FEASIBILITY AND PRELIMINARY SAFETY OF NITRIC OXIDE RELEASING SOLUTION AS A TREATMENT FOR BOVINE MASTITIS

by

James Anthony Vieira Martins

B.Sc., The University of British Columbia, 2013

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate and Postdoctoral Studies

(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

August 2020

© James Anthony Vieira Martins, 2020
The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, a thesis entitled:

Feasibility and preliminary safety of nitric oxide releasing solution as a treatment for bovine mastitis

submitted by James Anthony Vieira Martins in partial fulfillment of the requirements for

the degree of Master of Science

In Experimental Medicine

Examining Committee:

Christopher Miller, Department of Medicine
Supervisor

Ronaldo Cerri, Faculty of Land and Food Systems
Supervisory Committee Member

Jeremy Carver, CEO of the International Consortium on Anti-Virals
Additional Examiner
Abstract

The risk of antibiotic resistance in bovine mastitis, one of the most widespread and significant diseases affecting dairy farming, both in terms of animal welfare and economic cost has become a concern, leading to a push for alternative treatments. Nitric oxide releasing solution (NORS) is a liquid formulation that releases nitric oxide, a broad-spectrum antimicrobial, single electron nitroxide radical. This solution was investigated as a potential antimicrobial treatment for bovine mastitis. Three main experiments were performed: 1) the development of NORS so that it is compatible in milk; 2) the antimicrobial effect of NORS in milk with in-vitro and ex-vivo samples; and 3) the consequences of administering NORS to healthy milking cattle using a dose-escalating in-vivo study. NORS was successfully created to be compatible in milk without causing curdling while continuing to release nitric oxide. Additionally, the formula lowered bacterial concentration in all infected samples, in a time and milk-diluted dependant fashion. In the preliminary safety study, metabolite concentrations were measured in their blood for methaemoglobin and nitrite; also, milk nitrite concentration was analyzed to determine a potential clearance time for the drug. Separately, somatic cell count was determined in order to ascertain possible mammary gland inflammation following NORS treatment. Blood methemoglobin concentrations following treatment were all within the normal range for cattle. However, blood and milk nitrite concentrations increased initially but, during the next 24 hours, returned to normal range, as did the somatic cell count after one week, without any clinical signs of mammary gland inflammation. NORS, if shown to be effective, could be an alternative treatment for mastitis with a shorter clearance time.
Lay Abstract

Nitric oxide releasing solution (NORS) is a liquid which releases nitric oxide, a molecule with antimicrobial properties. With antibiotic resistance becoming a worldwide concern, we evaluated whether this liquid would have the potential for an alternative treatment for bovine mastitis, the microbial causing inflammation of the udder. Three main experiments were performed: 1) we created a workable in milk recipe for NORS; 2) we tested whether it can kill common bacteria associated with bovine mastitis; and 3) we tested NORS on healthy cows to observe any poor drug side effects. These studies found that NORS could be successfully created to work in milk, while retaining its antibacterial properties when tested in milk samples. Additionally, the use of NORS on healthy animals showed no side effects. Overall, NORS could have the potential to be used as an alternative treatment to antibiotics for the treatment of mastitis in dairy cows.
Preface

Chapters 1, 2 and 5 (sections 5.1 and 5.2) is original, unpublished, independent work by the author, James Martins with feedback from my supervisor, Chris Miller and my supervisory committee.

Chapters 3, 4 and 5 (section 5.3) is based on published data obtained during my Master’s program; Regev, G., Martins, J., Sheridan, M.P., Leemhuis, J., Thompson, J., Miller, C. 2018. Feasibility and preliminary safety of nitric oxide releasing solution as a treatment for bovine mastitis, Research in Veterinary Science 118, 247-253. My contribution included the antimicrobial work seen in chapter 3, all the data analysis, including production of all tables and figures as well as the original manuscript composition. Drs Regev and Miller were involved in the major areas of concept and contributed to manuscript edits as did Sheridan. Leemhuis was responsible for nitrite and methemoglobin data collection and Thompson and his team at the University of British Columbia Dairy Education and Research Center were responsible for animal identification and handling, milk collection and performed the well-being assessments on treated animals.

All data obtained from this research project was approved by Health Canada Veterinary Drug Directorate, Experimental certificate Number (ESC): 192892 and UBC Animal Care Committee Research Ethics Board titled, “Phase I, placebo controlled, challenge trial for the safety and efficacy of Mastinex™, a nitric oxide releasing solution (NORS) to treat Mastitis in Dairy cows,” Animal care certificate number: A16-0058.
Table of Contents

Abstract.......................................................................................................................... iii
Lay Abstract...................................................................................................................... iv
Preface............................................................................................................................. v
Table of Contents........................................................................................................... vi
List of Tables................................................................................................................... viii
List of Figures............................................................................................................... ix
List of Symbols, Abbreviations and Others................................................................. x
Acknowledgements ..................................................................................................... xiii
Dedication....................................................................................................................... xiv
1. Introduction............................................................................................................... 1

1.1. What is Bovine Mastitis ....................................................................................... 1
    1.1.1. Epidemiology of Bovine Mastitis ................................................................. 1
    1.1.1.1. Microbes Associated with Bovine Mastitis ............................................. 4
    1.1.1.2. Bovine Mammary Defense to Microbes ............................................... 6
    1.1.1.3. Transmission of Bovine Mastitis ............................................................ 9
    1.1.2. Costs to the Dairy Industry ....................................................................... 12
    1.1.3. Current Practices and Treatment ............................................................... 13
        1.1.3.1. General Prevention Techniques ......................................................... 13
        1.1.3.2. The Five Point Plan ......................................................................... 15
        1.1.3.3. Tests to Determine Subclinical Bovine Mastitis ............................. 17
        1.1.3.4. Treatment of Bovine Mastitis .............................................................. 19
        1.1.3.5. The Push for Other Treatments ......................................................... 24

1.2. What is Nitric Oxide............................................................................................ 25
    1.2.1. History ........................................................................................................ 26
        1.2.1.1. Endothelium-Derived Relaxing Factor ............................................ 26
    1.2.2. Nitric Oxide Synthases ............................................................................. 27
        1.2.2.1. Endothelial Nitric Oxide Synthase ..................................................... 28
        1.2.2.2. Neuronal Nitric Oxide Synthase ....................................................... 29
        1.2.2.3. Inducible Nitric Oxide Synthase ...................................................... 30
    1.2.3. Nitric Oxide and the Innate Immune System ........................................... 31
    1.2.4. Nitric Oxide and the Adaptive Immune System ....................................... 34
List of Tables

Table 1: Bovine clinical mastitis prevalence.................................................................2

Table 2: Bovine subclinical mastitis prevalence. ............................................................3

Table 3: Initial NORS pH of when curdling was observed..............................................52

Table 4: List of bacteria isolated from mastitis milk. .....................................................61

Table 5: Milk nitrite concentration after NORS infusion. ............................................73

Table 6: Somatic cell count after NORS infusion..........................................................74
List of Figures

Fig. 1: Nitric oxide production of NORS mixed with milk..........................................................53

Fig. 2: Bacteria concentration in inoculated milk after NORS treatment.................................60

Fig. 3: Bacteria concentration in mastitis milk after NORS treatment......................................61

Fig. 4: Methaemoglobin percent after NORS infusion.............................................................71

Fig. 5: Plasma nitrite concentration after NORS infusion.........................................................71
List of Symbols, Abbreviations and Others

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Abbreviation/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A:T</td>
<td>Adenine : Thymine</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>bNOS</td>
<td>Bacterial nitric oxide synthase</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>CAD</td>
<td>Canadian dollars</td>
</tr>
<tr>
<td>CD14</td>
<td>Cluster of Differentiation 14</td>
</tr>
<tr>
<td>cells/mL</td>
<td>Cells per milliliter</td>
</tr>
<tr>
<td>cfu/mL</td>
<td>Colony forming units per milliliter</td>
</tr>
<tr>
<td>Cgb</td>
<td>Cytoglobin</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>Ctb</td>
<td><em>Campylobacter jejuni</em> type III truncated globin</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDRF</td>
<td>Endothelium-derived relaxing factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMB</td>
<td>Eosin methylene blue</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
</tr>
<tr>
<td>g</td>
<td>Grams</td>
</tr>
<tr>
<td>g/mol</td>
<td>Grams per mole</td>
</tr>
<tr>
<td>G:C</td>
<td>Guanine : Cytosine</td>
</tr>
<tr>
<td>h</td>
<td>Hours</td>
</tr>
<tr>
<td>H+</td>
<td>Hydrogen ion</td>
</tr>
<tr>
<td>H₂O</td>
<td>Water</td>
</tr>
<tr>
<td>HNO₂</td>
<td>Nitrous acid</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IgA</td>
<td>Immunoglobulin A</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor-1</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Il-</td>
<td>Interleukin-</td>
</tr>
<tr>
<td>Il-12 p40</td>
<td>Interleukin-12 subunit beta</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IRF-1</td>
<td>Interferon regulatory factor 1</td>
</tr>
<tr>
<td>kPa</td>
<td>Kilopascal</td>
</tr>
<tr>
<td>L/min</td>
<td>Liters per minute</td>
</tr>
<tr>
<td>Mcal</td>
<td>Megacalorie</td>
</tr>
<tr>
<td>MetHb (%)</td>
<td>Percent methemoglobin</td>
</tr>
<tr>
<td>mg/kg</td>
<td>Milligram per kilogram</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin-resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>N&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Dinitrogen trioxide</td>
</tr>
<tr>
<td>N&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Dinitrogen tetroxide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometers</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Nitrogen dioxide</td>
</tr>
<tr>
<td>NO&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Nitrite</td>
</tr>
<tr>
<td>NO&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Nitrate</td>
</tr>
<tr>
<td>NONOates</td>
<td>Diazeniumdiolate nitric oxide donors</td>
</tr>
<tr>
<td>NORS</td>
<td>Nitric oxide releasing solution</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>Optical density at wavelength 600 nm</td>
</tr>
<tr>
<td>OONO&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Peroxynitrite</td>
</tr>
</tbody>
</table>
PCR: Polymerase chain reaction
PDGF: Platelet-derived growth factor
PKA: Protein kinase A
PKC: Protein kinase C
ppm: Parts per million
RSNO: S-Nitrosothiol
SCCmec: Staphylococcal cassette chromosome mec
spp.: Species
Th1: Type 1 T helper cell
Th2: Type 2 T helper cell
TNF-α: Tumor necrosis factor alpha
µL: Microliter
µm: Micrometer
µM: Micomolar
USD: United States dollars
w/v: Weight by volume
w/w: Weight by weight
x g: Times gravitational force
Acknowledgements

I would like to give my heartfelt thanks to my supervisor, Dr. Chris Miller for his mentorship, guidance and support and to my supervisory committee member Dr. Ronaldo Cerri. I also thank Dr. Gilly Regev for her work and support for this project. This study was funded through a service agreement between University of British Columbia and Bovicor Pharmatech Inc. I would also like to thank all of my lab mates whom helped me with this project, particularly Jonathan Leemhuis and to all the people at the University of British Columbia Education and Research Center.
To my family, for their support and love.

Thank you
1. Introduction

1.1 What is Bovine Mastitis

Bovine mastitis, defined as the inflammation of the mammary gland, is one of the most common and significant diseases found on dairy farms, both in terms of animal welfare and reduced milk yield and quality (Seegers, Fourichon and Beaudeau 2003). It is a common global disease among all breeds of bovine, especially those which yield high amounts of milk (Bhosale et al. 2014, Gentilini et al. 2002, Leelahapongsathorn, Schukken and Suriyasathaporn 2014, McDougall 1998, Mungube et al. 2004, Zadoks et al. 2001). The main cause of bovine mastitis is due to intramammary infections from microbes (Leelahapongsathorn et al. 2016) which results in the inflammatory yielding symptoms associated with mastitis.

1.1.1 Epidemiology of Bovine Mastitis

There are two main types of mastitis; clinical and the observantly symptomless subclinical mastitis. Clinical mastitis is often diagnosed either through the observation of changes in milk composition such as discoloration or clots or the changes to the mammary gland such as inflammation, swelling, heat, redness and pain (McDougall 1998). Subclinical mastitis is more prevalent than clinical mastitis and can be diagnosed through culturing of bacteria from milk samples, an increase of somatic cell count or a decrease production of milk (Bradley 2002, Yohannis and Molla 2013). Infections can also occur at any time from post-partum (Pyörälä 2008), late lactation (Moosavi et al. 2014) and dry periods (Oliver and Mitchell 1983). If left untreated, bovine mastitis can cause permanent tissue damage of the mammary gland (Zhao and Lacasse 2008).
Bovine mastitis can be found across the world with different incidence of the disease. Due to the visible confirmation and treatment of clinical mastitis, subclinical mastitis is often left undiagnosed and therefore more prevalent. Tables 1 and 2 shows the prevalence of clinical and subclinical mastitis respectively at the cow-level across multiple farms around the world. While each study differs in the way the data was obtained, the general picture showcases the far-reaching scale of the disease, affecting many nations, no matter their economic status.

**Table 1: Bovine clinical mastitis cow-level prevalence from around the world.**

<table>
<thead>
<tr>
<th>Country</th>
<th>Region</th>
<th>Size</th>
<th>Clinical Mastitis Prevalence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Algeria</td>
<td>Northeast</td>
<td>450</td>
<td>15.1%</td>
<td>(Bouzid et al. 2011)</td>
</tr>
<tr>
<td>Brazil</td>
<td>Bahia</td>
<td>187</td>
<td>1.07%</td>
<td>(Oliveira et al. 2010)</td>
</tr>
<tr>
<td>Canada</td>
<td>Ontario</td>
<td>2840</td>
<td>19.8%</td>
<td>(Sargeant et al. 1998)</td>
</tr>
<tr>
<td>China</td>
<td>Huanggang</td>
<td>1374</td>
<td>3.86%</td>
<td>(Yang et al. 2015)</td>
</tr>
<tr>
<td>China</td>
<td>Guangxi</td>
<td>1089</td>
<td>8.7%</td>
<td>(Yang et al. 2011)</td>
</tr>
<tr>
<td>Ecuador</td>
<td>El Oro</td>
<td>250</td>
<td>12%</td>
<td>(Amer et al. 2018)</td>
</tr>
<tr>
<td>Egypt</td>
<td>Benisuef</td>
<td>233</td>
<td>9.87%</td>
<td>(Elbably, Emeash and Asmaa 2013)</td>
</tr>
<tr>
<td>Ethiopia</td>
<td>Hawassa</td>
<td>529</td>
<td>3.4%</td>
<td>(Abebe et al. 2016)</td>
</tr>
<tr>
<td>Ethiopia</td>
<td>Hararghe</td>
<td>384</td>
<td>12.5%</td>
<td>(Zeryehun and Abera 2017)</td>
</tr>
<tr>
<td>India</td>
<td>Nationwide</td>
<td>32433</td>
<td>27%</td>
<td>(Krishnamoorthy et al. 2017)</td>
</tr>
<tr>
<td>Iran</td>
<td>Fars</td>
<td>1545</td>
<td>2.2%</td>
<td>(Hashemi, Kafi and Safdarian 2011)</td>
</tr>
<tr>
<td>New Zealand</td>
<td>Northland</td>
<td>3765</td>
<td>14.8%</td>
<td>(Petrovski et al. 2009)</td>
</tr>
<tr>
<td>Spain</td>
<td>Basque &amp; Gerona</td>
<td>2593</td>
<td>24.8%</td>
<td>(Perez-Cabal, Yaici and Alenda 2008)</td>
</tr>
<tr>
<td>Tanzania</td>
<td>Mvomero &amp; Njombe</td>
<td>91</td>
<td>21.7%</td>
<td>(Mdegela et al. 2009)</td>
</tr>
<tr>
<td>Zimbabwe</td>
<td>Marirangwe, Nharira, Guruve &amp; Dowa</td>
<td>584</td>
<td>4.8%</td>
<td>(Katsande et al. 2013)</td>
</tr>
</tbody>
</table>
Table 2: Bovine subclinical mastitis cow-level prevalence from around the world. Percent with an asterisk indicates studies where bovines with clinical mastitis were excluded.

<table>
<thead>
<tr>
<th>Country</th>
<th>Region</th>
<th>Size</th>
<th>Subclinical Mastitis Prevalence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Algeria</td>
<td>Northeast</td>
<td>450</td>
<td>29.7%</td>
<td>(Bouzid et al. 2011)</td>
</tr>
<tr>
<td>Argentina</td>
<td>Cordoba</td>
<td>2228</td>
<td>53.9%*</td>
<td>(Dieser et al. 2014)</td>
</tr>
<tr>
<td>Bangladesh</td>
<td>Dhaka</td>
<td>560</td>
<td>68%*</td>
<td>(Quaderi et al. 2013)</td>
</tr>
<tr>
<td>Belgium</td>
<td>Flanders</td>
<td>44667</td>
<td>41.3%</td>
<td>(Piepers et al. 2007)</td>
</tr>
<tr>
<td>Brazil</td>
<td>Bahia</td>
<td>187</td>
<td>38.50%</td>
<td>(Oliveira et al. 2010)</td>
</tr>
<tr>
<td>Brazil</td>
<td>Minas Gerais</td>
<td>617</td>
<td>55.4%*</td>
<td>(Cunha et al. 2015)</td>
</tr>
<tr>
<td>China</td>
<td>Huanggang</td>
<td>1374</td>
<td>18.78%</td>
<td>(Yang et al. 2015)</td>
</tr>
<tr>
<td>China</td>
<td>Guangxi</td>
<td>1089</td>
<td>48.8%</td>
<td>(Yang et al. 2011)</td>
</tr>
<tr>
<td>Costa Rica</td>
<td>Alajuela &amp; Heredia</td>
<td>2186</td>
<td>24.2%*</td>
<td>(de Graaf and Dwinger 1996)</td>
</tr>
<tr>
<td>Ecuador</td>
<td>El Oro</td>
<td>250</td>
<td>60%</td>
<td>(Amer et al. 2018)</td>
</tr>
<tr>
<td>Egypt</td>
<td>Benisuef</td>
<td>233</td>
<td>33.05%</td>
<td>(Elbably et al. 2013)</td>
</tr>
<tr>
<td>Ethiopia</td>
<td>Hawassa</td>
<td>529</td>
<td>59.2%</td>
<td>(Abebe et al. 2016)</td>
</tr>
<tr>
<td>Finland</td>
<td>Nationwide</td>
<td>273012</td>
<td>19.0%*</td>
<td>(Hiitiö et al. 2017)</td>
</tr>
<tr>
<td>India</td>
<td>Nationwide</td>
<td>32433</td>
<td>41%</td>
<td>(Krishnamoorthy et al. 2017)</td>
</tr>
<tr>
<td>Iran</td>
<td>Fars</td>
<td>1545</td>
<td>42.5%</td>
<td>(Hashemi et al. 2011)</td>
</tr>
<tr>
<td>Kosovo</td>
<td>Prishtina, Prizren &amp; Ferizaj</td>
<td>156</td>
<td>25.6%*</td>
<td>(Sylejmani et al. 2016)</td>
</tr>
<tr>
<td>Mexico</td>
<td>Tierra Caliente, Guerrero</td>
<td>259</td>
<td>20.5%*</td>
<td>(Olivares-Pérez et al. 2015)</td>
</tr>
<tr>
<td>Netherlands</td>
<td>Nationwide</td>
<td>7966</td>
<td>25.5%</td>
<td>(Santman-Berends et al. 2012)</td>
</tr>
<tr>
<td>Pakistan</td>
<td>Muzaffar Garh</td>
<td>500</td>
<td>45%*</td>
<td>(Bachaya et al. 2011)</td>
</tr>
<tr>
<td>Rwanda</td>
<td>Rubavu &amp; Nyabihu</td>
<td>123</td>
<td>50.4%*</td>
<td>(Mpatswenumugabo et al. 2017)</td>
</tr>
<tr>
<td>Serbia</td>
<td>Northwest</td>
<td>1026</td>
<td>20.7%*</td>
<td>(Zutic et al. 2012)</td>
</tr>
<tr>
<td>Sri Lanka</td>
<td>Batticaloa</td>
<td>152</td>
<td>43%*</td>
<td>(Sanotharan, Pangthinathan and Nafees 2016)</td>
</tr>
<tr>
<td>Switzerland</td>
<td>Bern</td>
<td>480</td>
<td>34.2%*</td>
<td>(Roesch et al. 2007)</td>
</tr>
<tr>
<td>Tanzania</td>
<td>Mvomero &amp; Njombe</td>
<td>91</td>
<td>51.6%</td>
<td>(Mdegela et al. 2009)</td>
</tr>
<tr>
<td>Turkey</td>
<td>Kars</td>
<td>500</td>
<td>24%</td>
<td>(Saglam et al. 2018)</td>
</tr>
<tr>
<td>Uganda</td>
<td>Kampala area</td>
<td>195</td>
<td>86.2%*</td>
<td>(Abrahmsën et al. 2014)</td>
</tr>
<tr>
<td>United States</td>
<td>North Carolina</td>
<td>652</td>
<td>20.8%*</td>
<td>(Mullen et al. 2013)</td>
</tr>
<tr>
<td>Uruguay</td>
<td>West Littoral Region</td>
<td>1077</td>
<td>52.4%*</td>
<td>(Gianneechini et al. 2002)</td>
</tr>
<tr>
<td>Zimbabwe</td>
<td>Marirangwe, Nharira, Guruve &amp; Dowa</td>
<td>584</td>
<td>16.3%</td>
<td>(Katsande et al. 2013)</td>
</tr>
</tbody>
</table>
1.1.1.1 Microbes Associated with Bovine Mastitis

More than 100 different micro-organisms have been identified to cause mastitis including fungi, yeast and prototheca, but the most common causes are bacteria, typically *Staphylococcus* spp., *Streptococcus* spp. and *Escherichia coli* (Bradley 2002, Canadian Bovine Mastitis Research Network 2010, Schroeder 2012, Yohannis and Molla 2013). A multi-study analysis in France found *Staphylococcus* spp. to be the most prevalent pathogen in subclinical mastitis while *E. coli* and *Streptococcus uberis* were more prevalent in clinical mastitis (Poutrel et al. 2018). While not as common, other pathogens which do obtain some focus in studies include, *Klebsiella* spp. (Davidson, Whitney and Tahlan 2015, Gorden et al. 2018), *Corynebacterium* spp. (Gonçalves et al. 2016, Hahne et al. 2018) and *Enterococcus* spp. (Gao et al. 2019, Różańska et al. 2019).

*Staphylococcus* is one of the most common intramammary infection microbe. *Staphylococcus aureus* in particular is a common pathogen in bovine mastitis where it can hold the plurality or even majority of pathogens isolated from milk samples (Abebe et al. 2016, Olde Riekerink et al. 2008, Wang et al. 2018). The species has several virulence factors including 25 different toxins, 15 types of adhesion molecules and 20 immune evasion molecules (Monistero et al. 2018). More concerning, *S. aureus* can gain antibiotic immunity via the mobile genetic element SCCmec which can give immunity to methicillin, also known as methicillin-resistant *Staphylococcus aureus* (MRSA) and other β-lactam antibiotics which are used in mastitis treatment (Pantosti 2012, Sawant, Sordillo and Jayarao 2005).
Coagulase-negative staphylococci are often categorized as a group of bacterial pathogens in bovine mastitis with *Staphylococcus chromogenes*, *Staphylococcus epidermidis*, *Staphylococcus hyicus*, *Staphylococcus haemolyticus*, *Staphylococcus simulans* and *Staphylococcus xylosus* being the most commonly isolated species (Thorberg et al. 2009). They are often seen as a mild pathogen, often only causing subclinical mastitis (Pyörälä and Taponen 2009), but they are still notable as they do cause decrease production of milk (Timms and Schultz 1987) and have received more focus in some nations as prevalence for this pathogen increases (Vakkamäki et al. 2017, Vlkova et al. 2017). As with *S. aureus*, Coagulase-negative staphylococci have been found to contain virulence factors, including staphylococcal superantigens which can result in the inflammatory component of mastitis (Park et al. 2011).

Streptococcus is also a common pathogen found in intramammary infection. *S. uberis* was found to be the most prevalent microbe found in studies done in Australia (Shum et al. 2009), Belgium (Verbeke et al. 2014) and New Zealand (McDougall 2003). As an environmental, pathogen, *S. uberis* differs from other mastitis causing streptococci in that it can be isolated from the udder surface (Khan et al. 2003). Other species such as *Streptococcus agalactiae* and *Streptococcus dysgalactiae* are considered to be contagious bacteria where improper milking techniques allow for its transmission (Fox and Gay 1993). *S. dysgalactiae* has been shown to contain virulence genes such as Streptolysin S-associated protein, factors which are also found in Group A Streptococcus, suggesting that the bacteria may cause disease in humans (Rato et al. 2011). Similarly, there is some evidence that *S. agalactiae* may transmit between bovine and humans (Manning et al. 2010). While *S. agalactiae* can be found worldwide, it is more prevalent in South
Among coliform bacteria, *E. coli* is generally the most common pathogen for bovine mastitis (Breen, Green and Bradley 2009, Olde Riekerink et al. 2008). The species has been subdivided into phylogenetic subgroups: A, B1, B2, C, D, E and F (Clermont et al. 2013), of which the majority of isolates from bovine mastitis originate from clades A and B1 (Goldstone, Harris and Smith 2016). Some strains of *E. coli* can be quite pathogenic. In one study, bovines were injected with 50 colony forming units of a clinical strain and within 9 hours, elevated somatic cell count was observed (Oliver et al. 2012). Toxins, including the Shiga toxin which can cause serious symptoms to both bovines and humans if consumed have been found (Lira, Macedo and Marin 2004). Similar to *S. aureus*, β-lactam antibiotics resistance is an issue, due to isolates containing the Extended-spectrum β-lactamase plasmid (Freitag et al. 2017, Rao et al. 2014).

### 1.1.1.2 Bovine Mammary Defense to Microbes

**Udder Structure and Physical Barrier**

The udder contains two pairs of mammary glands which drain from the teats. Typically, the four quarters are separated from one another and can function independently. During lactation, milk is excreted and stored in alveoli-like compartments. It is this location where drugs administered systemically can cross the blood-milk barrier and diffuse into the gland. The milk in the alveoli drain into the intralobular duct which converges into the lactiferous ducts which lead into the gland cistern and then the teat cistern. The milk is secreted through the teat canal.
The teat canal is closed off by a sphincter and its lumen contains keratinized epithelium which provides a physical barrier to prevent bacteria and pathogens from entering (Gruet et al. 2001). The keratinized epithelium is a very important physical barrier against pathogens and removal of keratin has shown to increase susceptibility to bovine mastitis (Sordillo and Streicher 2002). During non-lactating times the entire opening would be covered (Nickerson 1987). Additionally, the teat contains lipids which are antimicrobial and further aides in the protection from infection (Treece, Morse and Levy 1966).

Innate Defense

The innate immune response is very important to avoid long-term microbial colonization in the teat cistern and mammary gland (Rainard and Riollet 2006). Should microbes enter the teat canal, the internal end is called the Furstenberg’s rosette, which contains high levels of lymphocytes and can provide a local innate immune response (Gruet et al. 2001). Similarly, should pathogens come into contact with somatic cells in the milk an innate immune response is triggered (Rainard and Riollet 2006). Factors that could influence the strength of the innate immune system include stage of lactation, age, somatic cell count and nutritional status (Burvenich et al. 2003). In general, younger bovines during the first stage of lactation tend to have lower immune response competency and thus develop a more severe case of mastitis (Burvenich et al. 2003). Additionally, while low somatic cell counts in milk are more desirable, very low counts have been associated with an increase risk for clinical mastitis (Paape et al. 2002).
One of the most important aspects of innate defense is the recognition and response towards pathogen associated molecular patterns via cellular pattern recognition receptors. CD14, which binds to lipopolysaccharide binding proteins is a common pattern recognition receptor where it causes the production of TNF-α which triggers the cytokine cascade response in the innate immune system (Paape et al. 2002). Likewise, toll-like receptors are another common receptor which can detect pathogen associated molecular patterns. As with many animals, several toll-like receptors are found in bovines (Menzies and Ingham 2006). As mentioned, once these receptors are activated a cascading chemokine/cytokine effect occurs with the help of NF-κB which allows for immunoregulation to occur (Brightbill and Modlin 2000). Macrophages can also release chemoattractants which attract neutrophils towards the site of infection (Burvenich et al. 1994). Overall this allows for nonspecific eradication of pathogens through various methods including: phagocytosis, inflammation, release of hydroxyl and oxygen radicals and other structures including defensins (Sordillo and Streicher 2002). While the innate response is important in eradicating pathogens, should prolonged activation continue then the inflammation will cause cellular damage and thus clinical mastitis will occur (Paape et al. 2002).

Adaptive Defense

Should the innate defense be insufficient in eradicating the pathogen the adaptive immune response occurs. The response takes advantage of antigen specific lymphocytes and memory cells (Sordillo 2016).

T cell lymphocytes can be differentiated into several subgroups including CD4+ and CD8+ T cells (Sordillo and Streicher 2002). The cells are activated by antigens on MHC complexes from
antigen presenting cells such as B cells, dendritic cells, and macrophages (Sordillo and Streicher 2002). CD4+ T cells interact with MHC class II molecules and help eliminate extracellular pathogens while CD8+ T cells interact with MHC class I molecules and eliminate intracellular pathogens (Çomakli and Özdemir 2019). Whereas CD8+ T cells directly kill cells infected with viruses and intracellular bacteria in an attempt to prevent more from forming, CD4+ T cells act more in a supporting role where they help activate B cells and CD8+ T cells along with directing macrophages to destroy microbes via cytokine release (Alberts, Johnson and Lewis 2002, Çomakli and Özdemir 2019, Gutcher and Becher 2007, Ladel et al. 1994).

B cells aid in the adaptive defense by primarily producing antibodies (Hoffman, Lakkis and Chalasani 2016). Antibodies such as IgM aid in opsonization while IgA cause pathogens to agglutinate, allowing for easier detection and phagocytosis (Aitken, Corl and Sordillo 2011, Sordillo 2016). Another function of B cells are to differentiate into memory cells (Hoffman et al. 2016). Memory cells are retained for a faster response should the same pathogen infect the organism again. This allows for future responses to be faster, more robust, long lasting and more effective (Sordillo 2016).

1.1.1.3 Transmission of Bovine Mastitis

Transmission of bovine mastitis is often classified into two groups, contagious and environmental. In general, contagious mastitis is caused by pathogens living in other bovines while environmental mastitis is caused by pathogens living in the environment. While researchers and managers tend to fit one bacterial species into a category that is not always the case as microbial infection can originate from either other bovines or the environment (Klaas and
Zadoks 2018). However, certain microbes are more likely to be transmitted through one method over the other. For contagious infections it is more typical to see *Streptococcus* spp., *S. aureus*, coagulase negative staphylococci and *Corynebacterium bovis* (Gruet et al. 2001). Among environmental infections it is more typical to see coliform bacteria such as, *E. coli* and *Klebsiella* spp., *S. uberis* and *Enterococcus faecalis* (Gruet et al. 2001).

*Contagious Transmission*

Contagious mastitis typically contains pathogens which are suitable in living within the host’s mammary glands where they are more likely to cause subclinical mastitis relative to environmental mastitis (Bradley 2002). Bovine to bovine transmission of mastitis pathogens are often caused as the result of poor management protocols. Improper use of equipment such as not using a teat dip, reusing wipes or improper cleaning of milking machines can lead to increase incidents of bovine mastitis (Leelahapongsathon et al. 2014).

Milk machine management is very important in reducing instances of contagious mastitis. The machines can aid in mastitis infection by being a carrier of pathogens, by serving as a way to cross-infect cows, through damaging the teat via misuse and through loss of vacuum, it may create forces which can allow pathogens to move past the teat canal defenses (Spencer 1989). The slippage of liners between the machine and udder have shown to increase the instances of bovine mastitis (O'Shea, O'Callaghan and Meaney 1979). Avoiding the use of liner slips is necessary for good udder health and thus increasing vacuum pressure is required, but not too much that the pressure would cause damage to the udder (Besier, Lind and Bruckmaier 2016).
general, physical factors such as mechanical, thermal and chemical trauma can increase the chances of the mammary gland becoming infected (Zhao and Lacasse 2008).

Another way for contagious transmission is via insects. Flies are able to transmit microbes from a bovine suffering from mastitis to other bovines, allowing for non-human related transmission as described above (Chirico et al. 1997). This can lead to a condition known as summer mastitis, a condition where permanent loss of function of the milking quarter occurs and culling the animal is common (Jousimies-Somer, Pyörälä and Kanervo 1996).

**Environmental Transmission**

As better farming techniques contribute to the decrease of contagious mastitis incidence, difficulties in controlling environmental mastitis have become a larger issue (Leigh 1999).

Potential environmental contaminants can range from bedding to water and vegetation (Bhosale et al. 2014). While microbes do naturally exist in the environment, humans also play a factor in contaminating places where bovines frequent. One study showed *S. agalactiae* transfer between humans and bovines (Lyhs et al. 2016). Other animals may also introduce pathogens which cause mastitis into the environment. In areas with high swine populations it was found bovines had mastitis caused by MRSA typically found in pigs (Locatelli et al. 2016). Similarly, *Streptococcus canis*, a pathogen for cats and dogs was found in bovine milk, causing mastitis (Richards et al. 2012).

Bedding can be a large reservoir for pathogens and poorly managed areas can increase incidences of bovine mastitis (Klaas and Zadoks 2018). Several types of materials for bedding
can be used for cattle; sand, sawdust, peat, straw and recycled manure solids (Klaas and Zadoks 2018, Lejeune and Kauffman 2005, Rowbotham and Ruegg 2016). While there has been work in trying to find the optimal bedding material to decrease bovine mastitis and certain types can be correlated with a decreased amount of a certain pathogen, good general management practices seem to be the most optimal method (Hogan et al. 1989, Rowbotham and Ruegg 2016, Zehner et al. 1986).

Often, bedding along with walkways, water troughs and other places can become contaminated via feces which can create reservoirs for environmental mastitis. Feces contain several mastitis causing pathogens which can transmit to the udder. The same strains of *S. uberis* was found in both feces and in milk of mastitis suffering bovines (Zadoks, Tikofsky and Boor 2005). Similarly, *Klebsiella pneumoniae* was shown to be a fecal contaminant in farms (Munoz et al. 2006).

### 1.1.2 Costs to the Dairy Industry

The cost for bovine mastitis to the industry can sometimes be difficult to determine. This stems from the asymptomatic phenotype of subclinical mastitis which can allow the disease to go undetected until testing by dairy cattle managers (Petrovski, Trajcev and Buneski 2006). However, studies have attempted to quantify the number by relating the inverse relationship of somatic cells and milk production and quality (Bradley 2002).

In general, losses from clinical and subclinical mastitis can be from rejected milk, lower milk quality, culling and replacement of cows, drug and veterinarian expenses, preventative measures
and increase labour costs (Gruet et al. 2001, Halasa et al. 2007). The largest cost for bovine
mastitis is reduced milk production where estimates of 70% of total losses to the farm was found
(Schroeder 2012, Zhao and Lacasse 2008). Additionally, bovine mastitis results as one of the
largest reasons to cull a cow in the United States (Gruet et al. 2001).

In Canada it was estimated that for each cow an average of $662 CAD would be the cost for
treating and preventing bovine mastitis with 48% being attributed to subclinical mastitis, 34%
being attributed to clinical mastitis and 15% being attributed to direct preventative measures
(Aghamohammadi et al. 2018). In 2018, the Canadian Dairy Information Centre estimated there
to be 1.4 million dairy cows and heifers living in Canada (Canadian Dairy Information Centre
2019). Through extrapolation, it is then calculated that the cost for the nation would be 926.8
million dollars per year. Similarly, it is estimated that the economic cost of bovine mastitis for
the United States to be 1.8 billion USD in losses to the industry per year (Middleton et al. 2014,
Schroeder 2012).

1.1.3 Current Practices and Treatments

1.1.3.1 General Prevention Techniques

Managers employ several controls to prevent mastitis from forming including proper personnel
training, maintaining a healthy environment for the animals, establishing proper milking
procedures and keeping milking equipment as clean as possible.

Maintaining good hygiene practices is an important way to lower the rate of transmission of
bovine mastitis. Poor udder hygiene scores have shown a positive relationship to increased
somatic cell counts (Schreiner and Ruegg 2003). As the presence of moisture, dirt and manure can be a reservoir for pathogens it is in the best interest to limit the exposure of such places. With proper drainage and removal of manure, managers can reduce the risk of pathogenic transmission (Abebe et al. 2016). Sand is often considered to be a good source of bedding as a comfortable laying surface and having typically a lower bacterial count relative to other surfaces (van Gastelen et al. 2011). Additionally, the application of a teat sealant can be used to enhance the physical barrier between the teat and environment. An internal teat sealant is created through the use of bismuth subnitrate and is usually administered at the end of the lactation period for bovines and has shown to be as effective at preventing mastitis as antibiotic dry cow therapy (Huxley et al. 2002). A different study saw a reduction of post-calving intramammary infection incidences when administered the internal teat sealant a median of 39 days before calving (Parker et al. 2008). An external teat sealant, such as a latex covering has also been used pre-calving and showed reduction in the rate of bovine mastitis (McDougall et al. 2008).

Proper pest control, typically towards flies can be effective in reducing the rate of bovine mastitis. In order to prevent resistances towards certain pesticides it is suggested to rotate different classes of treatments and to use alternative strategies depending on the stage of the insect’s life cycle (Broce 2006, Byford et al. 1999). Isolation, preventing calves from suckling other cows and properly disposing of infected milk by usage of a phenol can also reduce the spread of mastitis (Bhosale et al. 2014). Additionally, vaccination is another option and has shown reduction of somatic cell counts caused by *S. aureus*, *S. uberis* and *E. coli* (Giraudo et al. 1997, Hogan et al. 1999, Leigh 1999).
One of the most important preventative methods is through proper maintenance of the milking process. Milk machines can account for around 10% of new infections and up to 20% should the machine not be set up properly (Mein 2012). By simply cleaning and drying the teat prior to machine milking, this can lower the concentration of bacteria in milk, which can affect quality and taste and lower the incidence of mastitis. This can be done by cleaning the machinery and using teat dips and paper towels to decrease the incidence of mastitis (Galton et al. 1982). Teat dips containing ingredients such as iodophors, sodium hypochlorite, and sodium dichloro-s-triazenertrione have shown to be effective against *S. aureus*, *S. agalactiae*, *E. coli* and *Pseudomonas aeruginosa* (Philpot, Boddie and Pankey 1978).

On the machine itself, maintaining proper vacuum pressure during the milking process is very important. The International Organization of Standardization has a vacuum pressure range at the teat end between 32 and 42 kPa (International Organization for Standardization 2007). This creates a balance where it is not too low where liner slips occur and not too high where damage to the teat may occur (Besier et al. 2016). Additionally, the use of appropriate size liners is recommended (Besier et al. 2016). It is also suggested that managers should create an evaluation program to perform preventative maintenance on the milking machine every milking, weekly, monthly and half yearly, depending on the area of concern or part to ensure high efficiency milking and a decreased chance in transmitting microbes to other animals (Jones 1999).

**1.1.3.2 The Five Point Plan**

One of the ground breaking additions to bovine mastitis prevention and treatment was the Five Point Plan. It was developed in the 1960s by the National Institute for Research into Dairying in
the United Kingdom and provided a checklist for managers to reduce the incidence of mastitis (Middleton et al. 2014). The Five Point Plan included 1) the proper maintenance of the milking machine, 2) usage of an effective germicide on teats after milking, 3) appropriate therapy of clinical mastitis, 4) during the drying off period, treat every quarter of every cow with an intramammary antibiotic and 5) cull chronically infected animals (Middleton et al. 2014).

The Five Point Plan when incorporated with national plans saw dramatic decreases in the instances of clinical mastitis, including the United Kingdom where instances fell from 150 to 40 cases per 100 cows per year from 1967 to 1982 (Green and Bradley 2013, McDougall 2002, Vissio et al. 2013). However, this was mostly due to the decrease of contagious mastitis pathogens (Bradley 2002). Environmental pathogens remained a large problem while \textit{S. aureus} continued to be a major cause of subclinical mastitis (Bradley 2002). Additionally, there is disagreement on whether the plan is cost effective and whether the control measures are associated with lower bulk milk somatic cell counts (Anderson and Blackshaw 1977, Emanuelson and Nielsen 2017). Also, of concern is the use of prophylactic antibiotics which is known to increase drug resistance in bacteria such as \textit{S. aureus}.

Other groups have since expanded on the Five Point Plan including the National Mastitis Council where they expanded on certain aspects of farm management (Middleton et al. 2014). The Ten Point Plan includes 1) establishing goals for udder health, 2) maintaining a clean environment, 3) perform proper milking techniques, 4) perform proper milking machine maintenance, 5) keep good records, 6) perform proper management of mastitis during lactation, 7) perform effective dry cow therapy management, 8) have a biosecurity policy, 9) monitor udder health status and
10) review control program on a regular basis (National Mastitis Council 2006). This also helped to addressed the concern of the overuse of antibiotics.

### 1.1.3.3 Tests to Determine Subclinical Bovine Mastitis

While clinical mastitis can be easy to detect from visual inspections (inflammation, swelling and clotted milk) to diagnosis the visually asymptomatic subclinical mastitis tests are required (McDougall 1998).

Somatic cell counting can be used to determine whether an animal has mastitis, particularly subclinical ones. Typically, healthy animals have less than 100,000 cells/mL of milk and an increase from this level can cause a decreased production of milk and milk quality (Deb et al. 2013, McDougall 1998, Schroeder 2012). Two common devices used to count somatic cells is through the Coulter Milk Cell Counter, which counts cells passing through an electric field and the Fossomatic, which counts fluorescent cells after staining it with a dye (Sharma, Singh and Bhadwal 2011). While somatic cell counts are important in determining the health status of bovines, it does take time to obtain results and thus quicker on field tests were developed to be used in conjunction with this test.

One of the most common tests is the California Mastitis Test. The reagent, an anionic detergent (sodium alkyl aryl sulfonate) is mixed in equal portions with milk where DNA from somatic cells is released which form a gel that thickens in relation to the concentration of cells within the milk (Carroll and Schalm 1962, Lakshmi 2016, Muhammad et al. 2010). As subclinical mastitis can cause an increase of leukocytes in the milk this method can be a way to determine possible
intermammary infections (Lakshmi 2016). It is considered good farm practise to perform the California Mastitis Test on the other quarters after one quarter shows signs of mastitis so that treatment can occur simultaneously (Gruet et al. 2001).

Other similar cow-side tests include the Whiteside Test where after a reagent is added the sample is graded based on the amount of cloudiness or precipitation that appears (Whiteside 1939). A modified Whiteside Test can be performed to determine the possibility of mastitis within a herd where bulk milk is tested and managers can then subsequently test each individual animal should the result be positive (Temple 1963). In South Asia, the use of a popular detergent brand (Surf Excel) has been used as a cheaper alternative to the California Mastitis Test and Whiteside Test (Muhammad et al. 2010). The Surf Field Mastitis Test has shown to produce similar results to other tests which allows it to be a viable alternative (Muhammad et al. 2010). Additionally, a 3% solution containing sodium lauryl sulphate has been used where the formation of clots designates a positive result (Thakur et al. 2018).

One method that doesn’t indirectly measure the concentration of somatic cells is through the measurement of electrical conductivity of the milk. During an intermammary infection the destruction of tight junctions and ion-pumps cause an influx of sodium and chloride to be present within the milk (Kitchen et al. 1980). The advantage of this method is it can be incorporated into a milking machine and automatically measured at each milking (Maatje et al. 1992). However, differences in conductivity between individuals and stage of lactation make implementation of this technology difficult and requires the measurement of other factors including milk yield and milk flowrate (Khatun et al. 2018, Linzell and Peaker 1975).
Additionally, various molecular and proteomic methods including PCR, including Multiplex and real-time PCR, ELISAs, 2D gel electrophoresis and mass spectrometry have been developed, but are often genre or species-specific which limits detection of rare pathogens (Lakshmi 2016).

1.1.3.4 Treatment of Bovine Mastitis

Antibiotic Treatment

When a cow is infected with bovine mastitis, antibiotics are the most common choice of treatment (McDougall 1998). Antibiotics can be chosen based on a number of factors; the species of bacteria and penetration of the treatment to the site of infection while attempting to minimize the risk of bacterial antibiotic resistance and toxicity to the animal (McDougall 1998). For example, staphylococci are often treated using beta-lactams such as penicillin or methicillin (Gentilini et al. 2002). For the treatment to be considered successful a clinical response must be observed 5-7 days after antibiotic treatment or else it would be considered a therapeutic failure (Schukken and Deluyker 1995). Two routes of administration are often employed; systemic and intramammary treatments.

Systemic treatment occurs typically through intramuscular injections while intramammary treatment occurs when the drug is administered through the teat canal of the animal. Choosing the route of administration can often depend on the pharmacokinetics of the drug where selecting incorrectly could lead to therapeutic failure (Sandholm, Kaartinen and Pyörälä 1990). In a systemic drug treatment, the ideal drug candidate should be a weak base, have good bioavailability, a low plasma bound protein concentration and be lipid soluble (Erskine et al.
1993, Ziv 1980a). During intramammary treatment the solubility of the drug determines its
distribution with a hydrophilic molecule remaining closer to the cistern while a lipophilic
molecule would distribute towards the membrane rich ducts and alveoli (Gruet et al. 2001).

The choice of route of administration differs between countries where Northern European
countries prefer systemic treatments while in other countries the use of systemic antibiotics are
used only for severe cases of bovine mastitis (Sérieys et al. 2005). Comparison studies have been
conducted with differing results. One study, performed during the dry period showed a higher
cure rate of systemic use of norfloxacin nicotinate than intramammary treatment with cephalirin
benzathine (Soback et al. 1990). However, a higher cure rate was found using the intramammary
treatment of penicillin-dihydrostreptomycin than the systemic treatment of penethemate
hydriodide (McDougall 1998). Another study showed systemic use of penethamate hydriodide
had more success in reducing somatic cell count to below 250,000 cells/mL than an
intramammary administration of an ampicillin/cloxacillin combination, though one reason was
that the penethamate hydriodide also reduced somatic cell counts in adjacent quarters, likely
caused by sublevel infections (Sérieys et al. 2005). With withdrawal periods required for each
route of administration, perhaps the best practice for managers is choosing the best type of
antibiotic rather than its route (Burmańczuk et al. 2017).

Similarly, pharmacodynamics of the drug is important, particularly whether milk interferes with
its antimicrobial activities. Studies have shown reduced antimicrobial activity for
aminoglycosides, enrofloxacin, gentamicin, macrolides, novobiocin, sulfadoxine-trimethoprim,

**Dry Cow Therapy**

One popular treatment employed by managers is to treat animals during the post-lactation (dry) period, also known as Dry Cow Therapy. During the dry period cows may be treated with an antimicrobial agent injected into the mammary gland (Cameron et al. 2014). This is done to eliminate any current disease and preventing new infections during a period where the udder is most susceptible (Soback et al. 1990). Dry Cow Therapy has the advantage of allowing a more uniform concentration of the drug to be administered, allows for more drug treatment options, has a higher treatment success rate and a lower cost by not having to dispose of milk (Gruet et al. 2001). The therapy isn’t without controversy, notably the prophylactic use of antimicrobials which can promote resistance to such treatments (Afifi et al. 2018). Despite this, the prophylactic treatment of all cows is still recommended by the National Mastitis Council (National Mastitis Council 2006).

While first described as far back as the late 1970s, selective Dry Cow Therapy, the treatment of only cows suspected on having an intermammary infection has become an increasingly popular option to avoid the overuse of antibiotics in the dairy industry (Cameron et al. 2013, Rindsig et al. 1978). Additionally, the use of a teat sealant on all cows can aid in the success of the selective treatment (Huxley et al. 2002). If managers choose to perform selective Dry Cow Therapy, they must identify animals with intermammary infection via tests such as the California Mastitis Test or bacterial growth testing with growth media such as 3M Petrifilm plates (Cameron et al. 2013,
Sanford et al. 2006). While this may increase costs for managers, it can be argued that the decrease cost of using fewer antibiotics injections minus the future treatment of additional infected cows would ultimately save money (Browning et al. 1994).

**Issues with Antibiotic Treatment**

While the use of antibiotics is the most popular method in treating bovine mastitis there are a number of concerns for managers to take into account. One danger of repeated intramammary treatments is the erosion of keratin in the teat canal. With an increased diameter, the natural defense of the cow is lowered and thus there is an increase chance of subsequent infection (Gruet et al. 2001). Additionally, poor injection of the drug could cause contamination via propelling bacteria from the teat surface into the gland (Sandholm et al. 1990).

Moreover, in most countries, milk is not permitted to be sold until there is either zero or very limited (within the nation’s guidelines) detectable antibiotic residues in the milk (Bhosale et al. 2014). This is known as the withdrawal period. The combination of treatment days plus withdrawal period can typically cause loss of milk for around 6-10 days, further exacerbating economic losses, which can be increased even more in instances of therapeutic failures and retreatments (Gruet et al. 2001, Smith et al. 2005). Steeneveld et al. (2011) created multiple economic models for mastitis treatment regimens which calculated that milk withdrawal alone accounted for 11.9%-17.4% in losses per treatment.

The major concern of the use of antibiotics is that it can create added selective pressure towards antibiotic resistance which can cause therapeutic failure and more concerning, those pathogenic
bacteria could then be transferred to humans through food and water (White and McDermott 2001). This is a worldwide problem. In Argentina, 28.44% samples containing coagulase negative Staphylococcus were found to show antibiotic resistance to at least one antibiotic, mostly from penicillin (Gentilini et al. 2002). Gentilini (2002) related his findings to similar studies done in other nations and found penicillin resistance to coagulase negative Staphylococcus to be at 37.2% in Finland, 36.1% in Denmark and two studies in the United States showed 42.7% and 57% resistance to penicillin. As beta-lactam antibiotics are one of the most commonly used treatments for bovine mastitis, the cause of the resistance is often attribute to the production of beta-lactamase (Fejzic et al. 2014, Gentilini et al. 2002). In China, 541 isolates which included S. aureus, non-aureus staphylococci, Streptococcus spp., E. coli and Klebsiella spp. were recovered from 45 large dairy herd farms (>500 cows) where 27% of samples were resistant to at least three types of antibiotics (Cheng et al. 2019). Similarly, in Canada, 11.1% of E. coli and 20.1% of Klebsiella spp. isolates were resistant to at least two antibiotics (Saini et al. 2012).

The potential for increased prevalence of antibiotic resistance is also a concern as Myllys et al. (1998) found the antibiotic resistance of S. aureus and coagulase negative Staphylococcus increased from 37% to 64% and 27% to 50% respectively from 1988 to 1995 in Finland. However, with improved management and treatment protocols, VetPath, a pan-European antimicrobial susceptibility monitoring program found no change in the prevalence of antibiotic resistant bacteria between 2002-2006 and 2009-2012 (de Jong et al. 2018). In Nordic countries, the use of antibiotics is now strictly regulated where only veterinarians are allowed to administer antibiotic treatment (Valde et al. 2004). As antibiotic resistance becomes a concern for managers,
the options for treating bovine mastitis is becoming more challenging (Mehmeti et al. 2016, White and McDermott 2001, World Health Organization 2000).

1.1.3.5 The Push for Other Treatments

Several groups have explored the possibility of alternative treatments for bovine mastitis. One such treatment is the use of bacteriophages. Bacteriophage therapy identifies viral phages that specifically kill bacteria and then injecting them into the animal with the bacterial infection. One such is phage K which has bactericidal effect on staphylococci, including *S. aureus* (O'Flaherty et al. 2004). However, testing in raw milk has shown the inactivation of the bacteriophage due to whey proteins binding and bacterial aggregation, suggesting treatment may only be useful during the dry period of cows (Gill et al. 2006, O'Flaherty et al. 2005). A cocktail of bacteriophages, while still showing decreased activity in raw milk relative to broth showed more promising results suggesting more work in this field is needed (Breyne et al. 2017).

The use of metallic nanoparticles as antimicrobials has also been tested. More notably, silver nanoparticles have been shown to inhibit growth of bacteria isolated from bovine mastitis (Dehkordi, Hosseinpour and Kakrizangi 2011). At the same time, they have been shown to have low toxicity towards a bovine mammary epithelial cell line (Jagielski et al. 2018). More recently one group explored the possibility of using copper nanoparticles or a combined silver-copper nanoparticle and while it showed antimicrobial activity it was not as strong or encompassing as silver alone (Kalińska et al. 2019). There is also the possibility of using nanoparticles synergistically with antibiotics to create a more robust treatment (Devi and Joshi 2012). In an indirect role, nanoparticles can be used as carriers of drugs with poor bioavailability in the
mammary gland. Solid liquid nanoparticles have shown to increase the efficacy of tilmicosin treatment in a mouse mastitis model (Wang et al. 2012).

Natural compounds are another avenue being explored. While used in folk medicine for various ailments, plants and their compounds have become more scrutinized by the scientific community for their potential antimicrobial use. One example, is the examination of plants in Romania; *Evernia prunastri*, *Artemisia absinthium* and *Lavandula angustifolia* showed the best antimicrobial effects against 32 microorganisms isolated from milk (Pașca et al. 2017). In another study, extracts derived from *Angelica dahurica* and *Rheum officinale* showed both an anti-inflammatory and antimicrobial effect when used on bovines and may have a shorter treatment period than antibiotics (Yang et al. 2019). In terms of non-plant derived antimicrobials, lactoferrin, a glycoprotein found in tears and saliva is another molecule used. The protein, isolated in milk whey has shown to inhibit the growth of *S. aureus*, *E. coli*, *S. agalactiae* and *P. aeruginosa* (El Hafez et al. 2013). Finally, nitric oxide may prove to be an option as a treatment for bovine mastitis.

1.2 What is Nitric Oxide

Chemically, nitric oxide is a short-lived, naturally occurring single electron nitroxide radical consisting of one nitrogen and one oxygen atom. The molecule has a molecular mass of 30.006 g/mol, a melting point of -164°C and a boiling point of -152°C. Due to its unpaired electron, nitric oxide very rapidly reacts with other radicals such as superoxide (Lancaster 2006). It has a very short half-life of a few seconds (Hakim et al. 1996).
1.2.1 History

Nitric oxide was first classified by Joseph Priestley in 1772. The classification came about from the interest of an experiment performed by Stephen Hales where he mixed air with gas obtained from Walton pyrites and spirit of nitre (nitric acid) and obtained a red gaseous mixture which absorbed some of the air (Priestley 1775). After consulting Henry Cavendish, it was hypothesized that the cause of the red gaseous mixture was depended on the spirit of nitre only and that the same result would occur with combination of other pyrites and metals (Priestley 1775). Priestley was successfully able to obtain this red gaseous mixture and conclude it being from spirit of nitre (Priestley 1775). He gave it the name nitrous air, eventually becoming better known as nitric oxide. It was eventually shown that NO is an odorless colourless gas but is oxidized rapidly in a time concentration dependent manner to produce nitrogen dioxide. Nitrogen dioxide is a yellow to brownish coloured gas. Nitric oxide occurs naturally as a result of high combustion reactions (lightning and volcanoes) and is man made, produced in the exhaust from combustion engines. For much of its history since its discovery, nitric oxide was seen more or less as an atmospheric pollutant, but three decades ago it was discovered to be produced in mammals and today it is seen as a very versatile molecule in biology.

1.2.1.1 Endothelium-Derived Relaxing Factor

One of the first and largest discoveries regarding nitric oxide was when it was determined to be endothelium-derived relaxing factor (EDRF). EDRF was first described by Robert Furchgott who showed acetylcholine would dilate blood vessels only if the endothelium was intact, concluding that there was some kind of signal molecule that would make smooth muscles relax (Furchgott and Zawadzki 1980).
In 1977, Ferid Murad showed nitric oxide and other nitro-containing compounds increased cGMP levels in tissues and can cause relaxation in smooth muscle (Arnold et al. 1977). Similarly, Louis Ignarro noted nitric oxide possessed similar biological and chemical properties such as activating soluble guanylate cyclase from vascular smooth muscles (Ignarro et al. 1987). This led to an investigation on whether the two molecules were one and the same.

EDRF was found to be produced by both arteries and veins in a controlling manner and the effects of both EDRF and nitric oxide are inhibited by methylene blue and hemoproteins (Ignarro et al. 1987). Using arterial and veins, the half-life of EDRF and nitric oxide was calculated to be 3-5 seconds (Ignarro et al. 1987). Another link between EDRF and NO was that EDRF reacts with hemoglobin to produce nitrosyl-hemoglobin, causing a shift in the Soret region absorbance maximum from 433 nm to 406 nm (Ignarro et al. 1987). Independently, both Palmer (1987) and Ignarro (1987) demonstrated that NO accounts for the physiologic and pharmacologic properties of EDRF (Ignarro et al. 1987, Palmer et al. 1987). Due to their work with nitric oxide as a signalling molecule in the cardiovascular system, Furchgott, Ignarro and Murad would receive the 1998 Noble Prize in Physiology or Medicine.

1.2.2 Nitric Oxide Synthases

Since the 1980s, nitric oxide has been discovered to be a very versatile molecule in mammals with neurons, endothelial cells, and macrophages being the most well-known sources of NO. The rush of discoveries allowed nitric oxide to be declared as the molecule of the year in 1992 by
Science magazine (Koshland 1992). Interestingly, Dr Chris Miller, published the first review article on the potential of nitric oxide in medicine (Miller and Miller 1992).

In brief, biochemically, the nitric oxide molecule can modulate neuron function, regulate vasodilation and is involved in infection response (Croen 1993). In mammals, nitric oxide is synthesized from L-arginine by dimerized nitric oxide synthase (NOS). For all isoforms, an oxygen molecule and NADPH are required where L-arginine is converted to citrulline and nitric oxide is released (Marletta 1993). There are three isoforms of NOS that are found in mammals; endothelial, neuronal and inducible.

1.2.2.1 Endothelial Nitric Oxide Synthase

Endothelial nitric oxide synthase (eNOS) is mostly expressed in endothelial cells and its primary function is to act as a vasodilator. The production of nitric oxide via eNOS is sensitive to many stimuli, such as, shear stress, acetylcholine, bradykinin, histamine and 17β-estradiol (Zhao, Vanhoutte and Leung 2015). Unlike the other two isoforms of nitric oxide synthase, eNOS is predominately a membrane-bound protein, regularly attached to the Golgi apparatus, but can also be found on the rough endoplasmic reticulum and vesicles (O'Brien et al. 1995).

Many of these agonists cause an influx of calcium into the endothelial cell which then bind to calmodulin and then bind to eNOS and enhance nitric oxide production (Zhao et al. 2015). eNOS can also be regulated via phosphorylation of certain serine and threonine sites from cGMP-dependent protein kinase II (Butt et al. 2000).
Nitric oxide is able to cause vasodilation of smooth muscle cells by binding to soluble guanylate cyclase which catalyzes the conversion of guanosine 5’-triphosphate into cyclic guanosine 3’,5’-monophosphate (Denninger and Marletta 1999). The cGMP can then cause a reduction of intracellular calcium of the smooth muscle by activating cGMP-dependent protein kinases which then phosphorylate several proteins, including, ion channels, ion pumps, receptors and enzymes (Carvajal et al. 2000). This decrease of intracellular calcium will result in myosin light chain kinase remaining inactive and thus unable to phosphorylate myosin, leading to relaxation of smooth muscle (Webb 2003).

1.2.2.2 Neuronal Nitric Oxide Synthase

Neuronal nitric oxide synthase (nNOS) is predominantly expressed in neurons, both in the central nervous system and the peripheral nervous system. As with eNOS, nNOS is dependent on calcium and calmodulin to be activated (Andrew and Mayer 1999). Likewise, the synthase can be regulated through various phosphorylation sites via kinases and phosphatases such as PKA, calmodulin-dependent kinases, PKC and phosphatase 1 (Zhou and Zhu 2009). Unlike eNOS, nNOS is found in the cytosol of the cell and its location is determined based on protein interactions with its PDZ (post-synaptic density protein, discs-large, ZO-1) domain (Jiang et al. 2014). One example is PSD95 which targets nNOS to the post synaptc density and allows it to be in contact with a higher concentration of calcium and thus become more active (El-Mlili et al. 2008).

The mechanism of action for nNOS is similar to eNOS where soluble guanylate cyclase is bound, increasing the concentration of cGMP (El-Mlili et al. 2008). This pathway in the brain is
thought to modulate learning and memory behaviour and that its decrease may lead to memory impairment (Yamada et al. 1996a, Yamada et al. 1996b). It is also believed that abnormal signalling of nitric oxide may play a role in neurodegenerative diseases such as multiple sclerosis, excitotoxicity, Alzheimer’s and Parkinson’s disease (Steinert, Chernova and Forsythe 2010).

Additionally, nNOS at the central nervous system has been shown to reduce vascular sympathetic tone as does nNOS in the peripheral nervous system via nitrergic nerves (Toda, Ayajiki and Okamura 2009, Togashi et al. 1992). Interestingly, it is nNOS and not eNOS that is responsible for the relaxation of the corpus cavernosum smooth muscle via nitrergic nerves which can then result in penile erection (Förstermann and Sessa 2012).

1.2.2.3 Inducible Nitric Oxide Synthase

The final isoform of nitric oxide synthase found in mammalian cells is inducible nitric oxide synthase (iNOS). iNOS can be found in many types of cells including endothelium, hepatocytes, monocytes, mast cell and smooth muscle cells, but is most commonly known to be found in M1 macrophages (Aktan 2004, Förstermann et al. 1994, Lu et al. 2015). As with nNOS, iNOS is also found in the cytosol of cells (Liu, García-Cerdeña and Sessa 1995). iNOS is unique among its isoforms in that it is calcium independent, meaning the concentration of calcium in the cytosol does not control its activation. This is due to iNOS lacking a polypeptide insert in the FMN binding domain which inhibits its function in low levels of calcium and calmodulin (Salerno et al. 1997). This allows nitric oxide to keep being produced during periods of infection. Instead it is controlled at the transcriptional level (Cho et al. 1992) where toxins such as
lipopolysaccharides and cytokines such as IL-1, IFN-γ or TNF-α increase its transcription while cytokines such as IL-4, IL-8, IL-10, tumor growth factor-β, PDGFs and IGF-1 inhibit its transcription (Kuncewicz et al. 2003, Mayer and Hemmens 1997).

iNOS expression in different cells can sometimes have contradicting results which suggests that regulation of the protein is widely variable depending on the cell type and species (Rao 2000). For example, bovine macrophages are more sensitive to cytokine stimulation to induce iNOS than human macrophages (Jungi et al. 1996). In general, cellular receptors which bind with molecules such as lipopolysaccharides, IL-1 and TNF-α are known to activate the NF-κB pathway, leading to an increase of iNOS (Aktan 2004, Arias-Salvatierra et al. 2011, Beg et al. 1993). IFN-γ activates the JAK-STAT signalling pathway, more specifically STAT1α which in turn increases the expression of IRF-1 (De Stefano et al. 2006).

Conversely IL-4 activates the JAK-STAT signalling pathway, more specifically STAT6 to inhibit production of iNOS (Hiroi et al. 2013). TGF-β not only reduces the transcription level of iNOS via inhibiting the JAK-STAT signalling pathway, but it can also destabilize the protein, leading it to degrade at a quicker rate (Takaki et al. 2006). The production of iNOS can cause the phosphorylation of eIF-2α which interferes with translational protein synthesis and is thought of as a negative feedback mechanism to autoregulate iNOS (Petrov et al. 2001).

### 1.2.3 Nitric Oxide and the Innate Immune System

Nitric oxide plays a major role in the innate host response of mammals, including bovines (Liew and Cox 1991, Silanikove et al. 2005). While at low levels (nanomolar), nitric oxide is a
signalling molecule, targeting its physiological enzymes, at higher levels (micromolar) nitric oxide, other, non-specific enzymes become targets allowing it to exert its cytostatic and cytotoxic effects on pathogens and potentially the host itself (Vallance and Charles 1998). In animal models, there has been a correlation that the inactivation of the inducible nitric oxide synthase can cause an increase of the microbial load (Fang 1997).

During the early stages of infection and inflammation, immune cells via iNOS synthesize nitric oxide to help combat the foreign body. Due to nitric oxide’s non-polar properties it is predicted the molecule can enter microbes through crossing the cell membrane. A direct study showed that the diffusion behaviour resembles oxygen, thanks to their similar structures (Denicola et al. 1996). As a molecule with a free radical, nitric oxide reacts with reactive oxygen species such as superoxide and hydrogen peroxide to create other antimicrobial molecules such as peroxynitrite (OONO\(^-\)), S-nitrosothiols (RSNO), nitrogen dioxide (NO\(_2\)), dinitrogen trioxide (N\(_2\)O\(_3\)), and dinitrogen tetroxide (N\(_2\)O\(_4\)) (Jones et al. 2010b). While some bacteria such as Salmonella typhimurium, E. coli and Listeria monocytogenes have been shown to be resistant to nitric oxide directly, when combined with reactive oxygen species, its intermediates have been shown to be antimicrobial (Vallance and Charles 1998). Nitric oxide and its intermediates have been shown to have both cytostatic and cytotoxic effects against a variety of pathogens, including protozoans, flukes, fungi, and bacteria (Croen 1993).

Nitric oxide and its intermediates have been shown to have multiple targets in order to cause cell stasis or death. This is done through the modification of lipids, proteins and DNA (Fang 1997). While by itself nitric oxide is an inhibitor of lipid peroxidation due to its ability to react with
lipid peroxyl radicals, in the presence of reactive oxygen species it can react and become a strong oxidizer (Hogg and Kalyanaraman 1999). Peroxynitrite and nitrogen dioxide have been shown to cause lipid damage which can lead to cell toxicity (Fang 1997). For example, damage to the lipid bilayer can cause changes to its permeability and proton gradient (Hogg and Kalyanaraman 1999).

In proteins, nitric oxide is able to react with thiols, heme groups, iron-sulfur clusters, and tyrosyl radicals to aid in host defense (Fang 1997). S-nitrosylation of thiols, particularly cysteine residues are an important antimicrobial mechanism of nitric oxide which can alter the function or stability of the protein (Kuncewicz et al. 2003, Schairer et al. 2012). Nitric oxide is able to directly bind to heme groups and other metal complexes, especially ones where a disulfide bond is near which can also cause disruption in protein function (Drapier and Bouton 1996). In bacteria, metal complexes, such as catalase serve as a defense against hydrogen peroxide and other reactive oxygen species. Therefore, when nitric oxide inhibits catalase it allows the bacteria to become more susceptible to hydrogen peroxide while at the same time nitric oxide protects the host from reactive oxygen species during the innate immune response (Wink and Mitchell 1998). Reaction with tyrosyl radicals interferes with ribonucleotide reductase function and thus inhibits DNA replication and cell growth (Lepoivre, Flaman and Henry 1992).

Damage to DNA can possibly occur through two methods. Firstly, in conjunction with dinitrogen trioxide nitrosative deamination can occur where 5-methylcytosine can be formed into thymine which can lead to G:C to A:T conversion should it go unrepaired (Juedes and Wogan 1996). Secondly, peroxynitrite can lead to oxidative stress and damage, causing apurinic or apyrimidinic
sites or strand breaks (Juedes and Wogan 1996). Nitric oxide can also disrupt the DNA repair system (Fang 1997).

1.2.4 Nitric Oxide and the Adaptive Immune System

While the bulk of the studies are about the immunological role of nitric oxide and its cytotoxic properties in the innate immune system, it also has a role in the adaptive immune system as an immunoregulator and can be thought of as a link between the innate and adaptive immune system. Nitric oxide can induce biphasic responses depending on its concentration. At low levels, the Th1/Th2 balance favours Th1 differentiation in both murine and humans (Niedbala, Cai and Liew 2006). This is done through nitric oxide increasing cGMP production which leads to an increase production of IL-12Rβ2 and an increase of differentiation via IL-12 p70 binding (Niedbala et al. 2006). Overall, this encourages more production of pro-inflammatory cytokines.

At higher levels, nitric oxide can change the Th1/Th2 balance to favour Th2 responses through downregulating IL-2 and IFN-γ and upregulating IL-4, IL-12 p40 and prostaglandin E₂ (Kolb and Kolb-Bachofen 1998). This can be seen as a negative feedback loop to protect the host from over production of nitric oxide as iNOS is upregulated during Th1 responses (Kolb et al. 1996). Additionally, nitric oxide can also decrease the local level of Th1 cells at the site of inflammation by limiting platelets from producing P-selectins, an endothelial adhesion molecule (Austrup et al. 1997, Whiss, Andersson and Srinivas 1997).
1.2.5 Microbial Defenses Against Nitric Oxide

Since nitric oxide is a commonly used molecule in innate defense in mammals, microbes have developed various methods to combat or resist nitrosative stress. Some commonly used ones are increased production of scavenger molecules (thiols), producing detoxifying enzymes, increasing mechanisms of repair and depletion of L-arginine (Bogdan 2015, Miller et al. 2007).

Thiols are a group of organic compounds which contain a sulphur group attached to an alkyl group. Typically, thiols are used to combat oxidative stress, but can also be used to combat nitrosative stress (Vergauwen, Pauwels and Van Beeumen 2003). Some microbes can synthesize thiol structures such as glutathione which aid in sequestering nitric oxide and other molecules (Fang 1997). Bacteria such as *E. coli* and *S. typhimurium*, with high levels of glutathione have been shown to be more resistant to nitrosative damage (Vallance and Charles 1998). Other thiols which are used to combat nitric oxide stress include mycothiol, bacillithiol and potentially coenzyme A (Gout 2019, Miller et al. 2007, Perera, Newton and Pogliano 2015, Vargas et al. 2016).

One of the major tool microbes have at their disposals is the ability to produce enzymes such as globlins or reductases to resist or detoxify nitric oxide (Bowman et al. 2011). Some types of enzymes used by microbes include flavohemoglobin, flavorubredoxin and cytochrome c nitrite reductase (Karlinsey et al. 2012). Globins are hemeproteins that can bind to ligands such as, oxygen and nitric oxide (Vinogradov and Moens 2008). Flavohemoglobin is found in bacteria, yeast and other fungi and can bind to nitric oxide, converting it to nitrate aerobically or nitrous oxide anaerobically (Poole and Hughes 2000). In *Salmonella enterica* Typhimurium, the
production of flavohemoglobin is transcriptionally repressed by NsrR and at low levels of nitric oxide it becomes inactivated and thus an increased production of the detoxifying enzyme occurs (Karlinsey et al. 2012). NsrR is also important in regulating other non-flavohemoglobin such as the single domain globin Cgb in *Campylobacter jejuni* which similarly converts nitric oxide to nitrate (Smith et al. 2011). Truncated globins also have a similar detoxifying process, though some such as Ctb in *C. jejuni* do not (Koebke, Waletzko and Pacheco 2016, Tinajero-Trejo et al. 2013). More research is needed in this area.

The use of reductases is another option microbes use to detoxify nitric oxide. Flavorubredoxin in *E. coli* provides protection in anaerobic conditions by converting nitric oxide to nitrous oxide (Gardner, Helmick and Gardner 2002, Gomes et al. 2002). The enzyme is regulated by NorR which is activated in the presence of nitric oxide (Flatley et al. 2005). While the main function of cytochrome c nitrite reductase is to reduce nitrite to ammonium it can also reduce nitric oxide and thus also plays a role in microbial defense (van Wonderen et al. 2008). Nitric oxide reductase is another option used by microbes such as *Neisseria meningitidis* which inhibit new formation of S-nitrosothiol from macrophages (Laver et al. 2010).

Having a good DNA repair mechanism is very important for microbes in tolerating nitric oxide. One study found that *Salmonella typhi* with a mutated Base Excision Repair system had attenuated virulence against mice, but virulence was restored when the mice were given an iNOS inhibitor (Richardson et al. 2009). Another study showed that the repair protein *Mutation-Frequency-Decline*, typically known as a house keeping gene, is also important in maintaining DNA function (Darrigo et al. 2016).
Microbes, in a bid to acquire nutrients in a low glucose, high amino acid environment may utilise the arginine deiminase pathway as a method to metabolize L-arginine (Cusumano, Watson and Caparon 2014, Stadelmann et al. 2013). Another method, commonly used by all types of microbes that decreases the amount of available arginine is through arginase (Das, Lahiri and Chakravortty 2010). *Leishmania donovani*, are able to uptake L-arginine via the LdAAP3 transport pump, which can cause a decrease in the availability of the amino acid for the host (Darlyuk et al. 2009). As L-arginine is necessary for nitric oxide production via synthases, a decrease in its availability would lower the production of nitric oxide and potentially allow microbes to tolerate its effect (Marletta 1993).

Additionally, some bacteria are able to produce nitric oxide via their own nitric oxide synthase (bNOS) and use it to their own benefit. In *Bacillus anthracis*, Shatalin et al. use nitric oxide to combat the host’s use of reactive oxygen species, in particular hydrogen peroxide to form a less harmful molecule and to upregulate catalase which bolsters its defense (Shatalin et al. 2008). Bacteria may also use nitric oxide as a way to resist certain antibiotics. In MRSA bNOS derived nitric oxide made the bacteria more resistant to other drugs such as vancomycin and daptomycin, but surprisingly made it more susceptible to aminoglycosides (van Sorge et al. 2013).

1.2.6 Nitric Oxide Gas as a Treatment Option

1.2.6.1 Nitric Oxide Gas as a Vasodilating Treatment

With knowledge of the vasodilating and antimicrobial properties of nitric oxide, medical professionals have developed methods to exogenously deliver nitric oxide for the treatment of
various ailments and diseases. One of the initial treatment options using exogenous nitric oxide gas was for persistent pulmonary hypertension, particularly in newborn babies suffering from Blue Baby Syndrome. Several studies showed patients treated with low dose of gaseous nitric oxide saw an increase of oxygenation within the blood without a decrease in systemic blood pressure (Kinsella et al. 1992, Kinsella et al. 1993, Roberts et al. 1992). Systemic vasodilation is averted due to nitric oxide rapidly binding with haemoglobin to form methaemoglobin (Roberts et al. 1992). Nitric oxide is an approved drug for treating newborn babies suffering from Blue Baby Syndrome (FDA-FOI 2000, Nathan and Hibbs 1991).

Similarly, nitric oxide gas was used as a therapeutic intervention in Ebstein’s anomaly of the tricuspid valve (Bruckheimer et al. 1998). During treatment, the patient’s oxygen saturation level and blood pressure normalized while the heart and lung returned to a normal size (Bruckheimer et al. 1998). The patient was eventually discharged and six months later showed no symptoms (Bruckheimer et al. 1998). Infants who were at risk for pulmonary hypertension after cardiac operations also showed an increase in pulmonary vasodilation after being administrated 2-20 ppm of nitric oxide (Miller et al. 1994).

In adults, nitric oxide is also used as a treatment for persistent pulmonary hypertension where in one early study 40 ppm was used on patients, showing a decrease in pulmonary vascular resistance, but not in systemic vascular resistance (Pepke-Zaba et al. 1991). Likewise, similar results were seen in nitric oxide exposure to patients suffering from Adult Respiratory Disease Syndrome which included a prolonged study of 30 minute treatments of 5-20 ppm for 3-53 days that showed consistent lowering of pulmonary-arterial pressure (Rossaint et al. 1993). Dellinger
and colleagues confirmed the safety of the treatment where up to 80 ppm of gaseous nitric oxide was administered and showed similar survival rates with a methaemoglobin below 7% (Dellinger et al. 1998). While subsequent studies showed little improvement on survival rate, the oxygenation it provides makes it invaluable as a tool for oxygen compromised patients by improving ventilation to perfusion ratios (Albert et al. 2017). Inhaled nitric oxide has also been used to treat hypertension induced by various hemolytic diseases as the gas binds to free-floating haemoglobin, forming methaemoglobin and thus prevent the scavenging of nitric oxide produced naturally by endothelial cells, allowing blood vessels to vasodilate (Yu et al. 2019).

1.2.6.2 Nitric Oxide Gas as an Antimicrobial Treatment

Extensive research has shown that nitric oxide has the potential as a broad-spectrum antimicrobial treatment against viral, bacterial and fungal infections (Miller et al. 2009, Regev-Shoshani, Crowe and Miller 2013b, Regev-Shoshani et al. 2013c). Importantly, it has been shown that nitric oxide has antimicrobial properties against antibiotic resistant bacteria giving healthcare professionals an additional tool for treatment (Deppisch et al. 2016). Miller and colleagues have identified and demonstrated one of the mechanisms of bactericidal action of gaseous nitric oxide (Miller et al. 2007). As with endogenously produced nitric oxide, gaseous nitric oxide at much higher concentrations than endogenously produced nitric oxide, rapidly overwhelms the thiol (glutathione or mycothiol) defense reservoirs against oxidative/nitrosative stress of a broad spectrum of bacteria (Miller et al. 2009). This leads to electron transport complex asphyxia from nitric oxide binding to heme-containing enzymes, such as aconitase and other bactericidal damages such as DNA deamination due to oxygen radicals such as hydrogen peroxide and other nitrogen radicals (Miller et al. 2009).
One option for using gaseous nitric oxide as an antimicrobial treatment is for various respiratory diseases. In-vitro work has shown nitric oxide gas is able to eradicate bacteria associated with nosocomial pneumonia where exposures of 200 ppm reduced the bacterial load by five logs within five hours (McMullin et al. 2005). However, concerns for such long exposures in-vivo could lead to methemoglobinemia in humans. This led to Miller and colleagues to demonstrate in-vitro that intermittent exposure (30 minutes on, 3.5 hours off) at 160 ppm could also lead to bactericidal results while not decreasing sensitivity of microbes to nitric oxide (Miller et al. 2009).

Cystic fibrosis, an autosomal disorder has an established side effect of creating a more hospitable environment for pathogens such as *P. aeruginosa* and *S. aureus* to survive, multiply and encouraging the formation of biofilms (Ratjen and Döring 2003). While mucoid *P. aeruginosa* have been shown to be more resilient to antibiotic treatment it is still susceptible to nitric oxide (Yoon et al. 2006). Miller et al., demonstrated that this delivery regimen with 160 ppm inhaled nitric oxide was safe and effective in rats with pneumonia and in healthy adults (Miller et al. 2012, Miller et al. 2013). A feasibility and safety study where an intermittent dose of thirty minutes 160 ppm nitric oxide was given three times daily for two periods of five days each over two consecutive weeks showed a reduction of bacterial and fungal load and improved lung function in patients with high pulmonary bacterial loads that had chronic cystic fibrosis (Deppisch et al. 2016). Another method where nitric oxide was instead used as an anti-biofilm therapy in conjunction with antibiotics where 10 ppm was administered in eight-hour treatments per day for seven days showed a reduction in biofilm aggregates (Howlin et al. 2017).
Gaseous nitric oxide is another treatment that is currently being explored to treat non-tuberculous mycobacteria, another species that cause pulmonary infections, especially in patients with cystic fibrosis. Currently, non-tuberculous mycobacteria have intense antibiotic treatment regiments which could lead to toxicity of the host (Floto et al. 2016). Current work has shown that intermittent gaseous nitric oxide treatment is tolerable and early results have shown a decrease in non-tuberculous mycobacterial load (Miller et al. 2018, Yaacoby-Bianu et al. 2018).

Another option for gaseous nitric oxide is to use it as a topical antimicrobial. Chronic wound infections have been shown to house bacteria living in biofilms which inhibit healing (Edwards and Harding 2004). Gaseous nitric oxide has been shown to be antimicrobial at 200 ppm to various pathogens associated with chronic wound infections while not showing any cytotoxic effect to human dermal fibroblasts (Ghaffari et al. 2006). The same group would later use a rabbit wound model to show a reduction of S. aureus in the wound of the rabbit after eight hour exposure of 200 ppm nitric oxide for three days (Ghaffari et al. 2007). In a similar study, a non-healing leg ulcer was treated with 200 ppm gaseous nitric oxide in a plastic leg boot overnight for two weeks and showed significant decrease in size after treatment and was completely healed 26 weeks post-treatment (Miller et al. 2004). Nitric oxide has also been shown to increase wound healing in both infected and aseptic wounds (Shekhter et al. 2005).

1.2.6.3 Safety and Toxicity

The usage of nitric oxide is not without its risks. The National Institute for Occupational Safety and Health and the Environmental Protection Agency have given nitric oxide an inhalation
threshold limit value as a time-weighted average of 25 ppm (NIOSH 1988). The molecule is also a common air pollutant and is present in concentrations of 10-1000 ppm in cigarette smoke (Borland 1988). Nitric oxide is able to react with oxygen to form nitrogen dioxide which at uncontrolled levels can lead to chronic cases of pulmonary edema (Vassilyadi and Michel 1988). However, when carefully controlling nitrogen dioxide contamination, Stavert and Lehnert were able to expose rats to 1000 ppm nitric oxide for 30 minutes and showed no evidence of lung injury (Stavert and Lehnert 1990).

Several studies have shown the safe use of gaseous nitric oxide. For topical use, 10,000 ppm of nitric oxide was used for 30 minutes for five days in the treatment of cutaneous leishmaniasis and showed no adverse effects (Miller et al. 2011). In rabbits, 43 ppm nitric oxide and 3.6 ppm nitrogen dioxide were continuously delivered for six days without any pathological changes in the lungs (Hugod 1979). Similarly, nitric oxide was continuously delivered at 32, 64 or 128 ppm for 3 hours and 512 ppm for forty-five minutes to healthy volunteers without any adverse side effects (Young, Sear and Valvini 1996). The shorter exposure time at 512 ppm was due to methemoglobin, another toxicity issue reached 5% (Young et al. 1996). An intermittent study where healthy volunteers were exposed to 160 ppm of gaseous nitric oxide for 30 minutes every four hours, five times a day for five days showed low levels of methemoglobin (0.9%) and no changes to lung function or blood chemistry (Miller et al. 2012). It is believed that the high affinity of hemoglobin for nitric oxide make inhalation of nitric oxide relatively safe as nitric oxide binds to hemoglobin before reacting with oxygen to form nitrogen dioxide (Moncada and Higgs 1993). Methemoglobin safety and toxicity will be discussed at a later chapter.
1.2.7 Non-Gaseous Nitric Oxide Treatments

Besides gaseous nitric oxide other methods have been used to deliver nitric oxide exogenously. When the bond is cleaved, nitric oxide can be released from s-nitrosothiols. This can be done via photolysis or metal decomposition with metals such as copper or iron (Hogg 2000, Sexton et al. 1994). The rate can be increased in the presence of a reducing agent such as thiol or ascorbate (Hogg 2000). Exogenous s-nitrosothiols have been shown to have antimicrobial effects where S-nitrosoglutathione and S-nitroso-N-acetylcysteine were bactericidal to several species associated with bacterial keratitis (Cariello et al. 2012). The drawback for s-nitrosothiols is their relatively unstable forms, forcing cool, dry storage conditions (Schairer et al. 2012).

However recent studies have attempted to bridge that drawback. Li and Lee developed a polymer imbedding S-nitrosoglutathione to aid in diabetic wound healing (Li and Lee 2010). S-nitroso-N-acetylpenicillamine while not endogenously found is popular among researchers due to its increased stability relative to other s-nitrosothiols (Wo et al. 2015). When combined with a polymer, Wo and colleagues have shown S-nitroso-N-acetylpenicillamine can release nitric oxide for 28 days and can be used to remove biofilms in catheters (Wo et al. 2015). Other groups have used other methods to stabilize the molecule via polymers or nanoparticles (Singha et al. 2019, Sundaram et al. 2016).

Other nitric oxide donor drugs such as glyceryl trinitrate (nitroglycerin) and sodium nitroprusside are primarily used to lower blood pressure and to treat angina pectoris (Yu et al. 2019). Glyceryl trinitrate has also been shown to be an adjunctive theory in experimental cerebral malaria.
Glyceryl trinitrate has been explored as a possible treatment for cutaneous leishmaniasis where a patient was treated with glyceryl trinitrate patches for one week (Zeina, Banfield and al-Assad 1997). Similarly, s-nitrosothiols have been shown to kill both Leishmania major and Leishmania amazonensis (de Souza et al. 2006). Additionally, other s-nitrosothiols such as S-nitroso-acetyl-penicillamine have been shown to kill parasites such as Trypanosoma cruzi (Vespa, Cunha and Silva 1994). In conjunction with caprylic acid, glyceryl trinitrate has shown to eradicate biofilm forming bacteria and fungi (Rosenblatt, Reitzel and Raad 2015).

In-vitro studies have shown sodium nitroprusside to be bactericidal to Clostridium sporogenes at a concentration of 80 µM (Joannou et al. 1998). Likewise, the compound has been shown to be bactericidal to Bacillus subtilis and promote biofilm dispersion in P. aeruginosa (Barraud et al. 2006, Moore et al. 2004). The removal of biofilms and its ability to kill sulphate reducing bacteria has been suggested as a possibly additive along with sodium nitrate in pipeline maintenance (Fida et al. 2018).

Diazeniumdiolate, also known as NONOates can be another way to deliver nitric oxide exogenously. Initially it was shown that nitric oxide can react with certain nucleophiles to form a diolate group in the diazeniumdiolates, but later it was shown that the group can by hydrolyzed to release nitric oxide allowing it to have biomedical applications (Keefer 2011). One interesting aspect of diazeniumdiolates is that depending on the structure of the nucleophile the rate of nitric
oxide release can range from seconds to hours (Morley and Keefer 1993). This was highlighted by Shim and colleagues where different diazeniumdiolates had different antimicrobial effects against periodontal pathogens (Shim et al. 2018).

Overall, this has led to many unique ways to administrate nitric oxide to the site of infection. For example, the diazeniumdiolate PYRRO-C3D is specifically designed to release nitric oxide in the presence of β-lactamase produced by bacteria such as *Haemophilus influenzae* where biofilm formation is inhibited, allowing antibiotic treatment to be more effective (Collins et al. 2017). Using chitosan oligosaccharides to form diazeniumdiolate, the released nitric oxide was able to inhibit growth and biofilms of *P. aeruginosa*, allowing it to have possible use in inhalation therapeutics (Reighard and Schoenfisch 2015). Combining diazeniumdiolate with sol-gels to create a coating for orthopedic devices showed less microbial adhesion to the surface of the device which could be used to reduce the risk of implant infections (Nablo, Rothrock and Schoenfisch 2005). Additionally, diazeniumdiolates have been incorporated in silica based nanoparticles (Carpenter et al. 2011). Recently, a hydrogel containing diazeniumdiolate was produced and showed antimicrobial properties towards *E. coli* and increased collagen expression in human dermal fibroblasts (Durão et al. 2018).

Nitric oxide delivery using zeolites, a nanoporous non-soluble material has also been tested. In general, nitric oxide gas is bound to metal ions in the zeolite and is released when it comes into contact with an aqueous solution (Wheatley et al. 2006). The rate of release can be changed by using different chemical compositions of zeolites and by using polymers to change the contact with water (Wheatley et al. 2006). Nitric oxide bound zeolites have antimicrobial efficacy among
pathogens common in wounds while showing low cytotoxicity among fibroblasts (Neidrauer et al. 2014).

Other methods of nitric oxide delivery include a probiotic patch containing nitrite salts has been developed where it utilizes Lactobacillus fermentum’s metabolism of glucose to lactic acid to form nitric oxide (Jones et al. 2010a). Subsequent in-vitro studies showed its antimicrobial properties against a variety of bacterial and fungal pathogens over an application period of 4-8 hours (Jones et al. 2010a). Using the rabbit wound model the patch has been shown to increase the rate of wound closure (Jones et al. 2012). Additionally, a photodynamic hydrogel containing a nitric oxide donor was developed that showed antimicrobial activity when exposed to light, allowing control over nitric oxide delivery (Halpenny et al. 2009).

1.2.8 Acidified Nitrites

Another way to produce nitric oxide is through the chemical reaction using acidified nitrites. Briefly, nitrite and hydrogen ions form the unstable nitrous acid which then decomposes into water and dinitrogen trioxide which further decomposes into nitric oxide and nitrogen dioxide as seen below.

\[
\begin{align*}
\text{NO}_2^- + \text{H}^+ & \rightleftharpoons \text{HNO}_2 \\
2\text{HNO}_2 & \rightleftharpoons \text{H}_2\text{O} + \text{N}_2\text{O}_3 \\
\text{N}_2\text{O}_3 & \rightleftharpoons \text{NO} + \text{NO}_2
\end{align*}
\]

Additionally, nitrous acid may decompose straight to nitric oxide and nitrate as seen below.

\[
\begin{align*}
\text{NO}_2^- + \text{H}^+ & \rightleftharpoons \text{HNO}_2 \\
3\text{HNO}_2 & \rightleftharpoons 2\text{NO} + \text{NO}_3^- + \text{H}^+ + \text{H}_2\text{O}
\end{align*}
\]
The rate of nitric oxide production can therefore be controlled by the concentration of nitrites and the pH of the solution. This allows for easy adjustment of nitric oxide production and can be more specifically tailored to combat certain microbes with more robust nitric oxide defenses (Weller et al. 2001). Acidified nitrites can be made using various sources of nitrites such as potassium nitrite and sodium nitrite (Ormerod et al. 1999, Weller et al. 1998) while acids such as citric acid, ascorbic acid, lactic acid and salicylic acid have been used to drop the pH to activate the reaction (Davidson et al. 2000, Mühlig et al. 2014, Phillips et al. 2004, Weller et al. 1998).

In-vitro, using stomach acid as its acid source acidified nitrite has shown to have antimicrobial effects against *Helicobacter pylori*, one of the most common pathogens in the world (Dykhuizen et al. 1998).

Acidified nitrite creams have been used as topical antimicrobial where a nitrite cream and an acid cream are mixed together during application. A clinical trial using the cream as a treatment for tinea pedis was conducted and showed reduced infection and clinical symptom scores (Weller et al. 1998). A similar trial for the viral skin infection molluscum contagiosum was performed which saw a 75% cure rate in two months vs. 21% in the control (Ormerod et al. 1999). Acidified nitrite creams have been shown to be a possible treatment for Buruli ulcers caused by *Mycobacterium ulcerans* (Phillips et al. 2004), anogenital warts (Ormerod et al. 2015) and MRSA infected wounds (Ormerod et al. 2011).

Another form acidified nitrites are used in is through an aqueous solution. This has led to nitric oxide to be used as a disinfectant. In one study, acidified nitrites were tested against other
disinfectants as rapidly acting agents against *Clostridium difficile* spores in a hospital environment and showed a three-log reduction within 15 minutes (Wullt, Odenholt and Walder 2003). Additionally, acidified sodium nitrite in conjunction with hydrogen peroxide was used as a disinfectant for contact lens (Heaselgrave, Andrew and Kilvington 2010).

Using acidified nitrates, our group has evaluated a nitric oxide releasing solution (NORS), made from sodium nitrite and citric acid which releases nitric oxide in a concentration-controlled manner (Regev-Shoshani et al. 2013b). Previously, nitric oxide produced from NORS has shown to have anti-fungal properties against *Trichophyton rubrum* and *Trichophyton mentagrophytes* which can potentially be used as a footbath treatment (Regev-Shoshani et al. 2013b).

Among treatment for bovine diseases, nitric oxide has been used as an alternative to metaphylactic antibiotics use in bovine respiratory disease complex, a disease caused by a viral infection and an opportunistic and subsequent bacterial infection. A spray device containing nitric oxide release solution was sprayed nasally on young calves entering the feedlot and showed a decrease rate of symptoms of bovine respiratory disease complex vs. a control group (Regev-Shoshani et al. 2013a). In a later study, NORS has been shown to be safe to administer to the respiratory tract of cattle and that it had in-vitro antimicrobial effects against viruses and bacteria associated with bovine disease complex (Regev-Shoshani et al. 2014). When compared with an antibiotic (tilmicosin) treatment regiment, NORS was shown not to be inferior in the metaphylactic treatment of low to moderate risk beef cattle for bovine respiratory disease complex (Regev-Shoshani et al. 2017).
1.3 Hypothesis

Similarly, to bovine respiratory disease complex, we hypothesize that nitric oxide, specifically nitric oxide releasing solution can have the potential use as an alternative method to antibiotic treatment. This potential treatment is derived from the knowledge that nitric oxide is a potent antimicrobial agent (Miller et al. 2009). This novel way of treatment will be explored in several ways. First, we will explore the feasibility of creating a nitric oxide releasing solution that is compatible with the conditions seen in bovine mammary glands and whether it retains its antimicrobial properties. We further explored the antimicrobial effects of NORS in ex-vivo bacterial load in infected milk samples. Finally, a preliminary pilot phase I safety study was performed in healthy, lactating dairy cows to show whether or not it can be safely administered.
2. Determining the Choice of NORS Formulation and pH for Use with Milk

2.1 Purpose

One characteristic of NORS is the pH for the drug needs to be acidic for it to be activated. In an environment involving milk, one of the implications is the milk may curdle once it comes into contact with NORS. This could lead to blockage in the ducts which can not only aggravate symptoms of the ailing animal and potentially increase the withdrawal period, but it could also produce a barrier that would prevent nitric oxide from reaching the site of infection. It is also possible that clots may promote the odds of the infected quarter from going blind (no production of milk), leading to more economic impacts and uncertainty to food security (Sarba and Tola 2017).

The objective of this experiment is to create a formulation of NORS that won’t cause curdling of milk, yet remain active to produce sufficient nitric oxide.

2.2 Methods

*Milk Collection*

Animal use protocols were reviewed and approved by the University of British Columbia Animal Care Committee. Milk samples were collected from Holstein cows at the University of British Columbia’s Dairy Education and Research Centre in Agassiz, British Columbia, Canada. The cattle were milked twice daily in a Double 12 Boumatic parallel milking parlor and were group-housed in a free-stall cubicle that allowed 24-hour access to a ration of grass silage and concentrates that provided a net energy lactation of 39.5 Mcal.
500 mL of raw milk was collected aseptically, from a mix of 3 healthy cows (confirmed by no microbes in milk samples and somatic cell count lower than 100,000). The milk was transported to the laboratory on ice, within 3 hours of collection, then mixed and aliquoted into 50 mL tubes, and stored at -20°C.

NORS Preparation

Nitric oxide releasing solution was prepared as previously described (Regev-Shoshani et al. 2013b, Stenzler 2015). In brief, sodium nitrite (Sigma-Aldrich, Missouri, United States) was added to N-saline (Baxter, Illinois, United States) and was acidified using citric acid (Sigma-Aldrich, Missouri, United States). Three concentrations of nitrite (100 mM, 200 mM and 400 mM) at various pH levels were prepared. Once the solution was made, it was used within 1 hour.

Milk Curdle Test

NORS was mixed with milk in a 1:1 (600 μL to 600 μL), 2:1 (800 μL to 400 μL), 5:1 (1000 μL to 200 μL) and 10:1 (1000 μL to 100 μL) NORS to milk ratios in a 1.5 mL tube. After 5 minutes signs of curdling were ascertained by visually observing the tube. Additionally, the contents were dumped on a kitchen sieve to confirm no curdling.

Measuring Nitric Oxide

Nitric oxide was measured using the chemiluminescence nitric oxide analyzer (NOA, model 280i, GE Analytical Instruments, Boulder, Colorado, US). NORS of concentration 400 mM and pH 3.9 was added to milk at ratios of 1:1 and 2:1 NORS to milk, creating a final NORS concentration in milk of 200 mM and 267 mM respectively. Five milliliters of each solution were
injected with a syringe into a specialized glass device containing an input where medical grade nitrogen gas (Praxair, Connecticut, US) flowed at 1 L/min to the output which was attached to the chemiluminescence. Nitric oxide production was measured for 30 minutes.

2.3 Results

The curdling of milk occurred at initial NORS pH injections ranging from pH 3.50 to 3.80. In general, the results followed the pattern where the most susceptible to curdling samples were found in ratios with the least amount of milk and in higher nitrite concentrations (Table 3).

Nitric oxide was successfully measured using the chemiluminescence nitric oxide analyzer (Fig. 1). After 30 minutes the mixture was still producing 1.0 ppm and 3.0 ppm in the 1:1 and 2:1 NORS to milk ratios respectively.

Table 3: Initial NORS pH of when curdling of milk was observed. Three different NORS concentrations at four different NORS to milk ratios were used.

<table>
<thead>
<tr>
<th>NORS Concentration</th>
<th>Ratio (NORS:Milk)</th>
<th>10:1</th>
<th>5:1</th>
<th>2:1</th>
<th>1:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM</td>
<td></td>
<td>3.80</td>
<td>3.75</td>
<td>3.60</td>
<td>3.50</td>
</tr>
<tr>
<td>200 mM</td>
<td></td>
<td>3.80</td>
<td>3.75</td>
<td>3.70</td>
<td>3.60</td>
</tr>
<tr>
<td>400 mM</td>
<td></td>
<td>3.80</td>
<td>3.75</td>
<td>3.70</td>
<td>3.65</td>
</tr>
</tbody>
</table>
Fig. 1: Nitric oxide production of NORS mixed with milk. Five millilitres of 400 mM NORS at pH 3.9 mixed with milk in a) 1:1 and b) 2:1 NORS to milk ratios was injected into the glass device and nitric oxide was measured using the chemiluminescence nitric oxide analyzer for 30 minutes.

2.4 Discussion

Nitric oxide can be detected with acidified nitrites at a pH as high as 6, though the lower the pH the more nitric oxide is produced (Carlsson et al. 2001). The identification of the proper pH level which releases sufficient nitric oxide and avoiding curdling of milking is very important in the development of the drug to treat bovine mastitis.

In bovines, the protein content of milk is 3.4% with 82% of them belonging to a class of proteins known as caseins (Fox and McSweeney 1998). The caseins proteins form a large micelles structure which scatter light, giving milk its well-known white colour (Fox and McSweeney 1998). These structures are normally held together through hydrophobic interactions and have a repulsive barrier to prevent aggregation (Ausar et al. 2001). At pH 4.6 casein reaches its isoelectric point and begins to precipitate in solution due to its lost of its net negative charge (Fox and McSweeney 1998). The breakdown of the hydrophobic interactions allows casein to aggregate through Van der Waals interactions, giving a curdled appearance (Ausar et al. 2001).
To address this issue, we used a high nitrite concentration and a relative higher pH, which resulted in NORS releasing sufficient nitric oxide in milk while at the same time not causing the milk to curdle. No curdling was observed in NORS infusions with a pH greater than 3.8. As lactation is expected to continue after infusion, the pH of the NORS-milk combination is expected to increase back towards the normal pH of milk of around 6.5-6.7 (Anema and Li 2003) as the drug becomes diluted.

The NORS to milk ratios were decided in collaboration with farm managers. After machine milking and hand stripping, it is estimated that between 20 mL to 40 mL remain in each quarter. With a planned infusion of 40 mL NORS, a 2:1 and 1:1 NORS to milk ratio would be achieved. For further studies, a concentration of 400 mM and pH 3.9 will be investigated. This formulation of NORS provides similar nitric oxide release to previous work, allowing this formulation to achieve antimicrobial effects within minutes while at the same time it creates a bit of a buffer to minimize the risk of casein aggregation.
3. Determining Antimicrobial Properties of NORS in Milk

3.1 Purpose
When discussing the potential use of a drug for treatment it is important to take into account the environment in which the drug will be present in. Several times, a potential treatment for bovine mastitis looked promising only for it to be ineffective when tested in milk (Fang and Pyörälä 1996, Louhi et al. 1992, Owens and Watts 1987). As the presence of milk exists in the mammary gland of lactating cattle it is necessary to determine its role in therapeutic treatments.

While the antimicrobial properties of nitric oxide releasing solution has been discussed before (Regev-Shoshani et al. 2013b, Regev-Shoshani et al. 2014) whether that property remains in the presence of milk has yet to be explored. In this chapter, we will investigate whether NORS retained its antimicrobial property by treating seeded bacteria in milk (in-vitro) and milk from bovines suffering from mastitis (ex-vivo) with the NORS formulation developed in the previous chapter.

3.2 Methods
*Milk Collection*
Animal use protocols were reviewed and approved by the University of British Columbia Animal Care Committee. Milk samples were collected from Holstein cows at the University of British Columbia’s Dairy Education and Research Centre in Agassiz, British Columbia, Canada. The cattle were milked twice daily in a Double 12 Boumatic parallel milking parlor and were group-housed in a free-stall cubicle that allowed 24-hour access to a ration of grass silage and concentrates that provided a net energy lactation of 39.5 Mcal.
For the in-vitro study, a total of 200 mL of raw milk was collected aseptically, from a mix of three healthy cows (confirmed by no microbes in milk samples and somatic cell count lower than 100,000). The milk was transported to the laboratory on ice, within 3 hours of collection, then mixed and aliquoted into 15 mL tubes, and stored at -20°C.

For the ex-vivo study, ten cows presenting with signs of clinical mastitis (rectal temperature, appearance and clots in milk), and confirmed independently by high somatic cell counts (> 100,000) and presence of bacteria in milk, were identified. Milk samples (~20 mL) were manually collected aseptically in 50 mL tubes from these cows. The samples were transported to the laboratory on ice, within 3 hours of collection and then stored at -20°C.

**Bacterial Samples used for in-vitro testing**

*S. aureus* Newbould 305 was obtained from Dr. Bonnie Mallard at the Ontario Veterinary College, Guelph, Ontario. *E. coli* was isolated from a clinical case of bovine mastitis in our lab at the Jack Bell Research Centre, Vancouver, British Columbia.

**Bacteria Identification for ex-vivo testing**

The infected milk samples were thawed to room temperature (20–25 °C) and 100 μL from each was plated at 10-fold dilutions onto brain heart infusion agar plates, and placed into a 37 °C incubator overnight. Samples had to contain a bacterial concentration of greater than 100 cfu/mL to be included in this study.
Bacteria were identified by first performing a gram staining test (VWR, Pennsylvania, United States) and then grown on selective media. Gram positive, cocci shaped bacteria were grown on selective strep agar (Hardy Diagnostics, California, United States) to identify *Streptococcus* sp. and mannitol salt agar (Sigma-Aldrich, Missouri, United States) to identify *S. aureus*. *S. aureus* was further confirmed via catalase (hydrogen peroxide, Sigma-Aldrich, Missouri, United States) and coagulation (rabbit with EDTA Coagulase Plasma, Becton Dickinson, New Jersey, United States) tests.

Gram negative, bacilli shaped bacteria were first grown on MacConkey agar (Sigma-Aldrich, Missouri, United States) to determine whether they could ferment lactose. Lactose positive samples were then grown on Levin EMB agar (Becton Dickinson, New Jersey, United States) to identify *E. coli*.

**NORS Preparation**

Nitric oxide releasing solution was prepared as previously described (Regev-Shoshani et al. 2013b, Stenzler 2015). In brief, sodium nitrite (Sigma-Aldrich, Missouri, United States) was added to N-saline (Baxter, Illinois, United States) to create a concentration of 400 mM and was acidified using citric acid (Sigma-Aldrich, Missouri, United States) to reach a pH of 3.9. Once the solution was made, it was used within 1 hour.

**In-vitro Testing**

*S. aureus* Newbould 305 and *E. coli* were removed from storage in the -80°C freezer, defrosted and were grown in brain heart infusion (Becton Dickinson, New Jersey, United States) broth
overnight in a 37°C incubator shaker. Using a spectrophotometer (Optizen, Mecasys, South Korea), the concentration of bacteria was estimated by using a standard curve to correlate bacterial colony forming count with absorbance at OD\textsubscript{600}. The solution was adjusted to 10\textsuperscript{8} cfu/mL using brain heart infusion broth. Milk samples were defrosted at room temperature (20-25°C). For each data point, 10 μL of the bacterial solution was added to 990 μL of milk to create a final estimated bacteria concentration of 10\textsuperscript{6} cfu/mL.

400 mM NORS at pH 3.9 was added to bacteria-seeded milk at ratios of 1:1 (500 μL to 500 μL) and 2:1 (666 μL to 333 μL) NORS to milk. This created a final NORS concentration in milk of 200 mM and 267 mM respectively. Three exposure periods were used for each bacterial isolate and the two NORS to milk ratios ranging from 2 to 30 minutes. A control with saline was used with exposures of 10 and 30 minutes used for the 2:1 and 1:1 ratios respectively.

Following exposure, samples were diluted with phosphate buffered saline (Sigma-Aldrich, Missouri, US), plated on brain heart infusion agar plates, and incubated overnight at 37 °C. On the next day, the number of colony forming units growing on the plate were counted and the concentration of bacteria calculated. All experiments were done in triplicate and repeated three times (n=3).

*Ex-vivo Testing*

Infected milk samples were retrieved from storage and defrosted at room temperature (20-25°C). NORS of concentration 400 mM and pH 3.9 was added to the milk samples at ratios of 1:1 (500 μL to 500 μL) and 2:1 (666 μL to 333 μL) NORS to milk. This created a final NORS
concentration in milk of 200 mM and 267 mM respectively. Three exposure periods were used for the two NORS to milk ratios ranging from 2 to 30 minutes. A control with saline was used with exposures of 10 and 30 minutes used for the 2:1 and 1:1 ratios respectively.

Following exposure, samples were diluted with phosphate buffered saline, plated on brain heart infusion agar plates, and incubated overnight at 37 °C. On the next day, the number of colony forming units growing on the plate were counted and the concentration of bacteria calculated. Both in-vitro and ex-vivo experiments were done in triplicate and repeated three times (n=3).

Statistics
Statistics for the change in bacterial concentration (log$_{10}$ cfu/ml) was analyzed using GraphPad Prism 6 where a separate one-sample, paired t-tests at each time-point was performed. A p-value $< 0.05$ was considered significant.

3.3 Results
Raw milk samples, from three healthy cows, were inoculated with $E. coli$ or $S. aureus$ bacteria and subsequently treated with 400 mM NORS at two different volume ratios of NORS to milk for increasing periods of time. The 1:1 volume ratio treatment significantly reduced the concentration of both $E. coli$ ($p < 0.05$) and $S. aureus$ ($p < 0.01$) after 10 minutes (Fig. 2a). Following 20 minutes ($S. aureus$) and 30 minutes ($E. coli$) of NORS treatment there was no detectable growth of the bacteria in the milk samples.
In the samples treated with a 2:1 volume ratio of NORS to milk, a more rapid rate of bacterial clearance was achieved with significant reduction in the concentration of both *E. coli* (p < 0.05) and *S. aureus* (p < 0.01) was detected after 2 minutes of exposure time. No detectable growth of both bacteria occurred after 5 minutes (Fig. 2b).

**Fig. 2:** Bacterial concentration (log$_{10}$ [cfu/mL]) of *E. coli* and *S. aureus*, in inoculated milk at four intervals after the addition of 400 mM NORS at pH 3.9 at a) 1:1 and b) 2:1 NORS:milk ratios n=3. (*p < 0.05, **p < 0.01). NG = No Growth

The bacteria present in milk samples, collected from the ten cows presenting with signs of clinical mastitis, contained a concentration range of 100 to 40,000 cfu/mL bacteria and are described in Table 4. In general, *E. coli*, *S. aureus* and *Streptococcus* spp. were isolated. The milk samples were treated with NORS. A 1:1 volume ratio of 400 mM NORS to milk caused a significant decrease in bacterial concentration (p < 0.01) after 10 minutes (Fig. 3a). Following 30 minutes of NORS treatment there was no detectable growth of bacteria (cfu/mL) in the milk samples. In the samples treated with a 2:1 volume ratio of 400 mM NORS to milk a significant decrease in bacterial concentration (p < 0.001) was detected after 2 minutes (Fig. 3b). Following 5 minutes of NORS treatment there was no detectable growth of bacteria in the milk samples.
**Table 4:** Rate of bacterial elimination in ten cows presenting with clinical mastitis, associated with Gram-positive or Gram-negative organisms, following in-vitro treatment with 400 mM NORS.

<table>
<thead>
<tr>
<th>Cow ID</th>
<th>Primary bacterial isolate</th>
<th>Time for elimination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NORS 1:1</td>
</tr>
<tr>
<td>95</td>
<td><em>E. coli</em></td>
<td>30 min</td>
</tr>
<tr>
<td>132</td>
<td><em>E. coli</em></td>
<td>10 min</td>
</tr>
<tr>
<td>1116</td>
<td><em>S. aureus</em></td>
<td>10 min</td>
</tr>
<tr>
<td>2002</td>
<td><em>S. aureus</em></td>
<td>20 min</td>
</tr>
<tr>
<td>2045</td>
<td><em>Streptococcus</em> sp.</td>
<td>30 min</td>
</tr>
<tr>
<td>2282</td>
<td><em>Streptococcus</em> sp.</td>
<td>30 min</td>
</tr>
<tr>
<td>3068</td>
<td><em>S. aureus</em></td>
<td>10 min</td>
</tr>
<tr>
<td>3103</td>
<td><em>E. coli</em></td>
<td>20 min</td>
</tr>
<tr>
<td>3129</td>
<td><em>S. aureus</em></td>
<td>20 min</td>
</tr>
<tr>
<td>7041</td>
<td><em>E. coli</em></td>
<td>30 min</td>
</tr>
</tbody>
</table>

**Fig. 3:** Bacterial concentration (log$_{10}$(cfu/mL)) in milk samples obtained from bovines with signs of clinical mastitis at four intervals after the addition of 400 mM NORS at pH 3.9 at a) 1:1 and b) 2:1 NORS:milk ratios. n=3.; (**p < 0.01, ***p < 0.001). NG = No Growth
3.4 Discussion

There are several reasons as to why milk deactivates the antimicrobial properties of a treatment. This can range from milk proteins, lipids, pH, and ionic characteristics (Ziv 1980b). For example, tetracycline performs relatively poorly in milk due to its irreversible binding with casein and other large proteins in milk (Kuang et al. 2009). Similarly, bacteriophage K showed reduced performance due to whey proteins binding and bacterial aggregation (Gill et al. 2006, O'Flaherty et al. 2005). The wide range of pH in milk from animals with bovine mastitis can affect the dissociation constant of the drug causing a reduction of bioavailability, particularly in drugs administered systemically (Gruet et al. 2001). In these experiments, this formulation of the nitric oxide releasing solution (NORS) was able to maintain its antimicrobial properties by reducing bacterial concentration to below detectable levels.

In review, nitric oxide can react with other molecules such as reactive oxygen species and sulphydryl groups, which can cause the formation of S-nitrosothiols. This can alter the function of bacterial proteins and cause cell stasis or death (Vallance and Charles 1998). Additionally, nitric oxide may also interfere with DNA repair mechanisms, by denaturing metabolic proteins through nitrosylating heme- or thiol groups (Wink and Mitchell 1998) and it can react with hydrogen peroxide and other oxygen radicals to create cytotoxic substances such as peroxynitrite (Beckman and Koppenol 1996). Microorganisms, such as E. coli are able to combat stress caused by nitric oxide by containing intracellular globins and reductases which can detoxify nitric oxide and its intermediates (Poole 2005). However, therapeutic nitric oxide concentrations are able to overwhelm the microbial thiol (i.e. gluathione or mycothiol) detoxification mechanism (Miller et al. 2007). The relatively stronger detoxification mechanism of E. coli could explain the longer
time needed for its complete eradication, compared to the time needed to eradicate *S. aureus* (Fig. 2).

A gram-positive (*S. aureus*) and a gram-negative (*E. coli*) bacteria were chosen for the in-vitro testing as they are commonly found in bovine mastitis (Bradley 2002), the latter of which was isolated in our lab from a bovine milk sample. The Newbould 305 strain used is a well researched strain that causes both mild and chronic bovine mastitis (Peton et al. 2014, Tartaglia et al. 2018).

It is also important to test NORS in milk from animals suffering from bovine mastitis as there is an elevated concentration of phagocytes, lysozymes, lactoferrin and immunoglobulins which may have altered the results due to possible interactions with nitric oxide (Fang and Pyörälä 1996). Additionally, microbes can display different phenotypes in milk. For example, *S. aureus* can have a polysaccharide capsule when growing in milk, but not when grown in general culture media broth (Opdebeeck, O'Boyle and Frost 1988). Based on the results of these ex-vivo samples there is no observable difference in the antimicrobial property of NORS relative to the in-vitro testing.

Given the role of nitric oxide in the mammalian immune system the results were not unexpected. Nitric oxide plays both a signalling and a cytotoxic role in the immune system (Vallance and Charles 1998). One study showed, inducible nitric oxide synthase can be activated in milk and when exposed to toxins, nitric oxide, through its products can be measured (Bouchard et al. 1999). Our study confirms that an acidified nitrite solution, delivered in this formulation,
provided sufficient nitric oxide to achieve antimicrobial effects in both in-vitro and ex-vivo simulations, which then allowed the rationale to further investigate its potential use in an in-vivo mastitis model.
4. Initial Phase I Safety Study in Bovines

4.1 Purpose

In the previous chapter, the feasibility was demonstrated for using NORS as a potential treatment for bovine mastitis. With these data in hand, moving from a pre-clinical stage to the beginnings of a phase I clinical trial was justified. In order to assure the safety of drugs, Health Canada (Veterinary Drug Directorate) and other regulatory organizations have created a list of objectives which drugs must meet before approval and release to the public. A phase I stage focuses on the safety and pharmacology of the compound, typically on healthy individuals (Lipsky and Sharp 2001).

Establishing the safety of the drug on animals is a very important for the approval process. Pertaining to NORS, one major aspect that was monitored was the effect on methemoglobin. Increased concentration of methemoglobin is a well-known side effect of nitric oxide exposure where the molecule binds to hemoglobin in the blood, causing an oxidation reaction of the iron molecule which at high enough levels could lead to the lack of oxygen being delivered to organs otherwise known as methemoglobinemia (Salguero and Cummings 2002). In the cattle industry this is better known as nitrate poisoning due to the potential presence of nitrate in water and feed which when converted to nitrites can also cause methemoglobinemia (Jainudeen, Hansel and Davison 1964, Ridder and Oehme 1974).

Additionally, an animal well-being assessment was performed to confirm no changes in health along with measuring changes in somatic cell counts. With one of nitric oxide’s role as a chemoattractant it was possible that somatic cell counts could temporarily increase as it would
during an infection (Burvenich et al. 2003, Zeiher et al. 1995). The length of time in which residues of NORS, specifically nitrite remains in the animal was also to be investigated.

In general, a dose escalation study was executed on a small group of healthy animals and the response was measured during and after the treatment interventions. Particularly, as it pertains towards methemoglobin, nitrite concentration in milk and blood and changes in somatic cell counts.

4.2 Methods

Study Site
Animal use protocols were reviewed and approved by the University of British Columbia Animal Care Committee. The study was conducted at the University of British Columbia’s Dairy Education and Research Centre in Agassiz, British Columbia, Canada. This milking herd contained Holstein cows aged 36-42 months and were in the second lactation stage. The cattle were milked twice daily in a Double 12 Boumatic parallel milking parlor, giving on average 25 L/day and were group-housed in a free-stall cubicle that allowed 24-hour access to a ration of grass silage and concentrates that provided a net energy lactation of 39.5 Mcal.

Throughout the study period, the health and well-being of the cows were assessed by a trained individual, once a day, in the afternoon. The well-being assessment criteria included the presence or absence of clots in milk, color of milk, rectal temperature, general appearance (i.e., visual evaluation of alertness/attentiveness and depression, or signs of discomfort and pain), hydration
status (i.e., assessment of skin elasticity in the neck region) and rumen fill (i.e. visual assessment and palpation of the rumen in the left flank of the cow).

**NORS Preparation**

Nitric oxide releasing solution was prepared as previously described (Regev-Shoshani et al. 2013b, Stenzler 2015). In brief, sodium nitrite (Sigma-Aldrich, Missouri, United States) was added to N-saline (Baxter, Illinois, United States) and was acidified using citric acid (Sigma-Aldrich, Missouri, United States) to reach a pH of 3.9. Three concentrations of nitrite (100 mM, 200 mM and 400 mM) were prepared. Once the solution was made, it was used within 1 hour.

**Metabolic Study**

A dose escalating study was performed. Three healthy bovines were selected to be part of the study where one computer randomized quarter was selected to be the treatment quarter. On the first day, after the morning milking the selected quarter was hand-stripped of residual milk and infused with 40 mL of saline at pH 3.9. Over the next 24 hours blood and milk samples were taken as described below. Afterwards, a 24 hour wash out period was observed where normal milking was performed without interference. In total, this was a 48 hour experimental protocol cycle. An escalating dose of 40 mL of NORS (100 mM then 200 mM then 400 mM, all at pH 3.9) was infused into the same quarter over each treatment cycle for a total of four cycles.

**Blood Samples**

Heparinised blood was obtained from the cocygeal vein at 5, 30, and 480 minute intervals following infusion. Within 30 minutes of collection, each blood sample was divided into two 1.5
mL tubes. The first tube was used to estimate methemoglobin percentage (MetHb(\%)) using a CO-Oximeter Analyzer (ABL80 Flex, Radiometer, Copenhagen, Denmark). The second tube was used for estimating nitrite concentration using chemiluminescence nitric oxide analyzer (NOA, model 280i, GE Analytical Instruments, Boulder, Colorado, US). The plasma from the second tube was harvested after centrifugation at 8600 x g in 4 °C for 1 minute and protein was removed by adding ethanol in a 3:2 ratio. After centrifugation at 15,200 x g in 4 °C for 1 min, the supernatant was stored at −20 °C for later nitrite analysis.

*Milk Samples*

Milk samples were obtained before the evening's milking (10 hours after infusion) where 20 mL milk was obtained aseptically from each of the four quarters. The cows were then milked as usual through the parlour. Subsequently, an additional sample from the treated quarter was taken aseptically. Additionally, a sample from the entire milk pool of the animal was collected. At 24 hours post-infusion a final sample was taken from the treated quarter, just prior to regular milking.

The milk samples were divided into 1.5 mL tubes, protein was removed by adding ethanol in a 3:2 ratio, and tubes were centrifuged at 15,200 x g in 4 °C for 1 min. The supernatant was collected and stored at −20 °C for later nitrite analysis using the chemiluminescence nitric oxide analyzer.
Nitrite Analysis via Chemiluminescence

Nitrite estimations in blood and milk samples were carried out on thawed samples using a chemiluminescence nitric oxide analyzer following the manufacture's protocol with slight modifications. 5 mL of 1% w/v potassium iodine in glacial acetic acid was placed into a purge vessel with a NaOH bubbler. 100 μL of each sample was injected into it, converting nitrite into nitric oxide. The nitric oxide gas was carried into the nitric oxide analyzer using medical grade nitrogen gas (Praxair, Connecticut, US) and the chemiluminescence was detected following the reaction with ozone. Results were compared to a previously established standard curve of known concentrations in blood and milk. Accuracy and linearity testing had been done previously to validate the method.

Changes in Somatic Cell Counts

A dose escalating study was performed. Three different healthy bovines were selected to be part of the study where one computer randomized quarter was selected to be the treatment quarter. Before the morning milking, a baseline sample was obtained by hand milking into a collection tube. After milking, 40 mL of 100 mM NORS at pH 3.9 was infused into the treatment quarter. Milk samples were obtained in a collection tube 14, 24, 38, 48 and 168 hours after infusion, prior to routine milking. The protocol was repeated, using the same quarter for 200 mM and 400 mM infusions. Milk samples were analysed for fat, protein, and lactose concentrations (w/w) and somatic cell counts at Pacific Milk Analysis Laboratory (Chilliwack, British Columbia, Canada).
Statistics

All data was entered and analyzed using GraphPad Prism 6. Changes in MetHb(%) in blood, and concentration of nitrite in blood and milk were tested with separate one-sample, paired t-tests at each time-point. A p-value < 0.05 was considered significant. The paired differences were calculated by subtracting the value at each testing time/concentration from the baseline value for each animal.

4.3 Results

The animals tolerated the treatments well during and after the treatment. No abnormal findings were observed with regard to the color of milk, clots or general milk appearance. Animals were hydrated, visual assessment of rumen fill appeared normal to a trained observer and no changes in demeanour were observed. An average rectal temperature of the animals over the study was 38.6°C with a range of 38.2-39.2°C, which was similar to baseline rectal temperatures. No adverse events were observed during the study.

Forty mL of NORS, with increasing concentrations (100, 200 and 400 mM), was administered to three healthy cows by intra-mammary infusion. Methemoglobin percentage was measured in blood samples taken at 5, 30, and 480 minutes post-treatment. Methemoglobin percentage did not change significantly from baseline following the 100 nor 200 mM NORS treatments (Fig. 4). Conversely, the 400 mM NORS treatment caused a significant increase of methemoglobin percentage 5 minutes (p < 0.05) and 30 minutes (p < 0.01) post-treatment (Fig. 4). The highest methemoglobin percentage level detected was 4.5%. Interestingly, in the 480 minute blood
samples, post 200 mM and 400 mM NORS treatment, methemoglobin percentage values not only dropped but were found to be significantly lower (p < 0.05) than baseline (Fig. 4).

**Fig. 4:** Rate of change in peripheral blood Methaemoglobin percentage of three healthy milking bovines following 40 mL infusion of: 100 mM, 200 mM, and 400 mM NORS solution in the udder. n=3. (*p < 0.05, **p < 0.01).

**Fig. 5:** Rate of change in mean plasma nitrite concentrations of three healthy milking bovines following 40 mL infusion of: 100 mM, 200 mM, and 400 mM NORS solution in the udder. n=3. (*p < 0.05, **p < 0.01).
Blood samples were taken at 5, 30 and 480 minutes post-treatment. A significant increase in plasma nitrite concentration was detected at 5 minutes post-treatment for the 200 mM (p < 0.01) and 400 mM (p < 0.05) treatment concentrations (Fig. 5). At 30 minutes post-treatment, a significant increase in blood nitrite concentration was detected in all treatment concentrations (100 and 200 mM, p < 0.05; 400 mM, p < 0.01). All samples of blood nitrite concentration returned back to baseline levels in the samples taken 480 minutes post-treatment.

Milk samples were collected 10 and 24 hours post NORS treatment. The treated quarter showed an increase of milk nitrite concentration from the sample obtained 10 hours post infusion, but prior to evening milking in each of the three cows, for all treatment concentrations, while the non-treated quarters showed no significant increase (Table 5). In some instances, nitrite concentration remained elevated post evening milking. The bulk milk (including all quarters), machine milked 10 hours post-treatment, generally showed no significant increase in milk nitrite concentration. Unexpectedly, one of the three animals after the 100 mM treatment had a substantially higher nitrite concentration in the bulk milk (735 nM), compared to the measurements for the same animal post 200 and 400 mM (89 nM and 195 nM, respectively). Within 24 hours of treatment, nitrite concentrations were of a magnitude similar to baseline concentrations.
Table 5: Milk nitrite concentration (nM) after 40 mL injection of 100 mM, 200 mM, and 400 mM NORS solution in the healthy udder. Baseline was measured before experiments began.

<table>
<thead>
<tr>
<th>Cow ID</th>
<th>Treatment Concentration (mM)</th>
<th>Baseline</th>
<th>10 h after treatment with NORS</th>
<th>24 h after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Before Milking (Treated Quarter)</td>
<td>Before Milking (Non-treated Quarters)</td>
</tr>
<tr>
<td>1064</td>
<td>100</td>
<td>147</td>
<td>38,634</td>
<td>235</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td></td>
<td>547</td>
<td>311</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td></td>
<td>527</td>
<td>195</td>
</tr>
<tr>
<td>1108</td>
<td>100</td>
<td>163</td>
<td>2668</td>
<td>196</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td></td>
<td>1215</td>
<td>191</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td></td>
<td>309</td>
<td>140</td>
</tr>
<tr>
<td>1111</td>
<td>100</td>
<td>183</td>
<td>2306</td>
<td>197</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td></td>
<td>5891</td>
<td>155</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td></td>
<td>8088</td>
<td>157</td>
</tr>
</tbody>
</table>
Table 6: Somatic cell count (×1000) in the treated quarter, after 100 mM, 200 mM, and 400 mM treatments.

<table>
<thead>
<tr>
<th>Cow ID</th>
<th>Treatment concentration (mM)</th>
<th>0</th>
<th>14</th>
<th>24</th>
<th>38</th>
<th>48</th>
<th>168</th>
</tr>
</thead>
<tbody>
<tr>
<td>4072</td>
<td>100</td>
<td>22</td>
<td>21</td>
<td>99</td>
<td>23</td>
<td>27</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>55</td>
<td>218</td>
<td>305</td>
<td>78</td>
<td>79</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>21</td>
<td>165</td>
<td>86</td>
<td>82</td>
<td>56</td>
<td>30</td>
</tr>
<tr>
<td>4093</td>
<td>100</td>
<td>21</td>
<td>9</td>
<td>28</td>
<td>15</td>
<td>39</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>63</td>
<td>32</td>
<td>29</td>
<td>60</td>
<td>64</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>12</td>
<td>49</td>
<td>65</td>
<td>78</td>
<td>54</td>
<td>15</td>
</tr>
<tr>
<td>4106</td>
<td>100</td>
<td>39</td>
<td>50</td>
<td>175</td>
<td>40</td>
<td>128</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>39</td>
<td>46</td>
<td>42</td>
<td>32</td>
<td>31</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>29</td>
<td>323</td>
<td>957</td>
<td>510</td>
<td>935</td>
<td>22</td>
</tr>
</tbody>
</table>

Following NORS treatment, the somatic cell counts in the three milking bovines are summarised in Table 6. A clinically significant increase in somatic cell count was observed in one animal infused with 400 mM NORS 24 hours post-treatment (957*10^3 cells/mL). Additionally, another clinically meaningful result was seen with the 200 mM NORS infusion 24 hours post-treatment in a different animal (305*10^3 cells/mL). All values returned to baseline a week post treatment. No inflammation was visually observed. In addition, no significant changes occurred to the fat, protein, or lactose contents post treatment (data not shown).

4.4 Discussion

*Methemoglobin and Serum Nitrite*

Biologically speaking, nitric oxide has a half-life in the body of less than six seconds and a radius of action of approximately 200 μm from its site of origin (Lancaster 1994). The molecule is generally inactivated by binding onto various things such as sulfhydryl groups of cellular thiols or in blood via nitrosylation into the heme moieties of hemoglobin to form methemoglobin (Miller et al. 2007, Miller et al. 2009, Yoshida et al. 1980). The molecule eventually reduces to
nitrates via methaemoglobin reductase and is then expelled through the urine (Miller et al. 2007, Miller et al. 2009, Yoshida et al. 1980).

Nitrate poisoning among ruminants is a very serious issue which can lead to methemoglobinemia and has led to tight regulations in the feed and water for these animals (Bruning-Fann and Kaneene 1993). Clinical signs such as weakness, ataxia, trembling, hypersensitivity, gasping for breath and a high pulse rate appear at 30-40% methemoglobin and death within 2-10 hours can occur at >80% (Bruning-Fann and Kaneene 1993). Additionally, conception rates were lower and spontaneous abortion may occur (Davison et al. 1964).

Typically, cattle have a range of 1-3% methemoglobin (Adams, McCarty and Hutchinson 2012). Although delivered infused into the udder, it was anticipated that exogenous nitric oxide would enter into the blood stream to produce methemoglobin. In this study, the highest concentration of methemoglobin percent was found to be 4.5%, 30 minutes after treatment with 400 mM NORS. This was reduced eight hours after treatment to below the baseline measured at time zero (Fig. 4). One such explanation could be due to the difference of feeding times between measurements as nitrites/nitrates from the feed are absorbed into the blood stream, and produce methemoglobin (Lee and Beauchemin 2014). It is also possible we observed an increase concentration of methemoglobin reductase in the blood as homeostasis combat the sudden increase of methemoglobin. This increased reductase activity was seen in a nitrate supplementation study performed by Godwin and colleagues (Godwin et al. 2015). Future studies, of the biological effects of NORS will need to be conducted.
While no short-term assessment of tissue damage caused by methemoglobin has been studied there has been work done on sustained levels of methemoglobin. Using a rat model, methemoglobin levels of 10% saw normal tissue oxygen tension, but at 15% a decrease of tissue oxygen tension was observed which led to organ damage (Linberg et al. 1998). Therefore, it is possible to suggest a relatively short burst of less than 10% methemoglobin percentage would be safe for the animal. Additionally, in humans and rats an IV injection of 5.7 and 8.9 mg/kg sodium nitrite respectively saw an increase of methemoglobin to 10% (Kohn et al. 2002). In this study, the highest dose contained 1.1 g of sodium nitrite meaning the cow would have to weigh less than 193kg for the level to match what was found in humans and this is not considering that NORS was infused intramammarily rather than directly into the blood. Thus, these results suggest that the risk for methemoglobinemia in lactating cows will be nominal.

The methemoglobin percent results correlated with the measurements for plasma nitrite. However, a significant increase in plasma nitrite was detected at all concentrations of NORS whereas a significant increase of methemoglobin percent was only observed at the 400mM infusion. Possibly, the serum nitrite test via chemiluminescence was more sensitive to changes in blood nitrite concentrations compared to the measurements of methemoglobin percent. However, it is also possible that lower concentrations of NORS produced less nitric oxide and these were rapidly metabolized into nitrites by methemoglobin reductase.

*Milk Nitrite*

Milk was analyzed for nitrite concentrations in the treated quarter and in the adjacent and diagonal quarters. In general, a localized effect of the treatment was observed as only the treated
quarter showed an increase in nitrite. This is in contrast to antibiotic treatment as all four quarters would contain antibiotic drug residues and all of the cow’s milk would be unavailable for retail sale (Erskine 2014). While in theory it would be possible to use milk from non-treated quarters, currently, most regulatory agencies do not allow milk from untreated quarters to be sold for human consumption during antibiotic treatment. Regardless, 24 hours after treatment, the nitrite concentration in all treated quarters returned to baseline, suggesting a withdrawal period of only a day after completion of treatment. As most withdrawal periods for drugs typically last 1-5 days after treatment ends NORS could be beneficial because of a short washout period resulting in a reduction in economic losses (Bhosale et al. 2014, Conzuelo et al. 2013, Smith et al. 2005, Steeneveld et al. 2011). However, the quality of milk after treatment would have to be investigated to assure it meets standards set by countries and companies.

For commercial sale, milk typically has a nitrite concentration of below 5 μM, though some authors have reported up to 38 μM of nitrite in marketable milk (Silanikove, Merin and Leitner 2014). In this study, the highest concentration of milk nitrite was 38.6 μM. However, outside of the 20 mL stripped from the treated quarter before the first milking (10 hours post NORS infusion) no value above 4.3 μM was measured. No official standard testing is used for nitrite in milk in the United States, but using drinking water as a standard for comparison, the amount of nitrite would be limited to 1 mg/L, which is 21.7 μM (US Environmental Protection Agency 2016). Interestingly, the milk nitrite concentration of breastfeeding women in early postpartum was measured to be higher than what was found in our study (82.20 μM in colostrum and 53.28 μM on the 30th day postpartum) (Cekmen et al. 2004). It is suggested that the high nitrite
concentrations in breast milk is beneficial to neonates by protecting them from pathogenic invasions through oral ingestion (Iizuka et al. 1999).

In this study, we found that one animal (#1064) had a high nitrite concentration in the milk in both before and after milking, ten hours after infusion with 100 mM NORS. This finding was inconsistent with other results as the same animal had a much lower milk nitrite concentration from the same quarter, even after treatment with higher concentrations of NORS (200 and 400 mM). Perhaps improper distribution of NORS within the udder occurred. Future studies with larger sample sizes are needed.

**Somatic Cell Count**

While the traditional definition for somatic cell includes almost all biological cells in a multicellular organism. When referring to somatic cells in milk, it is typically understood to include only polymorphonuclear neutrophils, lymphocytes, eosinophils, macrophages and epithelial cells (Paape et al. 2002). Somatic cell counts are one of the most common ways to identify subclinical mastitis and it defines milk quality standards. The most important factor affecting the somatic cell count of milk from an individual quarter is the infection status of that quarter. Infection results in an inflammatory reaction and recruits somatic cells. The maximum somatic cell count threshold for milk acceptance in dairy industries varies in different countries, though typically ranges from 1–7.5*10^5 cells/mL (Li et al. 2014).

In this study, it was observed in some cases that NORS treatment may have caused an increase in somatic cell count, however, the increases were not consistent. All increases were temporary and
returned back to baseline after one week. This is consistent with others reporting increased inflammation due to nitric oxide. In those studies, an increase of somatic cell counts correlated with an increase concentration of nitric oxide during mastitis infection (Atakisi et al. 2010, Bouchard et al. 1999). One function of nitric oxide is it can regulate the production of chemoattractants such as macrophage inflammatory protein-2 and monocyte chemoattractant protein-1 which can attract immune cells such as leukocytes to aggregate and thus lead to an increase of the somatic cell count (Trifilieff et al. 2000, Vallance and Charles 1998). Overall, this happens at relatively lower nitric oxide concentrations as higher levels lead to an anti-inflammatory response (Kolb et al. 1996). Perhaps the differences in results could be the rate at which milk is produced and how quickly NORS is being diluted. Conversely, NORS could have decreased the bacterial load and reduced the inflammatory response or acted as a free-radical scavenger. Future directions would be to investigate the amount of residual milk in the udder and balance it with an effective concentration of NORS to avoid udder inflammation. Another alternative is that NORS might be effective as a prophylactic dry cow therapy instead of antibiotics.

Additionally, the differences in somatic cell counts can also be of a result of the rebalancing of the microbiota of the udder. As nitric oxide has general antimicrobial properties, it is likely that a total reduction of the microbiota has occurred. The reestablishment of commensal bacteria in the udder may then cause a cellular immune response. While the potential change of commensal bacteria may be concerning, in antibiotic plus teat sealant used for dry cow therapy, the microbiome is not altered relative to other individuals receiving the teat sealant alone (Bonsaglia
et al. 2017). Of course, further studies on the effect of NORS on the microbiome would have to be conducted.
5. Concluding Remarks

5.1 Implications and Limitations

The main purpose of this study was to investigate whether the use of nitric oxide via NORS could be used as a treatment towards bovine mastitis. Overall, it was shown that the proposed formulation of NORS can be mixed into milk without it curdling while continuing to release nitric oxide and for it to retain its antimicrobial properties in both bacteria seeded milk and in milk obtained from cattle suffering from bovine mastitis. The initial safety tests demonstrated that healthy cows were able to tolerate the treatment without serious long term adverse effects. Together, these data suggest that NORS could be evaluated for efficacy in the treatment of bovine mastitis or as a dry cow treatment to reduce the use of prophylactic antibiotics.

One main limitation of this work was the lack of animals used in the safety trial. Typically, phase I trials include 20-100 individuals while our study included three (Lipsky and Sharp 2001). The purpose of this experiment was not to perform a full safety trial, but to examine the feasibility of such a trial. In addition, while the current data may not be enough for us to progress to a phase II trial, the information provided allows further investigation. Another limitation was that the economic impact of NORS was not addressed. Economic feasibility of NORS treatment should be evaluated prior to large scale safety and efficacy studies.

5.2 Future Directions

This work can be further built upon through the initialization of a formal phase I safety study with the proper number of replicates. Additional histological work including analyzing tissue samples for damage or investigating the changes of the microbiome of the mammary gland can...
also be performed. This would provide more information towards the implications of NORS use for bovine mastitis. This information may require augmentation of the current formula should adverse effects be discovered. Another interesting direction would be to explore whether nitric oxide has an effect on the lactation rate of bovines. On a separate direction, it may be interesting to focus first on NORS as a dry cow treatment.

5.3 Conclusion

Nitric Oxide Releasing Solution, as an antibiotic alternative, could be beneficial in multiple ways. Nitric oxide has been shown to eradicate pathogens related to bovine mastitis. It is a helpful molecule in the fight against antibiotic-resistant bacteria (Deppisch et al. 2016) and thus, NORS may be effective against drug-resistant mastitis infections, when current antibiotic treatment practice fails. Additionally, the withdrawal period of nitric oxide is short and can potentially lead to a faster resolution of infection. The preliminary safety data, requires further studies, particularly a safety assessment in a larger number of animals as well as in-vivo efficacy. If shown to be safe and as effective as antibiotics, NORS might then be used as a mastitis treatment.
References


Canadian Dairy Information Centre. 2019. Number of farms, dairy cows and heifers.


Koebke, K. J., M. T. Waletzko & A. A. Pacheco (2016) Direct Monitoring of the Reaction between Photochemically Generated Nitric Oxide and Mycobacterium tuberculosis


