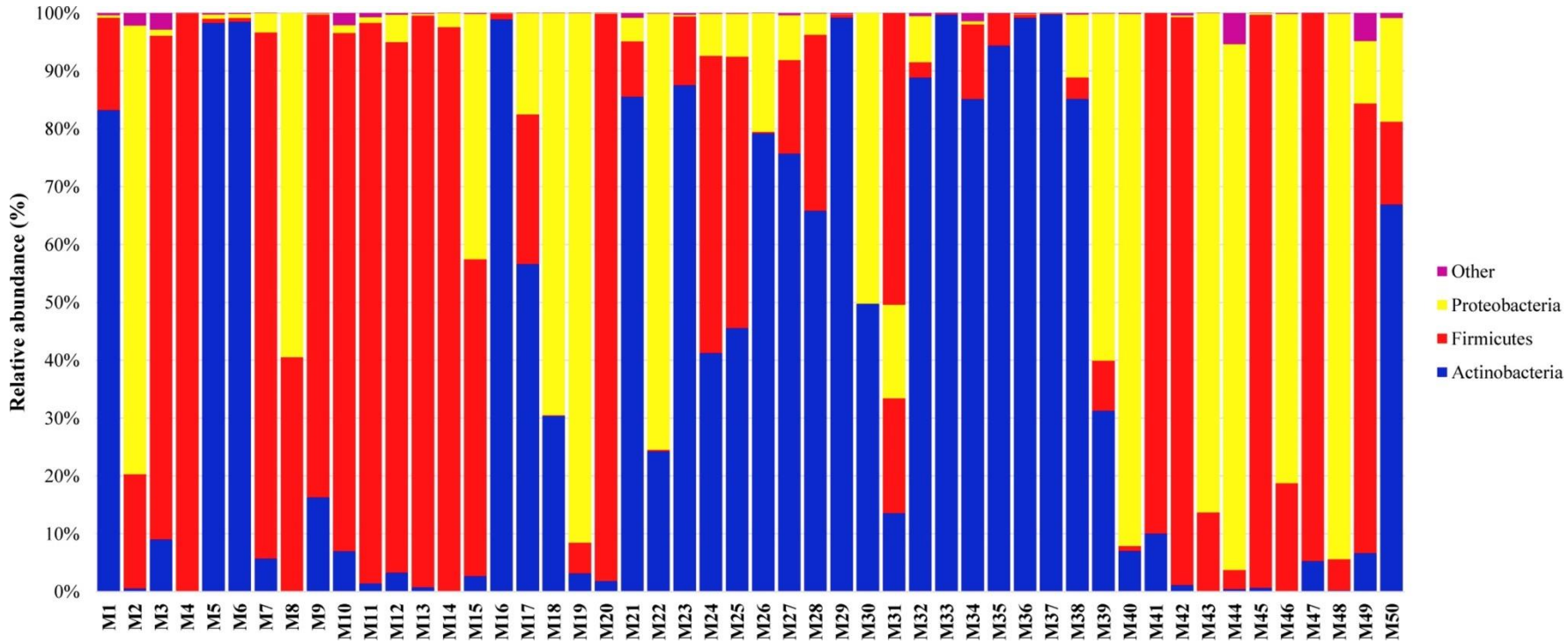

Figure 3.3. Phylum level assignments of average relative abundances of the microbiota in fifty bovine mastitic milk samples. Phyla with abundances below 1% are grouped as "Other".

At genus level *Rhodococcus* was the most abundant in most samples (38.0%), followed by *Pseudomonas* (16.0%), *Streptococcus* (12.0%) and *Staphylococcus* (8.0%) (Figure 3.4.).

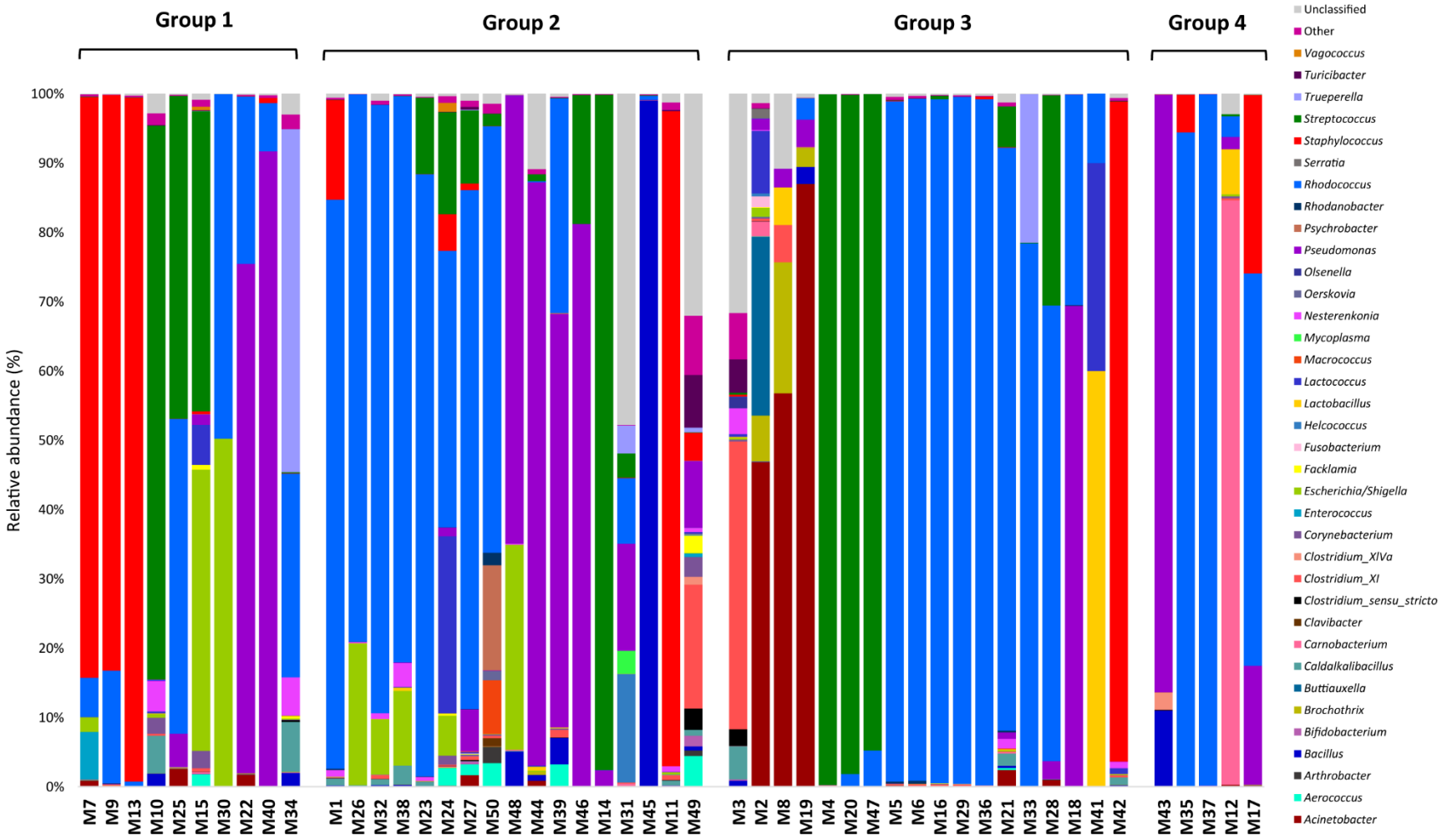
Group 1
Group 2
Group 3
Group 4
Relative abundance (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
M7 M9 M13 M10 M25 M15 M30 M22 M40 M34
M1 M26 M32 M38 M23 M24 M27 M50 M48 M44 M39 M46 M14 M31 M45 M11 M49
M3 M2 M8 M19 M4 M20 M47 M5 M6 M16 M29 M36 M21 M33 M28 M18 M41 M42
M43 M35 M37 M12 M17
Unclassified
Other
Vagococcus
Turicibacter
Trueperella
Streptococcus
Staphylococcus
Serratia
Rhodococcus
Rhodanobacter
Psychrobacter
Pseudomonas
Olsenella
Oerskovia
Nesterenkonia
Mycoplasma
Macrococcus
Lactococcus
Lactobacillus
Helcococcus
Fusobacterium
Facklamia
Escherichia/Shigella
Enterococcus
Corynebacterium
Clostridium_XIVa
Clostridium_XI
Clostridium_sensu_stricto
Clavibacter
Carnobacterium
Caldalkalibacillus
Buttiauxella
Brochothrix
Bifidobacterium
Bacillus
Arthrobacter
Aerococcus
Acinetobacter

Figure 3.4. Genus level assignments of average relative abundances of the microbiota in bovine mastitic milk samples. Bacterial genera under 1% are grouped as "Other". The samples are represented in 4 groups based on level of similarity between genus level assignments of relative abundances and results from 16S Sanger sequencing on blood agar plates. Group 1 is comprised of samples where in both cases the dominant genus is identified. Group 2 displays some similarities, group 3 has no similarities between the two data sets, and group 4 did not show any growth on blood agar plates.

The culture-based and metagenomics approaches displayed considerable divergence in their output. For example, approximately 50.0% of MiSeq samples had high levels of *Rhodococcus* while three samples had high levels of *Acinetobacter*, but we did not detect either genus using culture-dependent methods (discussed in detail below). Based on the results of both methods, we categorised our samples in four groups: Group 1 (M7, M9, M10, M13, M15, M22, M25, M30, M34 and M40) consisted of ten samples for which both 16S rRNA gene sequencing and 16S Sanger sequencing from blood agar plates resulted in the detection of the same dominant genus. More specifically, samples M7, M9 and M13 were dominated by *Staphylococcu*s whereas in samples M10, M15 and M25, the main genus detected was *Streptococcus*. Sample M22 and M40 were dominated by *Pseudomonas*, sample M34 was dominated by *Trueperella*, and sample M30 was dominated by *Escherichia*. However, this was not the case for the other samples. Group 2 consisted of 17 samples (M1, M11, M14, M23, M24, M26, M27, M31, M32, M38, M39, M44–M46 and M48–M50) displaying few similarities between the two data sets while the 18 samples in Group 3 displayed no similarities (M2–M6, M8, M16, M18–M21, M28, M29, M33,

M36, M41, M42 and M47). Finally, Group 4 (M12, M17, M34, M35 and M37) comprised of five samples that did not give rise to colonies on blood agar plates.

Of 36 genera detected in the bovine mastitic milk samples by 16S rRNA gene analysis, only eight were found in the culture-dependent analysis; namely *Staphylococcus*, *Bacillus*, *Carnobacterium*, *Escherichia/Shigella*, *Enterococcus*, *Streptococcus*, *Trueperella* and *Pseudomonas*. Moreover, from the culture-based approach we detected *Barnesiella* sp., *Kocuria*, *Microbacterium*, and *Raoultella* sp., which were not detected in the 16S rRNA gene analysis. Aerococci were detected in higher percentages in blood agar plates than via sequencing. It should be emphasised that 16S rRNA gene profiling only provides relative abundances and is not quantitative, albeit that *Corynebacterium* was found in similar relative abundances using the two methods. Furthermore, no colonies were obtained from samples M7, M11 and M24 on Baird Parker plates, even though staphylococci were found on blood agar plates for M7 and M11. This may demonstrate differences in nutritional requirements. 16S rRNA gene profiling indicated relative abundances for *Staphylococcus* at 83.81% (M7), 0.01% (M11) and 5.27% (M24).

Pseudomonas was detected in 6 samples (M1, M4, M5, M22, M41 and M47) by both cultivation and Sanger Sequencing, while it was predominant in the 16S profiling in 9 other samples (M18, M22, M31, M39, M40, M43, M44, M46 and M48). The detected differences could be due to the fact that *Pseudomonas* either could not grow in the cultivation conditions used or that it was there in different amounts, albeit 16S rRNA gene profiling only provides relative abundances and does not differentiate between viable and non-viable bacteria. *Pseudomonas* has been found in previous studies in raw milk [von Neubeck et al., 2015], bulk tank milk [Rodrigues et al., 2017] and is a member of the healthy core microbiome in human milk [Murphy et al., 2017].

Nevertheless, individual cases or sporadic outbreaks of mastitis may be caused by *Pseudomonas* sp., *T. pyogenes*, *Serratia* sp., or other unusual pathogens [Harmon, 1994]. *Pseudomonas* has also been associated with water contamination, including purified water systems [Ryan et al., 2011; Kuehn et al., 2013]. Water could be a significant source of microbial contamination considering that modern milking practices depend heavily on water for cleaning milking units. Indeed, mastitis caused by *P. aeruginosa* has been previously linked with contamination of water systems and teat disinfectants in the milking parlour [Kirk and Bartlett, 1984].

Eighteen out of fifty samples were dominated by *Rhodococcus* according to 16S rRNA profiling. In particular, 8 samples (M5, M6, M16, M21, M23, M29, M36 and M37) had over 80% *Rhodococcus* and were accompanied by low total mesophilic counts (up to 5 log cfu mL^{-1}). *Rhodococcus* was not detected from the colonies grown on Blood agar plates and it is likely that the species detected by the 16S rRNA gene sequencing were either anaerobic or could not grow under the conditions used in this study. *Rhodococcus* was previously misidentified in bovine mastitis milk samples as *Corynebacterium bovis* [Watts et al., 2000] while *Rhodococcus equi* was identified as the causative agent in an immunocompromised woman with granulomatous mastitis [Nath et al., 2013]. One study has identified *Rhodococcus* sp. as a causative agent in 4 out of 65 paired milk samples, collected from mastitic and healthy quarters of diseased dairy cows [Oultram et al., 2017].

Based on 16S rRNA gene profiling, 5 samples (M4, M10, M14, M20 and M47) were dominated by streptococci (ranging from 79.93 to 99.68%), however, three of those samples (M4, M20 and M47) were negative for streptococci on blood agar plates, possibly due to their anaerobicity. In other studies, streptococci not only have been linked with high SCC milk samples [Park et al., 2007; Zanardi et al., 2014;

Rodrigues et al., 2017], but they were also found in the healthy core microbiome of bovine [Quigley et al., 2013] and human milk [Murphy et al., 2017].

16S rRNA gene profiling identified *Trueperella* in 5 samples (M27, M31, M33, M34, M48 and M49 at 0.1%, 4.1%, 21.4%, 49.5% and 0.7%, respectively). Sample M34 was dominated by *Trueperella*, and *T. pyogenes* was the only member of this genus cultured. *T. pyogenes* has been shown to act synergistically with anaerobic bacteria, namely *Fusobacterium necrophorum*, *Bacteroides* sp., *Porphyromonas levii* in summer mastitis [Pyörälä et al., 1992]. Oikonomou et al. [2012] found that milk samples which were diagnosed as *T. pyogenes* mastitis, had a high prevalence of *F. necrophorum* subsp. *funduliforne*. We were unable to confirm this finding.

Samples M2, M8, and M19 were dominated by *Acinetobacter*. *Brochothrix* and *Pseudomonas* were detected in all three samples by 16S rRNA gene profiling. In sample M8, clostridia were detected while in sample M19 *Bacillus* was identified. Patel et al. [2017] demonstrated that 18 healthy mothers were rich in *Acinetobacter* compared with women with mastitis. Moreover, Kable et al. [2016] showed that *Acinetobacter* belongs to the core milk microbiota while Quigley et al. [2013] reported that *Acinetobacter* is often found in raw milk. *Acinetobacter* has the ability to adapt to various environmental conditions and several emerging pathogens have been described [Gurung et al., 2013]. *Acinetobacter* is also known to cause spoilage [Hantsis-Zacharov and Halpern, 2007] and is rarely a primary cause of mastitis [Oliver and Murinda, 2012].

Five samples contained *Escherichia/Shigella* at genus level (M15, M26, M30, M38, and M48 at 40.6%, 20.4%, 50.2%, 10.8%, and 29.6% respectively) which agreed with higher abundances of Enterobacteriaceae at family level and high abundances of Proteobacteria [Madigan et al., 2018]. These findings are in accordance with

previously published studies [Ganda et al., 2016; Lima et al., 2018; Vasquez et al., 2019] which also found the same pattern. *E. coli* was not detected in aerobic culture in sample M15, while for sample M26 only 25% of the colonies were identified *as E. coli*. Nonetheless, *E. coli* was the only bacterium recovered from samples M30 and M38 on blood agar plates.

16S rRNA gene profiling of samples M30 and M38 revealed a large diversity of taxonomic families, which is in accordance with previous characterisation of the microbiota of mastitic and healthy human and bovine milk, a finding that supports the possibility of an entero-mammary pathway [Perez et al., 2007; Angelopoulou et al., 2018]. This is a pathway in which bacteria from the gastrointestinal lumen reach the mammary gland with the help of dendritic cells and $CD18^{+}$ cells [Rescigno et al., 2001; Macphersno and Uhr 2004]. We detected many families in mastitic milk that are normally present in the gastrointestinal tract (GIT), such as Ruminococcaceae, Clostridiaceae, Peptostreptococcaceae and Lachnospiraceae. This is consistent with findings from normally sampled quarters and samples acquired via cannula [Jost et al., 2014; Oikonomou et al., 2014; Young et al., 2015; Ganda et al., 2015; Pang et al., 2018; Vasquez et al., 2019]. Members of these families have been previously detected in samples from different anatomical parts of the bovine GIT [Lima et al., 2015; Mao et al., 2015]. Ruminococcaceae, Clostridiaceae, Peptostreptococcaceae and Lachnospiraceae were also identified in faecal matter from cows; making it possible that their presence represents either contamination of samples or translocation into the udder [Young et al., 2015].

It is obvious that there are limitations to both culture-based and culture-independent diagnostics. Not all organisms causing infection can be cultivated and/or are recovered on culture, while 16S rRNA gene compositional profiling does not

provide sufficient resolution to pinpoint particular species and/or strains and furthermore, cannot differentiate between live and dead bacteria. Additionally, practical considerations such as price, time and labour intensity will influence the choice of method. On one hand, culturing bacteria is laborious, has a set price per sample (effect of sample number if limited) and can take up to a week to get results. On the other hand, 16S compositional sequencing is less laborious, its price per sample can be greatly affected by number of samples and can be very time consuming (outsourced sequencing usually takes 6 weeks, followed by data analysis). Nevertheless, metagenomic approaches are increasingly applied to acquire a detailed picture of the bacteria involved in the pathogenesis of mastitis.

Both strategies are almost certainly compromised by the low microbial biomass of most milk samples (and other types of samples). Salter et al. [2014] and Glassing et al. [2016] have both pointed out the potential for incorrect results in low biomass samples due to contamination or other artefacts that could lead to the discrepancies observed between the different studies examining the mastitic bovine milk microbiota [Oikonomou et al., 2012; Kuehn et al., 2013; Oikonomou et al., 2014; Oultram et al., 2017; Rodrigues et al., 2017; Lima et al., 2018; Pang et al., 2018; Vasquez et al., 2019]. A way to address the low biomass nature of the samples could be the inclusion of spike-in standards [Pollock et al., 2018] that can be added directly to the samples without affecting downstream bioinformatic analysis. However, we feel that this is less likely to be an issue in this study given that the majority of samples have a relatively high bacterial load. Storage conditions are another factor that should be taken into consideration as all samples were stored below 4 °C, overnight until they were further processed. However, psychrotrophs such as *Pseudomonas* sp. could proliferate at low temperature, complicating the interpretation of the finding that 16%

of samples were dominated by *Pseudomonas*. However, it should be emphasised that *Pseudomonas* has been found in the core microbiome of healthy human milk samples [Murphy et al., 2017] and therefore it remains possible for samples to be dominated by *Pseudomonas* at the time of sampling. Moreover, *S. aureus*, the main causative agent of bovine mastitis in Ireland has a temperature range for growth of 7–48 °C and so numbers should not increase on refrigeration. As storage conditions could influence the results of both culture-dependent and culture-independent methods, it is essential to minimise effects of sample handling, in particular collection method, time until sample processing, and sample storage.

We identified the microbiota composition of fifty bovine mastitic milks using both culture-dependent and -independent approaches with 20% (n = 10) of the tested samples giving similar outputs (Group 1). Group 2 displayed few similarities when 16S rRNA gene profiling was compared with culturing. Group 3 consisted of samples for which the two approaches were inconclusive and Group 4 samples gave no growth on Blood agar plates, indicating that the culture conditions used were not appropriate for the bacteria present in these samples. A high inter-sample diversity was noted in the 16S rRNA gene profiling, which was not always reflected in plating results. Thus, we suggest that the combination of the two methods sheds light into the microbial complexity of the disease and that symptoms might be driven or exacerbated by more than one insulting organism.

Acknowledgements

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Supplementary Files

Table S3.1. Bacteria identified with Sanger sequencing on Baird Parker and blood agar plates from 50 bovine mastitic milk samples. *S: Staphylococcus and St: Streptococcus*

Samples	Baird Parker agar plates	Blood agar plates
M1	*Staphylococcus aureus* (100%)	*Staphylococcus aureus* (80%); *Pseudomonas fluorescens* (20%)
M2	*S. argenteus* (100%)	*Staphylococcus sciuri* (100%)
M3	*S. argenteus* (100%)	*Carnobacterium maltaromaticum* (100%)
M4	*Staphylococcus epidermidis* (100%)	*Staphylococcus schleiferi* (40%); *Pseudomonas agarici* (60%)
M5	*Staphylococcus haemolyticus* (100%)	*Kocuria indica* (25%); *P. agarici* (50%); *Pseudomonas rhizosphaerae* (25%)
M6		*Streptococcus uberis* (100%)
M7		*S. schleiferi* (25%); *St. uberis (20%); S. devriesei* (35%); *S. haemolyticus* (15%); *Bacillus oryzaecorticis* (5%)
M8	*S. epidermidis* (35%); *Staphylococcus rostri* (25%); *S. argenteus (40%)*	*S. rostri* (10%); *E. faecalis* (90%)
M9	*S. aureus* (100%)	*S. aureus* (100%)
M10	*S. aureus* (30%); *S. argenteus* (30%); *S. haemolyticus* (30%); Uncultured bacterium (10%)	*Streptococcus dysgalactiae* (100%)

(continued)

Table S3.1. Continued

Samples	Baird Parker agar plates	Blood agar plates
M11		*Enterococcus faecalis* (20%); *St. dysgalactiae* (70%); *S. capitis (5%); S. argenteus* (5%)
M12	*S. haemolyticus* (5%); *C. maltaromaticum* (95%)	
M13	*S. aureus* (50%); *S. argenteus* (40%); *S. haemolyticus* (10%)	*Uncultured bacterium* (5%); *S. argenteus* (90%); *Lactobacillus pentosus* (5%)
M14	*Staphylococcus lugdunensis* (20%); *Staphylococcus equorum* (30%); *S. haemolyticus* (50%)	*Kocuria rosae* (50%); *Bacillus subtilis* (12.5%); *Barnesiella sp.* (12.5%); *Staphylococcus petrassi* (2.5%); *S. equorum* (12.5%); *St. uberis* (10%)
M15		*Enterococcus pseudoavium* (12.5%); *E. faecium* (10%); *St. uberis* (75%); *Aerococcus urinaeequi* (2.5%)
M16	*S. hominis* (40%); *S. petrassi* (10%); *S. haemolyticus* (50%)	*S. rostri* (15%); *S. epidermidis* (30%)*; K. rosae* (50%); Uncultured bacterium (5%)
M17	*S. aureus* (100%)	
M18	*S. aureus* (100%)	*Bacillus mycoides* (25%); *Bacillus weihanstephanensis* (25%); *Proteus vulgaris* (50%)

(continued)

Table S3.1. Continued

Samples	Baird Parker agar plates	Blood agar plates
M19	*S. chromogenes* (100%)	*St. uberis* (12.5%); *C. maltaromaticum* (12.5%); *E. faecalis* (75%)
M20	*Enterococcus lactis* (100%)	*Microbacterium maritypicum* (70%); *Raoultella terrigena* (20%); *Microbacterium oxidans* (10%)
M21	*E. lactis* (5%); *S. argenteus* (15%); *C. maltaromaticum* (10%); *Macrococcus caseolyticus* (10%); *E. faecium* (60%)	*R. terrigena* (85%); *M. maritypicum* (5%); *S. argenteus* (10%)
M22		*Pseudomonas rhodesiae* (10%), *P. azotoformans* (5%); *P. lactis* (10%); *P. gessardiii* (15%); *P. fluorescens* (25%); *P. paralactis* (10%); *P. brenneri* (15%); *P. simiae* (5%); *P. marginalis* (5%)
M23	*S. argenteus* (100%)	*St. dysgalactiae* (50%); *E. faecalis* (50%)
M24		*St. uberis* (100%)
M25	*S. epidermidis* (85%); *Staphylococcus capitis* (15%)	*St. uberis* (90%); *Corynebacterium lactis* (5%); *Streptococcus urinalis* (5%)

(continued)

Table S3.1. Continued

Samples	Baird Parker agar plates	Blood agar plates
M26	Uncultured bacterium (10%); *E. faecalis* (90%)	*E. coli* (25%); *E. faecalis* (50%); *St. dsygalactiae* (25%)
M27	*S. sciuri* (5%); *S. chromogens* (5%); *S. epidermidis* (90%)	*St. uberis* (100%)
M28	*S. schleiferi* (100%)	*E. faecalis* (1*00%)*
M29	*S. chromogenes* (100%)	*S. chromogenes* (100%)
M30		*E. coli* (100%)
M31		*St. uberis* (100%)
M32	*S. chromogenes* (100%)	*E. coli* (100%)
M33	*S. hominis* (90%); *S. petrassi* (10%)	*St. agalactiae* (100%)
M34		*Trueperella pyogenes* (100%)
M35	*S. argenteus* (100%)	
M36	*S. argenteus* (65%); *S. simiae* (35%)	*St. dysgalactiae* (100%)
M37		
M38		*E. coli* (100%)
M39	*S. schleiferi* (100%)	*A. urinaeequi* (100%)
M40	*C. maltaromaticum* (100%)	*Pseudomonas helmaticensis* (100%)
M41	*C. maltaromaticum* (100%)	*St. uberis* (100%)
M42	*C. maltaromaticum* (100%)	*E. coli* (70%); *St. uberis* (30%)
M43	*S. chromogenes* (35%); *S. argenteus* (65%)	
M44	*S. warneri* (100%)	*A. urinaeequi* (100%)
M45	*S. equorum* (100%)	*A. urinaeequi* (50%); *Psychrobacter pulmonis* (25%); *B. weihanstephanensis* (25%)
M46		*St. dysgalactiae* (100%)
M47	*C. maltaromaticum* (90%); *S. equorum* (10%)	*Pseudomonas weihenstephanensis* (20%); *A. urinaeequi* (20%); *C. maltaromaticum* (60%)
M48	*S. equorum* (40%); *S. sciuri* (60%)	*E. coli* (50%); *S. sciuri* (50%)

(continued)

Table S3.1. Continued

Samples	Baird Parker agar plates	Blood agar plates
M49	*S. argenteus* (65%); *S. haemolyticus* (35%)	*S. argenteus* (80%); *Kocuria gwangalliensis* (20%)
M50	*S. chromogenes* (100%)	*S. dysgalactiae* (65%); *S. chromogenes* (35%)

Table S3.2. Average genus and species identification on blood agar plates from fifty mastitic milk samples.

Genus	Percentage	Species	Percentage
Aerococcus	5.9%	*Aerococcus urinaeequi*	5.9%
Bacillus	2.0%	*Bacillus mycoides*	0.5%
		Bacillus oryzaecorticis	0.1%
		Bacillus subtilis	0.3%
		Bacillus weihanstephanensis	1.1%
Barnesiella	0.3%	*Barnesiella sp.*	0.3%
Corynebacterium	0.1%	*Corynebacterium lactis*	0.1%
Carnobacterium	3.8%	*Carnobacterium maltaromaticum*	3.8%
Escherichia	9.7%	*Escherichia coli*	9.7%
Enterococcus	8.9%	*Enterococcus faecalis*	8.4%
		Enterococcus faecium	0.2%
		Enterococcus pseudoavium	0.3%
Kocuria	3.2%	*Kocuria gwangalliensis*	0.4%
		Kocuria indica	0.5%
		Kocuria rosae	2.2%
Lactobacillus	0.1%	*Lactobacillus pentosus*	0.1%
Microbacterium	1.8%	*Microbacterium maritypicum*	1.6%
		Microbacterium oxidans	0.2%
New species	0.2%	New species	0.2%
Pseudomonas	8.2%	*Pseudomonas agarici*	2.4%
		Pseudomonas azotoformans	0.1%
		Pseudomonas brenneri	0.3%
		Pseudomonas fluorescens	1.0%
		Pseudomonas gessardii	0.3%
		Pseudomonas helmaticensis	2.2%
		Pseudomonas lactis	0.2%
		Pseudomonas marginalis	0.1%
		Pseudomonas paralactis	0.2%
		Pseudomonas rhizosphaere	0.5%
		Pseudomonas rhodesiae	0.2%
		Pseudomonas simiae	0.1%
		Pseudomonas weihenstephanensis	0.4%

(continued)

Table S3.2. Continued

Genus	Percentage	Species	Percentage
Proteus	1.1%	*Proteus vulgaris*	1.1%
Psychrobacter	0.5%	*Psychrobacter pulmonis*	0.5%
Raoultella	2.3%	*Raoultella terrigena*	2.3%
Streptococcus	31.6%	*Streptococcus agalactiae*	2.2%
		Streptococcus dysgalactiae	11.1%
		Streptococcus uberis	18.2%
		Streptococcus urinalis	0.1%
Staphylococcus	18.3%	*Staphylococcus argenteus*	4.0%
		Staphylococcus aureus	3.9%
		Staphylococcus capitis	0.1%
		Staphylococcus chromogenes	2.9%
		Staphylococcus devriesei	0.8%
		Staphylococcus epidermidis	0.7%
		Staphylococcus equorum	0.3%
		Staphylococcus haemolyticus	0.3%
		Staphylococcus rostri	0.5%
		Staphylococcus schleiferi	1.4%
		Staphylococcus sciuri	3.3%
		Staphylococcus petrassi	0.1%
Trueperella	2.2%	*Trueperella pyogenes*	2.2%

Table S3.3. Average genus and species identification on Baird Parker agar plates from fifty mastitic milk samples.

Genus	Percentage	Species	Percentage
Carnobacterium	12.00%	*Carnobacterium maltaromaticum*	12.00%
Staphylococcus	80.40%	*Staphylococcus aureus*	12.90%
		Staphylococcus argenteus	19.50%
		Staphylococcus sciuri	1.70%
		Staphylococcus epidermidis	8.30%
		Staphylococcus rostri	0.60%
		Staphylococcus schleiferi	5.40%
		Staphylococcus haemolyticus	7.40%
		Staphylococcus capitis	0.30%
		Staphylococcus lugdunensis	0.50%
		Staphylococcus equorum	4.80%
		Staphylococcus petrassi	0.50%
		Staphylococcus hominis	3.50%
		Staphylococcus chromogenes	11.80%
		Staphylococcus simiae	0.80%
		Staphylococcus warneri	2.40%
New species	0.50%	New species	0.50%
Enterococcus	6.90%	*Enterococcus faecalis*	2.40%
		Enterococcus durans	2.90%
		Enterococcus faecium	1.60%
Macrococcus	0.20%	*Macrococcus caseolyticus*	0.20%

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Chapter 7

General Discussion

Antimicrobial resistance (AMR) is a present-day crisis for human health with 700,000 deaths/year recorded due to AMR. It is predicted that 10 million deaths will be attributed to AMR every year after 2050 [O' Neil, 2014]. Taking this into consideration along with the dearth of new antibiotics, the World Health Organisation (WHO) has emphasised the need for alternative treatments [WHO, 2017]. Novel antimicrobials such as bacteriocins have potential to substitute or enhance conventional antibiotics for the treatment of conditions such as gastrointestinal diseases and mastitis. The latter is an inflammation of the mammary gland, reported both in lactating women and cows and causes significant pain in both.

Up to 33% of women are affected by clinical mastitis and it is a leading cause of precocious weaning [WHO, 2000] which can have lifelong health implications for the infant [Victora et al., 2016]. Breastfeeding is of equal importance for the mother as it reduces the risk of diabetes, ovarian, and breast cancer [Victora et al., 2016].

The Irish government in conjunction with the Irish health service adopted a key policy priority to increase the numbers of healthy people at all stages of life via the Healthy Ireland Framework, 'Healthy Ireland – A Framework for Improved Health and Wellbeing 2013-2025' [Department of Health, 2013]. This policy involves confronting risk factors and advocating protective factors during lifetime to promote well-being. Breastfeeding rates in Ireland are still the lowest in international comparisons despite the continuous increase during the last ten years [EuroPeristat, 2013; Victora et al., 2016]. Breastfeeding rates in Ireland on discharge from maternity hospitals are 46.3% [HSE, 2016] with 15% of children being exclusively breastfed for the first six months. In contrast, 38% of children globally are exclusively breastfed while the European average is 25% [WHO, 2013]. Moreover, breastfeeding initiation rates in Ireland are among the lowest globally compared to initiation rates of 90% in

Australia, 81% in the UK and 79% in the USA [PHAA, 2010; NHS, 2011; CDC, 2014; HSE, 2016] with breastfeeding being associated with maternal education and social status [Williams et al., 2010]. The WHO Global Targets for advancing maternal, infant and child nutrition have introduced the goal to increase exclusive breastfeeding to a minimum rate of 50% in the first six months by 2025. Therefore, breastfeeding promotion and elimination of breastfeeding cessation due to causes such as mastitis, are of utmost importance.

In cows, mastitis is the most persistent disease affecting dairy herds and is responsible for major economic losses. Indeed, mastitis accounts for a third of the direct costs of all dairy-related diseases and leads to ramifications in the health sector that are linked with immoderate antibiotic use for disease prevention and treatment (Hughes et al., 2018). Guaranteeing that milk is antibiotic residue free has important repercussions on public health and financial subsistence of producers. Milk containing antibiotic residues has the potential to transfer AMR bacteria to calves [EFSA, 2017] and consumers [Sharma et al., 2017] and at the same time, can be a potential allergen for consumers [Han et al., 2015; Olatoye et al., 2016]. Furthermore, the presence of antibiotic residues in milk can have a significant effect on infant health especially in countries where there is high incidence of diarrheal disease and malnutrition [Garcia et al., 2019]. In addition, consumption of contaminated milk can have a negative effect on the microbiome, leading to dysbiosis, an alteration in the microbiota composition which has been associated with disease states [Tanaka et al., 2009; Langdon et al., 2016].

In general, mastitis is perceived to be caused by bacterial infection with a great deal of research focused on the pathogenic organisms involved. This has led to an improved understanding of the pathogenesis of the disease, with bovine mastitis, as

discussed in Chapter 3, being a polymicrobial disease in which the symptoms might be directed or accentuated by more than one microbe. Staphylococci, streptococci and coliforms are the most often recorded causative agents for bovine mastitis, whereas *S. aureus*, *S. epidermidis* and *Corynebacterium* sp. are involved in human mastitis (Chapters 1-4). However, there is a recent theory according to which infection is not the only cause of the disease. More specifically, Ingman et al [2014] suggest that heightened host inflammatory signalling in conjunction with pathogenic or commensal bacteria may cause the inflammation and reduced milk synthesis associated with mastitis. Increased concentrations of cytokines are recorded in women suffering from mastitis which is indicative of the activation of the transcription factor nuclear factor kappa B (NFκB). Toll-like receptors (TLRs) are responsible for NFκB activation. Danger-associated molecular patterns (DAMPs) bind to pattern recognition receptors such as TLRs, triggering the inflammation cascade. Targeting TLR pathways by antagonists such as curcumin [Fu et al., 2014], could be a solution to sidestep inflammation.

Novel therapeutics for the prophylaxis and treatment of mastitis can include beneficial bacteria (probiotics), bacteriocins and phages (phage therapy). Probiotics, as discussed in Chapters 1 and 2, have surfaced as efficient agents in the prophylaxis and treatment of mastitis. However, in the case of human mastitis, only a handful of studies have assessed probiotic efficacy against the disease, thus there is a need for randomized controlled trials that simultaneously assess treatment effects on milk quality. Regarding bovine mastitis, a plethora of studies prove that probiotics are equally as effective as, or even better than, currently used antibiotics. Bacteriocins are another attractive therapeutic approach for both human and bovine mastitis. Teat washes and wipes with incorporated bacteriocin impede the spread of bovine mastitic

pathogens on the farm and commercial preparations containing nisin are available that are performing better than chemicals [Sears et al., 1992] such as iodine, which are commonly used. Nonetheless, there is no equivalent bacteriocin-related application for human mastitis. Indeed, only one study to date has investigated nisin as an alternative treatment for staphylococcal human mastitis [Fernández et al., 2008]. The major constraints in developing clinical applications for bacteriocins include difficulties achieving high production levels [Cotter et al., 2013], potency against selected pathogens and the concern of resistance development. Interest in phage therapy has been renewed due to the antibiotics resistance crisis and clinical trials are ongoing for various diseases. Yet the inability of some phages to replicate in raw milk [O'Flaherty et al., 2005] and limitations posed by current regulatory frameworks comprise major drawbacks for their use in mastitis treatment. To our knowledge, no phage or phage lysin has been assessed as an alternative treatment for human mastitis.

Research Thesis Contribution to Current Literature and Future Work

In the present research thesis, Chapter 1 discussed alternative treatments for bovine mastitis i.e. probiotics, bacteriocins, phages, and phage lysins, providing the latest updates on microbe-based solutions in light of the antimicrobial resistance crisis. Chapter 2 provided a synopsis of the most recent findings in terms of the bacteria involved in human mastitis and simultaneously reviewed available therapies. At the same time, the chapter explored probiotics and bacteriocins as credible alternatives to antibiotics for treatment and prevention of the disease.

In Chapter 3, a combinatorial approach was employed which comprised cultivation, 16S rRNA gene sequencing and culturomics to determine the complete microbial content of 50 bovine mastitic milk samples. Only 20% of the tested samples provided similar outputs, with both 16S rRNA Illumina MiSeq sequencing and 16S Sanger sequencing identifying the same dominant genus. An interesting adjustment in the study design for future experiments would be the inclusion of a mock community control and/or spike-in standards for both identification and quantification purposes as a major drawback in 16S rRNA profiling is that the results provide relative bacterial abundances and not quantitative. Milk is a low biomass biological fluid that contains PCR inhibitors, consequently spiking samples with non-milk bacteria would increase confidence in bioinformatic analysis. Mock communities have proven useful for benchmarking a variety of technologies, quantifying a sequencing error, identification of bias introduced during the sampling, and library preparation [Hardwick et al., 2018]. Nonetheless, a key limitation of mock communities is that they cannot be added directly to samples without the risk of contaminating downstream analysis [Pollock et al., 2018]. In contrast, spike-in standards are added directly to the samples thus allowing for controlling the quality per sample. Moreover, throughout the thesis, the V3 and V4 hypervariable regions of the 16S rRNA gene were targeted for Illumina MiSeq sequencing based on the platform suggestions. In contrast, other groups have utilized other regions such as V4 [Oultram et al., 2017] and V1-V2 [Oikonomou et al., 2012]. Therefore, the scientific community should reach a consensus on hypervariable regions to target, especially when the desired outcome is to make comparisons across sampling sites, time scales or treatments, or to compare results produced by different laboratories. Additionally, the usage of different platforms (as in the case of Oikonomou et al., 2012), complicates further the comparison between different data

sets. Next, the virulence genes and the antibiotic resistance profiles of these isolates could be investigated allowing for better understanding of the pathogenicity of these isolates and at the same time uncover genetic interactions between virulence and resistance. The latter could be critical in the identification of drug targets providing a drug discovery and development pathway to improve treatment options for chronic and recurrent infections.

In Chapter 4, subclinical mastitis (SM) emerged as a high frequency condition (37.8%) among asymptomatic lactating women using a combination of culture-based and culture-independent approaches. The aim of the study was to gain deeper insight into the microbiota composition of mastitic milk in conjunction with assessment of the immune response in each health condition with a view to ameliorate maternal post-natal health and improve the breastfeeding experience. The latter is crucial as breastfeeding can strengthen the mother-infant bond. Furthermore, improvement of the breastfeeding experience is important in Ireland due to the low breastfeeding rate. The core microbiota of healthy milk and subclinical mastitic milk samples comprised of eight genera including *Serratia* and *Stenotrophomonas*, whereas clinical milks were depleted of these two genera. In the literature, the term SM is used to imply breast inflammation that does not lead to clinically evident symptoms. SM is suggested to be linked with reduced milk production in lactating mothers and decreased weight gain in infants [Gomo et al., 2003]. Somatic cell count (SCC) is the pillar of SM diagnosis in cows [Ferronato et al., 2018] and direct microscopic SCC (DMSCC) was employed in order to enumerate SCC in the tested samples. DMSCC is based on the counting of stained cell nuclei, using an optical microscope [Orlandini and van de Bijgaart, 2011]. While DMSCC is a standardized method [ISO 13366-1|IDF 148-1], its performance relies greatly on the training and skill of the analyst. To eliminate uncertainty on the

accuracy of the DMSCC method, fresh bovine milk samples were acquired, and SCC were enumerated using DMSCC and the fluoro-optical method (as discussed in Chapter 4). It was shown that there is a strong correlation between the two aforementioned methods, reinforcing confidence in DMSCC. A larger study is required to investigate further the prevalence of SM in asymptomatic lactating women and the immune response caused by it. In particular, the immunological factors that have the potential to modulate inflammation in the mammary alveoli and are increased during mastitis such as lysozyme, secretory leukocyte protease inhibitor (SLPI), RANTES (Regulated on Activation Normal T Cell Expressed and Secreted), IL-6, IL-17 and IFN-γ [Tuaillon et al., 2017] could be studied. SLPI protects tissues from degradation by proteases that are released by neutrophils (Thompson and Ohlsson, 1986) whereas RANTES is a chemokine produced by $CD8^+$ lymphocytes, natural killer cells and mammary epithelial cells, that participates in the chemotaxis of the macrophages [Hughes and Watson, 2018]. This could provide us with additional information on the immunological status of human milk which could potentially be used as a diagnostic tool, thus improving maternal and infant health.

In Chapter 5, *S. aureus* (n=18) was isolated from milk donated by healthy, subclinical and clinical mastitic mothers. The impact of vancomycin and nisin A alone and in combination was assessed on biofilm formation and eradication of a selection of *S. aureus* strains (n=8). The combination treatment managed to significantly inhibit biofilm formation for seven of eight tested *S. aureus* strains. Nonetheless, this was not the case when the eradication of pre-formed biofilms was evaluated with none of the treatments being able to eliminate pre-formed biofilms. Kvist et al [2008] demonstrated that mastitic women had higher *S. aureus* and Group B streptococci (GBS) in their breast milk compared to healthy women, yet 31% of healthy lactating

women harboured *S. aureus* and 10% had GBS. The latter is in agreement with the findings in Chapter 5. All *S. aureus* strains were able to form biofilms which play a crucial role in bacterial pathogenesis both in human and bovine mastitis [Marín et al., 2017; Notcovich et al., 2018]. Administration of vancomycin to treat serious *S. aureus* infections is the last resort but it is unable to eradicate *S. aureus* biofilms as discussed in Chapter 5. Recently, the National Institute of Health (NIH) suggested a complementary approach to bypass drug resistance, where the activity of traditional antibiotics can be reinforced when combined with new antimicrobials [Algburi et al., 2017]. This could potentially negate drug resistance among target strains due to two distinct modes of action of the two antimicrobials such as in the case of nisin A and vancomycin. Moreover, combinatorial treatments can relieve the financial burden linked with the prescription of high-priced antibiotics. In order to efficiently combat drug resistance, a comprehensive approach is vital that will underscore therapeutic approaches employing both novel drugs and other new means to eliminate deleterious microbes. Future work on this chapter could include the sequencing of the genomes of the *S. aureus* isolates to study potential virulence factors such as panton-valentine leucocidin (pvl). Furthermore, Multilocus Sequence Typing (MLST) and *S. aureus*-specific staphylococcal protein A (spa) typing would aid strain characterization. The combination of these methods would facilitate the classification of the isolates as hospital-acquired, community-acquired or livestock-acquired thus contributing to the curb of AMR. Moreover, *in vivo* experiments in mice could be performed to study the infection capacity of the strains isolated from healthy subjects. The latter will provide us with information regarding the capacity of these strains to be actually pathogenic and potentially allow us to understand the fine line between commensal and pathogen.

In Chapter 6, human breast milk was demonstrated to contain a wide diversity of bacteriocin-producing strains with the ability to compete among the developing gut microbiota of the infant. Thirty-seven asymptomatic human milk samples were screened, and 80 isolates were detected with antimicrobial activity. An *in silico* screen was employed that provided an overview of the genetic capacity of these strains to produce bacteriocins and detected 61 putative gene clusters for bacteriocins of all known sub-classes, including 16 new prepeptides. In this chapter, it was shown that *S. lugdunensis* APC 3758 and *P. protegens* APC 3760 were able to inhibit two MRSA strains. Taking this into consideration, the antimicrobials produced by the aforementioned strains could be purified and their efficacy at inhibiting the *S. aureus* isolates from Chapter 5 could be assessed. For example, an *ex vivo* skin model could be used to mimic infection to which purified bacteriocin is applied and pathogen(s) clearance is then assessed. Carson et al. [2017], isolated 441 non-*aureus* staphylococci from bovine milk in which they detected 25 novel prepeptides from all known bacteriocin sub-classes agreeing with our study that milk is a rich source of bacteriocin-producing strains. More recently, O'Sullivan and collaborators [2019] investigated human skin as a source of bacteriocin-producing isolates and isolated 13 novel bacteriocin-producers. These finding showcase that both milk and skin are rich sources of bacteriocins. Given the scarcity of novel antibiotics and the ongoing antibiotic resistance crisis, bacteriocins are a realistic substitute, worthy of further investigation.

Summarised Plan for Future Work

1. Strategies to improve confidence in bioinformatic findings from low biomass samples
    a. Mock communities/spike-in standards
    b. Consensus on hypervariable regions
2. Evaluate additional immunologic factors – Potential diagnostic tool
3. Investigate alternative causes of mastitis apart from infection
4. Strain characterization of multidrug resistant S. aureus
5. Infection modelling for multidrug resistant S. aureus
6. Purification and characterization of predicted bacteriocins
7. *ex vivo* skin model to assess the pathogen clearance capacity of the purified bacteriocin

Overall, the work presented in this thesis seeks to drive and generate interest in the microbiology and treatment of bovine and human mastitis. It represents a significant stepping stone to advance our knowledge of (i) the pathogens involved in the disease in both species, (ii) the microbiota composition in diseased cows and women and (iii) the potential deployment of bacteriocins to treat the infection in both bovines and humans.

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