A retrospective analysis of the *in vitro* antimicrobial resistance of Gram-negative mastitis pathogens in Bavaria, Germany from 2014 – 2023

von Lea Hannah Pirner

Inaugural-Dissertation zur Erlangung der Doktorwürde
der Tierärztlichen Fakultät der Ludwigs-Maximilians-Universität
München

A retrospective analysis of the *in vitro* antimicrobial resistance of Gram-negative mastitis pathogens in Bavaria, Germany from 2014 – 2023

von Lea Hannah Pirner
aus Lauf an der Pegnitz

München 2025

Aus dem Zentrum für Klinische Tiermedizin der Tierärztlichen Fakultät
der Ludwigs-Maximilians-Universität München

Lehrstuhl für Physiologie und Pathologie der Fortpflanzung

Arbeit angefertigt unter der Leitung von:
Univ.-Prof. Dr. Wolfram Petzl

Angefertigt beim
Tiergesundheitsdienst Bayern e.V.
Eutergesundheitsdienst
Poing-Grub
Mentorin: Dr. med. vet., MSc, Ph.D, Diplomate ACVPM Ulrike Sorge

Gedruckt mit der Genehmigung der Tierärztlichen Fakultät
der Ludwig-Maximilians-Universität München

Dekan:	Univ.-Prof. Dr. Reinhard K. Straubinger, Ph. D.
Berichterstatter:	Univ.-Prof. Dr. Wolfram Petzl
Koreferent:	Univ.-Prof. Dr. Hermann Ammer

Tag der Promotion:
08. Februar 2025

Die vorliegende Arbeit wurde gemäß § 6 Abs. 2 der Promotionsordnung für die Tierärztliche Fakultät der Ludwig-Maximilians-Universität München in kumulativer Form verfasst.

Folgende wissenschaftliche Arbeiten sind in dieser Dissertationsschrift enthalten:

1. Publication I:

   Pirner, L. H.; Petzl, W.; Gangl, A.; Huber-Schlenstedt, R.; Sorge, U.S.

   ***In vitro* antimicrobial resistance of**
   Escherichia coli, Serratia marcescens, Klebsiella oxytoca,* and *Klebsiella pneumoniae
   on Bavarian dairy farms between 2014-2022

   https://doi.org/10.3168/jds.2023-24536
   Journal of Dairy Science Vol. 107 Issue 10, 2024

   Received December 12, 2023; Accepted April 26, 2024; Published September 20, 2024

2. Publication II:

   Pirner, L. H.; Petzl, W; Huber-Schlenstedt, R.; Sorge, U. S.

   ***In vitro* antimicrobial resistance of**
   ***Pasteurella multocida* and *Mannheimia haemolytica* from bovine mastitis**
   on Bavarian dairy farms between 2015 and 2023

   https://doi.org/10.48435/MSI.2025.3

   Milk Science International (78) 2025 P. 13-18, ISSN 2567-9538

   Submitted July 20, 2024; Accepted March 25, 2025

Meiner lieben Familie

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AMR	Antimicrobial resistance
ARG	Antimicrobial resistance gene
BHV 1	Bovine herpesvirus 1
BRD	Bovine respiratory disease
C.	Corynebacterium
Cfu	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
CM	Clinical mastitis
CMT	California Mastitis Test
CNS	Coagulase negative Staphylococci
E.	Escherichia
e. g.	Exempli gratia/ example given
HGT	Horizontal gene transfer
HPAI A H5N1	Highly pathogenic avian influenza virus serotype H5N1
IL	Interleukin
IR	Intrinsic resistance
K.	Klebsiella
LPS	Lipopolysaccharide
M.	Mannheimia
MALDI-TOF	Matrix-assisted laser desorption/ ionization – time of flight
MDR	Multidrug resistance
MGE	Mobile gene element
MHK	Mittlere Hemmstoffkonzentration
MIC	Minimum inhibitory concentration
NSAID	Non-steroidal anti-inflammatory drug
P.	Pasteurella
S.	Serratia
Sc.	Streptococcus
SCC	Somatic cell count
SCM	Subclinical mastitis
St.	Staphylococcus
TäHAV	German veterinary dispensary law Tierärztliche Hausaptothekenverordnung
TGD	Bavarian animal health services (Tiergesundheitsdienst Bayern e.V.)
TNF	Tumor necrosis factor
TSI	Triple-sugar-iron
USD	US Dollars

I. INTRODUCTION

Bovine mastitis is known all over the world as an important and prevalent issue in dairy cows. It can present itself as subclinical mastitis as well as clinical, showing a wide range of signs, from mild ones like changed milk character to severe generalized signs and even cow-loss (HERTL et al., 2011; SCHMENGER & KRÖMKER, 2020).

The biggest cause of mastitis are bacterial pathogens (HERTL et al., 2011; DUFOUR et al., 2019). There are many bacterial species that can infect the udder. They can be classified in different ways, one of which is to group bacterial mastitis pathogens by their Gram stain. Gram-positive mastitis pathogens commonly include *Staphylococcus (St.)* spp, like *St. aureus* and various *Streptococcus (Sc.)* spp., amongst many others (JAIN, 1979; DUFOUR et al., 2019). But in this thesis, the focus will be on gram-negative mastitis pathogens, and their antimicrobial resistance (AMR).

In general, gram-negatives or even just *Enterobacteriaceae* make up from 11.6% to 40.5% anywhere up to 55.5% of all cases of clinical mastitis and have gained importance over the last years, especially in high performing dairy cows on well-managed dairy farms (BRADLEY & GREENE, 2000; HOGAN & SMITH, 2003; SCHMENGER & KRÖMKER, 2020; ABDI et al., 2021). Of those pathogens, *Escherichia* (*E.) coli* is the most prevalent, followed by *Serratia (S.)* spp. and *Klebsiella (K.)* spp. (BRADLEY & GREENE, 2000).

Most gram-negative mastitis pathogens are classed as environmental mastitis pathogens. They can be found in manure, bedding, water and even milking equipment, from where they can reach the teat and enter the udder through the teat canal (HOGAN & SMITH, 2003).

Even though the majority of infections with gram-negatives do not result in severe clinical signs, they still cause a big percentage of clinical mastitis cases (HOGAN & SMITH, 2003; SCHMENGER & KRÖMKER, 2020). The reason gram-negative pathogens are the cause to

such a high percentage of severe clinical mastitis cases are their virulence factors: all gram-negative bacteria contain the endotoxins Lipopolysaccharides (LPS), in their outer membrane which can evoke a strong, sometimes excessive immune reaction resulting in substantial tissue damage in the mammary gland (BURVENICH et al., 2003; BRADFORD et al., 2015).

The strong inflammatory reaction along with the tissue damage has many consequences. A quarter infected with gram-negative mastitis pathogens has an overall slimmer chance for total recovery during the lactation period (SCHUKKEN et al., 2009; SHINOZUKA et al., 2016). This is associated with a significantly decreased milk yield, which can cause big economical losses for the farmer as well as the dairy industry in general (GROHN et al., 2004).

At first glance, the key to minimize economic losses due to clinical mastitis might seem to be quick and effective therapy, with the aim to eliminate the pathogens from the udder. To this day the treatment of choice for many practicing veterinarians is the intramammary application of antibiotics (SORGE et al., 2019). However, intramammarily applied antibiotics have often been proven ineffective in treating gram-negative pathogens over the last decades. There are many theories as to why local antibiotic treatment of gram-negative mastitis rarely works, for example high tissue damage, swelling, low bioavailability (DU PREEZ, 2000), or the transient and self-limiting nature of the infection (HOGAN & SMITH, 2003). In addition, gram-negative bacteria tend to be more resistant than gram-positives and many isolates of gram-negative bacteria, especially *E. coli*, show multidrug resistances (GUERRA et al., 2020). Those resistances can vary strongly between different regions and influence antimicrobial use even beyond mastitis therapy (KEHRENBERG et al., 2001). Therefore, the objective of this study was to analyze the prevalence and development of antimicrobial resistances (AMR) of gram-negative mastitis pathogens in Bavaria, Germany, between 2014 and 2023.

II. LITERATURE REVIEW

1. Bovine mastitis

1.1 Definition, forms, and prevalence

The word mastitis stems from the Greek word *mastos*, meaning breast, and the suffix *-itis* which means inflammation, and is defined as the inflammation of the mammary gland. With the udder being at the center of a dairy cow´s production value, mastitis is one of the most important diseases in dairy cattle (RUEGG, 2017; HEIKKILA et al., 2018; MORALES-UBALDO et al., 2023).

It is mainly caused by mastitis pathogens, including yeast fungi (e.g. *Candida* spp.), some algae like *Prototheca zopfii*, and even certain viruses (e.g. BHV1, foot-and-mouth disease virus, Parainfluenza-3-virus, HPAI A H5N1) that have the ability to invade the udder and cause inflammation (WELLENBERG et al., 2002; RICCHI et al., 2010; DWORECKA-KASZAK et al., 2012; NELLI RK et al., 2024). But the most prevalent and important group of mastitis pathogens are bacteria (HERTL et al., 2011).

The prevalence of mastitis depends greatly on the management and hygiene practices of a dairy farm (GREEN et al., 2007). And an average incidence of 41.6 cases of clinical mastitis per 100 cows per year, ranging between 13 and 75 cases has been reported (BRADLEY & GREEN, 2001).

Mastitis can present itself as clinical or subclinical. Clinical mastitis can range from abnormal milk to moderate symptoms, including swelling of the affected quarter and fever. Acute clinical mastitis is associated with severe, systemic signs, like high fever or hypothermia, and depression of the cow, and might even go along with shock-like symptoms. Subclinical mastitis (SCM), on the other hand, is not as easily recognizable, since there are no clinical signs. It is usually associated with an elevated milk somatic cell count (SCC) and decreased milk

production. Due to the “invisible” nature of subclinical mastitis, infected cows often spread pathogens throughout the herd (COBIRKA et al., 2020; MORALES-UBALDO et al., 2023).

1.2 Mastitis pathogens

There have been roughly 150 bacterial mastitis pathogens discovered so far (WATTS, 1988; MORALES-UBALDO et al., 2023). These bacterial mastitis pathogens can be categorized in different ways.

First, they can be grouped by their gram stain, into gram-positive and gram-negative pathogens. Examples for common gram-positive mastitis pathogens are *St. aureus, Sc. agalactiae, Sc. dysgalactiae,* and *Sc. uberis*, among many others. Known gram-negative mastitis pathogens include *E. coli*, *Klebsiella* spp., and *Serratia* spp. (COBIRKA et al., 2020).

Other categories are major and minor mastitis pathogens. Major pathogens include those, that are most frequently isolated from mastitis, such as *St. aureus, E. coli, Sc. agalactiae, Sc. dysgalactiae,* and *Mycoplasma bovis*. Minor pathogens are less often found in mastitis and are often opportunistic pathogens. Some well-known minor pathogens include coagulase-negative staphylococci (CNS: e.g. *St. chromogenes, St. epidermidis, St. sciuri*), *Corynebacterium (C.) bovis*, yeast, and fungi (HEIKKILA et al., 2018; COBIRKA et al., 2020).

The third common categorization of mastitis pathogens is based on their source and their ability to persist within the udder: contagious and environmental pathogens. Contagious pathogens (such as *St. aureus, Sc. agalactiae, Sc. canis,* or *Mycoplasma bovis*) have adapted to survive and particularly multiply within the mammary gland (COBIRKA et al., 2020; MORALES-UBALDO et al., 2023). With this ability, an infected udder serves as the reservoir, contaminating milking equipment and infecting other cows. If not promptly detected and treated, contagious mastitis pathogens are prone to cause chronic infections with subclinical

mastitis, which can turn into clinical flare-ups, when the cow is experiencing stress, that impacts her immune system (JAIN, 1979; RUEGG, 2017). In contrast, environmental pathogens are ubiquitously found in the environment (e.g. soil, bedding, or manure) and are often only transient in the udder. The most common environmental mastitis pathogens are *E. coli, K. pneumoniae, Sc. dysgalactiae*, and *Sc. uberis* (KLAAS & ZADOKS, 2018; MORALES-UBALDO et al., 2023). They predominantly cause clinical mastitis. They enter the mammary gland through the teat canal, causing tissue irritation and subsequently inflammation. They are rare to cause chronic infections and are typically not spread from cow to cow but contracted by the cows through contact with reservoirs in the environment.

There have been discussions around whether some pathogens are strictly environmental in the recent years. For example, *Sc. uberis* has been found to become contagious when the temperature rises in the summer months, causing heat-stress. Then, the pathogen is able to survive in the udder for extended periods of time, and even multiply and shed in the milk, allowing it to be spread to other cows through the milking equipment (ZADOKS et al., 2001; ZADOKS, 2007).

2. Gram-negative mastitis pathogens

2.1 Coliform mastitis and other gram-negative mastitis pathogens

The mastitis pathogens mainly discussed in this study will be gram-negatives. Though there are many gram-negative pathogens, that can cause mastitis, we will mainly be focusing on *E. coli, Klebsiella* spp., *Serratia* spp., *Pasteurella (P.) multocida,* and *Mannheimia (M.) haemolytica*.

Most common gram-negative mastitis pathogens are coliforms, including *Escherichia* spp., *Klebsiella* spp., *Serratia* spp., and *Enterobacter* species. Those originally gave mastitis caused by gram-negatives the name “Coliform Mastitis”. This has since been updated with other gram-

negative bacteria such as *Pseudomonas* spp. and *Proteus* spp., which were more recently introduced into the group of gram-negative mastitis pathogens (HOGAN & SMITH, 2003; SCHUKKEN et al., 2012). Gram-negatives can cause up to 40% of clinical mastitis (CM) cases, and up to 25% of cows in well-managed herds are diagnosed with "coliform mastitis" per year. In a study on clinical mastitis in Northern Germany, 30.5% of severe clinical mastitis cases were caused by coliforms (SCHMENGER & KRÖMKER, 2020). In Southern Germany, gram-negative pathogens have shown an increased prevalence in culture positive samples from clinically affected quarters in recent years (BECHTOLD et al., 2024b). A study from the UK reported 26.7% of sampled CM cases to be caused by *E. coli* (BREEN et al., 2009).

They are more common in well-managed modern dairies, as SCC is inversely related to the incidence of CM caused by gram-negative bacteria (ERSKINE et al., 1988; BARKEMA et al., 1998; OLDE RIEKERINK et al., 2008; SCHUKKEN et al., 2012).

E. coli is the most frequently isolated gram-negative mastitis pathogen, usually followed by *K. pneumoniae*, which is known for causing particularly severe infections, drastically decreasing milk production and quality. Despite being classified as environmental pathogen, *K. pneumoniae* has been found to be predominantly transmittable from udder to udder (KANEVSKY-MULLARKY et al., 2014; MORALES-UBALDO et al., 2023).

2.2 Overview of the pathogens

2.2.1 *E. coli* mastitis

E. coli is the most common amongst gram-negative pathogens. They are mostly associated with severe, sometimes even systemic clinical signs and are the most common cause of fatal mastitis and are therefore an important topic, especially in well-managed low SCC dairy herds (BURVENICH et al., 2003; COBIRKA et al., 2020). The outcome of *E. coli* mastitis depends

on many factors, such as clinical severity, age and lactation stage, energy balance of the cow, vitamin deficiency, and vaccination status. Particularly severe cases are often associated with an overshooting immune response of the host to bacterial endotoxins, a reaction that strongly depends on external, as well as internal factors and varies between each cow (BRENNECKE et al., 2021; FREDEBEUL-KREIN et al., 2022). *E. coli*, like many environmental pathogens, can be found in manure, bedding, and soil, which act as the main reservoir of infection, as shown by the genetic diversity of the mastitis-causing strains (CAMPOS et al., 2022; GOULART & MELLATA, 2022). They enter the udder through the teat canal and mostly multiply in the milk fraction, without adhering to the endothelial cells of the cisterns. Most intramammary infections with *E. coli* occur during the dry period and in early lactation, and particularly in the first and last two weeks of the dry period. This underlines the importance of adequate dry cow management as prevention for *E. coli* mastitis (HOGAN & SMITH, 2003; COBIRKA et al., 2020).

2.2.2 *Klebsiella* mastitis

Clinical mastitis caused by *Klebsiella* spp. has been shown to be more severe than most CM cases by other gram-negatives (SCHUKKEN et al., 2012), and, like other gram-negative mastitis, more prevalent in herds with low bulk milk SCC. Its importance is in part due to the severity of its infections (CHENG et al., 2020), and a low efficacy of vaccination and treatments (SCHUKKEN et al., 2012). Its economic losses per case exceed even those of CM caused by *E. coli*, because the duration of milk production loss and risk of culling are higher in cases of CM caused by *Klebsiella* spp. (ERSKINE et al., 2002; SCHUKKEN et al., 2012). Some of the most common reservoirs of *Klebsiella* spp. are wood-based beddings (e.g. sawdust) (HOGAN et al., 1989) and manure. In cases of outbreaks, *Klebsiella* spp. have also been found on milking equipment, furthering the spread of infection (HOGAN & SMITH, 2003; SCHUKKEN et al.,

2012). A high prevalence of *Klebsiella* CM is usually positively correlated with poor udder hygiene, and even cleaning the teat in preparation for milking does not negate bad udder hygiene scores (SCHUKKEN et al., 2012).

2.2.3 *Serratia* mastitis

Serratia spp. are ubiquitous environmental pathogens. Their reservoirs range from bedding to manure and the parlor environment. Multiple outbreaks could be traced back to open containers of teat dip, such as chlorhexidine teat disinfectant, that lead to the dissemination of the pathogen at milking (OLLIS & SCHOONDERWOERD, 1989; FRIMAN et al., 2019). *Serratia* spp. have also been found in chlorhexidine disinfectant in other environments (MARRIE TJ & JW., 1981; DE FRUTOS et al., 2017) and are considered generally resistant to biocides (SCHUKKEN et al., 2012). They are reported to cause CM less frequently than other gram-negatives and tend to cause chronic, subclinical infections, that often alternate with CM. *Serratia* spp. mastitis is associated with a long duration of infection, with 55 days to 4 months on average, but some reports even talk about durations of up to 3 years (BARNUM et al., 1958; TODHUNTER et al., 1991; FRIMAN et al., 2019). The innate immune response to *Serratia* is also lower and shorter than with the other gram-negatives, which may contribute to the pathogen´s survival in the gland for long periods of time (BANNERMAN et al., 2004b). Infection with *Serratia* spp. commonly results in increased SCC but is not associated with a decrease in milk production. An increased culling risk is mostly due to recurrent episodes of mastitis, rather than severe CM. Treatment of *Serratia* mastitis is difficult, and although positive results with neomycin have been reported, multidrug resistance has been frequently reported (BUSH et al., 1991; SCHUKKEN et al., 2012; LIANG et al., 2023). Most infections with *Serratia* spp. seem to be cured spontaneously (SCHUKKEN et al., 2012).

2.2.4 Other gram-negatives

Apart from coliforms, other gram-negative pathogens can also cause mastitis, although they are rarely detected. These include, among others, *P. multocida* and *M. haemolytica*. Both are more known for their role in causing respiratory infections in cattle, as part of the bovine respiratory disease (BRD) -complex (SCHONECKER et al., 2020). *P. multocida* has been reported to occasionally cause mastitis as an opportunistic environmental pathogen. Infections mostly cause mild to moderate CM, with symptoms usually staying limited to the udder (BARNUM, 1954; ASFOUR & EL-METWALLY, 2016; MILANOV et al., 2017). In contrast to ewes, *M. haemolytica* very seldomly cause mastitis in cows. This is likely due to the reservoir being the respiratory tract of lambs, with the transfer taking place during suckling (OMALEKI et al., 2011). Infections by *M. haemolytica* also mainly present as clinical mastitis (MAPLESDEN & CARTER, 1955).

2.3 Pathogenesis

2.3.1 Reservoir and path of infection

Gram-negative mastitis pathogens inhabit many places in a cow´s environment and also contribute to many other infections besides the udder. In general, these environmental pathogens can be found in manure, bedding, or soil. For example, *E. coli* are part of the gut microbiome and thus can be found in manure. Much like *Klebsiella* spp. and *Enterobacter* spp., which also inhabit soil, grains, and water (HOGAN & SMITH, 2012). Outbreaks of *K. pneumoniae* are commonly correlated with the use of fine sawdust as bedding and *Klebsiella* spp. are also more frequently found in recycled manure solids as bedding, as opposed to frequently replaced bedding material (SORTER et al., 2014). *Serratia* spp. have been isolated from hoses, water tanks, and other parts of milking systems (HOGAN & SMITH, 2003;

SCHUKKEN et al., 2012).

Since gram-negative bacteria cannot grow on teat skin, the number of pathogens on teat skin is an indication of the cow´s exposure in its recent environment. This is especially linked with the number of bacteria found in the bedding and often correlates with rates of clinical mastitis. Clean, inorganic bedding materials, such as sand or limestone tend to house fewer gram-negative bacteria. The typical path of infection is through the teat canal, from where the bacteria travel into the gland, causing infection (HOGAN & SMITH, 2003).

2.3.2 Inflammation and virulence factors

2.3.2.1 Immune response and evasion of host defense

Inflammation is part of the very definition of the word “mastitis” and the central point in its development. First and foremost, it is a response to the invading pathogens, causing a host reaction in the form of swelling, heat, redness, pain, and diminished function (BRADFORD et al., 2015). The severity of these signs depends heavily on the external influence that triggers the inflammation and is often positively correlated with the number of pathogens in the udder (HOGAN & SMITH, 2003).

The innate immune response in the udder is pathogen specific. Upon infiltration of the mammary gland, bacteria activate pattern recognition receptors, such as TLR4, which can be found on many cell types and recognizes the lipid A-fraction of bacterial lipopolysaccharides. This activation leads to inflammation, a part of which is neutrophil infiltration. Inflammation is modulated by cytokines. Proinflammatory cytokines (e.g. tumor necrosis factor (TNF)-alpha, interleukin (IL)-1 beta) induce an acute-phase immune response, while other cytokines, like IL-10, inhibit proinflammatory cytokine production, modulating the inflammatory response (SCHUKKEN et al., 2012; BRADFORD et al., 2015). Several studies by Bannerman et al. in

2004 compared the activation of the innate immune response by different gram-negative species. They found that *K. pneumoniae* caused the most severe immune response, while the one by *S. marcescens* was the mildest one observed (BANNERMAN et al., 2004b; BANNERMAN et al., 2004a; BANNERMAN et al., 2004c).

In order to sustain in the mammary gland, the pathogens must first evade the first-line cellular host defenses, consisting of neutrophils and other phagocytes. One way is through producing capsules that prevent phagocytosis, or other surface components that make the bacteria less susceptible, like antiphagocytic factors that are related to the O serotypes of *E. coli* (HOGAN & SMITH, 2003).

2.2.2.2 Lipopolysaccharides

The primary virulence factors of gram-negative bacteria responsible for tissue damage in the udder are endotoxins. Endotoxin is the lipopolysaccharide portion of the bacterial wall, which is specific to gram-negatives. LPS increases cell wall integrity and serves as a barrier from environmental stressors. Structurally, LPS consists of macromolecules with 3 components: a lipid A fraction, an inner and outer core oligosaccharide, and a polysaccharide, called the "O-chain". Bacteria containing O-strains are referred to as "smooth types", as opposed to "rough types" without the O-strain (CAROFF & KARIBIAN, 2003; SCHUKKEN et al., 2012).

LPS is released during cell death and initiates the main inflammatory response, by causing vasoconstriction, thus disrupting blood flow (HOGAN & SMITH, 2003). This reaction leads to severe inflammation in the udder, causing tissue-damage. Large amounts of LPS can also induce a systemic inflammatory response with high fever, increased heart rate, and can even lead to septic shock. Those severe signs are mainly caused by the lipid A-fraction of LPS (SCHUKKEN et al., 2012; GUNTHER et al., 2017). There is an almost infinite amount of

virulence factors between all gram-negative mastitis pathogens, the review of all of which would go far beyond the scope of this study. But the one prerequisite needed in order to cause mastitis, is the ability to multiply in the mammary gland (HOGAN & SMITH, 2003).

2.2.2.3 Survival inside the mammary gland

Coliforms used to be considered opportunistic pathogens, without the ability to invade the mammary glands´ cells or to persist inside the udder for an extended amount of time. Recent studies have contested those opinions, proving that some strains indeed have adapted to the conditions inside the mammary gland and can cause persistent infections, by evading host defenses, invading endothelial cells and even replicating in the milk. Especially *E. coli* have developed many mechanisms to survive inside the udder, as well as *Serratia* spp., which have even been associated with mostly causing chronic infections, and *Klebsiella* spp. (TODHUNTER et al., 1991; BRADLEY & GREEN, 2001; KANEVSKY-MULLARKY et al., 2014).

Strains that do not have those mechanisms are said to multiply in the secretion of the mammary gland, without attaching themselves to the endothelial cells. In order to do this, the bacteria must be able to ferment lactose as a source of energy and grow in microaerobic conditions. In addition, some pathogens can bypass the inhibitory effects of lactoferrin, which is more active in involuted mammary glands and would stop bacterial growth by removing iron from the secretion, which is the limiting factor in the mammary gland. *Klebsiella* spp. are said to be more successful in circumvent lactoferrin, than e.g. *E. coli*, by using high affinity iron acquisition systems and therefore can also infect involuted mammary glands (HOGAN & SMITH, 2003).

2.3.3 Effect on organ systems outside of the udder

Unfortunately, the economic losses do not stop with milk yield and costs for treatment. Clinical mastitis cases can also influence organ systems outside the mammary gland, especially when paired with acute or even peracute systemic symptoms. Mastitis has also been proven to affect a cow´s reproductive system. Clinical mastitis occurring any time between 14 days before to 35 days after artificial insemination may decrease a cow´s probability to conceive (HERTL et al., 2010). Again, gram-negative bacteria showed the greatest impact, effecting in an 80% reduction of the probability of conception, with clinical mastitis by gram-negative bacteria one week after artificial insemination. They also had a greater impact overall, as compared to gram-positive pathogens, and have been more heavily associated to pregnancy loss due to CM (HERTL et al., 2010; DAHL et al., 2018).

2.4 Economic impact of gram-negative mastitis

Economic losses due to mastitis are made up of different components. The first, most obvious, is milk loss. Both in the form of reduced output due to the disease and discarded milk because of changed milk character or treatment. Then, there are veterinary or treatment costs, premature culling, and other, indirect factors like pregnancy loss due to mastitis, and preventive costs, which can be difficult to evaluate financially (HOGEVEEN & VAN DER VOORT, 2017; MORALES-UBALDO et al., 2023).

In the US, total economic costs of mastitis in the first 30 days of lactation were 444 USD. The parameters used were direct costs (diagnostics, therapeutics, non-saleable milk, veterinary services, labor, and fatalities) and indirect costs (premature culling, milk production loss, and future reproductive loss). These numbers are similar to those of a study from Canada, where financial losses were estimated between $386 to $779 (PUERTO et al., 2021). In Dutch farms,

the total cost of mastitis was an average of 240€ per lactating cow per year (VAN SOEST et al., 2016).

According to Cha, Bar et al. (2011), gram-negative mastitis cases were the most cost-intensive overall with $211.03 costs per case on average, followed by gram-positives with $133.73 and other organisms with $95.31. The main contributor for those costs in gram-negative cases was milk loss (72.4% of the cost per case), whereas the majority of costs in the other mastitis cases were spent on treatment (CHA et al., 2011). In 2018 a study by Heikkila et al. concluded that mastitis caused by *E. coli* resulted in the highest daily milk-losses (4.6 kg/d) throughout the 6 most common pathogens investigated in said study (HEIKKILA et al., 2018). In accordance with this, other studies also found gram-negatives to be the costliest mastitis pathogens (HOGEVEEN & VAN DER VOORT, 2017; FU et al., 2022).

2.5 Detection and Treatment

2.5.1 Detection

The usual method for identifying gram-negative mastitis pathogens is to take an aseptic milk sample of the affected quarter and incubate in one or several specific culture media for at least 18 hours at 37°C, until colonies are formed. To identify the pathogen, one may look at the colonies formed, Gram stain, KOH reaction, Cytochrome oxidase, Lactose fermentation, as well as many other tests (ADKINS & N.M.C., 2017). Selective culture media can be used, e.g. McConkey agar, which help distinguish between different species by indicating lactose fermentation with the appearance of pinkish-red colonies when the pH drops below 6.8 (SCHUKKEN et al., 2012). In addition, different methods of biochemical identification can be used to ascertain the pathogen´s ability to ferment lactose and produce gas or acid, (e.g. by triple-sugar-iron (TSI) reaction) (HOGAN & SMITH, 2003). The method used in this particular study is the identification with the help of MALDI-TOF MS (Bruker Corp.), which is part of

the Bavarian animal health services (Tiergesundheitsdienst Bayern e.V., TGD) laboratory since 2014.

However, isolating gram-negative mastitis pathogens comes with its own challenges. Very often, mammary infections with these pathogens are transient, meaning the pathogens will be eliminated from the udder very quickly after onset of mastitis, which makes them difficult to detect in the later stages. Additionally, gram-negatives are common contaminants of milk samples, as they are very abundant in the cows´ environment. For this reason, very careful aseptic sampling is key to avoid false diagnoses (HOGAN & SMITH, 2003).

2.5.2 Antimicrobial therapy

The majority of antimicrobials on a dairy farm are applied intramammarily, either in the course of dry-cow or mastitis treatment (DU PREEZ, 2000; SORGE et al., 2019). Antimicrobials typically used on dairy farms include beta-lactams (e.g. penicillin, ampicillin, oxacillin), extended-spectrum beta-lactams (e.g. ceftiofur), aminoglycosides (e.g. streptomycin), macrolides (e.g. erythromycin), lincosamides (e.g. pirlimycin), tetracycline, sulfonamides, and fluorquinolones (REDDING et al., 2019; ABDI et al., 2021). Many vouch for the efficacy of certain antimicrobials and though many isolates of gram-negative mastitis pathogens are sensitive to certain antibiotics *in vitro*, the treatment efficacy *in vivo* is often questionable (ABDI et al. 2021). Intramammarily applied antibiotics have often been proven ineffective in treating gram-negative pathogens, yet it still is the most frequently used treatment method. As mostly used antibiotics in coliform mastitis cases enrofloxacin and trimethoprim-sulfonamide have been described (DU PREEZ, 2000), however their application is not approved in all countries. There are many theories as to why local antibiotic treatment of gram-negative mastitis rarely works. These hypotheses include that high tissue damage or swelling prevents the antibiotic to distribute properly inside the udder resulting in insufficient drug concentration

or low bioavailability (DU PREEZ, 2000). Another contributing factor is that gram-negative mastitis intramammary infections can be transient, which means the infection is self-limiting. Therefore, intramammary antibiotic treatments become unnecessary, as the pathogen will be eliminated from the mammary gland regardless of antibiotic treatment, which had shown at best minimal effect on the duration of the infections (PYÖRÄLÄ et al. 1993, HOGAN & SMITH, 2003).

Antimicrobial treatment has only been proven helpful when administered systemically after the blood-milk barrier has already been broken down. In cases of bacteriemia, antibiotic therapy improved the chances of the cow's survival. But even here there seems to be no effect on the outcome of the mastitis affected quarter, only on eliminating the bacteria in the cow´s blood stream (ERSKINE et al., 2002). On top of this, systemic signs of gram-negative CM have been proven to be mostly caused by endotoxins or other virulence factors released from the pathogens inside the udder. Whereas bacteria themselves are rarely found in the bloodstream (NOBREGA et al., 2020; BRENNECKE et al., 2021; KREBS et al., 2023).

Still, for some gram-negative pathogens like *P. multocida* and *M. haemolytica*, antimicrobial treatment has been reported to successfully eliminate the bacteria from the udder. In these case studies, an intramammary injection of penicillin was administered, resulting in the bacteriological cure of the affected udders (BARNUM, 1954; D. C. MAPLESDEN, 1955; MILANOV et al., 2017). These studies did, however, not discuss the possibility of self-cure, a phenomenon which is prevalent in most other gram-negative mastitis pathogens (DU PREEZ, 2000; RUEGG, 2021).

Based on evidence, one could conclude that intramammary antimicrobial treatment will rarely increase the cure rate of mild to moderate intramammary infections with gram-negative mastitis pathogens in a meaningful manner and alternative treatments should be explored.

2.5.3 Supportive therapy and alternative treatment

Supportive therapy is especially recommended in acute and peracute clinical mastitis cases and can be an important tool in reducing systemic signs. It can consist of an addition of anti-inflammatory drugs, like corticosteroids or non-steroidal anti-inflammatory drugs (NSAID, such as meloxicam or carprofen), or an addition of fluids via intravenous infusion or drenching the cow, which is particularly important in severe cases with shock-like signs. Combining antibiotics with corticosteroids has shown beneficial effects by suppressing phagocytosis, reducing overshooting inflammation (DU PREEZ, 2000; RUEGG, 2021).

Supportive therapy has been confirmed to improve the chances of a cows survival in severe, gram-negative mastitis, by reducing inflammation and systemic signs, such as fever (ANDERSON & HUNT, 1989; KROMKER et al., 2011; RUEGG, 2021). Anti-inflammatory therapy can even bring economic benefits like lower culling rates and higher conception rates (VAN SOEST et al., 2018).

In search of alternative treatments, many other options have been explored so far, including medicinal plants or their extracts, essential oils, nanotechnology, and peptides, among others. Those alternative treatments by themselves have shown promising effects, such as bacteriostatic and bactericidal properties, antibiofilm activity, and anti-adhesive activity. In combination with antimicrobials, some plant-based treatments have been proven synergistic in reducing minimum inhibitory concentrations (MIC) significantly (PROCOPIO et al., 2019; CHENG & HAN, 2020; MORALES-UBALDO et al., 2023).

2.6 Prevention and management

2.6.1 Management and risk factors

Due to treatment costs and long-term economic losses caused by gram-negative mastitis, more focus has been laid on management and prevention methods to counteract mastitis. The

goal is to identify cow risk factors and minimize those by appropriate herd-management.

Some important risk factors are udder and leg hygiene and teat-end hyperkeratosis, most of the time caused by inappropriate milking equipment, high SCC (>200.000) at the time of drying off, and increasing parity (BREEN et al., 2009). Other cow risk factors for CM include rising parity, heat stress in the summer months, and the dry period, where the udder is particularly vulnerable to gram-negative pathogens, as well as during early lactation. Previous CM cases also significantly increase the incidence rate of CM and high producing cows seem more susceptible to CM by gram-negatives (SMITH et al., 1985; BURVENICH et al., 2003; WHIST et al., 2006; GREEN et al., 2007; STEENEVELD et al., 2008; BREEN et al., 2009).

There are many measures that can be taken to minimize the risk of an udder infection. Proper hygiene is key, especially in the dry-cow and calving areas, where the cows are especially prone to become infected due to decreased immune functions and increased stress. Milking hygiene is also very important, combined with proper equipment, as to prevent hyperkeratosis or injuries on the teat ends. Methods like pre-dipping have also shown a significant decrease in risk of CM (WHIST et al., 2006; SKOWRON et al., 2019).

2.6.2 Vaccination

Another way of preventing severe clinical mastitis caused by gram-negatives is vaccination. Commercially available vaccines use the core antigen, the exposed core oligosaccharide and lipid A of LPS. They alert factors of the innate immune response and induce antibodies that are reactive to all LPS, regardless of bacterial species. This makes them effective in reducing the severity of clinical mastitis by gram-negatives, but not preventing intramammary infections. Most of them use either *Escherichia coli* J5 (mutant *E. coli* O111:B4) or *Salmonella typhimurium* Re17 as antigens. Although these vaccines only claim efficacy against *E. coli*-mastitis, field studies have also found them to reduce severity of clinical mastitis cases by

Klebsiella spp., *Pseudomonas* spp., *Serratia* spp., and *Proteus* spp., and generally improving herd survival (GONZALEZ et al., 1989; HOGAN & SMITH, 2003; SCHUKKEN et al., 2012; BRADLEY et al., 2015). Here, it is important to note that the mainly observed function of commercially available vaccines is to reduce the severity of occurring CM cases (HOGAN et al., 1992; BRADLEY et al., 2015). And although vaccinated cows have no reduced risk of developing CM, a significant reduction in CM cases presenting with more than changed milk could be observed, and vaccinated cows overall produced more milk and milk solids (BRADLEY et al., 2015). There has also been an increased efficacy of the vaccine reported when local immunization in the form of an intramammary injection was added (HERRY et al., 2017).

Generally, vaccination is a valuable means of management, that should definitely be considered in herds with frequent gram-negative CM. It is not only effective in decreasing clinical symptoms in single cases, but also comes with economic advantages (KESSELS et al., 2016).

3. Antimicrobial resistance

Antimicrobial resistance occurs when microorganisms, such as bacteria, are not susceptible to antimicrobial substances. This resistance can be naturally present (inert or intrinsic resistance: IR) or develop through evolutionary selection of microorganisms. For the latter, pathogens genetically mutate to become resistant against those antimicrobials and therefore are able to adapt to this hostile environment. Especially in bacteria, AMR can also be transferred through the exchange of the genetic element that encode the resistance mechanisms, so called antimicrobial resistance genes (ARGs). This horizontal gene transfer (HGT) has been described many times and is a strong promoter of AMR, as those resistance genes can not only be passed on between bacteria from different strains, but also between bacteria of different species (SAN

MILLAN, 2018; MADDAMSETTI et al., 2024; WANG et al., 2024b). One of the most important vehicles for ARGs are plasmids, which have the ability to transfer between bacteria, and to whom the appearance of new AMR is often attributed. It has been hypothesized that plasmids can also exchange ARGs themselves through mobile genetic elements (MGEs) and that therefore a single plasmid can hold multiple ARGs (SAN MILLAN, 2018; MADDAMSETTI et al., 2024; WANG et al., 2024b).

There are many resistance mechanisms, that can either be intrinsic, adaptive, or acquired. Some examples for resistance mechanisms are drug inactivation by enzymatic degradation (e.g. through beta-lactamase or carbapenemase production), drug efflux through the expression of efflux pumps, limiting drug uptake through porin mutations decreasing membrane permeability, and modification of drug target sites (EICHENBERGER & THADEN, 2019; DAVIN-REGLI et al., 2021; GAUBA & RAHMAN, 2023).

Gram-negatives are generally considered more resistant than gram-positive mastitis pathogens, (ABDI et al., 2021), with typical resistance phenotypes including ampicillin, streptomycin, tetracycline, trimethoprim-sulfamethoxalone, nalidixic acid, chloramphenicol, and spectinomycin (AHMED & SHIMAMOTO, 2011).

3.1 Intrinsic resistance

AMR can result from inherent features of different bacteria that make them less or not at all susceptible to antimicrobials than other bacterial species would be affected by. This is called intrinsic resistance and can be found in most gram-negative, but also many gram-positive pathogens. One example for IR, that is widespread amongst gram-negative pathogens, is due to the structure of their cell walls. The outer membrane has two main barriers protecting gram-negative bacteria from certain antimicrobials: lipid-mediated barriers that block hydrophobic

substances and general diffusion porins blocking hydrophobic substances. This constitutes the IR of gram-negative bacteria against beta-lactams, like penicillin, ampicillin, and oxacillin, amongst other antimicrobials like macrolides (e.g. erythromycin), lincosamides (like pirlimycin), and streptogramin (LECLERCQ & COURVALIN, 1991; IMPEY et al., 2020; ABDI et al., 2021; GAUBA & RAHMAN, 2023).

3.2 AMR of gram-negative mastitis pathogens

Antimicrobial resistance of bacteria depends greatly on geographical region (KEHRENBERG et al., 2001). While rising prevalences of gram-negative mastitis pathogens have been described (BARKEMA et al., 1998; SCHUKKEN et al., 2012), other studies have reported low AMR of those pathogens, and even decreases in resistance overall (NUESCH-INDERBINEN et al., 2019). This can in part be attributed to the use of different antimicrobials – and the restriction thereof (SAINI et al., 2013).

3.2.1 AMR in *E. coli*

In different studies *E. coli* have mainly shown resistances to ampicillin, amoxicillin, procaine penicillin, streptomycin, oxytetracycline/ tetracycline, sulfamethoxazole-trimethoprim (SAINI et al., 2013; NUESCH-INDERBINEN et al., 2019; ISMAIL & ABUTARBUSH, 2020). *E. coli* have been described to be mostly resistant to erythromycin (97.1%), and in part resistant against tetracycline (41.2%), cephalothin (32.4%), and sulfadimethoxine (29.4%) (NAM et al., 2009; ABDI et al., 2021). *E. coli* are often classed as multidrug resistant (MDR), and isolates from mastitis can range from 62.8% (SAINI et al., 2012) to 98.2% MDR (GUERRA et al., 2020).

Resistance of *E. coli* against beta-lactams (e.g. amoxicillin, procaine penicillin) can be classed

as intrinsic resistance, due to the structure of the outer membrane of gram-negative bacteria (IMPEY et al., 2020). Resistances against erythromycin are also widespread and may even be classed as intrinsic (LECLERCQ & COURVALIN, 1991). They can be attributed in part to the impermeability of the cell membrane, but also to the expression of resistance genes ereA, ereB, and erxA, encoding the synthesis of erythromycin esterase, which hydrolyzes the antimicrobial´s lactone ring (ARTHUR & COURVALIN, 1986; LECLERCQ & COURVALIN, 1991).

3.2.2 AMR in *Klebsiella* spp.

According to a study by Abdi et al. (2021), most of the *K. oxytoca* and *K. pneumoniae* isolates were resistant to ampicillin, (75% and 92%, respectively). In addition, 100% of both *Klebsiella* species were resistant to erythromycin. A significant amount were also resistant against tetracycline, cephalothin, ceftiofur, and sulfadimethoxine (ABDI et al., 2021). Furthermore, *Klebsiella* spp. have been found resistant to streptomycin and kanamycin in other studies (NAM et al., 2009; SAINI et al., 2012). *Klebsiella* spp. are often described as multidrug-resistant (AHMED & SHIMAMOTO, 2011), especially when counting intrinsic resistances, with up to 55% of *Klebsiella* spp. isolated being MDR (SAINI et al., 2012).

However, most studies have not reported rising incidences of AMR in *Klebsiella* spp. isolates from mastitis cases (NAM et al., 2009; SAINI et al., 2012; FUENZALIDA et al., 2021).

3.2.3 AMR in *Serratia marcescens*

Like other gram-negative bacteria, *S. marcescens* is considered intrinsically resistant against beta-lactams and macrolides (e.g. erythromycin). But the CLSI guidelines also describe IR against amoxicillin/clavulanate and cefazolin, which could be confirmed in other studies (NAM

et al., 2009). In addition, *Serratia* spp. have also been found resistant against cephalotin, tetracycline, and streptomycin (NAM et al., 2009), rifampicin, cefamandole, polymyxin B/colistin, lincosamides, streptogramins, and others (FUSTÉ et al., 2012).

3.2.4 AMR in *Pasteurella multocida* and *Mannheimia haemolytica*

In contrast to the other afore discussed gram-negative bacteria, both *P. multocida* and *M. haemolytica* seem to be mostly susceptible against beta-lactams (BARNUM, 1954). Although there are very few studies on these pathogens isolated from mastitis, case reports usually describe them as susceptible to penicillin. At the same time, they are reported to be resistant against macrolides, tetracycline, and lincosamides - much like the other gram-negative pathogens discussed in this study (MILANOV et al., 2017; ALHAMAMI et al., 2021; VOLLWEIDER, 2023).

However, the possibility of self-limiting infections has not been discussed in regard to mastitis by *P. multocida* and *M. haemolytica* (BARNUM, 1954; MILANOV et al., 2017), and therefore cannot be ruled out completely.

3.3 Impact of AMR

With the spread of AMR depending in great part on the spread of ARGs, restricting the use of especially critically important antibiotics is crucial in preventing the development and spread of AMR mechanisms. This is why, as part of AMR prevention, monitoring existing and emerging AMR is incredibly important. Thus, a main objective of this study was analyzing the development of *in vitro* antimicrobial resistances in Bavaria for several gram-negative mastitis pathogens over a timespan of nine years, the results of which will be presented in the following articles.

III. PUBLICATIONS

1. Publication I

Kumulative Promotionsleistung: Publikation

***In vitro* antimicrobial resistance of *Escherichia coli, Serratia marcescens, Klebsiella oxytoca,* and *Klebsiella pneumoniae* on Bavarian dairy farms between 2014-2022**

Pirner, L. H.; Petzl, W.; Gangl, A.; Huber-Schlendtedt, R., Sorge, U. S.

Journal of Dairy Science, Accepted April 26, 2024

J. Dairy Sci. 107:8402–8412
https://doi.org/10.3168/jds.2023-24536

In vitro antimicrobial resistance of *Escherichia coli*, *Serratia marcescens*, *Klebsiella oxytoca*, and *Klebsiella pneumoniae* on Bavarian dairy farms between 2014 and 2022

Lea H. Pirner,[1,2]* Wolfram Petzl,[2] Armin Gangl,[1] Reglindis Huber-Schlenstedt,[1] and Ulrike S. Sorge[1]
[1]Department of Udder Health and Milk Quality, Bavarian Animal Health Services, 85586 Poing, Germany
[2]Clinic for Ruminants with Ambulatory and Herd Health Services, Centre for Clinical Veterinary Medicine, Ludwig Maximilian University of Munich, 85764 Oberschleissheim, Germany

ABSTRACT

The objective of this study was to describe the prevalence of antimicrobial resistance of *Escherichia coli*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, and *Serratia marcescens* from quarter milk samples submitted to the udder health laboratory of the Bavarian Animal Health Services (TGD) in Southern Germany between 2014 and 2022. All samples were tested with the California Mastitis Test and analyzed with a standard microbroth dilution to determine the MIC. The antimicrobials tested were amoxicillin/clavulanate, cefazoline, kanamycin/cefalexin, cefoperazone, cefquinome, and marbofloxacin. Breakpoints were chosen in accordance with the Clinical and Laboratory Standards Institute (CLSI). Over the study period, *E. coli*, *K. oxytoca*, and *K. pneumoniae* showed only few resistances to all antimicrobials tested. For those pathogens MIC 50 and MIC 90 were below breakpoint for all antimicrobials except cefoperazone over the 9 years. A decrease in MIC could be seen for *E. coli* and *K. oxytoca* for all of the antimicrobials. While the MIC for *K. pneumoniae* stayed more stagnant, the prevalence of resistance still decreased overall. *Serratia marcescens* isolates were proven intrinsically resistant to amoxicillin/clavulanate and cefazolin, and while in vitro resistances were low for all other antimicrobials tested, *S. marcescens* tended toward higher MIC for most of the antimicrobials over the years. Over time, there was also an overall increase in the number of isolates for all 4 pathogens per year. Starting 2018 there was a steep increase in the number of isolates particularly from clinical cases. This jump in numbers coincided with a change of the regulation for veterinary drug prescriptions in Germany in 2018 that required, among other things, antimicrobial resistance testing before a change of antibiotics in the course of treatment and the use of critically important antimicrobials. Overall, although the pathogens increased in numbers, the prevalence of their antimicrobial resistance remained low.
Key words: mastitis, antimicrobial resistance, gram-negative pathogens

INTRODUCTION

Bovine mastitis is considered to be the most economically relevant disease for the dairy industry. The disease is mostly caused by bacteria that can be grouped by their Gram stain into either gram-positive (e.g., *Streptococcus* spp., *Staphylococcus* spp.) or gram-negative mastitis pathogens (e.g., *Escherichia coli*, *Serratia* spp.).

The group of gram-negative mastitis pathogens were formerly known as "coliform mastitis." Although especially *E. coli* plays the biggest role, other coliforms such as *Klebsiella* spp., and *Serratia* spp., but also non-coliform gram-negatives, such as *Pasteurella multocida*, have been brought more into focus recently as well, with their impact on udder health and difficult treatment being at the center of discussion (Hogan and Smith, 2003; Schukken et al., 2012).

Gram-negative pathogens are commonly known for being able to cause severe clinical signs, including drastic drops in milk yield (Schukken et al., 2009), lower conception rates (Hertl et al., 2010), pregnancy losses (Dahl et al., 2018), loss of function of the affected quarter (Shinozuka et al., 2016), and even death of the animal (Hertl et al., 2011). This is resulting in higher economic losses than gram-positive mastitis cases (Heikkilä et al., 2018).

Most veterinarians will apply intramammary or parenteral antibiotics to treat gram-negative mastitis cases (Du Preez, 2000). However, antimicrobial treatment will often be unnecessary because these pathogens are commonly eliminated from the mammary gland without antimicrobial therapy (Du Preez, 2000; Ruegg, 2021).

Received December 12, 2023.
Accepted April 28, 2024.
*Corresponding author: leapirner@outlook.de

The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

Especially the application of critically important antibiotics is not advisable, since it brings little to no advantage in recovery and may cause an increase in antimicrobial resistance (**AMR**; Nobrega et al., 2020).

Nonetheless, intramammarily applied antimicrobials are still the most commonly used method of treatment in mastitis cases in Bavaria (Sorge et al., 2020). Germany is one of the foremost milk producers in the European Union and has a large number of dairy cattle (Popescu et al., 2019). Within Germany, Bavaria is the federal state with the highest number of dairy farms (16,788), dairy cattle (1.1 million), and milk production (8,050 thousand tonnes; LKV Bayern, 2020; BLE, 2023). Therefore, the use of antibiotics for mastitis therapy is an important issue in that state. Yet little is known about AMR in gram-negative mastitis pathogens in Germany or Bavaria. Only a few studies described the current situation and mostly focused on gram-positive or mastitis pathogens in general (Tenhagen et al., 2006; Bolte et al., 2020; Sorge et al., 2021). Europe-wide susceptibility monitoring programs, such as VetPath, collect milk samples on a large scale, but they only collect samples from clinical cases and from animals that are not currently being treated (Thomas et al., 2015; de Jong et al., 2018). Furthermore, the effect of legislative changes regarding the prescription of antimicrobial substances (BTK, 2018a) or the increasing implementation of selective dry cow treatments on dairy farms can only be evaluated over time in a large population that also includes isolates of healthy animals. As the Bavarian Animal Health Services e.V. (**TGD**) cultures a lot of quarter milk samples from roughly over 4,000 dairy herds per year and the TGD technicians also collect milk samples from animals that are not clinically ill, the changes in antimicrobial resistance of various gram-negative pathogens could be evaluated over time.

Therefore, the objective of this retrospective study was to describe the in vitro antimicrobial resistance of *E. coli*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, and *Serratia marcescens* from quarter milk samples of Bavarian dairy farms between 2014 and 2022.

MATERIALS AND METHODS

Because the samples for this retrospective study were collected only for herd health management and diagnostic purposes, IACUC approval was not necessary.

Sample Population

All quarter milk samples from Bavarian dairy farms that were submitted to the laboratory of the TGD between 2014 and 2022 and that had isolates of either *E. coli*, *K. oxytoca*, *K. pneumoniae*, or *S. marcescens* were included in this analysis. The samples were collected either by TGD technicians during herd screenings, or from individual cows by veterinarians or farmers. Herd screenings were carried out for various reasons: mostly for herd health improvement, but also in cows pre-dry-off for selective dry cow therapy. The average herd size in Bavaria is 44 cows (BLE, 2023). Therefore, herds with fewer than 60 cows were commonly examined in full, whereas for larger herds the sample size was selected individually depending on the number of cows and the reason for sampling. At the sampling on farm or upon arrival of the samples in the laboratory, abnormal milk (clinical mastitis) as well as the score of a California Mastitis Test (**CMT**) were recorded by either on-farm or laboratory TGD personnel, respectively. The milk was collected in 9-mL sample tubes with 1 mL of boric acid and either shipped cooled or uncooled (individual samples) to the laboratory.

Laboratory Analysis

The samples were processed in the TGD laboratory in accordance with the methods of the German Veterinary Association's (DVG) Guidelines (DVG, 2018, or respective edition).

Upon arrival of the quarter milk samples in the laboratory, they were warmed up to 16°C to 18°C and mixed thoroughly before inoculation onto one-quarter of an esculin-blood-agar plate. The inoculation loops were calibrated according to the DVG guidelines. The plates were then incubated at 36 ± 1°C for 18 to 24 h and monitored for cultural growth, as recommended by the DVG guidelines.

Colonies formed were evaluated according to an obligatory testing method of the TGD laboratory and the DVG guidelines, classifying cfu and morphology. For coliform isolates all pure cultures with 10 or more cfu or samples with a positive CMT and a pure culture with 2 or more cfu were considered as a positive result and the isolate was classified as the lead pathogen. In mixed cultures, in cases of reported mastitis and a positive CMT, the predominant isolate on the plate (e.g., forming more than half of the colonies on the plate) was classified as pathogenic and included in the study. Pathogens that did not meet these criteria were classified as contaminants and excluded from the study. Gram-negative rods were then further differentiated with Oxoid Brilliance Eco Colichrome-Agar (Thermo Fisher Scientific Inc.) and MALDI-TOF-MS (Bruker Corporation) to determine the bacterial species.

Because MALDI-TOF-MS evaluations were first implemented in 2014 at the TGD, that year was chosen as the first study year. The pathogens' AMR were as-

sessed by breakpoint analysis using a broth microdilution (breakpoint method, Micronaut-S-System, Merlin Diagnostica GmbH). For the analysis, microtiter plates Micronaut-S Mastitis 3 (penicillin, ampicillin, cefazolin, cefoperazone, cefquinome, oxacillin, pirlimycin, erythromycin, amoxicillin/clavulanate, kanamycin/cefalexin, and marbofloxacin) or Micronaut-S Mastitis 4 (ampicillin, cefoperazone, amoxicillin/clavulanate, kanamycin/cefalexin, oxacillin, erythromycin, marbofloxacin, and pirlimycin) were used. Each microtiter plate also contained predestined wells for growth control. The program used for MIC interpretation was MCN 6 (version MCN 6.00–08.01.2018 Rel. 89 and preceding versions; Demo Computer GmBH and Merlin Diagnostica GmBH).

Breakpoints were evaluated with a photometer (Tecan Sunrise, Demo Computer GmBH) and the program MCN6 version 6.00 and visual post-control and chosen in accordance with Clinical and Laboratory Standards Institute (**CLSI**) documents (CLSI Vet 01S-ED6; CLSI, 2023), where available. Breakpoints for the bacteria and indication of mastitis in dairy cows that were missing from the CLSI documents were taken from values for human medicine, similar pathogens, or different indications in the DVG guidelines (DVG, 2018, or respective edition). Typical intrinsic resistance patterns were used by the program. Intermediate results were included as resistant

Antibiotics that were included on the commercial plate but to which these pathogens have known intrinsic resistances (penicillin, ampicillin, pirlimycin, oxacillin, and erythromycin) were excluded from further analysis (Olivares et al., 2013). According to the CLSI guidelines for human medicine, *S. marcescens* is considered intrinsically resistant to both amoxicillin/clavulanate and cefalexin and therefore resistances of *S. marcescens* against these antimicrobials were not reported. Consequently, the pathogens were evaluated against amoxicillin/clavulanate, kanamycin/cefalexin, cefazolin, cefoperazone, cefquinome, and marbofloxacin

Statistical Analysis

The statistical analysis was done with SAS 9.4 (SAS Analytics Software Institute Inc., SAS Institute GmBH, Heidelberg, Germany). The distribution of MIC observations was summarized for each pathogen and antimicrobial (PROC FREQ) over time (i.e., by year) as well as across mastitis status. A logistic mixed model (PROC GLIMMIX) with resistance (0/1) against an antimicrobial (e.g., amoxicillin/clavulanate) as outcome and year as fixed effect and herd as random effect was attempted for each pathogen. However, the models did not converge because there were too many herds (n > 6,000) with mostly only 1 to 2 isolates. Therefore, differences between the distribution of MIC categories as well as the odds ratio of each pathogen-antimicrobial combination were compared in pairwise comparisons between categories (chi-squared) and only the unadjusted *P*-values of the PROC FREQ procedures are reported. The trend analysis was done with a Cochran Armitage trend analysis across all years (PROC FREQ). All figures were created in Excel (Microsoft Excel for Microsoft 365 MSO, Version 2302, Microsoft Corp.). Missing data were ignored and α was set at 0.05.

RESULTS

Sample Population Description

In total, 3,541,713 quarter milk samples from 902,185 cows from 15,285 herds were submitted to the TGD laboratory between 2014 and 2022, of which 765,894 cows were only sampled once in the 9-year period. Of those, a total of 21,738 quarter milk samples from 5,809 Bavarian dairy farms contained either *E. coli*, *K. oxytoca*, *K. pneumoniae*, or *S. marcescens* isolates that were analyzed with breakpoint analysis between 2014 and 2022 (Table 1). In roughly 90% (n = 16,619) of samples there was only one cow with positive quarter milk samples within the herd and year. In 7.2% (n = 1,331) of cases there were 2 isolates sampled per herd. When 3 or more pathogens were isolated during one sampling, the resistance patterns were comparable to those of isolates from samplings with only 1 or 2 positive quarter milk samples (results not shown).

Among the included isolates, the most frequently isolated pathogen was *E. coli* (71%), followed by *S. marcescens* (20%), *K. pneumoniae* (5%), and *K. oxytoca* (4%).

Most included quarter milk samples (64%) were from subclinical mastitis (CMT 1–3) and 33% from clinical mastitis cases. Only 3% of samples were from healthy quarters (CMT = 0; Table 1). *Serratia marcescens* had a higher percentage in subclinical (77%) and lower percentage in clinical mastitis cases (22%) than the other pathogens ($P < 0.01$).

The number of available isolates increased over the study period. In 2018, an especially steep increase was observed for each of the pathogens, respectively ($P < 0.01$; Table 1). Concurrently, the overall percentage of pathogens isolated from clinical mastitis cases in 2018 increased as well, especially in *E. coli* ($P < 0.01$) and *K. pneumoniae* ($P = 0.01$) isolates, while the number of isolates from subclinical mastitis cases and healthy udder quarters decreased proportionally. Similar to this change in CMT results, a change in sample origin (herd screenings vs. submissions from individual cows) was observed

Table 1. Distribution of isolates of *Escherichia coli*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, and *Serratia marcescens* from quarter milk samples by health status and year analyzed with broth microdilution between 2014 and 2022

Pathogen	Year	All isolates (N)	Healthy[1] (%)	Subclinical mastitis (%)	Clinical mastitis (%)
All		21,738	3	64	33
E. coli	All	15,388	3	61	36
	2014	957	4	70	26
	2015	1,314	4	72	24
	2016	1,428	3	68	29
	2017	1,363	4	66	30
	2018	2,470	4	57	39
	2019	2,182	2	54	44
	2020	1,995	2	57	41
	2021	2,006	2	57	41
	2022	1,673	2	58	40
K. oxytoca	All	921	4	66	30
	2014	58	—	74	26
	2015	118	5	72	23
	2016	70	3	74	23
	2017	71	4	58	38
	2018	130	5	67	28
	2019	117	3	67	30
	2020	101	5	58	37
	2021	111	4	66	30
	2022	145	6	64	30
K. pneumoniae	All	1,005	5	58	37
	2014	41	10	61	29
	2015	52	6	60	34
	2016	46	2	61	37
	2017	71	8	54	38
	2018	125	12	59	29
	2019	107	5	56	39
	2020	133	4	53	43
	2021	198	2	62	36
	2022	323	2	59	39
S. marcescens	All	4,424	1	77	22
	2014	24	—	92	8
	2015	266	1	81	18
	2016	360	2	79	19
	2017	401	2	80	18
	2018	551	2	76	22
	2019	668	1	76	23
	2020	732	1	78	21
	2021	726	1	74	25
	2022	696	—	78	22

[1]Negative California Mastitis Test results.

in 2018 (Table 2). Whereas the distribution between samples was fairly stable until 2017, a 10% increase of individual submissions was observed from 2018 onward ($P < 0.01$). This increase was seen for all 3 categories of udder health status, but was most evident for clinical mastitis cases, where individual submissions increased by roughly 10% in 2018 ($P < 0.01$). When looking at the data of all quarter milk samples submitted to the TGD by year, there was not as steep an increase in numbers in 2018. However, there was a rise in samples from clinical mastitis cases. Additionally, the percentages of individually submitted samples and samples from herd screenings were stable at roughly 20% and 80% over the 9 years (results not shown).

MIC Between 2014 and 2022

The distribution of the MIC for amoxicillin/clavulanate, kanamycin/cefalexin, cefazolin, cefquinome, cefaporazone and marbofloxacin are provided in Supplemental Tables S1 to S4 (see Notes).

All pathogens showed very few resistances to the tested antimicrobials and the MIC 50 and MIC 90 were below the respective breakpoint for all antibiotics (except cefoperazone, Supplemental Tables S2 and S4). There were no differences in resistances between isolates from clinical, subclinical, and nonmastitic cases for *K. oxytoca*, *K. pneumoniae*, or *S. marcescens* for any of the antimicrobials tested ($P = 0.50$). *Escherichia coli*

Table 2. Distribution of isolates by year and udder health status (from healthy quarters [healthy], subclinical mastitis [SCM], and clinical mastitis [CM] cases), based on circumstances of sampling, grouped as individual cow submissions by farmers or veterinarians and routine herd screenings done by TGD technicians

	Year								
Samples	2014	2015	2016	2017	2018	2019	2020	2021	2022
Individual submission									
All (N)	600	962	1,009	1,048	2,198	2,034	1,833	1,938	1,740
All (%)	56	55	53	55	67	66	62	64	63
Healthy[1] (%)	6	5	5	5	4	2	3	2	3
SCM (%)	66	69	66	64	55	54	53	56	55
CM (%)	28	25	29	31	41	44	44	42	42
Herd screening									
All (N)	480	788	895	858	1,078	1,040	1,128	1,103	1,006
All (%)	44	45	47	45	33	34	38	36	37
Healthy[1] (%)	1	2	1	2	4	1	—	—	1
SCM (%)	76	78	75	74	72	72	76	73	77
CM (%)	23	20	24	24	24	27	24	27	22

[1]Negative California Mastitis Test results.

isolated from clinical mastitis cases had a tendency to be more resistant against kanamycin/cefalexin and marbofloxacin than those isolated from subclinical cases and healthy quarters, especially in the years 2018 to 2022 ($P < 0.01$, results not shown). However, there was no difference for any of the other antimicrobials ($P = 0.50$). Individually sampled *E. coli* isolated from subclinical cases showed a tendency to be more resistant to kanamycin/cefalexin, cefazolin, cefoperazone, and marbofloxacin than samples from herd screenings, with no change over the years ($P < 0.01$, results not shown). Individually sampled *S. marcescens* from subclinical cases had fewer resistances against cefquinome and marbofloxacin than isolates from herd screenings, with no change over the years ($P = 0.01$, results not shown). There were no differences in resistances for *K. oxytoca* or *K. pneumoniae*, when comparing sample origin and mastitis status.

Escherichia coli, *K. oxytoca*, and *K. pneumoniae* were only seldomly resistant against both amoxicillin/clavulanate and cefazolin (Supplemental Tables S1 to S3). Over the years the proportion of isolates inhibited at the lowest concentration increased for all 3 antimicrobials ($P < 0.01$).

For kanamycin/cefalexin (Supplemental Tables S1 to S4), there were only few resistances from any of the 4 pathogens. For *S. marcescens* ($P = 0.20$) and *K. pneumoniae* ($P = 0.12$), the resistance patterns stayed mostly similar throughout the 9 years. There was a trend toward higher MIC for *E. coli* and *K. oxytoca* ($P = 0.01$).

Likewise, all pathogens were similarly sensitive to cefoperazone (Supplemental Tables S1 to S4). While the MIC 90 for both *K. oxytoca* and *S. marcescens* were above breakpoint, at the MIC of 4, they still showed an upward trend in the percentage of isolates with the MIC of ≤2 µg/mL ($P < 0.01$). *Klebsiella pneumoniae* only showed slight changes toward lower MIC ($P = 0.03$) and resistance of *E. coli* against cefoperazone decreased over the years ($P < 0.01$).

All pathogens were rarely resistant to cefquinome or marbofloxacin (Supplemental Tables S1 to S4). The distribution of resistant isolates of *K. pneumoniae* stayed consistent from 2014 to 2022 for both antimicrobials (cefquinome, $P = 0.15$; marbofloxacin, $P = 0.47$). *Escherichia coli* and *K. oxytoca* had an increase of the lowest MIC of ≤1 and ≤0.25 µg/mL, respectively ($P < 0.01$). In contrast, *S. marcescens*, though staying highly sensitive to both cefquinome and marbofloxacin, showed a slight tendency toward higher MIC ($P = 0.04$), although its MIC 50 and 90 stayed at the lowest concentration for all 9 years for both antibiotics

Overall *K. pneumoniae* showed the most consistent patterns throughout the years with only minimal changes, mostly toward lower MIC. Similar trends could be observed in *E. coli* and *K. oxytoca*, both tending toward lower MIC and showing only few resistances. In contrast, *S. marcescens* inclined toward higher MIC for cefquinome and marbofloxacin over the years and only showed a trend toward lower MIC for cefoperazone

In Vitro Resistance to Multiple Antimicrobials

Figure 1 provides an overview over the distribution of numbers of antimicrobials to which isolates of different pathogens were in vitro resistant for the years 2014 to 2022.

Overall, 82% (n = 17,899) of all isolates had no in vitro resistances, 9% (n = 1,981) were resistant to only 1, and 4% (n = 884) to 2 antimicrobials, with the numbers decreasing with every added AMR.

Eighty-two percent of *E. coli*, 79% of *K. oxytoca*, 89% of *K. pneumoniae*, and 83% of *S. marcescens* showed no in vitro resistance to any of the tested antimicrobials. The

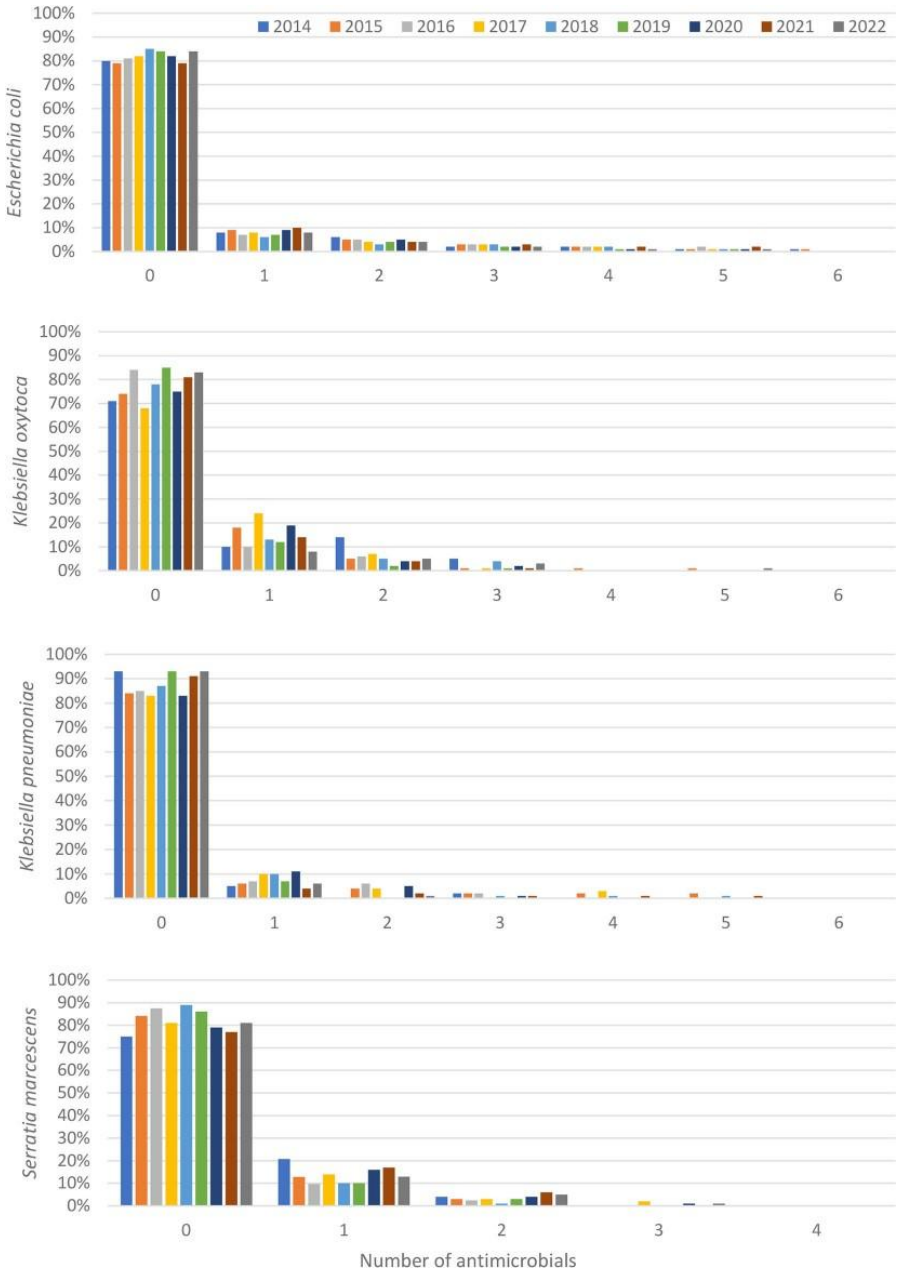

Figure 1. Number of antimicrobial substances tested (0–6) that mastitis pathogen isolates tested in vitro resistant to by year. *Serratia marcescens* was only tested against 4 antimicrobials because of intrinsic resistances against amoxicillin/clavulanate and cefazolin.

percentages of isolates with multiple AMR were low, with 7%, 14%, 6%, and 13% of isolates resistant to 1 antimicrobial, respectively. Here the 3 most common antimicrobials that the isolated pathogens had the most in-vitro resistances to were cefazolin, kanamycin/cefalexin, and cefoperazone; 4% of *E. coli*, 2% of *K. oxytoca*, 5%

of *K. pneumoniae*, and 4% of *S. marcescens* isolates were resistant to 2 antimicrobials. The most common combinations of 2 AMR were cefazoline and cefoperazone (n = 146), as well as kanamycin/cefalexin and cefoperazone (n = 114), and amoxicillin/clavulanate and kanamycin/cefalexin (n = 104). Roughly 2% of all isolates were resistant to 3 (n = 447) antimicrobials, the most common being the combination of cefazolin, cefoperazone, and cefquinome (n = 169). Even fewer isolates were resistant to 4 (n = 272) and 5 (n = 200) antimicrobials.

Throughout the 9 years, the number of isolates with multiple resistances decreased ($P < 0.01$) and only 0.3% of all isolates were resistant to all 6 antimicrobials tested (n = 55).

DISCUSSION

The strength of this study was the large number of isolates available for one laboratory, multiple farms, and udder health scores over a 9-year timespan (2014 to 2022). This allowed us to have a comprehensive overview of the distribution and progression of resistance of the different pathogens.

Among the 4 pathogens, *E. coli* was the most common one, followed by *S. marcescens*, *K. pneumoniae*, and *K. oxytoca*. *Escherichia coli* being the most common gram-negative mastitis pathogen concurs with the findings of other studies (Hogan and Smith, 2003; Malinowski et al., 2006; Younis et al., 2017; Bertolini et al., 2022). Most other studies report *K. pneumoniae* as the second most commonly isolated gram-negative pathogen (Malinowski et al., 2006; Morales-Ubaldo et al., 2023). However, our data included more *S. marcescens* isolates. One reason for this observation could be related to the nature of the infection and the samples of this study. *Serratia* spp. has been associated with subclinical mastitis more than other gram-negative pathogens (Schukken et al., 2012). In their study, Todhunter et al. (1991) reported 82.7% of *Serratia* spp. being isolated from subclinical mastitis cases (Todhunter et al., 1991). This concurs with the roughly 77% of subclinical mastitis cases found in this study for *S. marcescens*, exceeding the other pathogens by more than 10% (Table 1). Unfortunately, there is no other detailed German study about *Serratia* spp. to directly compare with the results of this study. In addition to subclinical mastitis, *Serratia* spp. has also been known for its long duration of infection (Barnum et al., 1958; Hogan and Smith, 2003; Schukken et al., 2012). New infections with *Serratia* spp. have been furthermore associated with the dry period (Todhunter et al., 1991; Hogan and Smith, 2003). Thus, the probability of detection of *Serratia* spp. infections might have been higher in this study because the isolates were not only taken from acute mastitis cases, but mostly routine sampling of the (sometimes entire) lactating herd by TGD technicians that would have included a higher proportion of potentially chronic cases. Contaminated samples were excluded from the study and the lead pathogen of each sample was determined according to strict rules. Although *Serratia* spp. are frequently reported to cause herd outbreaks (Barnum et al., 1958; Ollis and Schoonderwoerd, 1989), we detected no increased quantities of specific isolates within individual herds in our data. Therefore, increased numbers due to contaminated samples as well as herd outbreaks can be ruled out for the most part. Most likely, the above-mentioned factors allowed more *S. marcescens* to be isolated from subclinical infections that would otherwise have gone undiagnosed.

Table 1 showed an overall increase of all 4 pathogens over the 9 years, which aligns with increasing numbers of gram-negative mastitis pathogens observed in other studies (Pitkälä et al., 2004). It has been argued that environmental mastitis pathogens, such as *E. coli*, *S. marcescens*, and *Klebsiella* spp. might be more common in well-managed dairy herds with low bulk-SCC (Hogan and Smith, 2003) and have increased, as classic contagious mastitis pathogens have been decreasing (Schukken et al., 2012). This could explain the continuously rising numbers over the last years, as Bavarian dairy farms have become larger, with their performance increasing (Muñoz et al., 2011; LKV Bayern, 2020).

However, the biggest change in our data was the increase in isolates in 2018, in particular from clinical cases and individual cow submissions. This coincided with changes in the German veterinary dispensary law (Verordnung über tierärztliche Hausapotheken, TÄHAV; BTK, 2018a,b). The change of law came into force at the end of February 2018 and included mandatory antimicrobial sensitivity testing, if critical antimicrobials (e.g., third- or fourth-generation cephalosporins) were selected, when antibiotics were changed during the course of a therapy, if antibiotic treatments lasted longer than 7 d, and if a combination of different antibiotics was the chosen therapy by the veterinarian. The changed characteristics of samples in 2018 was therefore most likely a result of the updated regulation. Interestingly, when looking at the distribution of individually submitted isolates and samples from herd screenings in this study, as compared with the overall sample pool submitted to the TGD by year, there was quite a large difference in percentages. Samples with *E. coli*, *K. oxytoca*, *K. pneumoniae*, and *S. marcescens* were submitted individually by farmers or veterinarians in 55% of cases before, and 65% after 2018 (Table 2). In comparison, only about 20% of all quarter milk samples submitted to the TGD between 2014 and 2022 were individual submissions, with no change over time. This could be explained by gram-positive pathogens making up the majority of the overall

sample population. Because gram-positive pathogens are more likely to cause subclinical infections (Pitkälä et al., 2004; Dyson et al., 2022) than our gram-negative pathogens were, they were likely more commonly isolated in herd screenings, even before the change in legislation in 2018. Because most gram-negative mastitis pathogens are environmental pathogens and will be eliminated from the udder quickly, they are often difficult to detect in the later phase of mastitis (Smith et al., 1985; Hogan and Smith, 2003). With the time of sampling during the course of an infection playing such a critical role in the detection of these pathogens, the likelihood of isolating them increased. Veterinarians were required to take samples from affected quarters immediately after the first clinical signs occurred, if a specific combination of antibiotics was selected for treatment. This can be seen in the big jump in clinical mastitis cases from individually submitted samples in 2018 (Table 2). Mastitis cases that before this change in legislation would have been treated without further diagnostics or that would have been sampled later during the course of mastitis (resulting in "no growth"; Taponen et al., 2009) were now included in the sample pool. One can assume that samples that were taken before a change in therapy, when the initial therapeutic approach failed, also often resulted in "no growth" due to the self-limiting nature of the infections (Smith et al., 1985; Hogan and Smith, 2003). Thus, if therapy failed because of AMR against the initially applied antimicrobials and those pathogens could not be isolated, then those AMR were not taken into consideration in this study. This may have resulted in a possible bias in sample selection, which unfortunately is an unavoidable limitation in this study.

However, even with the numbers of these isolates rising, there was no indication of increasing resistances for most pathogens isolated from clinical cases in our data. Across all pathogens tested, only *S. marcescens* showed an increase in resistances against cefquinome and marbofloxacin. As for the other 3 pathogens, resistances either stayed consistent or declined over the 9 years. Other studies also concur with these findings; they described that over differing numbers of years, there was no indication of decreasing sensitivity to antimicrobials typically used in mastitis treatment (Erskine et al., 2002b; Nam et al., 2009). Much like the results shown in this article, Nüesch-Inderbinen et al. (2019) found, that even though *E. coli* isolates had increased in number, they had also tended toward lower MIC, with resistances decreasing accordingly (Nüesch-Inderbinen et al., 2019). The European VetPath initiative reported similar results: *E. coli* was largely susceptible to antibiotics commonly used for mastitis treatment (Thomas et al., 2015). For *Klebsiella* spp., the percentages for high MIC for *K. pneumoniae* remained stagnant or decreased in our data, and resistances for *K. oxytoca* decreased also for most antimicrobials tested, while the numbers of both pathogens increased from 2014 onward. Fuenzalida et al. (2021) described an increase in detection of *Klebsiella* spp. submitted to the Wisconsin Veterinary Diagnostic Laboratory from 2008 to 2019 as well. Also, there were no observed changes in antimicrobial resistance (Fuenzalida et al., 2021). Generally, *Klebsiella* spp. have been described to have low resistances to commonly used antibiotics (Massé et al., 2020).

However, whereas the other pathogens all tended toward lower MIC, *S. marcescens* did the opposite for all antimicrobials except kanamycin/cefalexin and cefoperazone. On top of rising percentages for higher MIC, *S. marcescens* also showed a high resistance (including a high MIC 90; results not shown) to amoxicillin/clavulanate as well as cefazolin. As noted above, according to the CLSI guidelines for human medicine, *S. marcescens* is considered resistant to both of those antimicrobials, which we have found reflected in our data. Similarly, other studies have found *S. marcescens* isolates to be predominantly resistant to amoxicillin/clavulanate and cefazolin (Nam et al., 2009; Fusté et al., 2012; Liang et al., 2023). Liang et al. (2023) reported all *S. marcescens* isolates in their study to have the plasmid-transferred TEM-resistance gene, which in combination with the CTX-M gene is responsible for β-lactam resistances. This TEM-β-lactamase has also been found by other authors, who associated the TEM-β-lactamase to an inducible, chromosomal cephalosporinase in *S. marcescens* (Farrar and O'Dell, 1976; Bush et al., 1991). It has been discussed that a hyperproduction of this TEM-β-lactamase could partly be responsible for resistance against β-lactam-β-lactamase combinations (Zhao et al., 2008). Additionally, *S. marcescens* has been observed to overproduce an AmpC-β-lactamase in high quantities (Yang et al., 2012). This mechanism has also been shown to overwhelm β-lactamase inhibitors, such as clavulanate, resulting in resistance to β-lactam-β-lactamase combinations as well (Jacoby, 2009; Weindorf et al., 1998). This could potentially explain the resistance against both amoxicillin/clavulanate and cefazolin

Considering our current knowledge, if CLSI were to include *S. marcescens* in bovine mastitis in the future, the classification should be considered intrinsically resistant to amoxicillin/clavulanate and cefazolin, as has already been established in the guidelines for human medicine

But in addition to those known intrinsic resistances, resistance patterns overall have not worsened according to our study, as well as many others from various countries (Erskine et al., 2002b; Nam et al., 2009; Nüesch-Inderbinen et al., 2019; Fuenzalida et al., 2021). Nevertheless, these numbers are still only an indication of in vitro antimicrobial susceptibility. Unfortunately,

in vitro susceptibility does not equate to therapeutic success in the field, as there are many other factors influencing the progression of treatment. Cure rates still tend to be lower for mastitis caused by gram-negative bacteria (Schmenger and Kromker, 2020), and although in theory certain antibiotics should be able to eliminate these pathogens, antibiotic therapy often still fails (Hogan et al., 1994; Du Preez, 2000). It has been argued that antibiotic therapy might not be necessary at all for mild or intermediate mastitis cases caused by gram-negatives. Although some studies have shown less culling in mastitis cases caused by gram-negatives that received antibiotic treatment, as compared with cows that received no treatment (Erskine et al., 2002a; Schukken et al., 2011), in other studies there were no reported differences in gram-negative cases that were treated antibiotically or not at all (Pyörälä et al., 1994; Nobrega et al., 2020). Sometimes antimicrobial treatment even resulted in more days of discarded milk, although according to Fuenzalida and Ruegg (2019) cases caused by *K. pneumoniae* seemed to have worse outcomes when not treated (Fuenzalida and Ruegg, 2019). Even the necessity of antibiotic treatment in highly acute gram-negative mastitis cases is questionable, as success has mostly been correlated with the treatment of bacteremia or toxemia-related pathogenesis (Erskine et al., 2002a; Fuenzalida and Ruegg, 2019; Nobrega et al., 2020). At the same time, even in severe cases of gram-negative mastitis, bacteremia has only been found to occur in around 15% of cases (Krebs et al., 2023) and severe clinical mastitis has been proven to be caused almost as often by gram-positives as it has by gram-negatives (Schmenger and Kromker, 2020), making immediate treatment of severe clinical mastitis in the field all the more difficult. In the end, the treatment of gram-negative mastitis remains a demanding task, and a subject of much discussion in the veterinary field.

CONCLUSIONS

An overall increase in numbers of all 4 pathogens, *E. coli*, *K. pneumoniae*, *K. oxytoca*, and *S. marcescens*, could be observed from 2014 to 2022. A change in the regulation of mandatory antibiograms in 2018 resulted in a steep increase in the number of pathogens, number of isolates from clinical mastitis cases, and individual submissions. *Escherichia coli*, *K. pneumoniae*, and *K. oxytoca* even showed lowering MIC over the years. In spite of intrinsic resistances of *S. marcescens* against amoxicillin/clavulanate and cefazolin, they were mostly sensitive to the other antibiotics. These observations fall in line with many studies from other countries. In summary, despite the increase in isolates and clinical cases, the level of antimicrobial resistance remains low and no noteworthy increase in antimicrobial resistance was observed.

NOTES

This study was made possible with the financial support of the Free State of Bavaria and the Bavarian Joint Founding Scheme for the Control and Eradication of Contagious Livestock Diseases (Bayerische Tierseuchenkasse, Munich, Germany). Supplemental material for this article is available at https://doi.org/10.5282/ubm/data.475 (Pirner et al., 2024). Because the samples for this retrospective study were collected only for herd health management and diagnostic purposes, IACUC approval was not necessary. The authors have not stated any conflicts of interest.

Nonstandard abbreviations used: AMR = antimicrobial resistance; CLSI = Clinical and Laboratory Standards Institute; CM = clinical mastitis; CMT = California Mastitis Test; SCM = subclinical mastitis; TGD = Bavarian Animal Health Services.

REFERENCES

Barnum, D. A., E. L. Thackeray, and N. A. Fish. 1958. An outbreak of mastitis caused by *Serratia marcescens*. Can. J. Comp. Med. Vet. Sci. 22:392–395.

Bertolini, A. B., A. M. Prado, P. J. Thyssen, M. de Souza Ribeiro Mioni, F. L. R. de Gouvea, D. da Silva Leite, H. Langoni, J. C. de Figueiredo Pantoja, V. M. Rall, F. F. Guimaraes, S. F. Joaquim, S. T. Guerra, R. T. Hernandes, S. B. Lucheis, and M. G. Ribeiro. 2022. Prevalence of bovine mastitis-related pathogens, identified by mass spectrometry in flies (Insecta, Diptera) captured in the milking environment. Lett. Appl. Microbiol. 75:1232–1245. https://doi.org/10.1111/lam.13791.

BLE (Bundesanstalt für Landwirtschaft und Ernährung). 2023. Bericht zur Markt- und Versorgungslage mit Milch und Milcherzeugnissen.

Bolte, J., Y. Zhang, N. Wente, and V. Kromker. 2020. In vitro susceptibility of mastitis pathogens isolated from clinical mastitis cases on northern German dairy farms. Vet. Sci. 7:10. https://doi.org/10.3390/vetsci7010010.

BTK. 2018a. Die neue TÄHAV ist in Kraft. Deutsches Tierärzteblatt. 66:484–489.

BTK. 2018b. Synopse zur zweiten Verordnung zur Änderung der Verordnung über tierärztliche Hausapotheken vom 21. February 2018 (BGBl. I S. 213–6).

Bush, K., R. K. Flamm, S. Ohringer, S. B. Singer, R. Summerill, and D. P. Bonner. 1991. Effect of clavulanic acid on activity of beta-lactam antibiotics in *Serratia marcescens* isolates producing both a TEM beta-lactamase and a chromosomal cephalosporinase. Antimicrob. Agents Chemother. 35:2203–2208. https://doi.org/10.1128/AAC.35.11.2203.

CLSI. 2023. CLSI VET01S ED6. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals. 6th ed. CLSI standard VET01S. Clinical and Laboratory Standards Institute, Wayne, PA.

Dahl, M. O., A. De Vries, F. P. Maunsell, K. N. Galvao, C. A. Risco, and J. A. Hernandez. 2018. Epidemiologic and economic analyses of pregnancy loss attributable to mastitis in primiparous Holstein cows. J. Dairy Sci. 101:10142–10150. https://doi.org/10.3168/jds.2018-14619.

de Jong, A., F. El Garch, S. Simjee, H. Moyaert, M. Rose, M. Youala, and E. Siegwart. 2018. Monitoring of antimicrobial susceptibility of

udder pathogens recovered from cases of clinical mastitis in dairy cows across Europe: VetPath results. Vet. Microbiol. 213:73–81. 10.1016/j.vetmic.2017.11.021

Du Preez, J. H. 2000. Bovine mastitis therapy and why it fails. J. S. Afr. Vet. Assoc. 71:201–208. https://doi.org/10.4102/jsava.v71i3.714.

DVG. 2018. Leitlinien zur Labordiagnostik der Mastitis - Probenahme und mikrobiologische Untersuchung. 2018 | 3. Auflage DVG Service (Verlag).

Dyson, R., N. Charman, A. Hodge, S. M. Rowe, and L. F. Taylor. 2022. A survey of mastitis pathogens including antimicrobial susceptibility in southeastern Australian dairy herds. J. Dairy Sci. 105:1504–1518. https://doi.org/10.3168/jds.2021-20955.

Erskine, R. J., P. C. Bartlett, J. L. VanLente, and C. R. Phipps. 2002a. Efficacy of systemic ceftiofur as a therapy for severe clinical mastitis in dairy cattle. J. Dairy Sci. 85:2571–2575. https://doi.org/10.3168/jds.S0022-0302(02)74340-3.

Erskine, R. J., R. D. Walker, C. A. Bolin, P. C. Bartlett, and D. G. White. 2002b. Trends in antibacterial susceptibility of mastitis pathogens during a seven-year period. J. Dairy Sci. 85:1111–1118. https://doi.org/10.3168/jds.S0022-0302(02)74172-6.

Farrar, W. E., and N. M. O'Dell. 1976. β-Lactamases and resistance to penicillins and cephalosporins in *Serratia marcescens*. J. Infect. Dis. 134:245–251. https://doi.org/10.1093/infdis/134.3.245.

Fuenzalida, M. J., E. Furmaga, and N. Aulik. 2021. Antimicrobial resistance in *Klebsiella* species from milk specimens submitted for bovine mastitis testing at the Wisconsin Veterinary Diagnostic Laboratory, 2008–2019. JDS Commun. 2:148–152. https://doi.org/10.3168/jdsc.2020-0031.

Fuenzalida, M. J., and P. L. Ruegg. 2019. Negatively controlled, randomized clinical trial to evaluate intramammary treatment of nonsevere, gram-negative clinical mastitis. J. Dairy Sci. 102:5438–5457. https://doi.org/10.3168/jds.2018-16156.

Fusté, E., G. J. Galisteo, L. Jover, T. Vinuesa, T. G. Villa, and M. Viñas. 2012. Comparison of antibiotic susceptibility of old and current *Serratia*. Future Microbiol. 7:781–786. https://doi.org/10.2217/fmb.12.40.

Heikkilä, A. M., E. Liski, S. Pyorala, and S. Taponen. 2018. Pathogen-specific production losses in bovine mastitis. J. Dairy Sci. 101:9493–9504. https://doi.org/10.3168/jds.2018-14824.

Hertl, J. A., Y. T. Grohn, J. D. Leach, D. Bar, G. J. Bennett, R. N. Gonzalez, B. J. Rauch, F. L. Welcome, L. W. Tauer, and Y. H. Schukken. 2010. Effects of clinical mastitis caused by gram-positive and gram-negative bacteria and other organisms on the probability of conception in New York State Holstein dairy cows. J. Dairy Sci. 93:1551–1560. https://doi.org/10.3168/jds.2009-2599.

Hertl, J. A., Y. H. Schukken, D. Bar, G. J. Bennett, R. N. Gonzalez, B. J. Rauch, F. L. Welcome, L. W. Tauer, and Y. T. Grohn. 2011. The effect of recurrent episodes of clinical mastitis caused by gram-positive and gram-negative bacteria and other organisms on mortality and culling in Holstein dairy cows. J. Dairy Sci. 94:4863–4877. https://doi.org/10.3168/jds.2010-4000.

Hogan, J., and K. L. Smith. 2003. Coliform mastitis. Vet. Res. 34:507–519. https://doi.org/10.1051/vetres:2003022.

Hogan, J. S., K. L. Smith, D. A. Todhunter, and P. S. Schoenberger. 1994. Therapy of experimentally induced coliform mastitis with a *Propionibacterium acnes* product. J. Dairy Sci. 77:462–467. https://doi.org/10.3168/jds.S0022-0302(94)76974-5.

Jacoby, G. A. 2009. AmpC β-lactamases. Clin. Microbiol. Rev. 22:161–182. https://doi.org/10.1128/CMR.00036-08

Krebs, I., Y. Zhang, N. Wente, S. Leimbach, and V. Kromker. 2023. Bacteremia in severe mastitis of dairy cows. Microorganisms 11:1639. https://doi.org/10.3390/microorganisms11071639.

Liang, Z., J. Shen, J. Liu, X. Sun, Y. Yang, Y. Lv, J. Zheng, X. Mou, H. Li, X. Ding, and F. Yang. 2023. Prevalence and characterization of *Serratia marcescens* isolated from clinical bovine mastitis cases in Ningxia Hui Autonomous Region of China. Infect. Drug Resist. 16:2727–2735. https://doi.org/10.2147/IDR.S408632.

LKV Bayern. 2020. Leistungsprüfung und Beratung in der Milchviehhaltung in Bayern 2020. LKV Milchleistungsprüfung 2020.

Malinowski, E., H. Lassa, A. Kllossowska, S. Smulski, H. Markiewicz, and M. Kaczmarowski. 2006. Etiological agents of dairy cows' mastitis in western part of Poland. Pol. J. Vet. Sci. 9:191–194.

Massé, J., S. Dufour, and M. Archambault. 2020. Characterization of *Klebsiella* isolates obtained from clinical mastitis cases in dairy cattle. J. Dairy Sci. 103:3392–3400. https://doi.org/10.3168/jds.2019-17324.

Morales-Ubaldo, A. L., N. Rivero-Perez, B. Valladares-Carranza, V. Velazquez-Ordonez, L. Delgadillo-Ruiz, and A. Zaragoza-Bastida. 2023. Bovine mastitis, a worldwide impact disease: Prevalence, antimicrobial resistance, and viable alternative approaches. Vet. Anim. Sci. 21:100306. https://doi.org/10.1016/j.vas.2023.100306.

Munoz, B., S. Lakner, and B. Brümmer. 2011. Determinants of Economic Growth in Organic Farming: The Case of Bavaria and Baden-Wuerttemberg. 2011 International Congress, Zurich, Switzerland.

Nam, H. M., S. K. Lim, H. M. Kang, J. M. Kim, J. S. Moon, K. C. Jang, J. M. Kim, Y. S. Joo, and S. C. Jung. 2009. Prevalence and antimicrobial susceptibility of gram-negative bacteria isolated from bovine mastitis between 2003 and 2008 in Korea. J. Dairy Sci. 92:2020–2026. https://doi.org/10.3168/jds.2008-1739.

Nobrega, D. B., S. A. Naqvi, S. Dufour, R. Deardon, J. P. Kastelic, J. De Buck, and H. W. Barkema. 2020. Critically important antimicrobials are generally not needed to treat nonsevere clinical mastitis in lactating dairy cows: Results from a network meta-analysis. J. Dairy Sci. 103:10585–10603. https://doi.org/10.3168/jds.2020-18365.

Nüesch-Inderbinen, M., N. Kappeli, M. Morach, C. Eicher, S. Corti, and R. Stephan. 2019. Molecular types, virulence profiles and antimicrobial resistance of *Escherichia coli* causing bovine mastitis. Vet. Rec. Open 6:e000369. https://doi.org/10.1136/vetreco-2019-000369.

Olivares, J., A. Bernardini, G. Garcia-Leon, F. Corona, M. B. Sanchez, and J. L. Martinez. 2013. The intrinsic resistome of bacterial pathogens. Front. Microbiol. 4:103. https://doi.org/10.3389/fmicb.2013.00103.

Ollis, G., and M. Schoonderwoerd. 1989. Alberta. Subclinical mastitis associated with *Pseudomonas aeruginosa* and *Serratia marcescens*. Can. Vet. J. 30:525.

Pirner, L., W. Petzl, A. Gangl, R. Huber-Schlenstedt, and U. Sorge. 2024. Supplementary tables to: In vitro antimicrobial resistance of *Escherichia coli*, *Serratia marcescens*, *Klebsiella oxytoca* and *Klebsiella pneumoniae* on Bavarian dairy farms between 2014–2022. Open Data LMU. https://doi.org/10.5282/ubm/data.475.

Pitkälä, A., M. Haveri, S. Pyorala, V. Myllys, and T. Honkanen-Buzalski. 2004. Bovine mastitis in Finland 2001—Prevalence, distribution of bacteria, and antimicrobial resistance. J. Dairy Sci. 87:2433–2441. https://doi.org/10.3168/jds.S0022-0302(04)73366-4.

Popescu, A., E. Stoian, and V. Şerban. 2019. The EU-28 milk sector trends in the period 2009–2018. Sci. Papers Ser. Manag. Econom. Eng. Agric. Rural Dev. 19:249–264.

Pyörälä, S., L. Kaartinen, H. Käck, and V. Rainio. 1994. Efficacy of two therapy regimens for treatment of experimentally induced *Escherichia coli* mastitis in cows. J. Dairy Sci. 77:453–461. https://doi.org/10.3168/jds.S0022-0302(94)76973-3.

Ruegg, P. L. 2021. What is success? A narrative review of research evaluating outcomes of antibiotics used for treatment of clinical mastitis. Front. Vet. Sci. 8:639641. https://doi.org/10.3389/fvets.2021.639641.

Schmenger, A., and V. Kromker. 2020. Characterization, cure rates and associated risks of clinical mastitis in Northern Germany. Vet. Sci. 7:170. https://doi.org/10.3390/vetsci7040170.

Schukken, Y., M. Chuff, P. Moroni, A. Gurjar, C. Santisteban, F. Welcome, and R. Zadoks. 2012. The "other" gram-negative bacteria in mastitis: *Klebsiella*, *Serratia*, and more. Vet. Clin. North Am. Food Anim. Pract. 28:239–256. https://doi.org/10.1016/j.cvfa.2012.04.001.

Schukken, Y. H., G. J. Bennett, M. J. Zurakowski, H. L. Sharkey, B. J. Rauch, M. J. Thomas, B. Ceglowski, R. L. Saltman, N. Belomestnykh, and R. N. Zadoks. 2011. Randomized clinical trial to evaluate the efficacy of a 5-day ceftiofur hydrochloride intramammary treatment on nonsevere gram-negative clinical mastitis. J. Dairy Sci. 94:6203–6215. https://doi.org/10.3168/jds.2011-4290.

Schukken, Y. H., J. Hertl, D. Bar, G. J. Bennett, R. N. Gonzalez, B. J. Rauch, C. Santisteban, H. F. Schulte, L. Tauer, F. L. Welcome, and Y. T. Grohn. 2009. Effects of repeated gram-positive and gram-negative clinical mastitis episodes on milk yield loss in Holstein dairy cows. J. Dairy Sci. 92:3091–3105. https://doi.org/10.3168/jds.2008-1557.

Shinozuka, Y., S. Kaneko, T. Kurose, A. Watanabe, K. Kuruhara, and K. Kawai. 2016. Factors associated with marketable milk production recovery after treatment of naturally occurring acute coliform mastitis. J. Vet. Med. Sci. 78:917–920. https://doi.org/10.1292/jvms.15-0689.

Smith, K. L., D. A. Todhunter, and P. S. Schoenberger. 1985. Environmental mastitis: Cause, prevalence, prevention. J. Dairy Sci. 68:1531–1553. https://doi.org/10.3168/jds.S0022-0302(85)80993-0.

Sorge, U. S., C. Baumgartner, and M. Resch. 2020. A survey of mastitis detection practices and treatments on dairy farms in Bavaria, Southern Germany. Milk Sci. Int. 73:24–28.

Sorge, U. S., R. Huber-Schlenstedt, and K. Schierling. 2021. In vitro antimicrobial resistance profiles of *Streptococcus uberis*, *Lactococcus* spp., and *Enterococcus* spp. from quarter milk samples of cows between 2015 and 2019 in Southern Germany. J. Dairy Sci. 104:5998–6012. https://doi.org/10.3168/jds.2020-19896.

Taponen, S., L. Salmikivi, H. Simojoki, M. T. Koskinen, and S. Pyorala. 2009. Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing. J. Dairy Sci. 92:2610–2617. https://doi.org/10.3168/jds.2008-1729.

Tenhagen, B. A., G. Koster, J. Wallmann, and W. Heuwieser. 2006. Prevalence of mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Brandenburg, Germany. J. Dairy Sci. 89:2542–2551. https://doi.org/10.3168/jds.S0022-0302(06)72330-X.

Thomas, V., A. de Jong, H. Moyaert, S. Simjee, F. El Garch, I. Morrissey, H. Marion, and M. Vallé. 2015. Antimicrobial susceptibility monitoring of mastitis pathogens isolated from acute cases of clinical mastitis in dairy cows across Europe: VetPath results. Int. J. Antimicrob. Agents 46:13–20. https://doi.org/10.1016/j.ijantimicag.2015.03.013.

Todhunter, D. A., K. L. Smith, and J. S. Hogan. 1991. *Serratia* species isolated from bovine intramammary infections. J. Dairy Sci. 74:1860–1865. https://doi.org/10.3168/jds.S0022-0302(91)78351-3.

Weindorf, H., H. Schmidt, and H. H. Martin. 1998. Contribution of overproduced chromosomal beta-lactamase and defective outer membrane porins to resistance to extended-spectrum beta-lactam antibiotics in *Serratia marcescens*. J. Antimicrob. Chemother. 41:189–195. https://doi.org/10.1093/jac/41.2.189.

Yang, H., J. Cheng, L. Hu, Y. Zhu, and J. Li. 2012. Mechanisms of antimicrobial resistance in *Serratia marcescens*. Afr. J. Microbiol. Res. 6:4427–4437. https://doi.org/10.5897/ajmr11.1545.

Younis, G., A. Awad, and N. Ashraf. 2017. Molecular and phenotypic characterization of antimicrobial resistance in gram negative bacteria recovered from subclinical mastitis. Adv. Anim. Vet. Sci. 5:196–204. https://nexusacademicpublishers.com/uploads/files/AAVS_5_5_196-204.pdf.

Zhao, W.-H., Z.-Q. Hu, G. Chen, R. Ito, and T. Shimamura. 2008. Hyperproduction of inhibitor-susceptible TEM β-lactamase is responsible for resistance of *Serratia marcescens* to β-lactam-β-lactamase inhibitor combinations. Chemotherapy 54:31–37. https://doi.org/10.1159/000112315.

ORCIDS

Lea H. Pirner https://orcid.org/0009-0002-7491-4844
Wolfram Petzl https://orcid.org/0000-0002-7931-2642
Reglindis Huber-Schlenstedt https://orcid.org/0000-0002-4452-0468
Ulrike S. Sorge https://orcid.org/0000-0002-7709-4282

2. Publication II

In vitro antimicrobial resistance of *Pasteurella multocida* and *Mannheimia haemolytica* from bovine mastitis on Bavarian dairy farms between 2015 and 2023

Pirner, L. H.; Petzl, W; Huber-Schlenstedt, R.; Sorge, U. S.

Milk Science International, Accepted March 25, 2025

In-vitro antimicrobial resistance of *Pasteurella multocida* and *Mannheimia haemolytica* from bovine mastitis on Bavarian dairy farms between 2015 and 2023

Lea Pirner[1], [2], Wolfram Petzl[1], Reglindis Huber-Schlenstedt[2], Ulrike Sorge[3]*

[1] Clinic for Ruminants with Ambulatory and Herd Health Services, Centre for Clinical Veterinary Medicine, Ludwig Maximilians University Munich, 85764 Oberschleissheim, Germany; wpetzl@lmu.de

[2] Department of Udder Health and Milk Quality, Bavarian Animal Health Services, 85586 Poing, Germany; Reglindis.Huber@TGD-Bayern.de

[3] Department of Udder Health and Milk Quality, Bavarian Animal Health Services, 85586 Poing, Germany; Ulrike.sorge@tgd-bayern.de

**Lea Pirner, Sonnenstr. 16, 85764 Oberschleissheim GER, 0049899091240, leapirner@outlook.de*

Date submitted: 30/07/2024 *Date accepted: 25/03/2025* *Volume/Page(s): 13-x*

Abstract

As the leading disease in dairy cows, mastitis and its major pathogens have been extensively researched. However, mastitis can also be caused by other, opportunistic pathogens, such as *Pasteurella (P.) multocida* and *Mannheimia (M.) haemolytica*, which are usually associated with bovine respiratory disease. To better understand the effects of these as mastitis pathogens, the objective of this study was to describe the *in-vitro* antimicrobial resistance of *P. multocida* and *M. haemolytica* in quarter milk samples from Bavarian dairy farms between 2015 and 2023. *P. multocida* was isolated almost as frequently from clinical (48.6%), as from subclinical cases (51.1%), while samples with *M. haemolytica* came predominantly from clinical mastitis (82%). And while *P. multocida* was isolated in roughly equal parts (49.6% vs. 50.4%) from samples of herd screenings as well as individual submissions, *M. haemolytica* was more frequently found in individually submitted samples (87.2%). *P. multocida* was *in-vitro* mostly resistant against erythromycin (81.4%) and pirlimycin (95%), and *M. haemolytica* against erythromycin (89.7%), pirlimycin (87.2%), and oxacillin (58.9%). Yet they showed only few resistances to the other tested antimicrobials. The high occurrence of resistances against those few antimicrobials were also reflected in a high percentage of multiple resistances (83.7%). As antimicrobial resistances of those pathogens vary throughout different regions, the numbers in this study were mostly consistent with those from other studies from Germany or Austria. In general, low resistances to penicillin were reported when *P. multocida* and *M. haemolytica* were isolated from cases of mastitis, as well as a high success rate in eliminating the pathogens from the udder. However, the possibility of self-cure remains unexplored for these pathogens. When treatment with antimicrobials was selected, penicillin seemed to be the antimicrobial of choice for mastitis caused by *P. multocida* and *M. haemolytica*.

Keywords: bovine mastitis, Gram-negative mastitis pathogens, minor pathogens, antimicrobial resistance

Introduction

Mastitis is one of the leading diseases of dairy cows worldwide [1]. It has many causative agents, the most common being bacteria [2]. Best known for causing bovine mastitis are pathogens such as *Staphylococcus (St.) aureus, Streptococcus (S.) dysgalactiae, Streptococcus (S.) agalactiae, Streptococcus (S.) uberis*, and *Escherichia (E.) coli* [3]. But there are a many more pathogens that can cause mastitis. While some are acclimated to the udder, also known as contagious mastitis pathogens, others are environmental pathogens and can cause opportunistic infections [4, 5]. Those environmental pathogens can cause varying other diseases and can be found on different areas of the body. An example for this are *Pasteurella (P.) multocida* and *Mannheimia (M.) haemolytica*.

P. multocida and *M. haemolytica* are Gram-negative bacteria that are not primarily known as mastitis pathogens. Both are usually associated with bovine respiratory disease (BRD), a disease which can occur when factors such as stress weaken the immune system [6]. *P. multocida* are most known as bovine nasopharyngeal commensals and opportunistic pathogens [7], while *M. haemolytica* is considered the most important pathogen of the BRD complex, in part because of its virulence factors causing high morbidity [8].

Cases of the two pathogens causing mastitis are rare. *P. multocida* mastitis has been reported mostly in case-studies [7, 9], meanwhile, *M. haemolytica* is more known to cause mastitis in sheep [10]. Although the source of the infection often remains unknown, the upper respiratory system of calves and lambs has been discussed as an important reservoir for both pathogens, the transmission taking place during suckling [9, 10] and anecdotal reports describe rises in

intramammary infections with Pasteurella or Mannheimia spp. in herds with nurse-cows. Unfortunately, due to the rarity of the infections, data on the antimicrobial resistance (AMR) profiles of *P. multocida* and *M. haemolytica* isolated from bovine mastitis are hard to find. Most of the time, the cases were treated according to the results of susceptibility testing of the isolated pathogen with antibiotics (e.g., Penicillin for *P. multocida*) [7]. Conclusive data of AMR profiles are mostly of isolates from BRD [11].

The objective of this retrospective study was to assess the *in-vitro* AMR of *P. multocida* and *M. haemolytica* isolated from bovine mastitis in Bavaria, Germany, from 2015 to 2023.

Material and Methods

Sample Population: All quarter milk samples with either *P. multocida* or *M. haemolytica* isolates that were submitted to the laboratory of the Bavarian Animal Health Services e. V. (TGD) between 2015 and 2023 were included in the analysis. The samples were collected either by TGD technicians during herd screenings or by veterinarians and farmers from individual cows. Herd screenings were carried out for example to improve udder health, and pre-dry-off for selective dry-cow therapy. Herds with fewer than 60 cows were usually examined in full, while in larger herds sample sizes were chosen based on the number of lactating cows and the reason for sampling.

Visually abnormal milk, i.e. clinical mastitis, and the score of a California Mastitis Test (CMT) were recorded by either on-farm personnel at the time of sampling or by TGD staff upon arrival of the samples in the laboratory. The milk was aseptically collected in 9 ml sample tubes with boric acid and shipped cooled (herd tests) or uncooled to the laboratory.

Laboratory Analysis: In the TGD laboratory the samples were processed in accordance with the German Veterinary Association´s (DVG) Guidelines ([12], or respective edition). Since this as a retrospective study IACUC approval was not necessary. Upon arrival in the laboratory, the quarter milk samples were inoculated onto one quarter of an Aesculin-blood-agar plate. The inoculation loops used were calibrated according to DVG Guidelines. The plates were then incubated at 36 +/- 1°C for 18-24 hours and monitored for cultural growth. Colonies formed were evaluated by colony forming units (cfu) and morphology. For non-coliform Gram-negative isolates, cultures with two or more cfu and a positive CMT, or isolates that grew in pure culture, were classified as pathogenic. Gram-negative rods with colony morphology fitting *P. multocida* or *M. haemolytica*, were differentiated with classic biochemical differentiation methods (2015) and MALDI-TOF-MS (Bruker Corporation) (after 2015) to determine the bacterial species.

The pathogens´ AMR were assessed by breakpoint analysis using a broth microdilution (breakpoint method, Micronaut-S-System, Merlin Diagnostica GmBH). For the analysis microtiter plates Micronaut-S Mastitis 3 (Penicillin, ampicillin, oxacillin, amoxicillin/clavulanate, kanamycin/cefalexin, cefazolin, cefoperazone, cefquinome, marbofloxacin, pirlimycin, and erythromycin) or Micronaut-S Mastitis 4 (Ampicillin, cefoperazone, amoxicillin/clavulanate, kanamycin/cefalexin, oxacillin, erythromycin, marbofloxacin, and pirlimycin) were used. Each microtiter plate also contained predestined wells for growth control. The program used for MIC interpretation was MCN 6 (version MCN 6.00 – 08.01.2018 Rel. 89 or preceding versions; Demo Computer GmBH and Merlin Diagnostica GmBH).

Breakpoints were evaluated with a photometer (Tecan Sunrise, Demo Computer GmBH) and the program MCN6 version 6.00 and visual post-control and chosen in accordance with CLSI-documents [13], where available. Breakpoints that were not available for the specific bacteria and indication of mastitis in dairy cattle were taken from values for human medicine, similar pathogens, or different indications in the DVG guidelines. Intermediate results were included as resistant. Multidrug resistance (MDR) was defined as isolates that were resistant to more than one antimicrobial.

Statistical analysis: The statistical analysis was done in SAS 9.4 (SAS Analytics Software Institute Inc., SAS Institute GmbH Heidelberg). To summarize breakpoint observations, PROC FREQ procedures were used by year for each pathogen and mastitis status. Differences in MIC distributions and the odds ratio of each pathogen-antimicrobial-combination were compared by year (CHI SQUARE). Only unadjusted p-values of the PROC FREQ procedures were reported. Cochran Armitage was used for trend analysis across all years (PROC FREQ). All figures were created in Excel (Microsoft Excel for Microsoft 365 MSO, Version 2302). Missing data were ignored and α was set at 0.05.

Results

Sample Population Description: In total, 3,503,410 quarter milk samples from 757,562 cows and 17,929 herds were analyzed in the TGD laboratory between 2015 and 2023. Of those, 319 samples from 223 herds contained either *P. multocida* or *M. haemolytica* and were analyzed with breakpoint analysis during the 9-year-period (Table 1). All isolates of *M. haemolytica* came from a single cow per farm. In contrast, 95% (n=229) of *P. multocida* were isolated from one cow per herd. However, in 3.7% (n=9) there were two positive cows per herd and one herd had 3 cows with *P. multocida* isolates at the same sampling date. In short, the vast majority of isolates (94.4%, n=294) was only one isolate per cow, herd, and sampling.

Of the two pathogens, *P. multocida* was isolated more frequently (n=280), with a slight increase in the number of positive samples over the 9 years (p=0.05). *M. haemolytica* (n=39) had only a few isolates each year and no temporal change in the number of isolates was observed (p=0.88, table 1).

Table 1: Distribution of isolates of *Pasteurella multocida* and *Mannheimia haemolytica* from quarter milk samples by health status and year analyzed with broth microdilution between 2015 and 2023.

Pathogen	Year	All isolates (N)	Clinical status of quarter		
			Healthy[1] (%)	Subclinical mastitis (%)	Clinical mastitis (%)
Pasteurella multocida	**all**	**280**	**0.3**	**51.1**	**48.6**
	2015	21	-	52	48
	2016	18	-	50	50
	2017	27	-	44	56
	2018	31	-	74	26
	2019	36	3	39	58
	2020	40	-	40	60
	2021	37	-	49	51
	2022	38	-	50	50
	2023	32	-	66	34
Mannheimia haemolytica	**all**	**39**	**2.6**	**15.4**	**82.0**
	2015	3	-	-	100
	2016	5	-	20	80
	2017	4	3	25	75
	2018	2	50	-	50
	2019	4	-	25	75
	2020	7	-	29	71
	2021	4	-	-	100
	2022	5	-	-	100
	2023	5	-	20	80

[1] Negative California Mastitis Test results

Only three *P. multocida* isolates originated from healthy quarters, while nearly as many positive samples were from clinical mastitis cases (51.1%), as subclinical mastitis cases (48.6%). This did not change over

Table 2: Distribution of MIC, MIC50 and MIC90 for *Pasteurella multocida* of quarter milk samples by antimicrobial, vertical lines indicate breakpoints. The MIC50 and MIC90 (M50/90) denote the MIC where 50% or 90% of isolates were susceptible to tested antibiotics, respectively.

Antimicrobial	MIC (µg/mL)				
Penicillin	**<=0.125**	**0.25**	**>=0.5**		
	97.3% M50/90	2.0%	0.7%		
Ampicillin		**<=4**	**>16**		
		99.3% M50/90	0.7%		
Amoxicillin/ clavulanate	**<=4/2**	**8/4**	**16/8**	**>=32/16**	
	93.6% M50/90	4.6%	1.1%	0.7%	
Oxacillin	**<=1**	**2**	**>=4**		
	88.6% M50	5.4% M90	6.1%		
Kanamycin/ cefalexin	**<=4/0.4**	**8/0.8**	**16/1.6**	**>=32/3.2**	
	80.9% M50	10.8% M90	7.2%	1.1%	
Cefazolin	**<=4**	**8**	**16**	**>=32**	
	97.8% M50/90	0.4%	0.4%	1.4%	
Cefoperazone	**<=2**	**4**	**8**	**>=16**	
	98.0% M50/90	0.8%	0.4%	0.8%	
Cefquinome	**<=1**	**2**	**4**	**>=8**	
	96.8% M50/90	2.4%	0.4%	0.4%	
Marbofloxacin	**<=0.25**	**0.5**	**1**	**>=2**	
	91.4% M50/90	3.6%	4.6%	0.4%	
Erythromycin	**<=0.25**	**0.5**	**1**	**2**	**>=4**
	10.4%	8.2%	20.7%	39.6% M50	21.1% M90
Pirlimycin	**<=1**	**2**	**>=4**		
	4.6%	0.4%	95.0% M50/90		

time (p=0.2). In contrast, over the years (p=0.12) the majority of *M. haemolytica* isolates originated mainly from clinical mastitis cases (82%), while isolates from subclinical (15.4%) or healthy quarters (2.6%) were few (Table 1).

When looking at submitted samples that contained *P. multocida*, the quarter milk samples were fairly evenly distributed between individual submissions by farmers (49.6%) and herd screenings (50.4%), with no change over the years (p=0.58). *P. multocida* from individual submissions were isolated slightly more often from subclinical and less frequently from clinical cases, than those from herd screenings (p=0.03, results not shown).

M. haemolytica was more frequently isolated from individual cases (87.2%) than during herd screenings (12.8%), which also stayed consistent over the sample period (p=0.74). For *M. haemolytica*, the ratio of subclinical and clinical cases did not change depending on sample origin (p=0.89, results not shown).

MIC between 2015 and 2023: Tables 2 and 3 show the distribution of minimum inhibitory concentrations (MIC), as well as MIC 50 and 90, for *P. multocida* and *M. haemolytica* against penicillin, ampicillin, amoxicillin/clavulanate, oxacillin, kanamycin/cefalexin, cefazolin, cefoperazone, cefquinome marbofloxacin, erythromycin, and pirlimycin.

Few *P. multocida* were resistant against penicillin, ampicillin, and

Table 3: Distribution of MIC, MIC50 and MIC90 for *Mannheimia haemolytica* of quarter milk samples by antimicrobial, vertical lines indicate breakpoints. The MIC50 and MIC90 (M50/90) denote the MIC where 50% and 90% of isolates were susceptible to tested antibiotics, respectively.

Antimicrobial	MIC (µg/mL)				
Penicillin	**<=0.125**	**0.25**	**>=0.5**		
	76.2% M50	14.3% M90	9.5%		
Ampicillin		**<=4**	**>16**		
		100% M50/90	-		
Amoxicillin/clavulanate	**<=4/2**	**8/4**	**16/8**	**>=32/16**	
	92.3% M50/90	7.7%	-	-	
Oxacillin	**<=1**	**2**	**>=4**		
	38.5%	2.6%	58.9% M50/90		
Kanamycin/cefalexin	**<=4/0.4**	**8/0.8**	**16/1.6**	**>=32/3.2**	
	56.4% M50	28.2%	15.4% M90	-	
Cefazolin	**<=4**	**8**	**16**	**>=32**	
	100% M50/90	-	-	-	
Cefoperazone	**<=2**	**4**	**8**	**>=16**	
	97.4% M50/90	-	-	2.6%	
Cefquinome	**<=1**	**2**	**4**	**>=8**	
	92.3% M50/90	7.7%	-	-	
Marbofloxacin	**<=0.25**	**0.5**	**1**	**>=2**	
	87.2% M50	7.7% M90	5.1%	-	
Erythromycin	**<=0.25**	**0.5**	**1**	**2**	**>=4**
	7.7%	2.6%	15.4%	12.8%	61.5% M50/90
Pirlimycin	**<=1**	**2**	**>=4**		
	7.7%	5.1%	87.2% M50/90		

1

cefazolin. The presence of resistant *P. multocida* isolates against oxacillin, cefoperazone, and cefquinome was equally low, and the predominant MIC even decreased further from 2015 onward ($p<0.01$). The MIC against amoxicillin/clavulanate, kanamycin/cefalexin, and marbofloxacin increased over the years ($p<0.01$) - although, their MIC 50 and 90 remained below their respective breakpoints. Among the tested antimicrobials, most *P. multocida* isolates were resistant against erythromycin (81.4%) and pirlimycin (95.0%). Both MIC 50 and 90 were well above their respective breakpoints ($p<0.01$) (Table 2).

While only a few *M. haemolytica* isolates were resistant against penicillin, cefoperazone, or marbofloxacin, none of the tested isolates were resistant against ampicillin, amoxicillin/clavulanate, cefazolin, or cefquinome. However, *M. haemolytica* were increasingly resistant against kanamycin/cefalexin, as the MICs of 8/0.8 µg/mL and 16/1.6 µg/mL grew over the years ($p<0.01$). Throughout the study period, most *M. haemolytica* isolates were resistant against erythromycin (89.7%), pirlimycin (87.2%), and oxacillin (58.9%), (P= 0.; table 3).

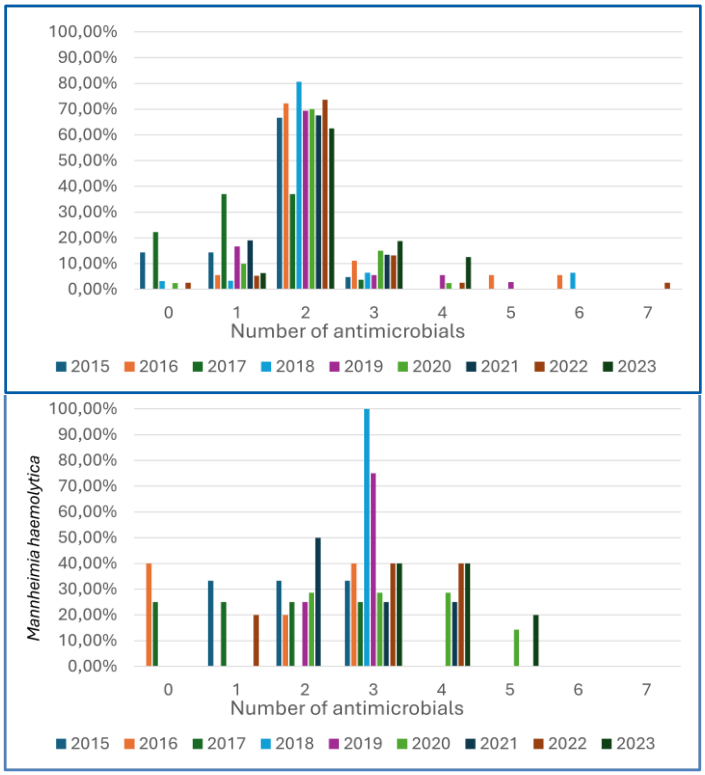

Figure 1: Number of antimicrobial substances tested that mastitis pathogen isolates tested *in-vitro* resistant to by year.

Multiple resistances: Figure 1 shows the number of isolates that were resistant against more than one antimicrobial. Most *P. multocida* isolates were resistant against two antimicrobials (67.1%, n=188), while roughly 13% were resistant to one (n=36), and 10.7% were resistant against 3 antimicrobials (n=30). Overall, only 4.3% (n=12) of *P. multocida* isolates showed no resistances, but their numbers declined over the years ($p<0.01$,). None of the *P. multocida* isolates was resistant against more than 7 antimicrobials at the same time.

The proportion of multi-resistant *M. haemolytica* stayed consistent from 2015 onward (p=0.59). Overall, 41% of *M. haemolytica* isolates were resistant to three (n=16), 20.5% isolates were resistant to two (n=8), and roughly 18% resistant to four antimicrobials (n=7). 7.7% of isolates were resistant to none (n=3) or one (n=3) antimicrobial, respectively, while none were resistant to more than 5 of the tested antimicrobials.

The most common combination of antimicrobials that *P. multocida* isolates were resistant against, was erythromycin and pirlimycin (n=182), which made up 67.9% of MDR by *P. multocida*. The next most common combinations were kanamycin/cefalexin, erythromycin, and pirlimycin (n=14, 5.2%) and oxacillin, erythromycin, and pirlimycin (n=11, 4.1%).

The combination of antimicrobials, that *M. haemolytica* was most commonly resistant to, was oxacillin, erythromycin, and pirlimycin (n=15), making up 41.7% of MDR by *M. haemolytica*. The second and third most common MDR were the combinations of erythromycin and pirlimycin (n=8, 22.2%) and oxacillin, kanamycin/cefalexin, erythromycin, and pirlimycin (n=4, 11.1%), respectively.

The antimicrobial both pathogens were most resistant to was pirlimycin (results not shown).

Discussion

The strength of this study is the number of samples collected over a long period of time. Both pathogens, especially *M. haemolytica,* are rarely isolated from milk samples and a continued isolation over time gives us more insight into the resistance patterns of those non-coliform Gram-negatives as mastitis pathogens.

Both *P. multocida* and *M. haemolytica* were often isolated from quarters affected with clinical mastitis. This high percentage in clinical mastitis aligned with a herd outbreak description by D. A. Barnum (1954). There, the infected quarters showed severe signs of clinical mastitis and eventually dried out completely, but none of the cows suffered systemic signs of inflammation [9]. Other studies also mention that clinical signs are very common in mastitis caused by *P. multocida* or *M. haemolytica* - with symptoms varying from abnormal milk with no macroscopical tissue damage to the infected quarter to severe clinical signs [7, 14, 15].

When looking at research on *P. multocida* and *M. haemolytica* isolated specifically from quarter milk samples, most studies report on isolated cases or herd outbreaks. In some of them, cases of shipping fever or pneumonia were documented before the mastitis cases occurred [9, 15]. However, there was no incidence in herd clustering in our data. And while the most discussed path of infection is suckling by infected calves [9, 10], we could not detect an increase in incidence of *P. multocida* or *M. haemolytica* mastitis in herds after they began using nurse cows (results not shown).

As a course of treatment, most of the isolates proved to be susceptible to penicillin and studies show that the pathogens were eliminated from the udder after treatment [7, 15]. None of those studies, however, describe the possibility of self-cure of the infected quarters, which is a phenomenon that can be frequently observed with other Gram-negative mastitis pathogens [16]. A study in Switzerland from 2023 that looked at mastitis in beef cows also found that *P. multocida* isolated from milk samples were susceptible to Penicillin, as well as Cefazolin [14], which aligns with our observations. In addition, most of the other MIC reported by Vollweider (2023) also coincided with ours. However, the MIC 90 against oxacillin and kanamycin/cefalexin was still below the respective breakpoint in our study, while the MIC 90 reported by Vollweider (2023) were above those breakpoints. Some antimicrobials were not included in our study, but isolates in other studies were frequently resistant to the following antimicrobials: tetracycline, chloramphenicol, neomycin, streptomycin spiramycin, and sulfonamides [7, 14]. Studies about AMR of bovine *P. multocida* and *M. haemolytica* from different sources often reported similar resistance patterns, especially when the data derived from the same geographic regions [11, 17]. Especially studies from Germany also reported low resistances toward penicillin, with isolates being the most resistant against spectinomycin and tetracycline, amongst other antimicrobials that differed in between studies [8, 11, 17].

P. multocida was largely resistant against erythromycin and pirlimycin in our study. Consequently, a large percentage of isolates

also showed multidrug resistance (MDR) against those two antimicrobials. In the same manner, the majority (41%) of *M. haemolytica* isolates showed MDR against three antimicrobials, those antimicrobials being oxacillin, erythromycin, and pirlimycin. Different studies on isolates from quarter milk samples, as well as samples from BRD report a rising incidence in MDR [7, 17], especially for *P. multocida* [11]. Again, MDR were different depending on the geographic regions.

These resistances may be explained by different resistance genes, that can often be transferred between pathogens [17]. A resistance against macrolides has been described in multiple studies from varying regions, in both *P. multocida* and *M. haemolytica*, caused by macrolide-resistance-genes erm(42), mph(E), and msr(E), expressed in different combinations [18, 19], as well as the mef(C) and mph(G) genes [20]. According to Desmolaize et al. (2011), two of the types of macrolide resistance coincide with a resistance against lincosamides [18], explaining the resistances of *P. multocida* and *M. haemolytica* isolates in this study against both erythromycin and pirlimycin. Though only shown here by *M. haemolytica* isolates, resistances against beta-lactams and aminoglycosides (especially kanamycin) have been described in other studies as well [8, 19, 20].

Despite the very clinical presentation and high incidence of MDR of mastitis due to *P. multocida* and *M. haemolytica*, the therapy seemed to be surprisingly simple. Although the resistances vary throughout different regions, in Germany at least penicillin only showed very few resistances. Unfortunately, the number of studies on mastitis by *P. multocida* and *M. haemolytica* is sparse and the question of self-cure remains. Whether antimicrobial therapy is completely necessary or if the pathogens would still be eliminated from the infected quarter, if untreated, ultimately has no conclusive answer at this point in time. In the end, the decision of treatment has to be left up to the practicing veterinarian in those rare cases.

Conclusion

In conclusion, roughly equal numbers of isolates originated from individual cases as herd sampling and were largely isolated from cases of clinical mastitis, especially *M. haemolytica*. *In-vitro* resistances remained mostly similar throughout the years, with *P. multocida* being largely *in-vitro* resistant against erythromycin and pirlimycin, and *M. haemolytica* against oxacillin, kanamycin/cefalexin, erythromycin, and pirlimycin, while both were over 90% sensitive to the other antimicrobials tested. Although the theory of spontaneous self-cure has yet to be explored, if antimicrobial treatment were elected, penicillin seemed to be the antimicrobial of choice.

Disclosure of conflicts of interest

The authors declare no potential conflicts of interest. xxxx

Compliance with Ethical Standards

This study has been conducted in compliance with ethical standards. xxxxx

Acknowledgments

This study was made possible with the financial support of the Free State of Bavaria and the Bavarian Joint Founding Scheme for the Control and Eradication of Contagious Livestock Diseases (Bayerische Tierseuchenkasse). The authors have not stated any conflicts of interest.

References

1. Puerto MA, et al., The hidden cost of disease: I. Impact of the first incidence of mastitis on production and economic indicators of primiparous dairy cows. J Dairy Sci, 2021. 104(7): p. 7932-7943. https://doi.org/10.3168/jds.2020-19584
2. Dufour, S., J. Labrie, and M. Jacques, The Mastitis Pathogens Culture Collection. Microbiol Resour Announc, 2019. 8(15). https://doi.org/10.1128/MRA.00133-19
3. Jain, N.C., Common mammary pathogens and factors in infection and mastitis. J Dairy Sci, 1979. 62(1): p. 128-34. https://doi.org/10.3168/jds.S0022-0302(79)83214-2
4. Klaas, I.C. and R.N. Zadoks, An update on environmental mastitis: Challenging perceptions. Transbound Emerg Dis, 2018. 65 Suppl 1: p. 166-185. https://doi.org/10.1111/tbed.12704
5. Morales-Ubaldo, A.L., et al., Bovine mastitis, a worldwide impact disease: Prevalence, antimicrobial resistance, and viable alternative approaches. Vet Anim Sci, 2023. 21: p. 100306. https://doi.org/10.1016/j.vas.2023.100306
6. Dabo SM, T.J., Confer AW, Pasteurella multocida and bovine respiratory disease. Anim Health Res Rev., 2007. Dec;8(2):129-50. doi: 10.1017/S1466252307001399
7. Milanov, D., et al., Pasteurella multocida mastitis in cow - case report. Veterinarski glasnik, 2017. 71(2): p. 117-122. https://doi.org/10.2298/vetgl170619011m
8. Noyes, N.R., et al., Mannheimia haemolytica in feedlot cattle: prevalence of recovery and associations with antimicrobial use, resistance, and health outcomes. J Vet Intern Med, 2015. 29(2): p. 705-13. https://doi.org/10.1111/jvim.12547
9. Barnum, D.A., A Herd Outbreak of Mastitis Caused by Pasteurella Multocida. Can J Comp Med Vet Sci, 1954. 18(4): p. 113-9. https://www.ncbi.nlm.nih.gov/pubmed/17648709
10. Omaleki, L., et al., The upper respiratory tract is a natural reservoir of haemolytic Mannheimia species associated with ovine mastitis. Veterinary Microbiology, 2015. Volume 181,(Issues 3–4,): p. Pages 308-312, https://doi.org/https://doi.org/10.1016/j.vetmic.2015.10.006
11. Melchner, A., et al., Antimicrobial Resistance in Isolates from Cattle with Bovine Respiratory Disease in Bavaria, Germany. Antibiotics (Basel), 2021. 10(12). https://doi.org/10.3390/antibiotics10121538
12. DVG, D.V.G., Leitlinien zur Labordiagnostik der Mastitis - Probenahme und mikrobiologische Untersuchung. 2018. 3. Auflage DVG Service (Verlag).
13. CLSI-VET01, CLSI VET01S ED6:2023 — Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals, 6th Edition, ed. M.T. Sweeney. 2023. 164. Clinical and Laboratory Standards Institute, Wayne, PA.
14. Vollweider, A., Mastitis pathogens and antibiotic resistance in beef cows in Switzerland, in Department for livestock , clinic for reproductive medicine 2023, University of Zurich: Vetsuisse-faculty of the University of Zurich. p. 20. Schweiz Arch 366 Tierheilkd. 2023 Jan;165(1):39-51. English. doi: 10.17236/sat00381.
15. Maplesden, D.C. and G.R. Carter, Bovine Mastitis Caused By Pasteurella Haemolytica: A Case Report. Can J Comp Med Vet Sci, 1955. 19(9): p. 295-6. https://www.ncbi.nlm.nih.gov/pubmed/17648849
16. Ruegg, P.L., What Is Success? A Narrative Review of Research Evaluating Outcomes of Antibiotics Used for Treatment of Clinical Mastitis. Front Vet Sci, 2021. 8: p. 639641. https://doi.org/10.3389/fvets.2021.639641

17. Kehrenberg, C., et al., Antimicrobial resistance in Pasteurella and Mannheimia: epidemiology and genetic basis. Veterinary Research, 2001. 32 (3-4): p. 323-339. https://doi.org/https://hal.science/hal-323 00902701
18. Desmolaize, B., et al., Combinations of macrolide resistance determinants in field isolates of Mannheimia haemolytica and Pasteurella multocida. Antimicrob Agents Chemother, 2011. 55(9): p. 4128-33. https://doi.org/10.1128/AAC.00450-11
19. Alhamami, T., et al., First Emergence of Resistance to Macrolides and Tetracycline Identified in Mannheimia haemolytica and Pasteurella multocida Isolates from Beef Feedlots in Australia. Microorganisms, 2021. 9(6). https://doi.org/10.3390/microorganisms9061322
20. Schink, A.K., et al., Novel multiresistance-mediating integrative and conjugative elements carrying unusual antimicrobial resistance genes in Mannheimia haemolytica and Pasteurella multocida. J Antimicrob Chemother, 2022. 77(7): p. 2033-2035. https://doi.org/10.1093/jac/dkac116

DISCUSSION

1. Gram-negative mastitis pathogens

In this retrospective study, an overview of *in vitro* antimicrobial resistance of several gram-negative mastitis pathogens in the state of Bavaria, Germany, could be obtained. This included antimicrobial resistance patterns of well-known mastitis pathogens *E. coli*, *Klebsiella* spp., and *Serratia marcescens*, as well as lesser-known mastitis pathogens *Pasteurella multocida* and *Mannheimia haemolytica*.

The biggest strength of this study was the high number of quarter milk samples provided by the Bavarian Udder Health Services. This was especially noticeable for the pathogens *P. multocida* and *M. haemolytica*, that were explored in the second publication, because the number of studies on this subject is sparse at best.

When looking at the total numbers of pathogens isolated at the TGD from 2014 –2023, the percentage of gram-negatives was relatively low with on average 8.1% per year, *E. coli* being the most prevalent with 4.3% per year. In addition, the prevalence amongst gram-negative mastitis pathogens in Bavaria could be observed. Most frequently isolated in this study was *E. coli*, followed by *S. marcescens*, *K. pneumoniae*, and *K. oxytoca*. Least often isolated were *P. multocida* und *M. haemolytica*. With most of the analyzed gram-negative pathogens a rise in incidence could be seen over the evaluated period of nine years. This coincides with observations of several studies, especially from countries with a well-developed, industrialized farming sector (HOGAN & SMITH, 2003; PITKALA et al., 2004). And although a high incidence of gram-negative CM can mostly be seen in the US, the structure change of the German dairy industry towards bigger, more automated dairy farms might introduce similar developments in the future (JONGENEEL et al., 2005). As latent gram-positive mastitis pathogen infections decrease, especially in modern, well-managed dairies, gram-negative pathogens are becoming more and more prevalent (SCHUKKEN et al., 2012). This rise in

isolates could mainly be seen for the more frequently isolated gram-negative pathogens, namely *E. coli* and *Klebsiella* spp., and *S. marcescens*. Most striking was a steep increase in isolates in 2018, especially in submissions from clinical cases in 2018, which overlapped with a legislative change regarding the prescription of antimicrobial substances in Germany (BTK, 2018). This rise in 2018 could not be seen in gram-positive pathogens isolated by the TGD (BECHTOLD et al., 2024a; KARELL et al., 2024). This may likely be caused by the high number of gram-negative pathogens sent in from samplings of individual cows, submitted by veterinarians and farmers, while gram-positives were predominantly isolated in herd screenings (69-80%) and stayed consistent over the years (BECHTOLD et al., 2024a; KARELL et al., 2024). In contrast, the individual submissions of largely clinical cases of gram-negatives rose by 10 % in 2018, when susceptibility testing in the form of an antibiogram became obligatory, e.g. before changes or combination of antimicrobials (BTK, 2018).

2. Antimicrobial resistance

At the core of this study was the antimicrobial resistance of the beforementioned pathogens. Although many studies have proven antimicrobials to have little effect against many gram-negatives in mastitis treatment (HOGAN et al., 1994; DU PREEZ, 2000; SCHMENGER & KRÖMKER, 2020), they are still the primary means of treatment internationally and in Germany (DU PREEZ, 2000; SORGE et al., 2019; PREINE et al., 2022). Based on this frequent use of antibiotics one would expect an increase in antimicrobial resistance of mastitis pathogens over the years. This, however, was not observed in this study. Only few combinations of pathogens and antimicrobials showed increased resistances over the years (e.g. *S. marcescens* and cefoperazone, *S. marcescens* and cefquinome, *K. oxytocin* and cefoperazone), while most stayed stagnant or even decreased in resistance over time. However, it must be noted that many of the first line antimicrobials typically used in mastitis treatment in Germany are beta-lactams,

against which coliforms are intrinsically resistant. With IR varying between different pathogens, such as the IR of *S. marcescens* against amoxicillin/clavulanate and cefazolin (Fusté et al., 2012; Liang et al., 2023; Nam et al., 2009), this further complicates the antimicrobial treatment of gram-negative mastitis pathogens.

But the AMR of mastitis pathogens does not only vary among species, but also between geographic regions. Specifically, the AMR of *P. multocida* and *M. haemolytica* in other regions differed from our results, while studies from Germany and Austria found similar resistance patterns to the isolates in this study (KEHRENBERG et al., 2001; VOLLWEIDER, 2023). This is possible through the exchange of resistance genes, which facilitates the transfer of resistances between bacterial strains, and even different species. It has also been confirmed that gene transfers accelerate the development of antibiotic resistances in several pathogens (MADDAMSETTI et al., 2024; WANG et al., 2024a; WANG et al., 2024b). This spread of resistance genes once again underlines the importance of monitoring antimicrobial resistance in order to better assess different pathogens´ susceptibility to standardly used antibiotics and its development over time. Of the antimicrobials used in this study, *P. multocida* and *M. haemolytica* were both resistant to pirlimycin and erythromycin. A resistance that has also been described in studies from various different countries (NOYES et al., 2015; MILANOV et al., 2017), and that can be attributed to a combined resistance against macrolides and lincosamides consisting of different resistance genes (DESMOLAIZE et al., 2011; ALHAMAMI et al., 2021; SCHINK et al., 2022). However, in this study only *M. haemolytica* showed a resistance against beta-lactams (oxacillin) and, in part, aminoglycosides (kanamycin/cefalexin), which has also been described for *P. multocida*, but could not be confirmed here (KEHRENBERG et al., 2001). An antimicrobial, that both *P. multocid*a and *M. haemolytica* were rarely resistant to, was penicillin, which was also described to lead to complete elimination of those pathogens from the udder, if used in other studies (MAPLESDEN & CARTER, 1955; MILANOV et al., 2017).

As other gram-negatives are notoriously intrinsically resistant against penicillin, this inevitably leads to the question of self-elimination of said pathogens (RUEGG, 2021). Especially, as the self-cure rate of udders infected with *P. multocida* or *M. haemolytica* is virtually unexplored.

When looking at *E. coli*, many studies have shown little to no benefit to antimicrobial treatment (PYÖRÄLÄ et al., 1993; FUENZALIDA & RUEGG, 2019). This has been true for other gram-negatives as well, like *S. marcescens*, which, like *E. coli*, can result in a self-limiting infection. But *S. marcescens* mastitis can also become highly chronic and almost impossible to treat, with pathogens persisting in the udder for months or even years, with the ability of turning into clinical mastitis flare-ups, when stressors occur (BARNUM et al., 1958; TODHUNTER et al., 1991; HOGAN & SMITH, 2003). Infections with *Klebsiella* spp. have also been found to be self-limiting, with some exceptions. In mastitis cases with severe clinical signs caused by *K. pneumoniae*, cows with antimicrobial therapy were reported to have better outcomes than those that were not treated, where non-treatment resulted in more cows culled due to the extreme severity of mastitis (FUENZALIDA & RUEGG, 2019).

But selective mastitis therapy does not have to mean a complete lack of treatment. Especially in severe cases, supporting therapy is still recommended (SCHMENGER & KRÖMKER, 2020; PREINE et al., 2022). Antiphlogistic and antipyretic treatment e.g. with NSAIDs, which can also help regulate excessive host immune reactions to endotoxins, and an infusion with fluids or drenching can greatly help combat inflammatory signs and has been proven beneficial, more so than antimicrobial therapy (DU PREEZ, 2000; SCHMENGER & KRÖMKER, 2020).

In the end, low *in vitro* antimicrobial resistance is only an indication of the general resistance situation of the various pathogens but does not guarantee therapeutic success. The current scientific knowledge should be taken into consideration in order to make evidence-based therapeutic decisions.

3. Conclusion

As many factors influence the occurrence of mastitis caused by gram-negatives, the landscape and specifics of animal husbandry play a big role. With the increased isolation of major gram-negative mastitis pathogens, especially from individual submissions and clinical cases, there was still a slight decrease in AMR of those pathogens overall. The AMR of minor gram-negative pathogens in this study stayed consistent over the years and coincided with reports from nearby regions. Low *in vitro* resistance does, however, not automatically equal *in vivo* treatment success. Because the marginal effect of antimicrobial treatment of gram-negative mastitis is low, benefits of mentioned treatment have only been proven in few, often severe cases. Instead, symptomatic, supportive therapy is recommended, since especially mild to moderate clinical cases have a high spontaneous cure rate. Gram-negatives are environmental and ubiquitous pathogens and are prone to exchange resistance-genes not only between strains, but also different bacterial species. Although there was little evidence of advancing AMR in gram-negative pathogens, the monitoring of their AMR patterns needs to continue in order to identify potential changes early.

IV. SUMMARY

Mastitis is the most economically important disease in dairy cattle worldwide and gram-negative mastitis pathogens cause a considerable percentage of infections leading to clinical mastitis. The objective of this study was to describe changes in the *in vitro* resistance *of E. coli, Klebsiella spp., Serratia marcescens, P. multocida,* and *M. haemolytica* to antimicrobials commonly used in mastitis treatment over several years (2014-2023). The secondary aim was to describe the percentage of isolates from healthy quarters, or those with clinical, or subclinical mastitis, and the effect of sample collection during herd screenings or from individual cows.

For this retrospective study, laboratory results of 22,057 quarter milk samples from 6,032 Bavarian dairy farms sampled over a 9-year-period were analyzed. A California Mastitis Test had been performed for each of the samples and bacterial species were determined with MALDI-TOF MS (Bruker Corp.). AMR had been assessed by breakpoint analysis using broth microdilution (breakpoint method, Micronaut-S-System, Merlin Diagnostica GmBH). Statistical analysis was done in SAS 9.4 (SAS Analytics Software Institute Inc., Heidelberg, Germany).

The most common of the gram-negative pathogens found was *E. coli*, followed by *S. marcescens, K. pneumoniae* and *K. oxytoca*. *P. multocida* and *M. haemolytica* were most rarely isolated. For the more common gram-negative mastitis pathogens the numbers increased in the year 2018. This steep incline could not be seen for the two minor pathogens. All gram-negatives displayed fairly high percentages of isolates from clinical mastitis cases. *S. marcescens* was the pathogen that caused the most cases of subclinical mastitis (77%), while *M. haemolytica* was isolated predominantly from cases of clinical mastitis (82%). Roughly 50% of the pathogens were isolated in submissions from individual cows and collected by practicing veterinarians or farmers. Interestingly, in 2018 the number of submissions of quarter milk samples deriving from clinical mastitis cases, and those collected by veterinarians and farmers also increased

greatly. This coincided with a change in the German veterinary dispensary law (TÄHAV), which included mandatory antimicrobial sensitivity testing.

Throughout the years and across tested antimicrobials, the AMR of the pathogens remained low. In addition, MIC decreased for many of the antimicrobials, especially in *E. coli*, and *Klebsiella* spp. isolates. AMR of *S. marcescens*, *P. multocida* and *M. haemolytica* stayed mostly consistent over the years. The antimicrobials the most isolates of *E. coli*, *S. marcescens*, and *Klebsiella* spp. were resistant against, were cefoperazone and cefazoline. However, even then only few pathogens were *in vitro* resistant against those antimicrobials. *P. multocida* and *M. haemolytica* were most resistant to erythromycin, pirlimycin, kanamycin/cefalexin, and oxacillin. While only 10% of *E. coli*, *S. marcescens* and *Klebsiella* spp. isolates showed low MDR, 82.7% of *P. multocida* and 84.6% of *M. haemolytica* isolates were multidrug-resistant.

Overall, the resistance patterns matched with those from many other studies, especially from those conducted in nearby regions. An influence of legislation with the purpose of reducing antimicrobial use on the overall number of isolates could be seen. *In vitro* resistances did not see a noteworthy increase over the study period. And although *in vitro* AMR of gram-negative mastitis pathogens is not an indicator for successful antimicrobial therapy in the field, its current status and progression are still important factors in determining antibiotic use and management practices in the future.

V. ZUSAMMENFASSUNG

Mastitis ist die wirtschaftlich wichtigste Krankheit von Milchkühen weltweit. Hierbei rufen Gram-negative Pathogene einen erheblichen Anteil der klinischen Mastitiden hervor. Ziel dieser Studie war es, die *In-vitro* Resistenzen von *E. coli, Klebsiella spp., S. marcensens,* sowie *P. multocida* und *M. haemolytica* gegen häufig verwendete Antibiotika der Mastitis-Therapie, sowie die zeitliche Entwicklung derer im Verlauf der Studie zu beschreiben. Zusätzlich wurde die Verteilung der Proben sowohl von Fällen klinischer und subklinischer Mastitis als auch aus gesunden Eutervierteln bestimmt und der Einfluss des Ursprungs der Proben, das heißt ob sie aus Herdenbeprobungen oder von einzeln beprobten Tieren stammten, untersucht.

Im Rahmen dieser retrospektiven Studie wurden 22.057 Viertelgemelksproben, welche über eine Zeitperiode von neun Jahren aus 6.032 bayrischen Milchviehbetrieben stammten, analysiert. Für jede Probe lag das Ergebnis eines Calfornia Mastitis Tests und bei makroskopischen Sekretveränderungen die Diagnose klinischer Mastitis vor. Die bakterielle Spezies wurde mithilfe eines MALDI-TOF-MS (Bruker Corp.) bestimmt. Antibiotikaresistenzen wurden mittels Breakpointanalyse durch ein Mikrodilutionsverfahren ermittelt (Breakpointmethode, Micronaut-S-Sysem, Merlin Diagnostica GmBH). Die statistische Analyse wurde in SAS 9.4 durchgeführt (SAS Analytics Software Institute Inc., Heidelberg, Germany).

Unter den hier analysierten Pathogenen wurde am häufigsten *E. coli* isoliert. In absteigender Häufigkeit folgten *S. marcescens, K. pneumoniae* und *K. oxytoca*. Am seltensten wurden *P. multocida* und *M. haemolytica* nachgewiesen. Die häufiger vorkommenden Pathogene erfuhren einen Anstieg an Isolaten ab 2018, als die neue Fassung der tierärztlichen Hausapothekenverordnung (TÄHAV) in Kraft trat, welche unter gewissen Voraussetzungen eine Antibiogrammpflicht vorschreibt. Dieser Anstieg konnte für die selten isolierten Erreger *P. multocida* und *M. haemolytica* nicht festgestellt werden. Alle hier untersuchten Gram-

negativen Erreger wurden verhältnismäßig häufig aus klinischen Fällen isoliert, allen voran *M. haemolytica* (82%). Im Gegensatz dazu zeigte *S. marcescens* den höchsten Anteil an Isolaten aus Fällen subklinischer Mastitiden (77%). Etwa die Hälfte der berücksichtigten Pathogene stammten aus Einzelbeprobungen, die von praktizierenden Tierärzten oder Landwirten eingesendet worden waren. Sowohl Proben klinischer Mastitiden als auch Einzeleinsendungen erfuhren 2018 ebenfalls einen Anstieg.

Generell betrachtet blieb die Anzahl von Antibiotikaresistenzen der untersuchten Pathogene gering, und erfuhr gegen viele der untersuchten Antibiotika sogar eine Abnahme der Minimalen Hemmstoffkonzentrationen (MHK). Hier waren besonders Isolate von *E. coli* und *Klebsiella* Isolate zu nennen. Die Resistenzen von *S. marcescens, P. multocida* und *M. haemolytica* blieben dagegen über den Studienzeitraum weitestgehend stabil. Während *E. coli, S. marcescens* und *Klebsiella* spp. am häufigsten gegen Cefoperazon und Cefazolin resistent waren, blieben deren MHK im Allgemeinen trotzdem gering. *P. multocida* und *M. haemolytica* waren überwiegend gegen Erythromycin, Pirlimycin, Kanamycin/Cefalexin und Oxacillin resistent. Während *E. coli, S. marcescens* und *Klebsiella* spp. mit circa 10% generell nur einen geringen Anteil and Multiresistenzen aufwiesen, waren jeweils 82.7% der Isolate von *P. multocida* und 84.6% der von *M. haeolytica* multiresistent.

Im Allgemeinen war die Resistenzlage vergleichbar der Beschreibungen anderer Studien, insbesondere, wenn diese aus geographisch naheliegenden Regionen kamen. Ein Einfluss der Änderung der TÄHAV auf die Gesamtmenge der isolierten Pathogene konnte beobachtet werden und die Resistenzlage verschlechterte sich über den Studienzeitraum nicht signifikant. Obwohl *In-vitro* Resistenzen bei Gram-negativen Mastitispathogenen keine Aussage hinsichtlich eines Therapieerfolges mittels Antibiotikatherapie zulassen, ist die Kenntnis über deren Resistenzlage doch ein wichtiger Faktor in der Wahl der in der Praxis angewandten Antibiotika und zukünftiger Managementmaßnahmen.

VII. REFERENCES

Abdi RD, Gillespie BE, Ivey S, Pighetti GM, Almeida RA, Kerro Dego O. 2021. Antimicrobial Resistance of Major Bacterial Pathogens from Dairy Cows with High Somatic Cell Count and Clinical Mastitis. *Animals (Basel)*. 2021;11(1):131. Published 2021 Jan 8. doi:10.3390/ani11010131

Adkins, P. R. F., & National Mastitis Council (N.M.C., U.S.). 2017. *Laboratory handbook on bovine mastitis* (J. R. Middleton, L. K. Fox, G. Pighetti, & C. Petersson-Wolfe, Eds.; Third edition). National Mastitis Council.

Ahmed, A. M., & Shimamoto, T. 2011. Molecular characterization of antimicrobial resistance in Gram-negative bacteria isolated from bovine mastitis in Egypt. *Microbiology and immunology*. *55*(5), 318-327.

Alhamami T, Chowdhury PR, Gomes N, Carr M, Veltman T, Khazandi M, Mollinger J, Deutscher AT, Turni C, Mahdi L, et al. 2021. First Emergence of Resistance to Macrolides and Tetracycline Identified in *Mannheimia haemolytica* and *Pasteurella multocida* Isolates from Beef Feedlots in Australia. *Microorganisms*. 9(6):1322. https://doi.org/10.3390/microorganisms9061322

Anderson, K.L., Hunt, E. 1989. Anti-inflammatory therapy in acute endotoxin-induced bovine mastitis. *Veterinary Research Communications.* 13, 17–26 https://doi.org/10.1007/BF00366848

Arthur M, Courvalin P. 1986. Contribution of two different mechanisms to erythromycin resistance in *Escherichia coli*. Antimicrob Agents Chemother. https://doi.org/10.1128/aac.30.5.694

Asfour HAE, Anwer, Abeer M., El-Metwally A. 2016. Some studies on *Pasteurella multocida* as a causative agent of mastitis in dairy cows and ewes. *Assiut Veterinary Medical Journal*, *62*(150), 143-156. doi: 10.21608/avmj.2016.170000

Bannerman, D.D., M.J. Paape, W.R. Hare, J.C. Hope. 2004a. Characterization of the Bovine Innate Immune Response to Intramammary Infection with *Klebsiella pneumoniae*. Journal of Dairy Science, 87 (8), 2420-2432, https://doi.org/10.3168/jds.S0022-0302(04)73365-2.

Bannerman, D.D., Paape, M.J., Goff, J., Kimura, K., Lippolis, J., & Hope, J. 2004b. Innate immune response to intramammary infection with *Serratia marcescens* and *Streptococcus uberis*. *Veterinary Research*, *35*(6), 681-700. DOI: 10.1051/vetres:2004040

Bannerman, D. D., Paape, M. J., Lee, J. W., Zhao, X., Hope, J. C., & Rainard, P. 2004c. *Escherichia coli* and *Staphylococcus aureus* elicit differential innate immune responses following intramammary infection. *Clinical and Vaccine Immunology*, *11*(3), 463-472. https://doi.org/10.1128/CDLI.11.3.463-472.2004

Barkema, H. W., Schukken, Y. H., Lam, T. J. G. M., Beiboer, M. L., Wilmink, H., Benedictus, G., & Brand, A. 1998. Incidence of clinical mastitis in dairy herds grouped in three categories by bulk milk somatic cell counts. *Journal of dairy science*, *81*(2), 411-419. https://doi.org/10.3168/jds.S0022-0302(98)75591-2

Barnum, D. A. 1954. A herd outbreak of mastitis caused by *Pasteurella Multocida*. *Canadian Journal of Comparative Medicine and Veterinary Science*, *18*(4), 113. PMCID: PMC1791674, PMID: 17648709

Barnum, D. A., Thackeray, E. L., & Fish, N. A. 1958. An Outbreak Of Mastitis Caused By *Serratia Marcescens*. *Canadian journal of comparative medicine and veterinary science*, *22*(11), 392–395. PMCID: PMC1614667, PMID: 17649094

Bechtold V, Petzl W, Huber-Schlenstedt R, Sorge US. 2024a. Distribution of Bovine Mastitis Pathogens in Quarter Milk Samples from Bavaria, Southern Germany, between 2014 and 2023—A Retrospective Study. *Animals*. 14(17):2504. https://doi.org/10.3390/ani14172504

Bechtold, V., Petzl, W., Huber-Schlenstedt, R., Gangl, A., & Sorge, U. S. 2024b. Antimicrobial resistance of *Streptococcus dysgalactiae*, *Streptococcus agalactiae*, and *Streptococcus canis* in quarter milk samples from Bavaria, Southern Germany, between 2012 and 2022. *Journal of Dairy Science*. https://doi.org/10.3168/jds.2023-24555

Bradford, B. J., Yuan, K., Farney, J. K., Mamedova, L. K., & Carpenter, A. J. 2015. Invited review: Inflammation during the transition to lactation: New adventures with an old flame. *Journal of dairy science*, *98*(10), 6631-6650. https://doi.org/10.3168/jds.2015-9683

Bradley, A. J., & Green, M. J. 2000. A study of the incidence and significance of intramammary enterobacterial infections acquired during the dry period. *Journal of dairy science*, *83*(9), 1957-1965. https://doi.org/10.3168/jds.S0022-0302(00)75072-7

Bradley, A. J., & Green, M. J. 2001. Adaptation of *Escherichia coli* to the bovine mammary gland. *Journal of Clinical Microbiology*, *39*(5), 1845-1849. https://doi.org/10.1128/jcm.39.5.1845-1849.2001

Bradley, A. J., Breen, J. E., Payne, B., White, V., & Green, M. J. 2015. An investigation of the efficacy of a polyvalent mastitis vaccine using different vaccination regimens under field conditions in the United Kingdom. *Journal of dairy science*, *98*(3), 1706-1720. https://doi.org/10.3168/jds.2014-8332

Breen, J. E., Green, M. J., & Bradley, A. J. 2009. Quarter and cow risk factors associated with the occurrence of clinical mastitis in dairy cows in the United Kingdom. *Journal of dairy Science*, *92*(6), 2551-2561. https://doi.org/10.3168/jds.2008-1369

Brennecke, J., Falkenberg, U., Wente, N., & Krömker, V. 2021. Are severe mastitis cases in dairy cows associated with bacteremia? *Animals*, *11*(2), 410. https://doi.org/10.3390/ani11020410

BTK e. V. 2018. Die neue TÄHAV ist in Kraft. Aus dem Ausschuss für Arzneimittel- und Futtermittelrecht der Bundestierärztekammer e. V. Ausgabe: 4/2018 S. 484-489

Burvenich, C., Van Merris, V., Mehrzad, J., Diez-Fraile, A., & Duchateau, L. 2003. Severity of *E. coli* mastitis is mainly determined by cow factors. *Veterinary research*, *34*(5), 521-564. DOI: 10.1051/vetres:2003023

Bush, K., Flamm, R. K., Ohringer, S., Singer, S. B., Summerill, R., & Bonner, D. P. 1991. Effect of clavulanic acid on activity of beta-lactam antibiotics in *Serratia marcescens* isolates producing both a TEM beta-lactamase and a chromosomal cephalosporinase. *Antimicrobial agents and chemotherapy*, *35*(11), 2203-2208. https://doi.org/10.1128/aac.35.11.2203

Campos, F. C., Castilho, I. G., Rossi, B. F., Bonsaglia, É. C., Dantas, S. T., Dias, R. C., Fernandes Junior A, Hernandes RT, Camargo CH, Ribeiro MG, Pantoja JCF, Langoni H & Rall, V. L. 2022. Genetic and antimicrobial resistance profiles of mammary pathogenic *E. coli* (MPEC) isolates from bovine clinical mastitis. *Pathogens*, *11*(12), 1435. https://doi.org/10.3390/pathogens11121435

Caroff, M., & Karibian, D. 2003. Structure of bacterial lipopolysaccharides. *Carbohydrate research*, *338*(23), 2431-2447. https://doi.org/10.1016/j.carres.2003.07.010

Cha, E., Bar, D., Hertl, J. A., Tauer, L. W., Bennett, G., González, R. N., Schukken YH, Welcome FL & Gröhn, Y. T. 2011. The cost and management of different types of clinical mastitis in dairy cows estimated by dynamic programming. *Journal of dairy science*, *94*(9), 4476-4487. https://doi.org/10.3168/jds.2010-4123

Cheng, J. Zhang Jv, Han Bo, Barkema Herman W., Cobo Eduardo R., Kastelic John P., Zhou Man, Shi Yuxiang, Wang Jianfang, Yang Rui, Gao Jian. 2020. *Klebsiella pneumoniae* isolated from bovine mastitis is cytopathogenic for bovine mammary epithelial cells. *Journal of dairy science*, *103*(4), 3493-3504. https://doi.org/10.3168/jds.2019-17458

Cheng, W. N., & Han, S. G. 2020. Bovine mastitis: Risk factors, therapeutic strategies, and alternative treatments—A review. *Asian-Australasian journal of animal sciences*, *33*(11), 1699. doi: 10.5713/ajas.20.0156

CLSI-VET01 2023. CLSI VET01S ED6:2023 — Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals, 6th Edition. 164

Cobirka, M., Tancin, V., & Slama, P. 2020. Epidemiology and classification of mastitis. *Animals*, *10*(12), 2212. https://doi.org/10.3390/ani10122212

Maplesden, D. C., & Carter, G. R. 1955. Bovine mastitis caused by *Pasteurella haemolytica*: A case report. *Canadian Journal of Comparative Medicine and Veterinary Science*, *19*(9), 295. PMCID: PMC1614149, PMID: 17648849

Dabo, S. M., Taylor, J. D., & Confer, A. W. 2007. *Pasteurella multocida* and bovine respiratory disease. Animal Health Research Reviews, 8(2), 129-150. doi:10.1017/S1466252307001399

Dahl, M. O., De Vries, A., Maunsell, F. P., Galvao, K. N., Risco, C. A., & Hernandez, J. A. 2018. Epidemiologic and economic analyses of pregnancy loss attributable to mastitis in primiparous Holstein cows. *Journal of Dairy Science*, *101*(11), 10142-10150. https://doi.org/10.3168/jds.2018-14619

Davin-Regli, A., Pages, J. M., & Ferrand, A. 2021. Clinical status of efflux resistance mechanisms in gram-negative bacteria. *Antibiotics*, *10*(9), 1117. https://doi.org/10.3390/antibiotics10091117

de Frutos, M., López-Urrutia, L., Domínguez-Gil, M., Arias, M., Muñoz-Bellido, J. L., Eiros, J. M., & Ramos, C. 2017. *Serratia marcescens* outbreak due to contaminated 2% aqueous chlorhexidine. *Enfermedades infecciosas y microbiologia clinica (English ed.)*, *35*(10), 624-629. https://doi.org/10.1016/j.eimce.2017.11.006

Desmolaize, B., Rose, S., Wilhelm, C., Warrass, R., & Douthwaite, S. 2011. Combinations of macrolide resistance determinants in field isolates of *Mannheimia haemolytica* and *Pasteurella*

multocida. *Antimicrobial agents and chemotherapy*, *55*(9), 4128-4133. https://doi.org/10.1128/aac.00450-11

Du Preez, J. H. 2000. Bovine mastitis therapy and why it fails: continuing education. *Journal of the South African Veterinary Association*, *71*(3), 201-208. https://hdl.handle.net/10520/EJC99350

Dufour, S., Labrie, J., & Jacques, M. 2019. The mastitis pathogens culture collection. *Microbiology resource announcements*, *8*(15), 10-1128. https://doi.org/10.1128/mra.00133-19

DVG. Deutsche Veterinärmedizinische Gesellschaft. 2018. *Leitlinien zur Labordiagnostik der Mastitis – Probenahme und mikrobiologische Untersuchung*. 2018 | 3. Auflage DVG Service (Verlag)

Dworecka-Kaszak, B., Krutkiewicz, A., Szopa, D., Kleczkowski, M., & Biegańska, M. 2012. High prevalence of *Candida* yeast in milk samples from cows suffering from mastitis in poland. *The Scientific World Journal*, *2012*(1), 196347. https://doi.org/10.1100/2012/196347

Eichenberger, E. M., & Thaden, J. T. 2019. Epidemiology and mechanisms of resistance of extensively drug resistant Gram-negative bacteria. *Antibiotics*, *8*(2), 37. https://doi.org/10.3390/antibiotics8020037

Erskine, R. J., Eberhart, R. J., Hutchinson, L. J., Spencer, S. B., & Campbell, M. A. 1988. Incidence and types of clinical mastitis in dairy herds with high and low somatic cell counts. *Journal of the American Veterinary Medical Association*, *192*(6), 761-765. PMID: 3281923

Erskine, R. J., Bartlett, P. C., VanLente, J. L., & Phipps, C. R. 2002. Efficacy of systemic ceftiofur as a therapy for severe clinical mastitis in dairy cattle. Journal of dairy science, 85(10), 2571-2575. https://doi.org/10.3168/jds.S0022-0302(02)74340-3

Fredebeul-Krein, F., Schmenger, A., Wente, N., Zhang, Y., & Krömker, V. 2022. Factors associated with the severity of clinical mastitis. *Pathogens*, *11*(10), 1089. https://doi.org/10.3390/pathogens11101089

Friman, M. J., Eklund, M. H., Pitkälä, A. H., Rajala-Schultz, P. J., & Rantala, M. H. J. 2019. Description of two *Serratia marcescens* associated mastitis outbreaks in Finnish dairy farms and a review of literature. *Acta Veterinaria Scandinavica*, *61*, 1-11. https://doi.org/10.1186/s13028-019-0488-7

Fu, S., Wen, C., Wang, Z., Qiu, Y., Zhang, Y., Zuo, J., Xu Y, Han X, Luo Z, Chen W & Miao, J. 2022. Molecular epidemiology and antimicrobial resistance of outbreaks of *Klebsiella pneumoniae* clinical mastitis in Chinese dairy farms. *Microbiology Spectrum*, *10*(6), e02997-22. https://doi.org/10.1128/spectrum.02997-22

Fuenzalida, M. J., & Ruegg, P. L. 2019. Negatively controlled, randomized clinical trial to evaluate intramammary treatment of nonsevere, gram-negative clinical mastitis. *Journal of dairy science*, *102*(6), 5438-5457. https://doi.org/10.3168/jds.2018-16156

Fuenzalida, M. J., Furmaga, E., & Aulik, N. 2021. Antimicrobial resistance in *Klebsiella* species from milk specimens submitted for bovine mastitis testing at the Wisconsin Veterinary Diagnostic Laboratory, 2008–2019. *JDS communications*, *2*(3), 148-152. https://doi.org/10.3168/jdsc.2020-0031

Fusté, E., Galisteo, G. J., Jover, L., Vinuesa, T., Villa, T. G., & Viñas, M. 2012. Comparison of antibiotic susceptibility of old and current *Serratia*. *Future Microbiology*, *7*(6), 781-786. https://doi.org/10.2217/fmb.12.40

Gauba, A., & Rahman, K. M. 2023. Evaluation of antibiotic resistance mechanisms in gram-negative bacteria. *Antibiotics*, *12*(11), 1590. https://doi.org/10.3390/antibiotics12111590

Gonzalez, R. N., Cullor, J. S., Jasper, D. E., Farver, T. B., Bushnell, R. B., & Oliver, M. N. 1989. Prevention of clinical coliform mastitis in dairy cows by a mutant *Escherichia coli* vaccine. *Canadian Journal of Veterinary Research*, *53*(3), 301. PMCID: PMC1255715, PMID: 2670166

Goulart, D. B., & Mellata, M. 2022. *Escherichia coli* mastitis in dairy cattle: etiology, diagnosis, and treatment challenges. *Frontiers in Microbiology*, *13*, 928346. DOI: 10.3389/fmicb.2022.928346

Green, M. J., Bradley, A. J., Medley, G. F., & Browne, W. J. 2007. Cow, farm, and management factors during the dry period that determine the rate of clinical mastitis after calving. *Journal of dairy science*, *90*(8), 3764-3776. https://doi.org/10.3168/jds.2007-0107

Grohn, Y. T., Wilson, D. J., González, R. N., Hertl, J. A., Schulte, H., Bennett, G., & Schukken, Y. H. 2004. Effect of pathogen-specific clinical mastitis on milk yield in dairy cows. *Journal of dairy science*, *87*(10), 3358-3374. https://doi.org/10.3168/jds.S0022-0302(04)73472-4

Guerra, S. T., Orsi, H., Joaquim, S. F., Guimarães, F. F., Lopes, B. C., Dalanezi, F. M., Leite DS, Langoni H, Pantoja JCF, Rall VLM, Hernandes RT, Lucheis SB, & Ribeiro, M. G. 2020. Investigation of extra-intestinal pathogenic *Escherichia coli* virulence genes, bacterial motility, and multidrug resistance pattern of strains isolated from dairy cows with different severity scores of clinical mastitis. *Journal of dairy science*, *103*(4), 3606-3614. https://doi.org/10.3168/jds.2019-17477

Günther, J., Petzl, W., Bauer, I., Ponsuksili, S., Zerbe, H., Schuberth, H. J., Brunner RM, & Seyfert, H. M. 2017. Differentiating *Staphylococcus aureus* from *Escherichia coli* mastitis: S. aureus triggers unbalanced immune-dampening and host cell invasion immediately after udder infection. *Scientific Reports*, *7*(1), 4811. https://doi.org/10.1038/s41598-017-05107-4

Heikkilä, A. M., Liski, E., Pyörälä, S., & Taponen, S. 2018. Pathogen-specific production losses in bovine mastitis. *Journal of dairy science*, *101*(10), 9493-9504. https://doi.org/10.3168/jds.2018-14824

Herry, V., Gitton, C., Tabouret, G., Répérant, M., Forge, L., Tasca, C., Gilbert FB, Guitton E, Barc C., Staub C., Smith D.G.E., Germon P., Foucras G. & Rainard, P. 2017. Local immunization impacts the response of dairy cows to *Escherichia coli* mastitis. *Scientific reports*, *7*(1), 3441. https://doi.org/10.1038/s41598-017-03724-7

Hertl, J. A., Gröhn, Y. T., Leach, J. D. G., Bar, D., Bennett, G. J., González, R. N., Rauch B.J., Welcome F.L., Tauer L.W., & Schukken, Y. H. 2010. Effects of clinical mastitis caused by gram-positive and gram-negative bacteria and other organisms on the probability of conception in New York State Holstein dairy cows. *Journal of dairy science*, *93*(4), 1551-1560. https://doi.org/10.3168/jds.2009-2599

Hertl, J. A., Schukken, Y. H., Bar, D., Bennett, G. J., González, R. N., Rauch, B. J., Welcome F.L., Tauer L..W & Gröhn, Y. T. 2011. The effect of recurrent episodes of clinical mastitis caused by gram-positive and gram-negative bacteria and other organisms on mortality and culling in

Holstein dairy cows. *Journal of dairy science*, *94*(10), 4863-4877. https://doi.org/10.3168/jds.2010-4000

Hogan, J., & Smith, K. L. 2003. Coliform mastitis. *Veterinary research*, *34*(5), 507-519. DOI: 10.1051/vetres:2003022

Hogan, J., & Smith, K. L. 2012. Managing environmental mastitis. *Veterinary Clinics: Food Animal Practice*, *28*(2), 217-224. DOI: 10.1016/j.cvfa.2012.03.009

Hogan, J. S., Smith, K. L., Hoblet, K. H., Todhunter, D. A., Schoenberger, P. S., Hueston, W. D., Pritchard D.E., Bowman G.L., Heider L.E., Brockett B.L. & Conrad, H. R. 1989. Bacterial counts in bedding materials used on nine commercial dairies. *Journal of dairy science*, *72*(1), 250-258. https://doi.org/10.3168/jds.S0022-0302(89)79103-7

Hogan, J. S., Smith, K. L., Todhunter, D. A., & Schoenberger, P. S. 1992. Field trial to determine efficacy of an *Escherichia coli* J5 mastitis vaccine. *Journal of dairy science*, *75*(1), 78-84. https://doi.org/10.3168/jds.S0022-0302(92)77741-8

Hogan, J. S., Smith, K. L., Todhunter, D. A., & Schoenberger, P. S. 1994. Therapy of experimentally induced coliform mastitis with a *Propionibacterium acnes* product. *Journal of dairy science*, *77*(2), 462-467. https://doi.org/10.3168/jds.S0022-0302(94)76974-5

Hogeveen, H., & Van Der Voort, M. 2017. Assessing the economic impact of an endemic disease: the case of mastitis. *Revue scientifique et technique/Office International des Epizooties*, *36*(1), 217-226. doi: 10.20506/rst.36.1.2623

Impey, R. E., Hawkins, D. A., Sutton, J. M., & Soares da Costa, T. P. 2020. Overcoming intrinsic and acquired resistance mechanisms associated with the cell wall of gram-negative bacteria. *Antibiotics*, 9(9), 623. https://doi.org/10.3390/antibiotics9090623

Ismail, Z. B., & Abutarbush, S. M. 2020. Molecular characterization of antimicrobial resistance and virulence genes of *Escherichia coli* isolates from bovine mastitis. *Veterinary World*, *13*(8), 1588. doi: 10.14202/vetworld.2020.1588-1593

Jain, N. C. 1979. Common mammary pathogens and factors in infection and mastitis. *Journal of Dairy Science*, *62*(1), 128-134. https://doi.org/10.3168/jds.S0022-0302(79)83214-2

Jongeneel, R. A., Longworth, N., & Huettel, S. 2005. Dairy farm size distribution in east and west: evolution and sensitivity to structural and policy variables: case-studies of the Netherlands, Germany, Poland and Hungary. DOI: 10.22004/ag.econ.24772

Kanevsky-Mullarky, I., Nedrow, A. J., Garst, S., Wark, W., Dickenson, M., Petersson-Wolfe, C. S., & Zadoks, R. N. 2014. Comparison of virulence factors in *Klebsiella pneumoniae* strains associated with multiple or single cases of mastitis. *Journal of dairy science*, *97*(4), 2213-2218. https://doi.org/10.3168/jds.2013-7140

Karell, J., Petzl, W., Gangl, A., Huber-Schlenstedt, R., & Sorge, U. S. 2024. Changes in antimicrobial resistance of *Staphylococcus aureus* in bovine quarter milk samples from southern Germany between 2012 and 2022. *Journal of Dairy Science*, *107*(6), 3802-3812. https://doi.org/10.3168/jds.2023-23997

Kehrenberg, C., Schulze-Tanzil, G., Martel, J. L., Chaslus-Dancla, E., & Schwarz, S. 2001. Antimicrobial resistance in *Pasteurella and Mannheimia*: epidemiology and genetic basis. *Veterinary research*, *32*(3-4), 323-339. DOI: 10.1051/vetres:2001128

Kessels, J. A., Cha, E., Johnson, S. K., Welcome, F. L., Kristensen, A. R., & Gröhn, Y. T. 2016. Economic comparison of common treatment protocols and J5 vaccination for clinical mastitis in dairy herds using optimized culling decisions. *Journal of Dairy Science*, *99*(5), 3838-3847. https://doi.org/10.3168/jds.2015-10385

Klaas, I. C., & Zadoks, R. N. 2018. An update on environmental mastitis: Challenging perceptions. *Transboundary and emerging diseases*, 65, 166-185. https://doi.org/10.1111/tbed.12704

Krebs, I., Zhang, Y., Wente, N., Leimbach, S., & Krömker, V. 2023. Bacteremia in severe mastitis of dairy cows. *Microorganisms*, *11*(7), 1639. https://doi.org/10.3390/microorganisms11071639

Krömker, V., Paduch, J. H., Abograra, I., Zinke, C., & Friedrich, J. 2011. Effects of an additional nonsteroidal anti-inflammatory therapy with carprofen (Rimadyl Rind) in cases of severe mastitis of high yielding cows. *Berliner und Münchener tierärztliche Wochenschrift*, *124*(3-4), 161-167. PMID: 21465772.

Leclercq, R., & Courvalin, P. 1991. Intrinsic and unusual resistance to macrolide, lincosamide, and streptogramin antibiotics in bacteria. *Antimicrobial agents and chemotherapy*, *35*(7), 1273-1276.

Liang, Z., Shen, J., Liu, J., Sun, X., Yang, Y., Lv, Y., Zheng J., Mou X., Li H., Ding X. & Yang, F. (2023). Prevalence and Characterization of *Serratia marcescens* Isolated from Clinical Bovine Mastitis Cases in Ningxia Hui Autonomous Region of China. *Infection and Drug Resistance*, 2727-2735. https://doi.org/10.2147/IDR.S408632

Maddamsetti, R., Yao, Y., Wang, T., Gao, J., Huang, V. T., Hamrick, G. S., Son H.I. & You, L. (2024). Duplicated antibiotic resistance genes reveal ongoing selection and horizontal gene transfer in bacteria. *Nature Communications*, *15*(1), 1449. https://doi.org/10.1038/s41467-024-45638-9

Maplesden, D. C., & Carter, G. R. 1955. Bovine mastitis caused by *Pasteurella haemolytica*: A case report. *Canadian Journal of Comparative Medicine and Veterinary Science*, *19*(9), 295. PMCID: PMC1614149, PMID: 17648849

Marrie, T. J., & Costerton, J. W. 1981. Prolonged survival of *Serratia marcescens* in chlorhexidine. *Applied and Environmental Microbiology*, *42*(6), 1093-1102. DOI: https://doi.org/10.1128/aem.42.6.1093-1102.1981

Melchner, A., van de Berg, S., Scuda, N., Feuerstein, A., Hanczaruk, M., Schumacher, M., Straubinger R.K., Marosevic D. & Riehm, J. M. 2021. Antimicrobial resistance in isolates from cattle with bovine respiratory disease in Bavaria, Germany. *Antibiotics*, *10*(12), 1538. https://doi.org/10.3390/antibiotics10121538

Milanov, D., Aleksić, N., Todorović, D., & Bugarski, D. 2017. *Pasteurlla multocida* mastitis in cow. *Veterinarski glasnik*, *71*(2), 117-122. https://doi.org/10.2298/VETGL170619011M

Morales-Ubaldo, A. L., Rivero-Perez, N., Valladares-Carranza, B., Velázquez-Ordoñez, V., Delgadillo-Ruiz, L., & Zaragoza-Bastida, A. 2023. Bovine mastitis, a worldwide impact disease: prevalence, antimicrobial resistance, and viable alternative approaches. *Veterinary and animal science*, 100306. https://doi.org/10.1016/j.vas.2023.100306

Nam, H. M., Lim, S. K., Kang, H. M., Kim, J. M., Moon, J. S., Jang, K. C., Kim J.M., Joo Y.S. & Jung, S. C. 2009. Prevalence and antimicrobial susceptibility of gram-negative bacteria isolated

from bovine mastitis between 2003 and 2008 in Korea. *Journal of dairy science, 92*(5), 2020-2026. https://doi.org/10.3168/jds.2008-1739

Nelli, R. K., Harm, T. A., Siepker, C., Groeltz-Thrush, J. M., Jones, B., Twu, N. C., Nenninger A.S., Magstadt D.R., Burrough E.R., Piñeyro P.E., Mainenti M., Carnaccini S., Plummer P.J. & Bell, T. M. 2024. Sialic acid receptor specificity in mammary gland of dairy cattle infected with highly pathogenic avian influenza A (H5N1) virus. *Emerging Infectious Diseases, 30*(7), 1361. doi: 10.3201/eid3007.240689

Nobrega, D. B., Naqvi, S. A., Dufour, S., Deardon, R., Kastelic, J. P., De Buck, J., & Barkema, H. W. 2020. Critically important antimicrobials are generally not needed to treat nonsevere clinical mastitis in lactating dairy cows: Results from a network meta-analysis. *Journal of dairy science, 103*(11), 10585-10603. https://doi.org/10.3168/jds.2020-18365

Noyes, N. R., Benedict, K. M., Gow, S. P., Booker, C. W., Hannon, S. J., McAllister, T. A., & Morley, P. S. 2015. *Mannheimia haemolytica* in feedlot cattle: prevalence of recovery and associations with antimicrobial use, resistance, and health outcomes. *Journal of veterinary internal medicine*, 29(2), 705-713. https://doi.org/10.1111/jvim.12547

Nüesch-Inderbinen, M., Käppeli, N., Morach, M., Eicher, C., Corti, S., & Stephan, R. 2019. Molecular types, virulence profiles and antimicrobial resistance of *Escherichia coli* causing bovine mastitis. *Veterinary record open, 6*(1), e000369. https://doi.org/10.1136/vetreco-2019-000369

Riekerink, R. O., Barkema, H. W., Kelton, D. F., & Scholl, D. T. 2008. Incidence rate of clinical mastitis on Canadian dairy farms. *Journal of dairy science, 91*(4), 1366-1377. https://doi.org/10.3168/jds.2007-0757

Ollis, G., & Schoonderwoerd, M. 1989. Alberta. Subclinical mastitis associated with *Pseudomonas aeruginosa* and *Serratia marcescens*. *The Canadian Veterinary Journal, 30*(6), 525. PMCID: PMC1681118, PMID: 17423359

Omaleki, L., Browning, G. F., Allen, J. L., & Barber, S. R. 2011. The role of *Mannheimia* species in ovine mastitis. *Veterinary microbiology, 153*(1-2), 67-72. https://doi.org/10.1016/j.vetmic.2011.03.024

Omaleki, L., Browning, G. F., Allen, J. L., Markham, P. F., & Barber, S. R. 2015. The upper respiratory tract is a natural reservoir of haemolytic *Mannheimia* species associated with ovine mastitis. *Veterinary Microbiology, 181*(3-4), 308-312. https://doi.org/10.1016/j.vetmic.2015.10.006

Pitkälä, A., Haveri, M., Pyörälä, S., Myllys, V., & Honkanen-Buzalski, T. 2004. Bovine mastitis in Finland 2001—prevalence, distribution of bacteria, and antimicrobial resistance. *Journal of dairy science, 87*(8), 2433-2441. https://doi.org/10.3168/jds.S0022-0302(04)73366-4

Preine, F., Herrera, D., Scherpenzeel, C., Kalmus, P., McCoy, F., Smulski, S., Rajala-Schultz P., Schmenger A., Moroni P. & Krömker, V. 2022. Different European perspectives on the treatment of clinical mastitis in lactation. *Antibiotics, 11*(8), 1107. https://doi.org/10.3390/antibiotics11081107

Procópio, T. F., Moura, M. C., Bento, E. F., Soares, T., Coelho, L. C., Bezerra, R. P., Mota R.A., Porto A…LF, Paiva PMG & Napoleão, T. H. 2019. Looking for alternative treatments for bovine and caprine mastitis: Evaluation of the potential of Calliandra surinamensis leaf pinnulae

lectin (CasuL), both alone and in combination with antibiotics. *Microbiologyopen*, *8*(11), e869. https://doi.org/10.1002/mbo3.869

Puerto, M. A., Shepley, E., Cue, R. I., Warner, D., Dubuc, J., & Vasseur, E. 2021. The hidden cost of disease: I. Impact of the first incidence of mastitis on production and economic indicators of primiparous dairy cows. *Journal of dairy science*, *104*(7), 7932-7943. https://doi.org/10.3168/jds.2020-19584

Pyörälä, S., Kaartinen, L., Käck, H., & Rainio, V. 1994. Efficacy of two therapy regimens for treatment of experimentally induced *Escherichia coli* mastitis in cows. *Journal of dairy science*, *77*(2), 453-461. https://doi.org/10.3168/jds.S0022-0302(94)76973-3

Redding, L. E., Bender, J., & Baker, L. 2019. Quantification of antibiotic use on dairy farms in Pennsylvania. *Journal of dairy science*, *102*(2), 1494-1507. https://doi.org/10.3168/jds.2018-15224

Ricchi, M., Goretti, M., Branda, E., Cammi, G., Garbarino, C. A., Turchetti, B., Moroni P., Arrigoni N. & Buzzini, P. (2010). Molecular characterization of *Prototheca* strains isolated from Italian dairy herds. *Journal of dairy science*, *93*(10), 4625-4631. https://doi.org/10.3168/jds.2010-3178

Ruegg, P. L. 2017. A 100-Year Review: Mastitis detection, management, and prevention. *Journal of dairy science*, *100*(12), 10381-10397. https://doi.org/10.3168/jds.2017-13023

Ruegg, P. L. 2021. What is success? A narrative review of research evaluating outcomes of antibiotics used for treatment of clinical mastitis. *Frontiers in veterinary science*, *8*, 639641. DOI: 10.3389/fvets.2021.639641

Saini, V., McClure, J. T., Léger, D., Keefe, G. P., Scholl, D. T., Morck, D. W., & Barkema, H. W. 2012. Antimicrobial resistance profiles of common mastitis pathogens on Canadian dairy farms. *Journal of dairy science*, *95*(8), 4319-4332. https://doi.org/10.3168/jds.2012-5373

Saini, V., McClure, J. T., Scholl, D. T., DeVries, T. J., & Barkema, H. W. 2013. Herd-level relationship between antimicrobial use and presence or absence of antimicrobial resistance in gram-negative bovine mastitis pathogens on Canadian dairy farms. *Journal of dairy science*, *96*(8), 4965-4976. https://doi.org/10.3168/jds.2012-5713

San Millan, A. 2018. Evolution of plasmid-mediated antibiotic resistance in the clinical context. *Trends in microbiology*, *26*(12), 978-985. DOI: 10.1016/j.tim.2018.06.007

Schink, A. K., Hanke, D., Semmler, T., Brombach, J., Bethe, A., Lübke-Becker, A., Teske K., Muller K.E. & Schwarz, S. 2022. Novel multiresistance-mediating integrative and conjugative elements carrying unusual antimicrobial resistance genes in *Mannheimia haemolytica* and *Pasteurella multocida*. *Journal of antimicrobial chemotherapy*, *77*(7), 2033-2035. https://doi.org/10.1093/jac/dkac116

Schmenger, A., & Krömker, V. 2020. Characterization, cure rates and associated risks of clinical mastitis in Northern Germany. *Veterinary sciences*, *7*(4), 170. https://doi.org/10.3390/vetsci7040170

Schönecker, L., Schnyder, P., Schüpbach-Regula, G., Meylan, M., & Overesch, G. 2020. Prevalence and antimicrobial resistance of opportunistic pathogens associated with bovine respiratory disease isolated from nasopharyngeal swabs of veal calves in Switzerland.

Preventive veterinary medicine, *185*, 105182. https://doi.org/10.1016/j.prevetmed.2020.105182

Schukken, Y., Chuff, M., Moroni, P., Gurjar, A., Santisteban, C., Welcome, F., & Zadoks, R. 2012. The "other" gram-negative bacteria in mastitis: *Klebsiella*, *Serratia*, and more. *Veterinary Clinics: Food Animal Practice*, 28(2), 239-256. DOI: 10.1016/j.cvfa.2012.04.001

Schukken, Y. H., Hertl, J., Bar, D., Bennett, G. J., González, R. N., Rauch, B. J., Santisteban C., Schulte H.F., Tauer L., Welcome F.L. & Gröhn, Y. T. 2009. Effects of repeated gram-positive and gram-negative clinical mastitis episodes on milk yield loss in Holstein dairy cows. *Journal of dairy science*, *92*(7), 3091-3105. https://doi.org/10.3168/jds.2008-1557

Shinozuka, Y., Kaneko, S., Kurose, T., Watanabe, A., Kuruhara, K., & Kawai, K. 2016. Factors associated with marketable milk production recovery after treatment of naturally occurring acute coliform mastitis. *Journal of Veterinary Medical Science*, 78(5), 917-920. https://doi.org/10.1292/jvms.15-0689

Skowron, K., Sękowska, A., Kaczmarek, A., Grudlewska, K., Budzyńska, A., Białucha, A., & Gospodarek-Komkowska, E. 2019. Comparison of the effectiveness of dipping agents on bacteria causing mastitis in cattle. *Annals of Agricultural and Environmental Medicine*, *26*(1), 39-45. DOI: https://doi.org/10.26444/aaem/82626

Smith, K. L., Todhunter, D. A., & Schoenberger, P. S. 1985. Environmental mastitis: cause, prevalence, prevention. *Journal of dairy science*, *68*(6), 1531-1553. https://doi.org/10.3168/jds.S0022-0302(85)80993-0

Sorge, U. S., Baumgartner, C., & Resch, M. 2020. A survey of mastitis detection practices and treatments on dairy farms in Bavaria, Southern Germany. *Milk Science International-Milchwissenschaft, 73*(5), 24-28. *ISSN 2567-9538*

Sorter, D. E., Kester, H. J., & Hogan, J. S. 2014. Bacterial counts in recycled manure solids bedding replaced daily or deep packed in freestalls. *Journal of Dairy Science*, *97*(5), 2965-2968. https://doi.org/10.3168/jds.2013-7814

Steeneveld, W., Hogeveen, H., Barkema, H. W., van den Broek, J., & Huirne, R. B. 2008. The influence of cow factors on the incidence of clinical mastitis in dairy cows. *Journal of dairy science*, *91*(4), 1391-1402. https://doi.org/10.3168/jds.2007-0705

Todhunter, D. A., Smith, K. L., & Hogan, J. S. 1991. *Serratia* species isolated from bovine intramammary infections. Journal of dairy science, 74(6), 1860-1865. https://doi.org/10.3168/jds.S0022-0302(91)78351-3

Van Soest, F. J., Santman-Berends, I. M., Lam, T. J., & Hogeveen, H. 2016. Failure and preventive costs of mastitis on Dutch dairy farms. *Journal of dairy science*, *99*(10), 8365-8374. https://doi.org/10.3168/jds.2015-10561

Van Soest, F. J., Abbeloos, E., McDougall, S., & Hogeveen, H. 2018. Addition of meloxicam to the treatment of bovine clinical mastitis results in a net economic benefit to the dairy farmer. Journal of Dairy Science, 101(4), 3387-3397. https://doi.org/10.3168/jds.2017-12869

Vollenweider, A. 2023. Mastitis pathogens and antibiotic resistance in beef cows in Switzerland. *Schweiz Arch Tierheilkd*. 2023 Jan;165(1):39-51. DOI: 10.17236/sat00381

Wang, Q., Li, X., Zhou, K., Li, Y., Wang, Y., Zhang, G., Guo H., Zhou J. & Wang, T. 2024a. Mechanisms of conjugative transfer of antibiotic resistance genes induced by extracellular polymeric substances: Insights into molecular diversities and electron transfer properties. *Journal of Hazardous Materials*, *476*, 135181. https://doi.org/10.1016/j.jhazmat.2024.135181

Wang, X., Zhang, H., Yu, S., Li, D., Gillings, M. R., Ren, H., Mao D., Guo J. & Luo, Y. 2024b. Inter-plasmid transfer of antibiotic resistance genes accelerates antibiotic resistance in bacterial pathogens. *The ISME journal*, *18*(1), wrad032. https://doi.org/10.1093/ismejo/wrad032

Watts, J. L. 1988. Etiological agents of bovine mastitis. *Veterinary microbiology*, *16*(1), 41-66. https://doi.org/10.1016/0378-1135(88)90126-5

Wellenberg, G. J., van der Poel, W. H., & Van Oirschot, J. T. 2002. Viral infections and bovine mastitis: a review. Veterinary microbiology, 88(1), 27-45. https://doi.org/10.1016/S0378-1135(02)00098-6

Whist, A. C., Østerås, O., & Sølverød, L. 2006. Clinical mastitis in Norwegian herds after a combined selective dry-cow therapy and teat-dipping trial. *Journal of dairy science*, *89*(12), 4649-4659. https://doi.org/10.3168/jds.S0022-0302(06)72515-2

Zadoks, R. N. 2007. Sources and epidemiology of *Streptococcus uberis*, with special emphasis on mastitis in dairy cattle. *CABI Reviews*, (2007), 15-pp. https://doi.org/10.1079/PAVSNNR20072030

Zadoks, R. N., Allore, H. G., Barkema, H. W., Sampimon, O. C., Gröhn, Y. T., & Schukken, Y. H. 2001. Analysis of an outbreak of *Streptococcus uberis* mastitis. *Journal of dairy science*, *84*(3), 590-599. https://doi.org/10.3168/jds.S0022-0302(01)74512-2

VIII. ACKNOWLEDGEMENTS

An erster Stelle möchte ich meinem Doktorvater, Dr. Wolfram Petzl, danken, welcher mich durch meine Doktorarbeit begleitet hat und stets mit Rat zur Stelle war. Deine ermutigenden Worte waren immer eine Quelle der Motivation und haben mich nach jeder Schreibflaute wieder an den Schreibtisch zurückgebracht, um neue Ideen zu Papier zu bringen.

Besonderen Dank möchte ich an meine Betreuerin Dr. Ulrike Sorge richten, die mir stets mit konstruktiver Kritik und Ermutigungen zur Seite stand. Für dich war keine Frage zu schwer und keine Antwort zu offensichtlich. Mit großer Geduld hast du mich durch das Erstellen meiner Artikel und dieser Arbeit begleitet und mir dabei die Grundzüge des wissenschaftlichen Arbeitens besser erklärt, als ich es in Hörsälen je verstanden habe. Trotz vieler Stolpersteine und vor allem technischer Schwierigkeiten habe ich dank dir nie aufgegeben. Gemeinsam haben wir selbst den SAS-Kundendienst bezwungen.

Dem Tiergesundheitsdienst Bayern e.V. möchte ich danken, dass er mir diese Doktorarbeit ermöglicht hat. Allen voran hat mich Dr. Reglindis Huber-Schlenstedt bei jeglichen Fragen zu den Erregern und Laborabläufen unterstützt, ebenso wie das ganze TGD-Laborteam mir immer hilfreich zur Seite stand.

Doch ohne die Hilfe meiner Kommilitoninnen, den anderen Doktorandinnen am TGD, hätte ich diese Arbeit nicht fertigstellen können. Unsere gegenseitige Unterstützung war mir Gold wert und hat mir über unzählige Probleme hinweggeholfen, genauso wie sie mir aus vielen Tiefpunkten in den letzten zweieinhalb Jahre herausgeholfen haben. Besonderen Dank möchte ich hier an Verena Bechtold richten, die mit mir gemeinsam auch ihre Doktorarbeit eingereicht hat. Ich danke dir für die gemeinsame Zeit, und dass ich mich auf dich stützen konnte.

Besonderer Dank gilt auch Thomas, der mir stets mit Rat und einem offenen Ohr zur Seite stand. Vielen Dank, dass du meinen Frust ertragen und erfolgreich mein SAS installiert hast, an dem ich beinahe verzweifelt bin.

Zu guter Letzte danke ich meiner Familie für ihre emotionale und finanzielle Unterstützung. Sie hat mir viele Sorgen erleichtert und mir erst ermöglicht, eine Doktorarbeit zu beginnen. Ohne eure Hilfe wäre ich noch in zwei Jahren nicht fertig geworden.