Retrospective analysis of quarter milk samples from Bavaria: Changes in the distribution of mastitis pathogens and antimicrobial resistance of *Streptococcus dysgalactiae*, *Streptococcus agalactiae*, and *Streptococcus canis* isolates between 2012 and 2023

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ABBREVIATIONS

AMR	Antimicrobial resistance
BMD	Broth microdilution
BTM	Bulk tank milk
DVG	German Veterinary Association (Deutsche Veterinärmedizinische Gesellschaft)
CAMP	Christie–Atkins–Munch-Peterson
CIA	Critically important antimicrobials
CLSI	Clinical and Laboratory Standards Institute
CMT	California mastitis test
DCT	Dry cow therapy
e.g.	Exempli gratia
erm	Erythromycin ribosome methylation gene family
E.	*Escherichia*
GBS	Group-B *Streptococcus*
i.e.	Id est (that is)
IMI	Intramammary infection
KAA-Agar	Kanamycin–esculin–azide agar,
L.	*Lactococcus*
MALDI-TOF MS	Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry
MDR	Multidrug resistance
MGEs	Mobile genetic elements
MIC	Minimum inhibitory concentration
MLSb	Macrolide-Lincosamide-Streptogramin B
NAS	Non-aureus staphylococci
NIRD	National Institute for Research in Dairying
NMC	National Mastitis Council
NSAIDs	Nonsteroidal anti-inflammatory drugs
OIE	World Organization for Animal Health
QMS	Quarter milk sample
PBP	Penicillin-binding-proteins
RNA	Ribonucleic acid
S.	*Staphylococcus*
Sc.	*Streptococcus*
SCC	Somatic cell count
Se.	*Serratia*
T.	*Trueperella*
TÄHAV	Veterinary Drug Prescription Legislation (Tierärztliche Hausapothekenverordnung)
tet	Tetracycline resistance determinants
TGD	Animal Health Service (Tiergesundheitsdienst)
THI	Temperature-Humidity-Index
VGP	Viertelgemelksprobe

I. INTRODUCTION

Bovine mastitis is one of the most common and economically significant diseases in the dairy industry, causing substantial losses due to decreased milk production, increased treatment costs, and the potential culling of affected animals (HALASA et al., 2007). In addition to its economic consequences, mastitis raises public health concerns due to the growing problem of antimicrobial resistance (AMR) (NARANJO-LUCENA & SLOWEY, 2023), as mastitis treatments account for the majority of antibiotic use in dairy herds worldwide (NOBREGA et al., 2020).

Effective mastitis control requires understanding of the distribution of mastitis pathogens, which can vary across regions influenced by differences in farm management, veterinary services, laboratory resources, and season (ZADOKS & FITZPATRICK, 2009; TOMAZI et al., 2018). This underscores the need for local data, especially in extensive dairy regions like Bavaria in Germany. One of the most common pathogens causing bovine mastitis are *Streptococcus (Sc.)* spp., which include species of the pyogenic group, such as *Sc. dysgalactiae*, *Sc. agalactiae*, and *Sc. canis*. These closely related species are capable of horizontal gene transfer, contributing to the emergence of new AMR (ALVES-BARROCO et al., 2020). In response to the increasing awareness of antibiotic-resistant bacteria, national and international organizations, such as the World Organization for Animal Health (OIE), have implemented surveillance programs to monitor antimicrobial resistance (TOUTAIN et al., 2017). However, these programs often lack comprehensive regional data, and rarer pathogens - such as *Sc. canis* - are not taken into account.

Therefore, the objectives of this work were to investigate the distribution of mastitis pathogens in Bavaria from quarter milk samples (QMS) submitted to the laboratory of the Bavarian Animal Health Service (TGD) between 2014 and 2023 in general, in relation to the clinical status of the quarters, and to determine seasonal differences in the detection risk of mastitis pathogens (Publication I). In addition, the objective was to investigate changes in antimicrobial resistances for *Sc. dysgalactiae*, *Sc. agalactiae*, and *Sc. canis* between 2012 and 2022 (Publication II).

II. LITERATURE REVIEW

1. Bovine mastitis

Bovine mastitis is an endemic disease, characterized by inflammation of the mammary gland, typically caused by intramammary infection (IMI) (HALASA et al., 2007; EFSA et al., 2023). While the causes can be non-infectious (e.g. physical trauma), bacterial infections are the main cause for mastitis (BRADLEY, 2002; CHENG & HAN, 2020). Bacteria usually enter through the teat canal, where they multiply or release toxins within the udder (WINTER & ZEHLE, 2009). In response, the mammary gland initiates an inflammatory reaction to fight the infection, leading to an increase in somatic cell count (SCC) (GARCÍA, 2004). This disease is globally associated with harmful effects on the welfare of dairy cows (SILVA et al., 2021). The importance of mastitis was early recognized and therefore a five-point plan was developed in the 1960s by the National Institute for Research in Dairying (NIRD) which included measures that aimed to control mastitis (RUEGG, 2017). These measures led to a significant reduction of contagious mastitis. However, environmental pathogens have since become more important and while subclinical mastitis has also decreased, there has been no corresponding decrease in clinical mastitis (TENHAGEN et al., 2006).

1.1. Mastitis classification

Bovine mastitis can be classified according to the appearance of symptoms into subclinical and clinical mastitis (AQIB et al., 2021). Clinical mastitis is characterized by observable changes in the milk, udder, or even the cow’s overall condition. The signs of inflammation include abnormalities of the milk character, such as discoloration of the milk, flakes, or lumps. In moderate mastitis cases swelling, heat, redness and pain of the udder can also be present. In severe clinical mastitis cases, cows may exhibit systemic symptoms such as fever, loss of appetite or even death (KIBEBEW, 2017; BRENNECKE et al., 2021). Clinical mastitis poses therefore a major concern for animal welfare (AQIB et al., 2021).

In contrast, subclinical mastitis shows no visible signs of inflammation. Instead its detection depends on diagnostic tools like the California-Mastitis-Test (CMT) (PAKRASHI et al., 2023) or direct determination of the SCC. The only indications are an increased SCC (≥ 100,000 cells/mL) and reduced milk yield, with no noticeable changes of the milk character (WINTER & ZEHLE, 2009). The increased SCC is part of the cows immune response and includes leukocytes (i.e., neutrophils, macrophages, lymphocytes) and epithelial cells (SHARMA et al., 2011) and is commonly used as indicator for intramammary infections (DOHOO & LESLIE, 1991). Due to the lack of obvious symptoms, subclinical mastitis often remains unnoticed and is therefore more prevalent, with an incidence estimated to be 15–40 times higher than clinical mastitis (KABELITZ et al., 2021). Consequently, cows with subclinical mastitis act as a pathogen reservoirs within the herd, from which the pathogens can be spread between or during milking (WINTER & ZEHLE, 2009). As a result, subclinical mastitis is responsible for greater financial losses than clinical mastitis (CHENG & HAN, 2020).

1.2. Mastitis pathogens

Mastitis can be caused by bacteria, viruses, fungi, molds, and algae (WELLENBERG et al., 2002; TOMANIĆ et al., 2022). However, it is mainly caused by bacteria invading the teat canal and causing IMI in one or more quarters of the udder (KIBEBEW, 2017). Once arrived in the gland the pathogens find ideal conditions to multiply and damage the udder tissue (GARCÍA, 2004). These pathogens can be broadly divided into either major and minor or contagious and environmental pathogens (HASSAN et al., 2009).

1.2.1. Major and minor pathogens

Bacteria can be classified in major and minor pathogens depending on the physical and biochemical changes that they induce in milk (HASSAN et al., 2009). Major pathogens are considered to be more virulent and damaging to the udder (REYHER et al., 2012). They include species as *Staphylococcus (S.) aureus, Sc. uberis, Sc. agalactiae, Sc. dysgalactiae, Escherichia (E.) coli, and Trueperella (T.) pyogenes.* Major pathogens often cause clinical mastitis, persist for a prolonged time in the udder, and may require longer antimicrobial therapy (REYHER et al., 2012). In contrast, minor pathogens include pathogens like non-aureus staphylococci (NAS) or *Corynebacterium* spp. They cause less severe reactions in the udder, but are also able to cause clinical mastitis in some cases (HASSAN et al., 2009). Furthermore, they are reported to have a negative impact on milk production, and should therefore not be underestimated causing reduction in milk yield (HEIKKILÄ et al., 2018). Nevertheless, minor pathogens, like NAS, are still considered of lesser importance for the dairy production (DE BUCK et al., 2021).

1.2.2. Contagious and environmental pathogens

This classification depends on the transmission of the causative pathogens. Contagious pathogens are known to spread from cow to cow, whereby cows with an infected udder can infect healthy cows (SHARIF et al., 2009). This usually happens during milking time, with the hands of the milker, towels or the milking machine being a reservoir of contagious pathogens (GARCÍA, 2004). Therefore new infections usually appear during the lactation period (SHARIF et al., 2009). The main contagious mastitis pathogens are *S. aureus* and *Sc. agalactiae* (FRANCOZ et al., 2012). Living on the udder and teat, these pathogens colonize and invade the teat canal, causing IMI (CHENG & HAN, 2020). They are adapted to survive in the udder and spread easily within the herd (KABELITZ et al., 2021).

Contrary to contagious pathogens, environmental pathogens such as *E. coli* or *Sc. uberis* do not usually live on the cow's udder and teat skin. As the name suggests they exist in the cow's environment like the bedding and housing of the herd, from where they invade the udder and multiply, inducing a host immune response. Environmental pathogens often cause clinical mastitis and are usually quickly eliminated by the immune system of the host (CHENG & HAN, 2020). Nevertheless, not all pathogens could be strictly classified into contagious or environmental. For example, *Sc. dysgalactiae* can spread through both environmental sources and contagious transmission (WENTE & KRÖMKER, 2020).

An important difference between contagious and environmental pathogens lies in their effects on subclinical mastitis. Research suggests that subclinical mastitis is more likely to occur with contagious pathogens (SØRENSEN et al., 2010). Additionally, mastitis caused by contagious pathogens tends to result in greater milk loss and therefore higher costs per case compared to mastitis caused by environmental pathogens (SØRENSEN et al., 2010).

1.2.3. Prevalence of mastitis pathogens

Historically, contagious pathogens such as *S. aureus* and *Sc. agalactiae* were the most prevalent causes of bovine mastitis (RUEGG, 2017). However, due to effective control measures, the prevalence has shifted towards environmental pathogens (KABELITZ et al., 2021). For example, a study in Northern Germany found that *Sc. uberis* and coliforms, such as *E. coli*, are now among the leading causes of severe mastitis (SCHMENGER & KRÖMKER, 2020). Other studies also found *Sc. uberis* to be the most common pathogen in clinical mastitis cases, e.g. in Belgium (VERBEKE et al., 2014) and an increasing prevalence for this pathogen has been reported around the world (PHUEKTES et al., 2001). Nowadays, the distribution of mastitis pathogens from culture positive quarter milk samples in Germany, as reported by the German Veterinary Association (DVG, 2024), in decreasing frequency is as follows: *Sc. uberis*, NAS, *E. coli,* coryneformes, esculin-positive streptococci, *S. aureus* and *Sc. dysgalactiae*. Despite overarching trends, the distribution of mastitis pathogens varies between countries. For example, WANG et al. (2022) reported a decreasing trend for *S. aures* in China, while ACHARYA et al. (2021) noted an increasing detection in Canada. In addition, SMISTAD et al. (2023) found a relatively stable prevalence of *S. aureus*. MORALES-UBALDO et al. (2023) also reported in their meta-analysis wide ranges of prevalences for example for *Sc. agalactiae*, with 0.1% in Canada and 10.3% in Ethiopia, or *Sc. uberis,* with 1.5% in Serbia and 74% in China. Therefore, local data are very important in the development of mastitis control programs, as knowing the specific pathogen distribution in each region allows for more effective and targeted measures.

1.3. Risk factors and control

Bovine mastitis is a complex disease influenced by multiple risk factors that can be categorized into intrinsic (host-related) and extrinsic (environmental and management-related) factors (AJOSE et al., 2022). The intensity of the inflammatory reaction is in addition also influenced by the invading pathogen (ASHRAF & IMRAN, 2020). Host factors include breeding and genetics, udder structure, age and stage of lactation, milk yield, transition period, host nutritional stress, and immune system (CHENG & HAN, 2020). High-yielding breeds like Holstein-Friesians are more prone to mastitis compared to lower-yielding breeds like Jerseys and Rendena cattle, which show greater disease resistance (CHENG & HAN, 2020). Structural factors such as teat-form, pendulous udders, and teat-lesions also increase the infection risk (SHARIF et al., 2009). Age is another factor, with older cows being more vulnerable due to the easier penetration of pathogens into the teat canal and the accumulation of existing pathogens (KITILA et al., 2021).

The transition period around calving is particularly risky due to immunosuppression from oxidative stress. Additionally, nutritional stress, particularly negative energy balance and deficiencies in essential nutrients, can weaken the immune system, increasing the likelihood of infection (CHENG & HAN, 2020).

Extrinsic factors include poor milking hygiene, inadequate sanitation and improper use of milking equipment, all of which can significantly increase the incidence of clinical and subclinical mastitis (MBINDYO et al., 2020). Environmental conditions, such as the cleanliness of housing and udder hygiene, also play a significant role. Overcrowding, contaminated surfaces, wet bedding, and poor ventilation can favor the growth of mastitis pathogens and increase the exposure of cows, leading to an increased incidence of mastitis (CHENG & HAN, 2020). Also climatic factors, such as hot and humid climate, detected through a high Temperature-Humidity-Index (THI) can lead to a higher shedding of mastitis causing pathogens, which increases the risk of mastitis (HAMEL et al., 2021). Studies have shown that high temperatures and high humidity can increase the likelihood of IMI, especially from environmental pathogens (RAKIB et al., 2020; GANTNER et al., 2023). This factor is particularly important in view of changing climate patterns and more frequent extreme weather events due to climate change (BOKHARAEIAN et al., 2023).

Controlling mastitis requires a comprehensive approach that includes improved herd management, regular testing and strict hygiene protocols to reduce the incidence and economic impact of mastitis in dairy herds. As stated above, a mastitis control program, known as the five-point-plan, was successfully introduced in the 1960’s (RUEGG, 2017). This plan included dipping teats after milking, treating all cows with antibiotics at drying off, appropriate treatment of clinical cases of mastitis, culling chronically ill cows and maintenance of milking equipment (HOGEVEEN et al., 2011). Later these measures were expanded to a 10-point plan, that included further practices such as, “establishment of goals for udder health, maintenance of a clean, dry, comfortable environment, proper milking procedures, good record-keeping, maintenance of biosecurity, regular monitoring of udder health status, and periodic review of the herd’s mastitis control program” (MIDDLETON et al., 2014). Depending on the causative pathogen, the most important control and prevention measures against contagious mastitis are aimed at limiting contact between infected and healthy cows (CHENG & HAN, 2020). The control of environmental mastitis can be achieved by minimizing the exposure of teat ends to environmental pathogens and strengthening the cow's resistance to intramammary infections (IMI), e.g. through vaccinations (SMITH & HOGAN, 1993; CHENG & HAN, 2020). However, mastitis control must be constantly adapted within a herd, as different risk factors and pathogens contribute to the varying effectiveness of the measures.

1.4. Treatment

The main treatment against bovine mastitis is antimicrobial therapy (GOMES & HENRIQUES, 2016). Whenever possible, mastitis treatment should be targeted at the causative bacteria. Accurate diagnosis of the causative pathogens, as well as susceptibility testing, is a crucial part of mastitis management (SHARUN et al., 2021). It guides the selection of appropriate antibiotics and reduces unnecessary antimicrobial use and thereby the risk of AMR (SHARUN et al., 2021). However, in acute cases, treatment is often initiated based on herd data and clinical experience (KIBEBEW, 2017). In some cases, particularly with pathogens such as *E. coli*, non-antimicrobial measures such as anti-inflammatory treatment with nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, supportive fluid therapy, and frequent milking should be used, especially in mild to moderate cases (SUOJALA et al., 2013). Moreover, mild mastitis cases involving "no-growth" or gram-negative pathogens rarely benefit from intramammary antibiotic treatments (SORGE et al., 2020). As to the antimicrobial therapy it can be done during lactation or as dry cow therapy (DCT). DCT is generally used at the end of lactation to treat existing infections and prevent new infections during the dry period (GRUET et al., 2001). This therapy may involve the use of antibiotics alone or in combination with internal teat sealants, which has been shown to significantly reduce the incidence of clinical and subclinical mastitis in the following lactation (RUNCIMAN et al., 2010). During lactation, the focus shifts to treating new infections when they occur, and prolonged antibiotic treatment can improve cure rates, particularly in cases involving *S. aureus* (BARKEMA et al., 2006). It has been noted, that bacteriological cure for clinical mastitis has been the highest in the first 100 days of lactation, regardless of pathogen (SCHMENGER & KRÖMKER, 2020). When choosing the antibiotic it should be evaluated if critically important antimicrobials (CIA) are necessary. CIA, such as fluoroquinolones, 3rd and 4th generation cephalosporins, and macrolides are essential for treating certain diseases in humans (MCEWEN & COLLIGNON, 2018). Hence, the use of CIA in food-producing animals may elevate the risk of untreatable infections in humans (NOBREGA et al., 2020). Furthermore, critical antimicrobials do not add benefit to the treatment of mild to moderate mastitis cases (NOBREGA et al., 2020). As the extensive use of antimicrobials raises significant public health concerns, for example almost 90% of antibiotic residues in milk are related to mastitis treatment (KABELITZ et al., 2021), current research deals with alternatives to antimicrobial therapy such as the use of herbal remedies, probiotics, bacteriophages, and nanotechnology-based treatments (GOMES & HENRIQUES, 2016; MUSHTAQ et al., 2018; ANGELOPOULOU et al., 2019).

1.5. Antimicrobial resistance

AMR refers to the ability of bacteria to withstand the effects of antimicrobial drugs, leading to treatment failure, increased mortality rates, and higher healthcare costs (PRESTINACI et al., 2015). Although, antimicrobial therapy is an important measure for treating bovine mastitis and human infections (REN et al., 2020), its use contributes to the development of AMR, which poses a serious threat to human and animal health (NOBREGA et al., 2020). Broad-spectrum antimicrobials are known to increase selection pressure and thus have a greater effect on the development of resistance than narrow-spectrum antimicrobials (BARBOSA & LEVY, 2000). Despite this, veterinarians often choose broad-spectrum antibiotics, even though most mastitis cases in southern Germany are caused by gram-positive pathogens that are penicillin-sensitive *in vitro*, and gram-negative mastitis does not benefit from antibiotic treatment (SORGE et al., 2020). The inappropriate prescription of antimicrobials promotes drug resistance in exposed bacteria and leads to the persistence of antibiotic resistance genes within bacteria that share the same ecological niches (ALVES-BARROCO et al., 2020). Consequently, the EUROPEAN COMMISSION (2015) recommends the prudent use of antimicrobials and emphasizes a rational and targeted approach.

Mechanisms of AMR include: enzymatic inactivation of antibiotics, e.g. the degradation of β-lactams by β-lactamases (GAUBA & RAHMAN, 2023); changes in the antimicrobial target that prevent effective binding of the antibiotic, often due to spontaneous mutations, including genomic and RNA (ribonucleic acid) changes (GOMEZ et al., 2017); and blocking the access of drugs to targets, e.g. by reducing cellular uptake through reduced membrane permeability and/or by using active efflux pumps that enhance the elimination of drugs from the cell (ALVES-BARROCO et al., 2020). Bacterial drug resistance can be divided into intrinsic and acquired resistance (REYGAERT, 2018). Intrinsic resistance is a naturally occurring phenomenon that inhibits antimicrobial activity and can be observed in most strains of a species. This resistance may be constitutive (existing independently of prior antibiotic exposure) or induced (by exposure to antibiotics or environmental stressors) (ALVES-BARROCO et al., 2020). Acquired resistance results from chromosomal point mutations or the acquisition of mobile resistance genes, which enable previously susceptible bacterial populations to develop resistant strains, usually following exposure to an antimicrobial agent (ALVES-BARROCO et al., 2020).

Continuous monitoring of resistant mastitis pathogens is essential for decision-making in bovine mastitis treatment. To address this need, various national and international organizations, including the World Organization for Animal Health and the European Commission, have introduced AMR surveillance programs to monitor the development of resistances (TOUTAIN et al., 2017). Unfortunately, these nationwide surveillance programs lack information on regional differences and do not consider rare pathogens that can also serve as reservoirs for resistance genes. In Germany, the use of antimicrobials is further regulated by law, e.g. by the 16th Act Amending the German Medicinal Products Act (16th AMG amendment) from 2014 or the amendment to the law in 2018 for the use of antimicrobials in veterinary medicine (Verordnung über tierärztliche Hausapotheken, TÄHAV) (BUNDESTIERÄRZTEKAMMER, 2018). These regulations aim to minimize the use of antimicrobials and CIA in livestock farming.

2. Streptococcal mastitis

One of the most common major pathogens causing bovine mastitis are *Streptococcus* spp. Streptococci are gram-positive, catalase-negative, facultatively aerobic pathogens with spherical or ovoid shape, which often build chains or diplococci in liquid medium (TOIT et al., 2014). They are microbiologically identified based on colony morphology, hemolysis, esculin hydrolysis and CAMP (Christie–Atkins–Munch-Peterson) factor and classified into Lancefield groups (DVG, 2018). This genus includes contagious and environmental bacteria, which can induce both, subclinical as well as clinical mastitis (KACZOREK et al., 2017).

Streptococcal mastitis usually lasts around 12 days, but can extend to over 300 days in chronic cases (KABELITZ et al., 2021). A higher risk of developing a mastitis due to streptococci has been associated with older cows and early lactation stages (AMIN, 2017). Symptoms vary widely depending on the causing species and may include abnormal milk parameters, swollen udder, systemic symptoms such as fever, anorexia or behavioral changes. But also, the absence of symptoms can occur alongside an increase in SCC (KABELITZ et al., 2021). The main strategy for the treatment of streptococcal mastitis is antimicrobial therapy, with β-lactams being the first choice, and despite the widespread use of penicillin, high susceptibility to these antimicrobials has been reported (HAENNI et al., 2018). The most important species causing streptococcal mastitis are *Sc. uberis, Sc. dysgalactiae*, *Sc. agalactiae* and *Sc. canis.* While *Sc. uberis* is known as esculin-positive, *Sc. dysgalactiae, Sc. agalactiae* and *Sc. canis* are esculin-negative (DVG, 2018). The last three species are major pathogens of the pyogenic group and are closely related (ALVES-BARROCO et al., 2021). *Sc. dysgalactiae, Sc. agalactiae,* and *Sc. canis* are described in more detail below.

2.1. ***Streptococcus dysgalactiae***

Sc. dysgalactiae is an esculin-negative CAMP-negative streptococcal pathogen and belongs to Lancefield-Group C (DVG, 2018). It is a significant cause of mastitis, ranked as the second most common streptococcal pathogen isolated from bovine mastitis in Germany (4.7%), following *Sc. uberis* (DVG, 2024), and the third most common pathogen responsible for clinical mastitis in Bavaria (HUBER-SCHLENSTEDT & SCHIERLING, 2016). In Norway, this species is even reported to be the second most common major mastitis pathogen, after *S. aureus* (SMISTAD et al., 2023). A wide range of prevalence is noted in other countries worldwide, e.g. 40% in North America (KABELITZ et al., 2021) and 7% in Northwest China (SHEN et al., 2021). *Sc. dysgalactiae* is considered an intermediate pathogen due to its ability to spread through both contagious and environmental routes, and it is assumed that transmission occurs from farm to farm (WENTE & KRÖMKER, 2020). The results of a study from WENTE and KRÖMKER (2020) indicated that carrying out strain comparisons at herd level could be a valuable strategy for selecting appropriate control measures against this pathogen. For example, the presence of a single strain in several cows in a herd indicates a contagious infection while multiple strains indicate an environmental transmission. The different transmission pathways would require different control strategies.

Infections caused by this pathogen can manifest as both, subclinical, and clinical mastitis (CALVINHO et al., 1998). The presence of *Sc. dysgalactiae* at the time of drying off, increased the risk of clinical mastitis during lactation (GREEN et al., 2002). Furthermore, high shedding rates (HAMEL et al., 2021) and milk losses (HEIKKILÄ et al., 2018) were also associated with this pathogen. *Sc. dysgalactiae* is furthermore involved in the multicausal disease known as summer mastitis, which mainly affects dry cows and heifers in summer in northern Europe and Japan, and has also been isolated from flies (CALVINHO et al., 1998). Intramammary antibiotic therapy has been shown to achieve cure rates of approximately 90% for subclinical mastitis caused by *Sc. dysgalactiae* (ERSKINE et al., 2003).

2.2. *Streptococcus agalactiae*

Sc. agalactiae is an esculin-negative, CAMP-positive streptococcal pathogen belonging to Lancefield-Group B (DVG, 2018). Due to its Lancefield-classification this species is also known as Group B *Streptococcus* (GBS) (RAABE & SHANE, 2019). *Sc. agalactiae* is widely known as a contagious pathogen, but JØRGENSEN et al. (2016) found in their study *Sc. agalactiae* strains also in the cows' environment, suggesting an oro-fecal transmission cycle. This has also been reported by other studies (COBO-ÁNGEL et al., 2018; HAN et al., 2022). *Sc. agalactiae* was first identified in bovines and was exclusively linked to bovine mastitis (HERNANDEZ et al., 2021). Later it was found out, that this pathogen is also a human pathogen that can cause severe infections in neonates, pregnant women, elderly people and immune-comprised adults (RATO et al., 2013). By 1927, *Sc. agalactiae* was identified as the cause of approximately 90% of intramammary infections, being a major factor in the degradation of milk quality (RUEGG, 2017). Nowadays, with introduction of the five-point plan and due to hygiene measures its incidence has drastically fallen and is now only rarely isolated from bovine mastitis cases (HAENNI et al., 2018). For example, in Germany *Sc. agalactiae* is only found in 2% of culture positive quarter milk samples (DVG, 2024). In other countries prevalences of 6% in Slovakia (COBIRKA et al., 2020), 0.1% in Canada (LEVISON et al., 2016), and 18% in Pakistan (MORALES-UBALDO et al., 2023) have been reported. In cattle this pathogen is known as a major streptococcal pathogen that causes subclinical and mild to moderate clinical mastitis (HAENNI et al., 2018). It causes high SCC (DJABRI et al., 2002) and has a low probability of self-cure (COBO-ÁNGEL et al., 2018). Untreated cases can even lead to udder atrophy. However, hygienic measures and β-lactams for treatment are usually effective against this pathogen (VALENTIN-WEIGAND, 2023).

2.3. *Streptococcus canis*

Sc. canis is a β-hemolytic, esculin-negative streptococcal species belonging to Lancefield-Group G (DVG, 2018). It is a widely known commensal of the mucous membranes and skin of carnivores and could be isolated of asymptomatic carriers as well as in pathological cases (TIKOFSKY & ZADOKS, 2005). *Sc. canis* used to be considered a pathogen of dogs and cattle, but has since been isolated from many mammals, making it the streptococcus species with the widest host range, causing mild to severe diseases with zoonotic potential (PAGNOSSIN et al., 2022).

In 2022, the average prevalence of *Sc. canis* was only 0.2% in Germany (DVG, 2024). A study by GROH et al. (2023) found *Sc. canis* in only 0.3 % of all quarter milk samples they examined in Bavaria, but in 16.5% of samples from clinical mastitis cases. In another study, *Sc. canis* was found in less than 1% of all samples tested in Canada (LEVISON et al., 2016). Unfortunately, comprehensive data on *Sc. canis* is scarce, which might be because it is relatively rare and therefore not as much of a focus in monitoring programs. Although, *Sc. canis* is described to be a relatively rare cause of bovine mastitis, multiple studies have documented outbreaks of mastitis caused by this pathogen (CHAFFER et al., 2005; TIKOFSKY & ZADOKS, 2005; EIBL et al., 2021; GROH et al., 2023).

In cows *Sc. canis* is described as a contagious pathogen that can cause outbreaks of subclinical mastitis with a marked increase of SCC (KRÓL et al., 2015). Furthermore, it has been shown that *Sc. canis* is capable of causing long-term subclinical mastitis with durations of up to 14 months (KRÓL et al., 2015). TIKOFSKY and ZADOKS (2005) found that *Sc. canis* can persist in untreated cows, with a low spontaneous self-cure rate of only 9%, but treatment with β-lactams showed cure rates of 67% during lactation and up to 87.5% in dry-treated cows. Persistently infected cows can be the source of *Sc. canis* infections for other animals, so effective prevention and control within a herd requires measures like those used in *Sc. agalactiae* eradication programs, such as detection and therapy of infected cows, identification of therapy-resistant cows, DCT, and post-milking teat dipping (KRÓL et al., 2015). In addition, when assessing biosecurity measures the potential spread from pets to cows should also be considered (PAGNOSSIN et al., 2022).

2.4. Antimicrobial resistance in esculin-negative streptococci

In veterinary medicine, a decreasing susceptibility to β-lactams has been observed in bovine streptococci, particularly in *Sc. uberis*, *Sc. dysgalactiae* and *Sc. agalactiae,* from full towards decreased susceptibility, requiring ongoing surveillance (HAENNI et al., 2018). For example, in Romania resistance against penicillin for *Sc. dysgalactiae* and *Sc. agalactiae* was reported by PASCU et al. (2022). In Brazil, resistance percentages of even 86% against penicillin were found in *Sc. agalactiae* isolates from bulk tank milk (BTM) samples (MESQUITA et al., 2019). In contrast, a European AMR monitoring program (EL GARCH et al., 2020) found no resistance of *Sc. dysgalactiae* and *Sc. agalactiae* against penicillin. They reported the highest resistance percentages in those pathogens against tetracycline. This aligns with other studies reporting high tetracycline-resistances in *Sc. dysgalactiae* - 64% in the Netherlands (MARAN, 2009), up to 100% in Portugal (RATO et al., 2013) and China (SHEN et al., 2021) - and in *Sc. agalactiae* - 55.5% in India (JAIN et al., 2012), 57% in Portugal (RATO et al., 2013), and 81% in China (LIU et al., 2022).

Unfortunately, data about AMR in *Sc. canis* from bovine mastitis cases are sparse. However, the most commonly observed AMR phenotype is tetracycline resistance. This is found in strains from different host species and is associated with the presence of the *tetM, tetO, tetS, tetK and tetL* genes (PAGNOSSIN et al., 2022). The widespread prevalence of tetracycline resistance may be attributed to the presence of tetracycline resistance determinants (tet genes) on conjugative transposons, which can efficiently transfer between related bacterial species (ALVES-BARROCO et al., 2020). Apart from tetracycline, the European monitoring program (EL GARCH et al., 2020) also reported resistances to erythromycin and pirlimycin in *Sc. dysgalactiae* and *Sc. agalactiae.* AMR against these antimicrobials has also been reported for *Sc. dysgalactiae*, e.g., in the Netherlands (MARAN, 2009), and for *Sc. agalactiae,* e.g., in Portugal (RATO et al., 2013). The combination of resistance to macrolides and lincosamides is a common AMR pattern in these species. Cross-resistance to macrolides, lincosamides, and streptogramin B is frequently observed in the MLSb phenotype

(PINTO et al., 2013; HAENNI et al., 2018), which is caused by a methylase encoded by the erm (erythromycin ribosome methylation) gene family (DZYUBAK & YAP, 2016). Additionally*,* *ermB* was found in nearly all erythromycin-pirlimycin resistant isolates, as reported by RATO et al. (2013). Furthermore, a significant association between *ermB* and *tetM* has been identified among the strains of pyogenic streptococci. Tetracycline resistance genes are often acquired through mobile genetic elements (MGEs) that also carry determinants for erythromycin resistance (ALVES-BARROCO et al., 2020).

In pyogenic streptococci also multidrug resistance (MDR) has been reported, complicating antimicrobial therapy and leading to treatment failure in humans and animals (ALVES-BARROCO et al., 2020). For example, a wide range of multidrug resistance has been observed all over the world with 82% of the tested isolates in a study from China (SHEN et al., 2021), but only 6% in Germany (MINST et al., 2012). This shows that AMR and MDR can vary greatly in different countries and regional surveillance is of great importance. Furthermore, although *Sc. canis* is known to be a rare mastitis pathogen and AMR seems to be low, it is also essential to monitor its distribution and resistance trends, since rare species may serve as resistance gene reservoirs and AMR emerges as result of horizontal gene transfer, among other mechanisms (ALVES-BARROCO et al., 2020).

3. Objectives

Bavaria is an important region for the German dairy industry, where 30% of the country's dairy cows are kept (BAYERISCHE LANDESAMT FÜR STATISTIK, 2023) and which produces a quarter of the annual milk volume (BITTLMAYER, 2015). Given the importance of mastitis for the dairy industry and the increasing concern about AMR, it can be concluded that knowledge about regional mastitis pathogen distribution and AMR trends is crucial to monitor trends and adapt appropriate management strategies and treatments. Therefore, the objectives of this work were to investigate the distribution of mastitis pathogens in Bavaria from QMS submitted to the laboratory of the TGD between 2014 and 2023 a) in general, b) in relation to the clinical status of the quarters, and c) to determine seasonal differences in the detection risk of mastitis pathogens. Furthermore, the objective was to investigate changes in antimicrobial resistances for *Sc. dysgalactiae*, *Sc. agalactiae*, and *Sc. canis* between 2012 and 2022.

III. PUBLICATIONS

1. Publication I

Cumulative doctoral achievement: Publication

Distribution of Bovine Mastitis Pathogens in Quarter Milk Samples from Bavaria, Southern Germany, between 2014 and 2023—A Retrospective Study

Bechtold, V.; Petzl, W.; Huber-Schlenstedt, R.; Sorge, U.S.

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Article

Distribution of Bovine Mastitis Pathogens in Quarter Milk Samples from Bavaria, Southern Germany, between 2014 and 2023—A Retrospective Study

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Simple Summary: Bovine mastitis is the most common disease affecting the dairy industry and is mostly caused by intramammary infections (IMIs) due to mastitis pathogens. In this retrospective study we investigated the distribution of mastitis pathogens in all quarter milk samples (QMSs) submitted to the Bavarian Animal Health Service (TGD) in Southern Germany between 2014 and 2023. Overall, 19% of the QMSs contained mastitis pathogens and the most frequently isolated pathogens, in decreasing frequency, were non-aureus staphylococci (NAS), *Staphylococcus (S.) aureus*, *Streptococcus (Sc.) uberis*, and *Sc. dysgalactiae*. However, differences were found in the distribution of the mastitis pathogens depending on the mastitis status of the quarter from which the samples originated and the time of year.

Abstract: The objective of this study was to investigate the distribution of mastitis pathogens in quarter milk samples (QMSs) submitted to the laboratory of the Bavarian Animal Health Service (TGD) between 2014 and 2023 in general, in relation to the clinical status of the quarters, and to analyze seasonal differences in the detection risk. Each QMS sent to the TGD during this period was analyzed and tested using the California Mastitis Test (CMT). Depending on the result, QMSs were classified as CMT-negative, subclinical, or clinical if the milk character showed abnormalities. Mastitis pathogens were detected in 19% of the QMSs. Non-aureus staphylococci (NAS) were the most common species isolated from the culture positive samples (30%), followed by *Staphylococcus (S.) aureus* (19%), *Streptococcus (Sc.) uberis* (19%), and *Sc. dysgalactiae* (9%). In culture-positive QMSs from CMT-negative and subclinically affected quarters, the most frequently isolated pathogens were NAS (44% and 27%, respectively), followed by *S. aureus* (25% and 17%, respectively) and *Sc. uberis* (8% and 22%, respectively). In QMSs from clinically affected quarters, the most frequently isolated pathogens were *Sc. uberis* (32%), *S. aureus* (13%), *Sc. dysgalactiae* (11%), and *Escherichia (E.) coli* (11%). The distribution of NAS and *Sc. uberis* increased throughout the study period, while that of *S. aureus* decreased. From June to October, QMSs from subclinically affected quarters increased and environmental pathogens, such as *Sc. uberis*, were detected more frequently. In conclusion, this study highlights the dynamic nature of the distribution of mastitis pathogens, influenced by mastitis status and seasonal factors. Environmental pathogens still play an important role, especially in clinical mastitis and seasonal dependency, with the number of positive samples continuing to increase. It is therefore essential to continue mastitis control measures and to regularly monitor the spread of mastitis pathogens in order to track trends and adapt targeted prevention measures.

Keywords: bovine mastitis; mastitis pathogens; incidence; season

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1. Introduction

Bovine mastitis is the most common disease in the dairy industry, leading to significant economic losses due to reduced milk production and quality [1]. Apart from the economic consequences, mastitis also affects animal welfare and raises public health concerns due to the increased use of antibiotics [2]. Typically, bovine mastitis is caused by pathogens like staphylococci, streptococci, or coliform species that induce intramammary infections (IMIs) [3]. These IMIs can manifest either clinically, with typical signs of inflammation, or subclinically, without visible signs [4]. The pathogens that cause mastitis are traditionally classified into contagious and environmental, or alternatively major and minor pathogens. The first classification depends on the transmission of the causative pathogens. Contagious pathogens like *Staphylococcus (S.) aureus* and *Streptococcus (Sc.) agalactiae* are thought to spread predominantly via milk droplets among cows [5]. This usually happens during milking time, where the hands of the milker, towels, or the milking machine serve as a fomite for the transmission of contagious pathogens [6]. In contrast, environmental pathogens such as *Sc. uberis* and coliforms as *Escherichia (E.) coli* are commonly found in the cow's environment, such as their bedding or lanes. From there, they can infect the udder [7]. Major pathogens often cause clinical mastitis and can remain in the udder for an extended period, whereas minor pathogens usually cause less severe reactions [8,9]. Historically, contagious pathogens such as *Sc. agalactiae* and *S. aureus* were the primary cause of mastitis [2]. In the 1960s, a five-point plan was developed by the National Institute for Research in Dairying, which was later expanded to a ten-point plan, that included practices such as teat dipping after milking, proper maintenance of milking equipment, and treating all cows with antibiotics at dry-off [2,10]. These measures led to a significant reduction in contagious mastitis pathogens and shifted the prevalence towards environmental pathogens [11]. Besides poor hygiene, environmental factors, such as hot and humid climate, can promote the growth of environmental mastitis pathogens [1]. For example, dairy cows are affected by heat stress in the warmer months, and a high temperature–humidity index may lead to a higher shedding of mastitis-causing pathogens [12]—even in moderate climates such as Germany's.

In Germany, environmental pathogens, *Sc. uberis* in particular, have the highest prevalence and are responsible for the majority of clinical mastitis cases in Northern Germany [13]. This was also reported for Bavaria, where *Sc. uberis* also accounts for the majority of clinical mastitis cases [14]. Bavaria is a significant dairy-producing region in Germany, housing 30% of German dairy cows. The predominant breed is Simmental cows and the average herd size is 44 cows per herd [15,16]. This differs from other parts of Germany, e.g., eastern Germany, where the predominant breed is Holstein-Friesian and the average herd size is 197 cows [17]. Regional differences of dairy production may impact the distribution of mastitis pathogens. Therefore, it is important to monitor trends of those pathogens for specific regions over a longer period. This knowledge may directly impact management practices and preventive measures on dairy farms and thus plays an essential role in improving dairy cattle health. Therefore, the objectives of this study were to investigate the distribution of mastitis pathogens in quarter milk samples (QMSs) submitted to the laboratory of the Bavarian Animal Health Service (TGD) between 2014 and 2023 (a) in general, (b) in relation to the clinical status of the quarters from which the QMS originated, and (c) to analyze possible seasonal differences in the detection of mastitis pathogens.

2. Materials and Methods

This retrospective study included all quarter milk samples (QMSs) sent to the milk quality laboratory of the Bavarian Animal Health Service (TGD) between 2014 and 2023 (n = 3,886,162). The samples used in this study were collected for herd health management and diagnostic purposes. Institutional Animal Care and Use Committee approval was therefore not necessary.

Most QMSs were taken from whole herd screenings by TGD technicians (around 83% across the years). Those herd screenings were requested by the farmer or veterinarian, and generally, TGD technicians collected aseptic QMSs from all functioning quarters of lactating cows. This was carried out to check the intramammary infection status within herds, improve udder health, or even to help avoid a milk delivery ban in rare instances. Due to the small herd size (on average around 45 cows), all lactating cows were usually sampled to identify the predominant mastitis pathogen within the herd. In some cases, e.g., larger herds or follow-up sampling, a subset of cows might have been chosen for examination. The remaining QMSs (about 17% across the years) were submitted by farmers or veterinarians for individual case screenings, such as cows with clinical mastitis or before drying off. In Germany, usually, all quarters of a cow are sampled because billing is on a per cow (not sample) basis and the sample is collected in sterile 9 mL vials with boric acid. A semiquantitative analysis of somatic cells in milk of each QMS was evaluated directly on farm by TGD technicians or upon arrival at the milk laboratory using the California Mastitis Test (CMT). Samples were classified as negative (N) if the CMT showed a negative or as subclinical (S) if the CMT showed a positive result. Clinical mastitis (C) was diagnosed if the milk showed abnormal characteristics or if the cow exhibited other signs of clinical mastitis (e.g., swollen udder). This classification was determined either by technicians during on-farm sampling or through visual examination of the milk in the laboratory.

2.1. Bacteriological Analysis

All QMSs were culturally tested in the TGD milk laboratory according to the (at the time) current guidelines of the German Veterinary Association (DVG) (e.g., [18]). Each QMS was cultured on esculin blood agar based on Columbia agar with sheep's blood additive using an inoculum volume of 0.01 mL or 0.05 mL for QMSs from clinically affected quarters. The plates were incubated aerobically at 36 ± 2 °C. Evaluations were performed after 18–24 h and 48 h of incubation. If it was not possible to evaluate the plates twice, they were only evaluated after 36 h of incubation. The determination of a positive result depended on the respective species in accordance with DVG guidelines.

The isolates were initially differentiated based on colony morphology, Gram stain, hemolysis, and hemotoxin zones. *S. aureus* was identified by colony morphology and hemolysis (clear zone of β-hemolysis). Clumping factor or coagulase were assessed only in isolates that did not exhibit a clear zone of β-hemolysis to distinguish them from non-aureus staphylococci (NAS). MALDI-TOF (microflex MALDI Biotyper, reference database V.3.3.1.0., Bruker Daltonik GmbH, Leipzig, Germany) was used for strains with unclear results or for further differentiation into individual species (e.g., *S. haemolyticus*, *S. chromogenes*, *S. epidermidis*). NAS were rarely further differentiated. To simplify matters, the identified staphylococcal species were divided into *S. aureus* and NAS.

Streptococcal strains were differentiated based on several criteria, including colony morphology, hemolysis pattern, esculin hydrolysis, CAMP (Christie–Atkins–Munch–Peterson) factor, and classification into Lancefield groups. To identify the according Lancefield group, a commercial test kit was used (Streptex™ Acid Extraction KT/50TST, ZL59/R30951301, Thermo Scientific™, Waltham, MA, USA). Esculin-negative strains were further differentiated using the CAMP factor test, in which a β-hemolytic *S. aureus* strain was used. *Sc. agalactiae* was identified as esculin-negative and CAMP factor-positive, in contrast to *Sc. dysgalactiae*, which was identified as esculin-negative and CAMP factor-negative, belonging to Lancefield Group C. Strains of streptococci displaying significant β-hemolysis within Lancefield Group G were identified as *Sc. canis*. Esculin-positive streptococci were cultured on KAA-Agar (kanamycin–esculin–azide agar, Merck 1.05222.0500, Darmstadt, Germany), an in-house method with an agar selective for enterococci, and a disk test against penicillin (10 µg, Oxoid CT0043B, Thermo Scientific™, Waltham, MA, USA) and rifampicin (2 µg, Oxoid CT0078B, Thermo Scientific™, Waltham, MA, USA). Depending on the results of this method, strains could be classified into *Sc. uberis*, *Enterococcus (E.)* spp., or other esculin-positive streptococci. Using MALDI-TOF, species could be further differentiated but were here summarized as *Enterococcus* spp. (*E. faecium*, *E. faecalis*), *Lactococcus (L.)* spp. (*L. lactis*, *L. garvieae*), and other esculin-positive streptococci (if not applicable to either enterococci or lactococci). *Trueperella (T.) pyogenes* was identified on the basis of colony morphology, hemolysis pattern and, if necessary, microscopy.

Further differentiation by MALDI-TOF was carried out for all Gram-negative rod bacteria. Here, species were summarized as *E. coli*, *Serratia (Se.)* spp. (incl. *Se. marcescens*), and Gram-negative species (e.g., *Pseudomonas* spp., *Pasteurella* spp., *Proteus* spp., *Klebsiella* spp., coliforms, *Raoultella* spp.).

Other rarely detected species that were not applicable to the other groups were classified as “others” (e.g., *Nocardia* spp., *Listeria* spp., *Mycoplasma* spp.).

In the following text, the pathogens are assigned as follows: Environmental pathogens: *Sc. uberis*, *Sc. dysgalactiae*, *E. coli*, *T. pyogenes*, NAS, other esculin-positive streptococci, *Serratia* spp., and Gram-negative pathogens. Contagious pathogens: *S. aureus*, *Sc. agalactiae*, and *Sc. canis.*

2.2. Statistical Analysis

SAS 9.4 software (SAS Analytics Software Institute Inc. SAS Institute GmbH, Heidelberg, Germany) was used for the statistical analysis. PROC FREQ procedures were used to display the individual pathogens by year, month, and mastitis status. Chi-square test was used to compare prevalence of the different mastitis status, and according to sample origin (herd/individual). The Cochran–Armitage trend was used to assess a prevalence trend for each pathogen over the years and months. The graphics were created using Microsoft Excel 2010 (Microsoft Excel for Microsoft 365 MSO, Version 2308, Redmond, WA, USA) and α was set at 0.01.

3. Results

Between 2014 and 2023, 3,886,162 QMSs from 634,022 cows and 15,609 herds were analyzed. Of the total, 1.5% of QMSs were contaminated and therefore excluded. Mastitis pathogens were cultured in only 19% of the QMSs, meaning that 81% showed no growth. One pathogen was detected in 95.5% of the culture-positive QMSs and two pathogens were detected in 4.5%. Overall, 729,459 isolates of mastitis pathogens were included in this analysis.

Table 1 provides an overview of all QMSs included in this study. Of the total, 83% of QMSs came from herd samplings, while the remaining were sent in by farmers or veterinarians from individual case investigations. Herd screenings contributed 85% of the samples from CMT-negative quarters and 67% from subclinically affected quarters. In contrast, QMSs from clinically affected quarters were more likely to come from individual samplings ($p < 0.01$). Furthermore, there was a sharp increase in individual submissions from clinically affected quarters from an average of 40% (2014–2017) to an average of 55% (2018–2023, $p < 0.01$). As the majority of samples were from herd screenings, the majority of isolates were detected in samples from herd screenings. Only *E. coli* (68%) and *T. pyogenes* (57%) were found more frequently in samples from individual submissions than herd screenings ($p < 0.01$).

Considering culture-negative and culture-positive QMSs, the apparent prevalences of the most frequently isolated pathogens were as follows: NAS (5.7%), *S. aureus* (3.6%), *Sc. uberis* (3.6%), and *Sc. dysgalactiae* (1.6%).

Figure 1 shows the distribution of pathogens in culture-positive samples over the years. The most frequently isolated pathogens in culture-positive samples were NAS (30%). Though the vast majority of NAS (95%) were not further differentiated, the rest consisted of 2% *S. chromogenes*, 0.9% *S. epidermidis*, 0.8% *S. haemolyticus*, 0.5% *S. simulans*, 0.4% *S. xylosus*, and others less than 0.2%. The second most common pathogen in culture-positive samples was *S. aureus* (19%). The distribution of *S. aureus* isolates declined from 26% in 2014 to 15% in 2023 ($p < 0.01$). In contrast, NAS (25% to 34%) and *Sc. uberis* (16% to 22%) isolates increased over the whole study period ($p < 0.01$). An increase in *E. coli* isolates was observed from 2018 onwards (2014–2017: 2% to 2018–2023: 3%, $p < 0.01$).

Table 1. All quarter milk samples (QMSs) between 2014 and 2023. The QMSs stemmed from entire or partial herd screenings (herd) or submissions from individual cows (individual).

Year	QMS (n)	Herds (n)	Cows (n)	Herd (%)	Individual (%)	CMT [1] Negative (%)	Subclinical Mastitis (%)	Clinical Mastitis (%)
All	3,886,162	15,609	634,022	83	17	71	27	2
2014	382,752	4957	97,364	87	13	74	25	2
2015	371,039	4820	94,878	86	14	72	27	2
2016	389,947	4646	99,713	83	17	72	27	2
2017	401,453	4719	103,335	81	19	71	27	2
2018	451,573	5767	116,397	81	19	71	27	2
2019	395,016	4899	101,980	82	18	73	25	2
2020	380,001	4689	98,385	82	18	67	31	2
2021	388,417	4479	99,747	82	18	69	29	2
2022	350,167	3773	89,689	82	18	70	27	2
2023	375,797	3774	96,993	83	17	72	26	2

[1] California Mastitis Test.

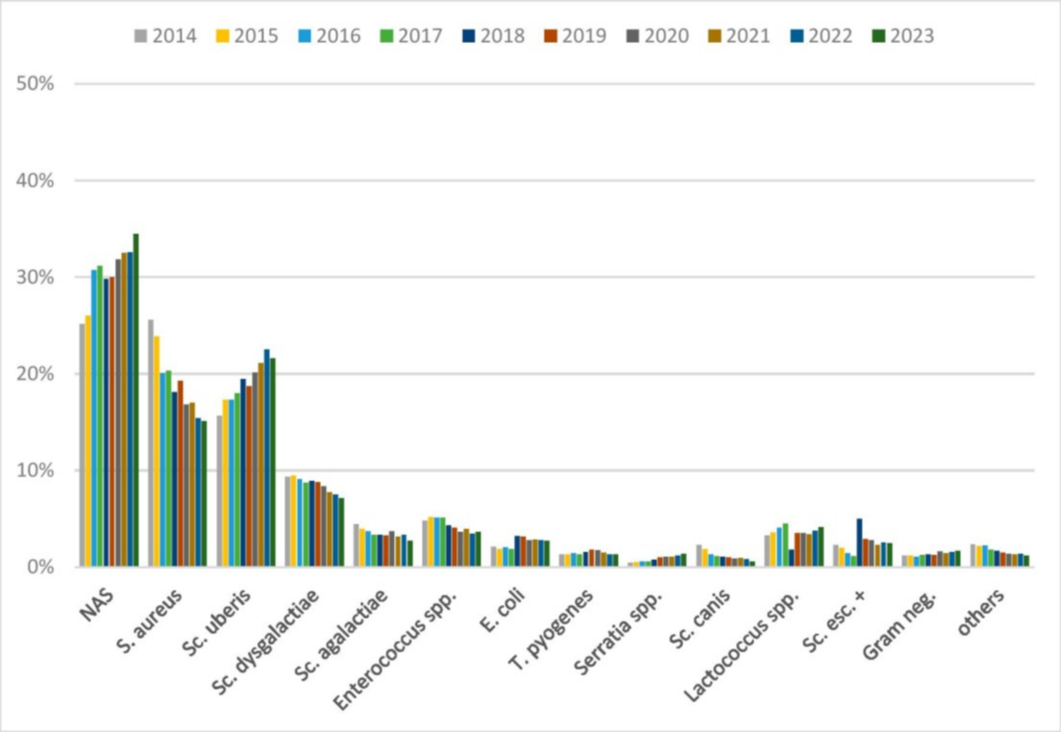

Figure 1. Distribution of mastitis pathogens in culture-positive samples per year. NAS = non-aureus staphylococci; S. = *Staphylococcus*; Sc. = *Streptococcus*; E. = *Escherichia*; T. = *Trueperella*; Sc. esc. + = other esculin-positive streptococci; Gram neg. = Gram negative pathogens.

3.1. Distribution in Dependence of Mastitis Status

The distribution of mastitis pathogens shifted depending on the mastitis status of the udder (Figure 2). Thus, pathogens were more likely cultured from QMSs from clinically affected quarters ($p < 0.01$), compared to CMT-negative or subclinically affected quarters. When considering all QMSs, the apparent prevalences of the most commonly detected mastitis pathogens in samples from CMT-negative quarters were as follows: NAS (3%), *S. aureus* (2%), *Sc. uberis* (0.6%), and *Sc. dysgalactiae* (0.4%). In QMSs from subclinically affected quarters, the apparent prevalences were NAS (12%), *Sc. uberis* (10%), *S. aureus* (8%), and *Sc. dysgalactiae* (4%). In QMSs from clinically affected quarters, the apparent prevalences were *Sc. uberis* (26%), *S. aureus* (11%), *Sc. dysgalactiae* (9%), and *E. coli* (9%).

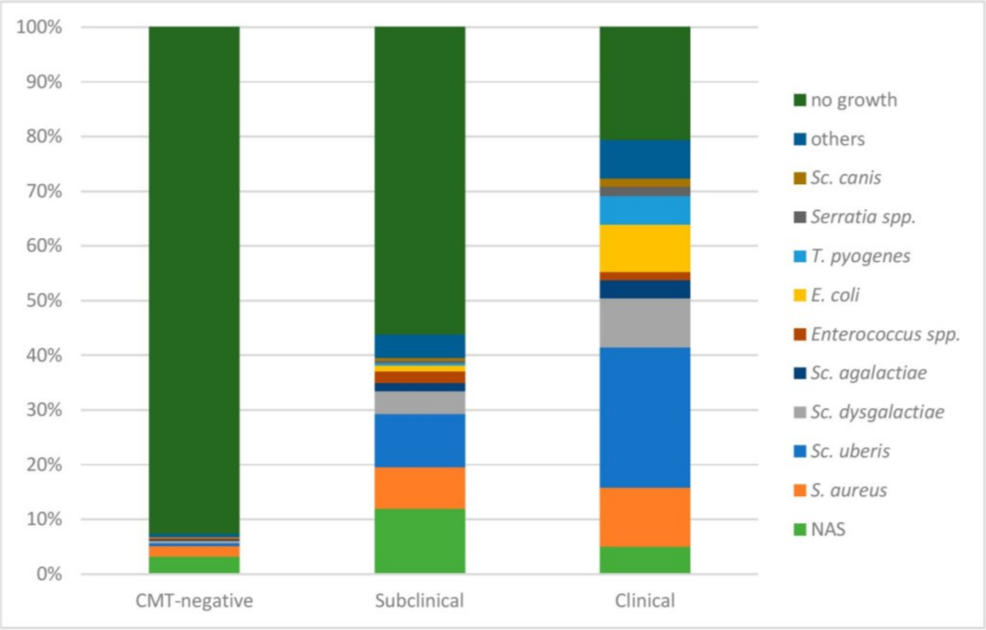

Figure 2. Distribution of no-growth samples and mastitis pathogens in CMT-negative, subclinically affected, and clinically affected quarters. NAS = non-aureus staphylococci; Sc. = *Streptococcus*; S. = *Staphylococcus*; E. = *Escherichia*; T. = *Trueperella*; all other detected species are included in "others".

Figure 3A–C shows the annual distribution of mastitis pathogens from culture-positive samples for each mastitis status. While NAS were the most common pathogens in QMSs from CMT-negative (44%) and subclinically affected quarters (27%), they were only isolated in 6% of QMSs from clinically affected quarters. The distribution of NAS from CMT-negative and subclinically affected quarters increased over the study period (Figure 3A,B, $p < 0.01$), while no trend was observed for the distribution of NAS from clinically affected quarters (Figure 3C, $p = 0.29$); 43% of NAS from clinically affected quarters were undifferentiated; the differentiated NAS included *S. chromogens* (16%), *S. epidermidis* (11%), *S. haemolyticus* (10%), *S. simluans* (8%), *S. xylosus* (3%), *S. sciuri* (3%), and others at less than 2.5% each. Similarly, *S. aureus* was detected more frequently in QMS from CMT-negative (25%) and subclinically affected quarters (17%) than in clinically affected quarters (13%). Overall, *S. aureus* isolates from all three mastitis classifications showed a decline in their detection in culture-positive samples during the study period (Figure 3A–C, $p < 0.01$, respectively), though the distribution of *S. aureus* isolates from clinically affected quarters remained at a constant level from 2018 onwards ($p = 0.02$).

In contrast, *Sc. uberis*, *Sc. dysgalactiae*, *E. coli*, *T. pyogenes*, and *Serratia* spp. were more common in QMSs from clinically affected quarters (Figure 3C, $p < 0.01$, respectively). *Sc. uberis* was the most frequently isolated pathogen in QMSs from clinically affected quarters (32%) and was detected in only 8% of QMSs from culture-positive and CMT-negative quarters. *Sc. dysgalactiae* was the third most frequent pathogen in clinically affected QMSs (11%)—after *Sc. uberis* and *S. aureus*. *E. coli* had 10% higher detection in samples from clinically affected quarters compared to CMT-negative quarters (1%, $p < 0.01$). *T. pyogenes* and *Serratia* spp. showed a 5% and 2% higher distribution, respectively, in

QMSs from clinically affected quarters than in QMSs from CMT-negative quarters ($p < 0.01$, respectively). *Sc. uberis* isolates showed an increase across all three mastitis classifications ($p < 0.01$, respectively), while *Sc. dysgalactiae* isolates showed a decrease across all three mastitis classifications ($p < 0.01$, respectively). *E. coli* showed a clear increase in culture-positive QMSs from clinically affected quarters (from 8% in 2014 to 12% in 2023, $p < 0.01$), with a sudden increase from 2017 (8%) to 2018 (13%, $p < 0.01$). *T. pyogenes* isolates showed no trend regardless of mastitis status ($p > 0.08$, respectively). In contrast, *Serratia* spp. showed a steady increase in QMSs from clinically affected quarters (2% to 4%, $p < 0.01$).

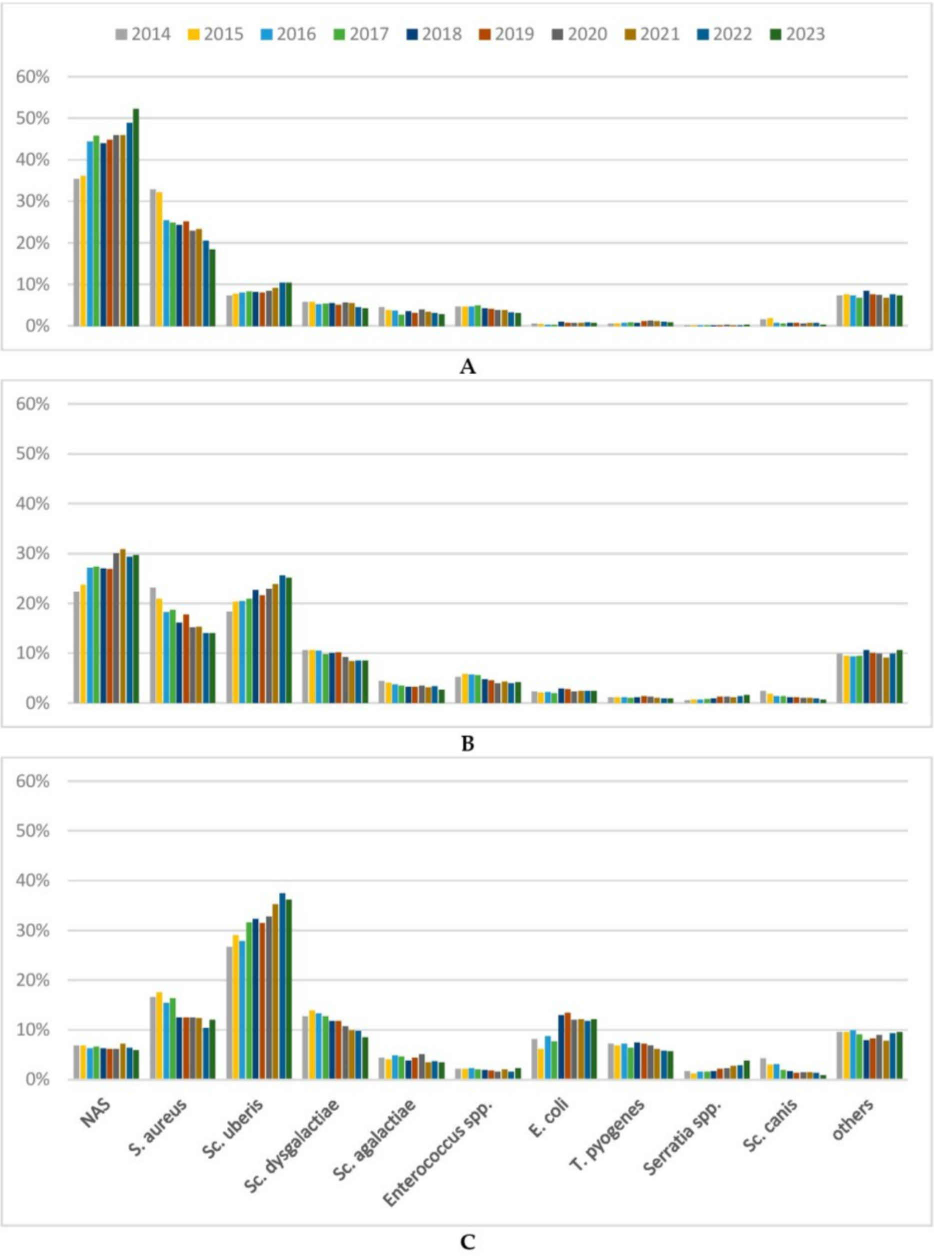

Figure 3. Distribution of mastitis pathogens from culture-positive samples from CMT-negative (**A**), subclinically affected (**B**), and clinically affected (**C**) quarters per year. NAS = non-aureus staphylococci; S. = *Staphylococcus*; Sc. = *Streptococcus*; E. = *Escherichia*; T. = *Trueperella*; all other detected species are included in “others”.

3.2. Seasonal Distribution of Mastitis Pathogens

Differences in the mastitis status of culture-positive QMSs were found depending on season. In the warmer months, from June to October, the frequency of CMT-negative samples decreased (on average from 30% to 26%, $p < 0.01$) and the frequency of subclinical samples increased (on average from 62% to 66%, $p < 0.01$). In contrast, the proportion of QMSs from clinically affected quarters remained constant at 8% across all months.

Figure 4 provides an overview of the distribution of mastitis pathogens from culture-positive samples over the months during the entire study period. The detection of environmental pathogens like *Sc. uberis*; other esculin-positive streptococci, including *Enterococcus* spp. and *Lactococcus* spp.; and Gram-negative pathogens, including *E. coli* and *Serratia* spp., increased during the warmer months from June to October (pooled average 37%) compared to the colder months from November to May (pooled average 32%, $p < 0.01$).

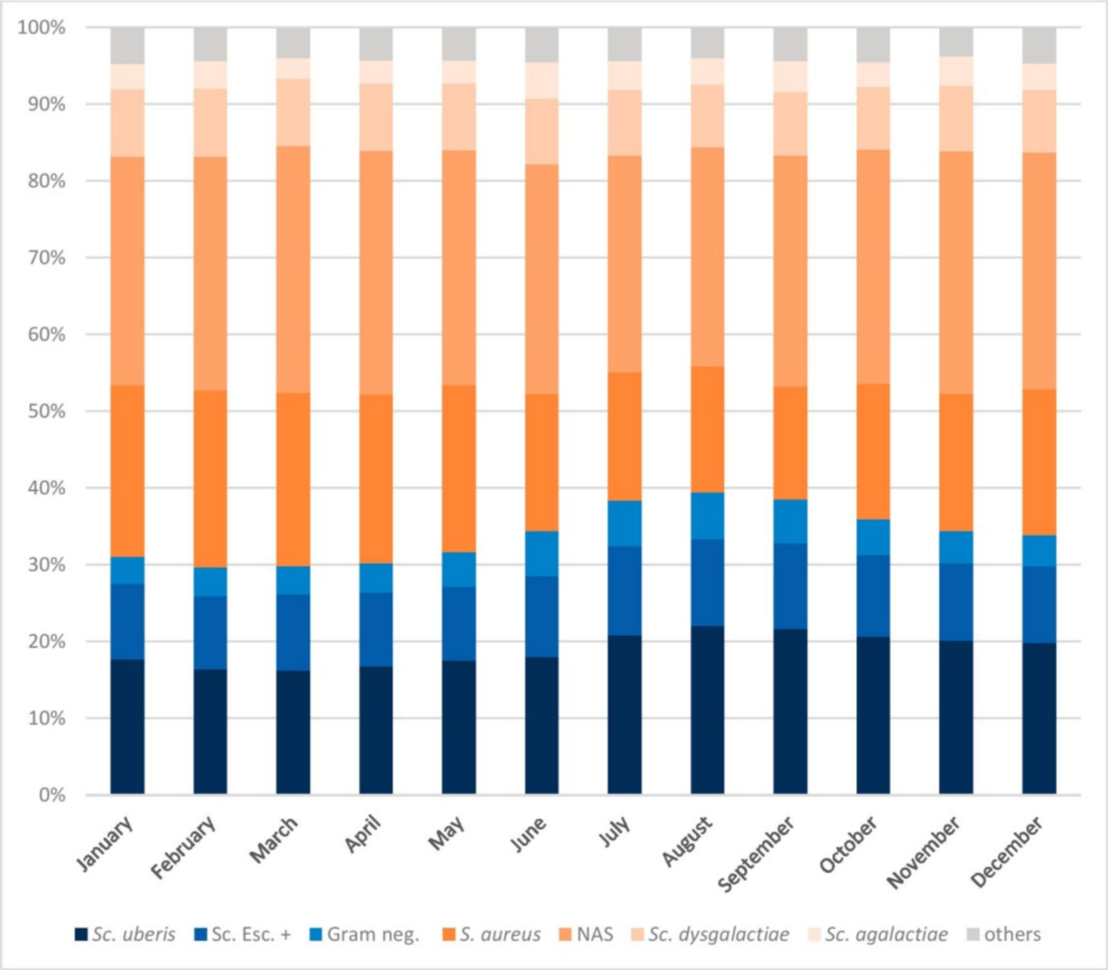

Figure 4. Distribution of mastitis pathogens in seasonal comparison per month in culture-positive samples. Sc. = *Streptococcus*; Sc. esc. + = other esculin-positive streptococci (incl. *Lactococcus* spp. and *Enterococcus* spp.); Gram neg. = Gram-negative pathogens (incl. *E. coli* and *Serratia* spp.); S. = *Staphylococcus*; NAS = non-aureus staphylococci. Others: all others, incl. *Sc. canis* and *T. pyogenes*.

Sc. uberis isolates were detected at an average of 21% of culture-positive QMSs from June to October, but at an average of 18% of QMSs in the colder months of November to May. The situation was similar with other esculin-positive streptococci and Gram-negative pathogens, with an average of 2% more isolates detected in the months of June to October in each case ($p < 0.01$, respectively). In contrast, the detection of *S. aureus* fell from an average of 21% during November to May to an average of 17% during the warmer months of June to October ($p < 0.01$). Similarly, the detection of NAS fell from 31% to 29% during the same period ($p < 0.01$). *Sc. dysgalactiae* and *Sc. agalactiae* showed no differences depending on the season (on average 9% and 3%, respectively, across months).

4. Discussion

A strength of this study is that a large number of isolates from numerous herds and cows were included over a 10-year period. In addition, QMSs from different clinical scores were analyzed in a single milk quality laboratory. This made it possible to evaluate trends in a specific region. Enrollment of herds or submission of individual samples was based on voluntary submissions rather than a random sample. Therefore, statements regarding the prevalence of pathogens within herds of this region should be avoided.

The majority of samples stemmed from herd screening, where TGD technicians took aseptic QMSs from all four quarters of every lactating cow of the herd (or a subset of cows in larger herds). Consequently, most samples in this study came from CMT-negative or subclinically affected quarters. Accordingly, a higher proportion of QMSs from clinically affected quarters came from individual submissions compared to herd samples, as veterinarians and farmers are more likely to submit samples to base their therapy on laboratory results. Furthermore, we found that *E. coli* and *T. pyogenes* were the only pathogens that were each more often found in samples from individual cows. An important factor that influences the prevalence is the duration of the infection [19]. *E. coli* is known to cause short infections (10–30 days) and is quickly eliminated by the host's immune response [20]. For *T. pyogenes*, Wente et al. [21] found an infection duration of one week to seven months. The timing of the sampling therefore plays an important role in the detection of mastitis pathogens, since sampling later during the infection likely leads to a no-growth result. As veterinarians and farmers often submitted acute mastitis cases, where the probability of detecting these pathogens is higher, these pathogens were detected more frequently in individual samples. When sampling entire herds at one time point, it is unlikely to catch clinical mastitis cases early during infection and therefore mastitis cases due to pathogens with a short infection period are more likely to end up in the "no-growth" category.

When interpreting our data, it should be taken into account that we only investigated the distribution of mastitis pathogens from QMSs sent to the TGD milk quality laboratory and the results may be biased by the high proportion of herd samplings and QMSs from CMT-negative and subclinically affected quarters. In our study, NAS were the most common pathogens in culture positive samples. Tenhagen et al. [22] found NAS to be the most common minor pathogens in QMSs from northern German dairy farms, and the German Veterinary Association observed the same Germany-wide [23]. Other studies also showed that NAS were most frequently detected in countries other than Germany, e.g., Norway [24], Slovakia [25], and Australia [26]. In addition, a study from Condas et al. [27] reported NAS to be the pathogens most frequently isolated from bovine milk worldwide. In most studies, NAS were the most frequently detected pathogens in quarters with subclinical mastitis [28]. This is consistent with our findings. However, we found NAS in 6% of QMSs from clinical mastitis, which is comparable to a study from Belgium, where they found NAS in 5% of all clinical samples [29], but different to a study from Finland, with 23.71% of NAS in clinical samples [30]. Furthermore, we found that the likelihood of NAS isolation increased throughout the study period, as also reported in Norway [24]. Zigo et al. [31] noted that this increase occurred after a reduction in main pathogens and that NAS are characterized by increased resistance to antibiotics and disinfectants, which may explain the rising distribution. Despite their surge, NAS are considered minor pathogens and therefore of lesser importance for dairy production [28].

The second most common pathogen in our study was *S. aureus*. We were more likely to find *S. aureus* than other German regions [22,23,32]. This may be explained by the sample selection (high number of QMSs from CMT-negative and subclinically affected quarters) and different herd structures in the regions. Fadlelmoula et al. [33] found that the risk of mastitis due to contagious pathogens is higher in small herds. Eastern German dairy herds are larger than Bavarian herds [22]. Furthermore, Wang et al. [34] noted that the prevalence of *S. aureus* varies greatly regional and worldwide. They further described that the prevalence of *S. aureus* has decreased over the last decade in China, which we also found in our study. Wang et al. [34] concluded that this development is due to rapid technological development and biosecurity measures taken by farms. Additionally, Munoz et al. [35] found that Bavarian farms have become larger and increased in their performance. In contrast, Acharya et al. [36] found an increasing proportion of *S. aureus* between 2008 and 2017 in Ontario. Similarly, Kortstegge and Krömker [32] noted an increasing risk of *S. aureus* in bulk tank milk with increasing herd sizes. Furthermore, Smistad et al. [24] observed a higher proportion of *S. aureus* (24.5%) in their study in Norway, and found *S. aureus* to be the most frequently detected major pathogen. They stated that the prevalence of *S. aureus* in Norway was relatively stable between 2000 and 2020 and explained that measures such as routine teat disinfection after milking are less consistently carried out in Norway. In addition to our results, Karell et al. [37] found an overall decreasing resistance trend in *S. aureus* in their study in Bavaria and concluded that this trend can be seen as a success of the measures taken in recent decades to tighten the use of antimicrobials and to control mastitis pathogens, which aimed to prevent new infections and eliminate existing infections. Therefore, the decreasing likelihood of detection and resistance of *S. aureus* can be seen as a success of the measures taken to combat mastitis pathogens in recent decades. Another notable observation was that *S. aureus* isolates were also fairly commonly detected in QMSs from CMT-negative and subclinically affected quarters. This is important, as Karell et al. [37] observed that *S. aureus* isolates from healthy or subclinical quarters were more likely to be in vitro resistant than isolates from clinical quarters. Woudstra et al. [9] found in their study that one *S. aureus* strain could cover 80% of the infections within a herd and that infected udder quarters are the main reservoir, underlining the contagious nature of this pathogen. Therefore, it could be concluded that cows with undetected infections act as a reservoir for within-herd transmission of *S. aureus* that also may carry virulence and resistance genes. The most important focus in preventing *S. aureus* infections is on reducing transmission from infected to uninfected quarters [9,38]. For this purpose, healthy cows or cows with subclinical mastitis should also be included in herd screenings in order to find potential sources of infection.

Sc. uberis was the third most common pathogen in culture positive samples in this study (19%). Its detection risk within culture-positive samples aligned with other reports from Germany (20.3%, [23]). It is noteworthy that it was the most common pathogen isolated from QMSs from clinically affected quarters (32%), which agrees with other studies from around the world [13,29,39]. In our study, *Sc. uberis* isolates showed an overall increase over the entire study period, which was also observed in Ontario by Acharya et al. [36] and previously by Phuektes et al. [40] for other parts of the world. Cobirka et al. [25] stated that *Sc. uberis* is mostly present in bedding material and that control measurements, such as post-milking teat disinfection and dry cow therapy, are far less effective against environmental pathogens, such as *Sc. uberis*. This may explain the increasing probability of *Sc. uberis* detection, since a decline in contagious pathogens (e.g., *S. aureus*), has been reported to go hand in hand with an increase in Gram-negative and therefore environmental pathogens [41]. In line with this, we found that the detection of other environmental pathogens such as *E. coli* and *Serratia* spp. also increased throughout the study period. Furthermore, the detection likelihood of *E. coli* jumped from 2018 onwards compared to previous years—especially in clinical cases. Since the number of individual submissions from clinical mastitis also increased at the same time, one can see the impact of a change in legislation in Germany in 2018 for the use of antimicrobials in veterinary medicine. It aimed to minimize the use of critically important antimicrobials (Verordnung über tierärztliche Hausapotheken, TÄHAV) [42] and included obligatory antimicrobial susceptibility testing, for example, if critically important antimicrobials were selected or antimicrobials were changed during therapy. Interestingly, this increase was especially observed for samples that tested positive for *E. coli*. This was also reported by Pirner et al. [43], who investigated the resistance of Gram-negative pathogens in Bavaria. One possible explanation could be that most of the pathogens detected in this study were Gram-positive pathogens. Those pathogens are more likely to cause subclinical mastitis and were commonly detected in herd screenings even before 2018 [43]. The change in legislation therefore did not impact their detection as much as it did for *E. coli* with its short infection duration. As QMSs now had to be sampled as soon as clinical signs appeared for treatment decisions [43], this increased the likelihood of detecting *E. coli*. *E. coli* is the most common Gram-negative pathogen to cause clinical mastitis worldwide [44]. In our study, *E. coli* was the most common Gram-negative pathogen in culture-positive QMSs from clinically affected quarters (11%). In Lower Saxony, Germany, Krebs et al. [45] reported in their study a much higher detection of *E. coli* from culture-positive clinical samples (35.2%). Other authors found *E. coli* to be associated with 19.8% of clinical mastitis cases in England and Wales [46], 15.5% in Belgium [29], or 27% of cases in China [47]. This underlines that the distribution of pathogens may vary depending on geographical region [48].

Besides region, a variety of factors can influence the prevalence of mastitis-causing pathogens, such as herd-size, housing system, and season [49]. Season also impacts cows in Germany, as they suffer from heat stress too [50]. The temperature–humidity index (THI) is widely used to assess heat stress, and a high THI is associated with an increased somatic cell score [51,52]. In our study, we found seasonal changes in the occurrence of QMSs from subclinically affected quarters, as they were slightly more common during the warmer months of June to October. Furthermore, we found differences in the detection of various mastitis pathogens depending on the month and therefore season. Environmental pathogens (e.g., *Sc. uberis*, Gram-negative as well as other esculin-positive pathogens) were more likely detected during the warmer months of June to October, while the proportion of *S. aureus* and NAS slightly decreased during that time. High temperatures and humidity may increase the probability of IMIs caused by environmental pathogens [53]. Other studies also reported that the prevalence of environmental mastitis due to, e.g., *Sc. uberis* [30,54] and *E. coli*, was the highest in summer and autumn [30], and the frequency of *S. aureus* and NAS mastitis cases was higher during winter [30,55]. All three studies noted a housing difference during the season, which may explain the observed distribution. Olde Riekerink et al. [54] stated that cows on pasture during summer are at an increased risk of environmental mastitis caused by pathogens such as *Sc. uberis.* The warm and humid conditions that prevail in summer, combined with the organic material on the pastures, create a favorable environment for these pathogens to thrive [54]. However, other studies reported an increased incidence of environmental mastitis or somatic cell count in hot and humid weather and explained this with immunosuppression due to heat stress and a higher pathogen load in the environment, leading to increased susceptibility to IMIs [56,57]. Furthermore, Hamel et al. [12] found higher shedding of *Sc. uberis* with a higher THI, which may be a possible explanation for the higher detection of *Sc. uberis* in summer. Other possible explanations are given by Kabelitz et al. [11], who suggest that high temperatures and thus the higher reproduction rate of pathogens in the environment could be a reason, as well as the transmission of bacteria by flies, which are particularly present in summer. In this study, however, we could only report the observed differences, as we did not have the climate or other risk factors like farming practices for all submissions. Therefore, we can only suggest explanations for the observed dynamics, and further research is needed. Nevertheless, increasing temperatures due to climate change, increasing heat stress, and therefore vulnerability to mastitis, as well as the influence on mastitis pathogens, likely bring new challenges for maintaining udder health [58].

Overall, the increase in environmental pathogens observed in this study emphasizes the need to continue and improve management practices. Effective control of environmental mastitis can be achieved by minimizing teat-end exposure to these pathogens and enhancing cow resistance to intramammary infections, e.g., through vaccination strategies [59]. Reducing the exposure of cows to environmental mastitis pathogens also involves maintaining clean and dry bedding, regular removal of manure, and avoiding overcrowding in barns and pastures [60]. In addition, season-dependent measures such as appropriate cooling can reduce heat stress for cows, thereby strengthening their immune response and reducing the likelihood of mastitis outbreaks [61].

5. Conclusions

This study underscores the dynamic nature of mastitis pathogen distribution, influenced by mastitis status and seasonal factors. Contagious pathogens such as *S. aureus* have decreased over the last decade, while environmental pathogens continue to play an important role for udder health in Bavaria. In addition, the study results emphasize that both healthy cows and those with subclinical mastitis can serve as a reservoir for mastitis pathogens. It is therefore essential to consider healthy cows and cows with subclinical mastitis as a reservoir for mastitis pathogens during monitoring and control efforts in order to track trends and adapt targeted prevention measures.

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2. Publication II

Cumulative doctoral achievement: Publication

Antimicrobial resistance of *Streptococcus dysgalactiae*, *Streptococcus agalactiae*, and *Streptococcus canis* in quarter milk samples from Bavaria, Southern Germany, between 2012 and 2022

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Antimicrobial resistance of *Streptococcus dysgalactiae*, *Streptococcus agalactiae*, and *Streptococcus canis* in quarter milk samples from Bavaria, Southern Germany, between 2012 and 2022

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ABSTRACT

The objective of this study was to analyze the in vitro antimicrobial resistance (AMR) of *Streptococcus dysgalactiae*, *Strep. agalactiae*, and *Strep. canis* over a 10-yr period from 2012 to 2022 against the most commonly used antimicrobial agents. For this purpose, all quarter milk samples (QMS) submitted to the milk laboratory of the Bavarian Animal Health Service were analyzed. Each QMS was tested using the California Mastitis Test and categorized as negative, subclinical, or clinical mastitis if the milk character was abnormal. Samples with *Strep. dysgalactiae*, *Strep. agalactiae*, or *Strep. canis* were included and a subset of isolates were further tested for in vitro AMR by breakpoint analysis with broth microdilution. *Streptococcus dysgalactiae* (61%, n = 65,750) was the most abundant pathogen among those 3 species, followed by *Strep. agalactiae* (28%, n = 30,486), and *Strep. canis* (11%, n = 11,336). All 3 species showed the highest resistance to the same 4 antimicrobial agents: erythromycin, marbofloxacin, pirlimycin, and cefalexin/kanamycin with varying degrees of resistance. Throughout the study period, *Strep. dysgalactiae*, *Strep. agalactiae*, and *Strep. canis* were largely susceptible to the remaining antimicrobial agents tested (penicillin, amoxicillin-clavulanate, oxacillin, cefazolin, cefoperazone, cefquinome). Only less than 14% of isolates of *Strep. dysgalactiae* and *Strep. canis* were resistant against any of the antimicrobials tested. *Streptococcus agalactiae* was the species with the highest percentage of resistant isolates. Although the percentage of resistant isolates from *Strep. canis* and *Strep. dysgalactiae* decreased, the percentage of resistant *Strep. agalactiae* isolates increased after 2017. In summary, most isolates were not resistant to the most commonly used antimicrobial agents for mastitis therapy, including β-lactam antibiotics and penicillin should remain the first-choice therapy against streptococcal mastitis.

Key words: *Streptococcus dysgalactiae*, *Streptococcus agalactiae*, *Streptococcus canis*, mastitis, antimicrobial resistance

INTRODUCTION

Mastitis is one of the most important diseases in the dairy industry around the world. It is responsible for economic losses of up to €125 billion per year worldwide due to lost milk and therapy costs and poses a problem for animal health and welfare (Kabelitz et al., 2021). This disease is caused by IMI, causing an inflammatory response in one or more quarters of the udder (Keane, 2019). One of the major causative pathogens for IMI are *Streptococcus* spp. (Kabelitz et al., 2021). These include major pathogens of the pyogenic group such as *Strep. dysgalactiae*, *Strep. agalactiae*, and *Strep. canis* (Alves-Barroco et al., 2021). Those species, which are known to infect not only cows but also occur as commensals in humans and different animals, can cause various diseases such as mastitis, pneumonia, meningitis, endocarditis, septicemia, and more (Zadoks et al., 2011; Haenni et al., 2018). Therefore, streptococci are important pathogens not only for cattle but also for humans and thus play an important role in public health.

The main treatment against mastitis pathogens is antimicrobial therapy (Cheng and Han, 2020). However, high antimicrobial usage is a cause for public health concern (Ruegg, 2017). In dairy cows and other food-producing animals excessive use of antimicrobials poses a risk to human health due to the emergence of food-borne multidrug-resistant pathogens (Kaczorek et al., 2017). For instance, mastitis therapy accounts for most antimicrobial treatments in dairy cows (Nobrega et al., 2020) and almost 90% of antibiotic residues in milk originate from the use of mastitis therapy (Kabelitz et al., 2021). Although streptococcal mastitis is mainly treated with β-lactams

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such as penicillin, oxacillin, and ampicillin, Kabelitz et al. (2021) reported that they are still very susceptible to these antimicrobials in vitro. Nonetheless, streptococci have reports of antimicrobial resistance (**AMR**), including to β-lactams (e.g., by Pascu et al. [2022] in Romania). In addition, multidrug resistance (**MDR**) has also been reported in pyogenic streptococcal species, which can complicate standard empirical antimicrobial therapy and lead to treatment failure in humans and animals (Alves-Barroco et al., 2020). Furthermore, horizontal gene transfer is one of the most important mechanisms for the development of new AMR. These gene transfers occur frequently in the pyogenic group and therefore also in *Strep. dysgalactiae*, *Strep. agalactiae*, and *Strep. canis* (Alves-Barroco et al., 2020). Hence, data on resistance in these species are therefore particularly important to investigate the development of resistance and to be able to recognize changes at an early stage. Various national and international organizations such as the World Organization for Animal Health, the European Commission, and so on have introduced AMR surveillance programs to observe resistance developments (Toutain et al., 2017). Although these programs, for example VetPath, an ongoing Europe-wide AMR surveillance program (El Garch et al., 2020), provide overview data for Germany, information on regional differences is lacking. In addition, relatively rare pathogens such as *Strep. canis* (Król et al., 2015) are not considered. Monitoring AMR in less common species such as *Strep. canis*, a zoonotic pathogen, is also crucial as these species can cause diseases ranging from mild to severe (Pagnossin et al., 2022). Furthermore, such species may also serve as reservoirs for resistance genes because AMR emerges as result of horizontal gene transfer, among other mechanisms (Alves-Barroco et al., 2020).

A potential effect on development of AMR in mastitis pathogens could be associated with changes in antimicrobial prescriptions. For instance, between 2011 and 2021 the sales of antimicrobials to veterinary practices declined in Germany by 65% (Sander et al., 2022) and many dairy farmers already implement, for example, selective dry cow therapy in Bavaria (Sorge et al., 2019). All of these changes in antimicrobial usage might affect antimicrobial resistance of mastitis pathogens—especially when investigated regionally. In Germany, Bavaria is a key state for the country's dairy industry—here 30% of German dairy cows are kept (Bayerische Landesamt für Statistik, 2023), and a quarter of the annual milk volume is produced (Bayerische Landesanstalt für Landwirtschaft, 2015). Bavaria has approximately as many dairy cows as Wisconsin, but Bavaria has 24,644 herds (Bayerische Landesamt für Statistik, 2023), whereas Wisconsin has only 5,696 herds (Dairy Farmers of Wisconsin, 2023). This smaller herd structure is also found in neighboring Alpine countries for example Austria (on average 24 cows/herd; Rinderzucht Austria, 2022), so that the results of this study may also be relevant in these countries beyond Bavaria. Therefore, the aim of this study was to describe changes in the in vitro resistance of *Strep. dysgalactiae*, *Strep. agalactiae*, and *Strep. canis* to commonly used antimicrobial classes in Bavaria, Southern Germany, between 2012 and 2022.

MATERIALS AND METHODS

Sample Population

All quarter milk samples (**QMS**) sent to the milk laboratory of the Bavarian Animal Health Service (Tiergesundheitsdienst; **TGD**) between 2012 and 2022 (n = 4,327,420 from 931,013 cows and 17,657 herds) were analyzed in this retrospective study. From those QMS a total of 65,750 (1.52%) *Strep. dysgalactiae*, 30,486 (0.7%) *Strep. agalactiae*, and 11,336 (0.26%) *Strep. canis* isolates were available for this study. They stemmed from 66,640 cows and 11,050 herds. Most QMS were from whole herd screenings collected by TGD technicians (69%–88% over the years). During these samplings, the technicians took QMS from each quarter of each cow in the herd. Because the average herd size was small (<45 lactating cows), in most instances all cows were sampled to investigate the predominant mastitis pathogen for the herd. For larger herds, a subset of cows might have been chosen instead of sampling the entire herd. Those herd screenings were requested by the farmer or veterinarian. The remaining QMS were sent in by farmers or veterinarians for screening of individual cases (e.g., cows suffering from clinical mastitis or before drying off). In Germany, all quarters of a cow are usually sampled because the billing is on a per cow (not sample) basis. The SCC value of each QMS was assessed directly on farm by TGD technicians or upon arrival at the milk laboratory by the California Mastitis Test (**CMT**). Samples were categorized as negative if the CMT was negative or subclinical if the CMT was positive. Clinical mastitis was present if the milk character was abnormal (e.g., flakes, watery). Information on the history of the cow (e.g., treatments) depended on the information provided by the farmer for each submission.

Laboratory Analysis

Bacteriological Analysis. The cultural testing of the QMS was carried out in the milk laboratory of the TGD. The laboratory methods complied with the guidelines of the German Veterinary Association (DVG, 2018), which partially refer to the procedures described in the (at the time) concurrent National Mastitis Council handbook (e.g., National Mastitis Council, 2004).

All QMS (0.01 or 0.05 mL for samples from clinical mastitis cases) were cultured on esculin-blood agar based on Columbia agar with sheep's blood additive and incubated aerobically at 36°C ± 2°C. Plates were evaluated according to DVG guidelines after 18 to 24 h and 48 h of incubation. If it was not possible to evaluate the plates twice, they were evaluated only after 36 h of incubation. A positive result was considered depending on the species. For example, esculin-negative streptococci were considered positive and pathogenic if at least 3 cfu were present on the plates in accordance with DVG guidelines. If the samples were taken by technicians and were from pretreated cows we knew of, they were also cultured on Sabouraud-agar.

Streptococcal isolates were identified by colony morphology, hemolysis, esculin hydrolysis, and Christie, Atkins, Munch-Peterson (**CAMP**) factor, as well as classification into Lancefield groups. Further differentiation of esculin-negative strains was conducted with CAMP factor testing, where a *Staphylococcus aureus* strain with β-hemolysis was used. Esculin-negative and CAMP-factor-positive strains were identified as *Strep. agalactiae*. Esculin-negative and CAMP-factor-negative strains, belonging to Lancefield group C, were identified as *Strep. dysgalactiae*. Streptococcal strains with pronounced β-hemolysis of Lancefield group G were identified as *Strep. canis*. From 2014 onward, strains with unclear results were further analyzed by MALDI-TOF (Microflex MALDI Biotyper, reference database V.3.3.1.0., Bruker Daltonik GmbH).

In Vitro Antimicrobial Susceptibility Testing. For in vitro antimicrobial susceptibility testing, the following inclusion criteria applied: For QMS from herd screenings, 2 to 3 randomly selected isolates were selected for a breakpoint analysis. In addition, samples from cows with subclinical or clinical mastitis, from pretreated quarters, or when the client had specifically requested an antibiogram underwent testing. For QMS from individual cows, a similar scheme was applied.

Streptococcal pathogens were tested for susceptibility to antimicrobials using the breakpoint method with a commercial broth microdilution system (Mastitis 3 plate, Merlin Diagnostika GmbH). Antimicrobial agents tested against on this commercial tray were β-lactam antibiotics (penicillin, amoxicillin-clavulanate, and the penicillinase-stable isoxazolylpenicillin oxacillin). Furthermore, the tray contained lincosamides (pirlimycin), macrolides (erythromycin), quinolones (marbofloxacin), aminoglycosides (kanamycin-cefalexin), as well as first- (cefazolin), third- (cefoperazone), and fourth- (cefquinome) generation cephalosporins. The antimicrobial agents ampicillin, gentamicin, and tetracycline were discarded because of incomplete data over the years or missing MIC values in the records. For the assessment of the respective breakpoints the program MCN 6 was used (version MCN 6.00–08.01.2018 Rel. 89; Demo Computer GmbH and Merlin Diagnostica GmbH). The program used the official breakpoints of the standards valid at that time (e.g., CLSI M100, CLSI VET01). Official current MIC breakpoints for the indication streptococcal mastitis in cattle are only available for pirlimycin, cefoperazone (2020 and after), and kanamycin-cefalexin (2023 and after; CLSI, 2023). For cefoperazone the breakpoint from Feßler et al. (2012) was used by the MCN6 program. The breakpoints for kanamycin-cefalexin as well as for ampicillin, erythromycin, marbofloxacin, and oxacillin were based on the then current mastitis layout for microplates from Feßler et al., which are identical to the currently valid ones (Feßler et al., 2022). The breakpoint for penicillin was based on the National Committee for Clinical Laboratory Standards guidelines (NCCLS, 2002).

If no breakpoint for the indication mastitis in cattle and the pathogen *Streptococcus* spp. existed based on the CLSI guidelines, Feßler et al. (2022), and the NCCLS guidelines, human breakpoints or breakpoints for other indications or animals based on DVG guidelines (2018, or respective edition) were used from the MCN 6 (version MCN 6.00–08.01.2018 Rel. 89; Demo Computer GmbH and Merlin Diagnostica GmbH) program. Intermediate results were classified as resistant.

Statistical Analysis

For the statistical analysis the software SAS 9.4 (SAS Analytics Software Institute Inc., SAS Institute GmbH Heidelberg) was used. PROC FREQ procedures were used for MIC observations by year and by isolate. Chi-squared test and PROC GLIMMIX procedures were used to compare prevalence of resistance of pathogens to different antimicrobials across years and clinical status (CMT negative to clinical). The Cochran-Armitage trend test was used to assess an AMR trend over time (years) for each pathogen and antimicrobial. Furthermore, a mixed logistic regression (PROC GLIMMIX) was implemented to assess changes in resistance per year for each pathogen-antimicrobial combination. Herd was included as random effect, and year and mastitis status were initially included as fixed effects. Backward elimination was used, and group comparisons were conducted using the LSMEANS statement. Unless otherwise stated the *P*-value of the odds ratio of the mixed logistic regression were reported. The MIC where 50% and 90% of isolates were inhibited by the tested antibiotics were MIC50 and MIC90, respectively. The number of antibiotics a pathogen was tested to resistant in vitro was summarized to determine the pathogens' MDR. Multidrug resistance was defined as a pathogen being resistant to 2 or more antimicrobial agents, where β-lactams (i.e., penicillin, amoxicillin-clavulanate, and oxacillin) were considered as a single antibiotic group. Graphics were created in Microsoft Excel 2010 (Microsoft Excel for Microsoft 365 MSO, Version 2308). Missing data were ignored per SAS default and α was set at 0.05.

RESULTS

Sample Description

Of all QMS sent to the TGD between 2012 and 2022 *Strep. dysgalactiae* were detected in 65,750 samples, *Strep. agalactiae* in 30,486 samples, and *Strep. canis* in 11,336 samples (Table 1). Most QMS were collected by technicians during herd screenings (69%–88% over the years). The remaining QMS were submitted by farmers or veterinarians for individual cows (12%–31%). Most isolates (64%–72%) stemmed from quarters with subclinical mastitis. Only ~9% to 13% of the isolates originated from quarters with clinical mastitis (Table 1).

A total of 9,150 *Strep. dysgalactiae*, 3,418 *Strep. agalactiae*, and 1,554 *Strep. canis* isolates were selected over the years for in vitro resistance testing with broth microdilution. Missing data from the resistance testing for the different isolates were ignored. In total, only 2 *Strep. dysgalactiae* isolates, 16 *Strep. agalactiae* isolates, and 1 *Strep. canis* isolate were not tested for each antimicrobial agent. On average (median), 1 isolate per herd was included for each evaluated *Streptococcus* spp., with a maximum per herd of 4 isolates for *Strep. canis* and 9 isolates for *Strep. dysgalactiae* and *Strep. agalactiae*. Approximately 56% of the in vitro resistance testing samples came from quarters with clinical mastitis and 41% from quarters with subclinical mastitis. Approximately 3% of the samples stemmed from cows with CMT-negative quarters. The mastitis status had no influence on the resistance in *Strep. dysgalactiae* and *Strep. agalactiae* ($P = 0.4$, $P = 0.6$, respectively). Only *Strep. canis* showed that isolates from quarters with clinical mastitis were less likely to be resistant in vitro than those from subclinically affected quarters ($P < 0.01$), whereas no significant difference was found compared with isolates from CMT-negative quarters ($P = 0.36$).

Supplemental Tables S1 to S10 (see Notes; Bechtold et al., 2024) show the distribution of the different isolates in the breakpoint analysis for the different antimicrobial agents over the years. Isolates from the 3 streptococcal species showed resistance to all antimicrobial agents tested, with very few exceptions. The highest resistance percentage was observed in all 3 species against the same 4 antimicrobial agents: erythromycin, marbofloxacin, pirlimycin, and cefalexin/kanamycin—only the level of resistance varied. However, all showed good in vitro susceptibility to the remaining antimicrobial agents—namely, penicillin, amoxicillin-clavulanate, oxacillin, cefazolin, cefoperazone, and cefquinome. For these antimicrobials, the MIC90 for all 3 streptococci remained at the lowest concentration tested throughout the study period.

In Vitro Resistance Testing of Strep. dysgalactiae

On average, 9% of all *Strep. dysgalactiae* isolates expressed resistance to erythromycin (Supplemental Table S5). For this antimicrobial agent a trend toward lower resistance was observed across the study period ($P < 0.01$).

Table 1. Overview of all quarter milk samples with *Streptococcus dysgalactiae*, *Streptococcus agalactiae*, and *Streptococcus canis* isolates and those analyzed with broth microdilution testing between 2012 and 2022; multiple references of single isolates possible

Item	Isolate (n)	Herd (n)	Cow (n)	CMT negative (%)	Subclinical mastitis (%)	Clinical mastitis (%)
Pathogen						
Strep. dysgalactiae	65,750	10,470	47,886	17.1	72.0	10.9
Strep. agalactiae	30,486	1,569	14,996	27.7	63.5	8.8
Strep. canis	11,336	846	5,603	18.7	68.1	13.2
Broth microdilution						
Strep. dysgalactiae	9,150	4,100	7,706	2.4	41.0	56.6
Resistant^{1,2}	1,206	382	977	2.7	48.0	49.3
Susceptible^{1}	7,944	3,718	6,729	2.4	39.9	57.7
Strep. agalactiae	3,418	764	2,423	3.5	41.3	55.2
Resistant^{1,2}	2,433	426	1,684	3.8	40.8	55.4
Susceptible^{1}	985	338	739	2.8	42.4	54.7
Strep. canis	1,554	406	940	3.0	41.7	55.3
Resistant^{1,2}	164	74	82	3.0	63.4	33.5
Susceptible^{1}	1,390	332	858	3.0	39.1	57.8

[1] Based on breakpoints used in this study.

[2] Resistant to at least one antimicrobial agent.

The odds for *Strep. dysgalactiae* isolates being resistant to erythromycin were ~4 times higher in 2012, 2013, and 2014 than in 2022. The highest percentage of resistance (up to 25%) against erythromycin was observed between 2012 and 2014, but it dropped afterward to below 12% (Figure 1, $P < 0.01$). This affected also the MIC50 and MIC90. Until 2014 less than 44% of *Strep. dysgalactiae* isolates were inhibited by erythromycin at the lowest MIC. After, the number of isolates inhibited at the lowest MIC increased ($P < 0.01$) and the MIC50 remained constant at the lowest concentration. From 2020, the MIC90 (≤0.125 µg/mL, Supplemental Table S5) followed.

The second most common resistance in *Strep. dysgalactiae* was against pirlimycin (6%). However, no clear trend was present over the years ($P = 0.1$, Figure 1) and the MIC90 was at the lowest concentration tested (≤1 µg/mL, Supplemental Table S4), throughout the entire study period. Therefore, no major differences were found in the odds of being resistant to pirlimycin over the years.

In addition to this, the only other resistances were observed against penicillin, marbofloxacin, and cefalexin/kanamycin. For those antimicrobials, on average, 1% of isolates were resistant against each of them, respectively (Supplemental Tables S1, S6, and S7). Although penicillin and cefalexin/kanamycin blocked the growth of, on average, 99% of the *Strep. dysgalactiae* isolates at the lowest concentration, MIC90 for marbofloxacin was 1 µg/mL.

In Vitro Resistance Testing of Strep. agalactiae

Streptococcus agalactiae was the isolate with the highest resistance prevalence against the antimicrobial agents (Figure 1B). Unlike *Strep. dysgalactiae* or *Strep. canis*, *Strep. agalactiae* isolates showed the highest percentage of resistance against cefalexin/kanamycin (43%). For this antimicrobial agent the proportion of resistant isolates increased from 14% in 2012 to 59% in 2022 ($P < 0.01$, Figure 1B). In addition, the odds for *Strep. agalactiae* of being resistant to cefalexin/kanamycin increased steadily. Therefore, the odds were ~8 times higher in 2022 compared with 2012, whereas they were only ~3 times higher in 2017. From 2013 until 2019 MIC50 was 8/0.8 µg/mL, which is also the breakpoint in our study (Supplemental Table S7). From then until 2022, the MIC50 changed to 16/1.6 µg/mL ($P < 0.01$), which is also where the MIC90 was throughout the study period.

The next most common resistance was against marbofloxacin (41%, Supplemental Table S6). During the study period MIC50 remained at the concentration of 1 µg/mL (i.e., the breakpoint), just as MIC90 remained at 2 µg/mL. It had a peak of resistance in 2013 (69%), where also the odds ratio of being resistant was ~2.5 times higher compared with 2022. Although the percentage of resistance decreased between 2013 (69%) and 2016 (29%, $P < 0.01$), an upward trend in resistant isolates was observed again from then until the end of the study, with small peaks in 2017 (42%) and 2019 (46%, $P < 0.01$, Figure 1B).

Twenty-six percent of *Strep. agalactiae* isolates were resistant to erythromycin throughout the study period (Supplemental Table S5). The peak in resistance occurred in 2013 (72%, Figure 1B), where the odds of being resistant to erythromycin were also 46 times higher than in 2022. In 2012 and 2014 the odds were ~13 times higher than in 2022, whereas subsequent years had no major differences. The overall number of resistant isolates fell from 2012 (54%) to 2022 (24%, $P < 0.01$). Furthermore, an increase in isolates inhibited at the lowest MIC can be observed from 2012 (22%) to 2017 (75%, $P < 0.01$, Supplemental Table S5). As of 2016, MIC50 was at the lowest MIC (≤0.125 µg/mL), whereas MIC90 remained mostly around the highest MIC tested (≥4 µg/mL),

In addition, 14% of all *Strep. agalactiae* isolates showed resistance against pirlimycin (Supplemental Table S4). No clear trend was observed for this antimicrobial agent during the entire study period ($P = 0.20$, Figure 1B) and therefore the year had no effect on the percentage of resistance ($P = 0.23$). In addition, the odds ratio did not vary greatly (~1 over the years). The MIC50 was consistently at the lowest MIC (≤1 µg/mL), although MIC90 remained at the highest MIC tested (≥4 µg/mL), with the only exception in 2017 (MIC50+MIC90 at ≤1 µg/mL).

In Vitro Resistance Testing of Strep. canis

Streptococcus canis was the least likely to be resistant against all antimicrobial agents tested. Less than 11% of the isolates were resistant to any of the antimicrobials. Similar to *Strep. dysgalactiae* it showed the highest resistance against erythromycin (Supplemental Table S5). In addition, a peak in resistance to erythromycin was recorded in 2013 (28%), similar to *Strep. dysgalactiae* and *Strep. agalactiae*, whereas resistance decreased in the following years ($P < 0.01$) and was 0% in 2022. Therefore, the odds of *Strep. canis* isolates being resistant to erythromycin were ~53 times higher in 2013 compared with 2021. In this context, percentage of isolates inhibited at the lowest MIC steadily increased from 2012 (22%) to 2022 (100%, $P < 0.01$). Therefore, MIC50 and MIC90 shifted toward lowest tested MIC (in 2015, 2017, respectively, $P < 0.01$). Hence, over the whole study period a trend toward fewer resistant isolates against erythromycin was observable ($P < 0.01$, Figure 1C).

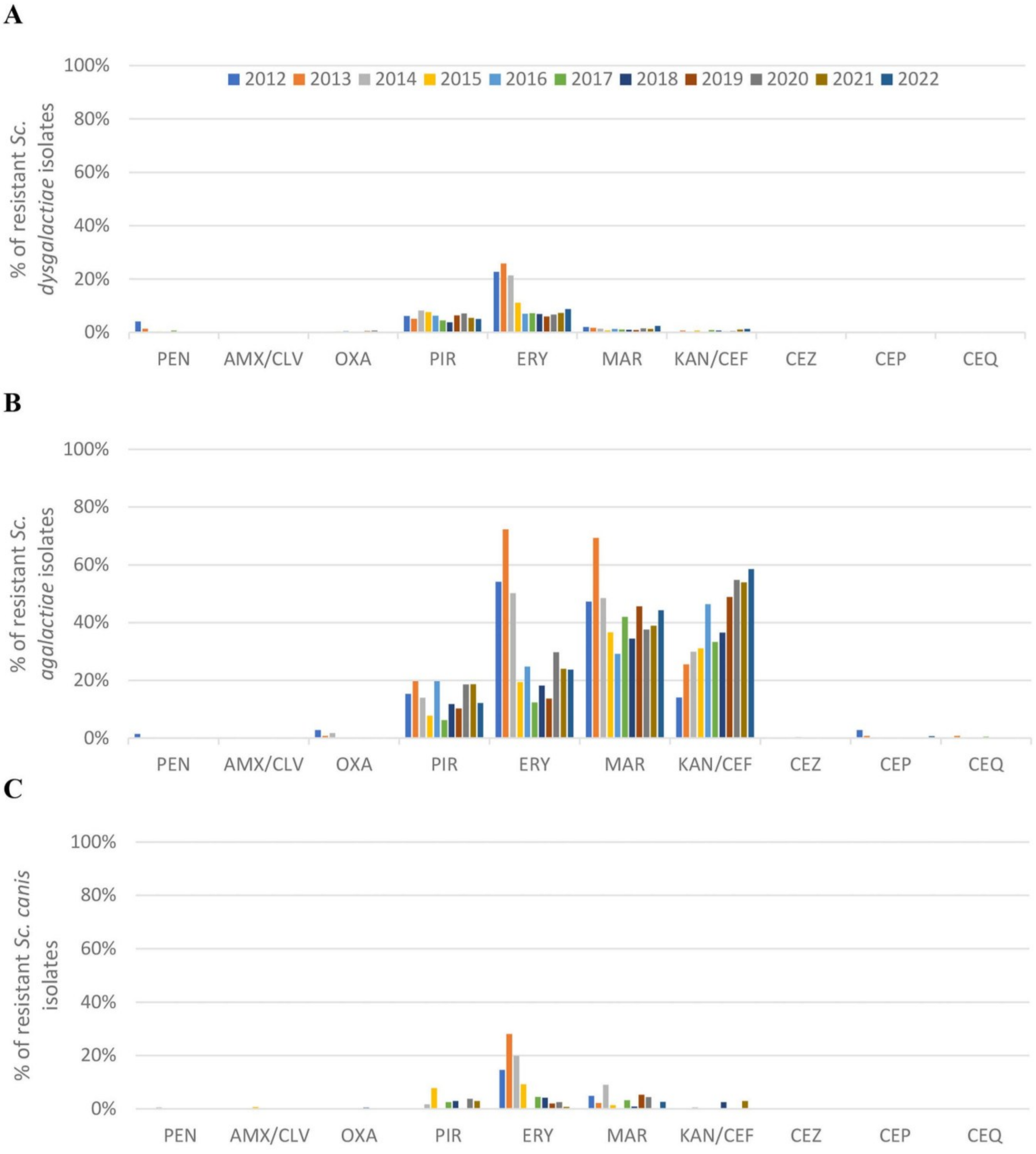

Figure 1. Percentage of resistant *Streptococcus dysgalactiae* (A), *Streptococcus agalactiae* (B), and *Streptococcus canis* (C) by antimicrobial agent and year. PEN = penicillin; AMX/CLV = amoxicillin-clavulanate; OXA = oxacillin; PIR = pirlimycin; ERY = erythromycin; MAR = marbofloxacin; KAN/CEF = kanamycin-cefalexin; CEZ = cefazolin; CEP = cefoperazone; CEQ = cefquinome.

In addition to erythromycin, antimicrobial agents with some level of resistance were marbofloxacin (3%), pirlimycin (2%), and cefalexin/kanamycin (<1%, Supplemental Tables S6, S4, and S7). Overall, no clear trend for *Strep. canis* was observed for these 3 antimicrobials ($P = 0.06$, $P = 0.4$, $P = 0.06$, respectively, Figure 1C). Although for pirlimycin and cefalexin/kanamycin the MIC50 and MIC90 remained at the lowest MIC tested from 2012 to 2022, the MIC50 and MIC90 for marbofloxacin remained at 1 μg/mL—the breakpoint used in our study.

In Vitro Multidrug Resistance

A positive trend of fully susceptible isolates was observed for *Strep. dysgalactiae* and *Strep. canis* ($P < 0.01$, Figure 2). Figures 2A and 2C show that the majority of *Strep. dysgalactiae* (87%) and *Strep. canis* (89%) isolates were susceptible to all antimicrobials tested. Over the study period fully susceptible *Strep. canis* isolates steadily increased from 2013 (71%) to 2022 (97%, $P < 0.01$). Similarly, susceptible *Strep. dysgalactiae* isolates also increased until 2019 ($P < 0.01$) but remained at a constant level from 2019 until the end of the study ($P = 0.14$). In contrast, fully susceptible *Strep. agalactiae* isolates initially increased to 39% by 2017 ($P < 0.01$), but then began to decline until the end of the study period (20%, $P < 0.01$, Figure 2B).

Only 4% of *Strep. dysgalactiae* isolates and 2% of *Strep. canis* isolates were resistant to 2 or more antimicrobials. Both species showed the highest percentage of multidrug resistance to the combination of erythromycin and pirlimycin, with 3% in *Strep. dysgalactiae* and 2% in *Strep. canis*. The next multidrug resistance observed was against the combination of erythromycin and marbofloxacin, to which less than 0.5% of the isolates for each of both species were resistant, respectively.

Streptococcus agalactiae showed a higher percentage of multidrug resistance (36%) than *Strep. dysgalactiae* (3%) and *Strep. canis* (2%, $P < 0.01$, Figure 2B). Overall, only 29% of all *Strep. agalactiae* isolates were susceptible to all tested antimicrobial agents. 36% of isolates were resistant to one antimicrobial agent and another 36% were multidrug resistant. Here, resistance against both cefalexin/kanamycin and marbofloxacin was most common and observed for 15% of isolates. The combination of marbofloxacin-erythromycin as well as cefalexin/kanamycin-marbofloxacin-erythromycin-pirlimycin, or cefalexin/kanamycin-erythromycin-pirlimycin accounted each for 4% of multidrug-resistant isolates. The remaining multidrug-resistant isolates were distributed to less than 3% each among the other antimicrobial agents and their combinations. Starting in 2018, the percentage of *Strep. agalactiae* isolates resistant against one or more antimicrobial agents increased from 61% in 2018 to 80% in 2022 ($P < 0.01$).

DISCUSSION

A strength of this study was the large number of isolates from a large number of farms and cows. In addition, all samples were tested in a single laboratory over a 10-yr period and originated from quarters with different clinical scores (healthy to clinical).

As expected, *Strep. dysgalactiae* was the most frequently isolated pathogen among those 3 species, followed by *Strep. agalactiae* and *Strep. canis*. Our findings were similar to the report of the DVG (2019), where *Strep. dysgalactiae* was, on average, ~2 times or 5 times as frequent (4.6%) as *Strep. agalactiae* (2%) or *Strep. canis* (0.9%), respectively. Groh et al. (2023) also found similar results of the distribution of mastitis pathogens in Bavaria. It is well known that these streptococci usually cause subclinical mastitis, which is consistent with our findings that ~64% to 72% of the samples originated from cows with subclinical mastitis (Baumgartner et al., 2022). However, it should be noted that we only observed the frequency of these streptococci isolated from our laboratory submissions and not the prevalence within the Bavarian dairy population.

The most commonly used antimicrobial agents against streptococcal mastitis are β-lactams, and the high susceptibility to these agents, that we observed in our isolates, has been found in several other studies (Haenni et al., 2018; Hernandez et al., 2021; Kabelitz et al., 2021). Monitoring programs in Germany and Europe found also almost no resistance against penicillin in streptococci, which agrees with our findings (El Garch et al., 2020; BVL, 2021). In addition to the generally good in vitro susceptibility to β-lactams, we observed some resistance to erythromycin, marbofloxacin, pirlimycin, and cefalexin/kanamycin. A similar outcome was described by Kabelitz et al. (2021), where the most common resistance in streptococcal mastitis species was against tetracycline, erythromycin, pirlimycin, and gentamicin.

For *Strep. dysgalactiae* and *Strep. canis* the highest resistance percentage was against erythromycin with 9% and 7%, respectively. This is comparable to the 11% resistance percentage against erythromycin for *Strep. dysgalactiae* observed by the European Monitoring program (El Garch et al., 2020). Antimicrobial resistance monitoring conducted from 2002 to 2008 in the Netherlands (MARAN, 2009) showed similar results for *Strep. dysgalactiae* (8% resistant in 2008). Furthermore BVL, the German federal office for consumer protection and food safety (BVL, 2021), tested MIC90 for different antimicrobial agents from 2002 to 2021 and described for *Strep. dysgalactiae* also a decrease in MIC90 for eryth-

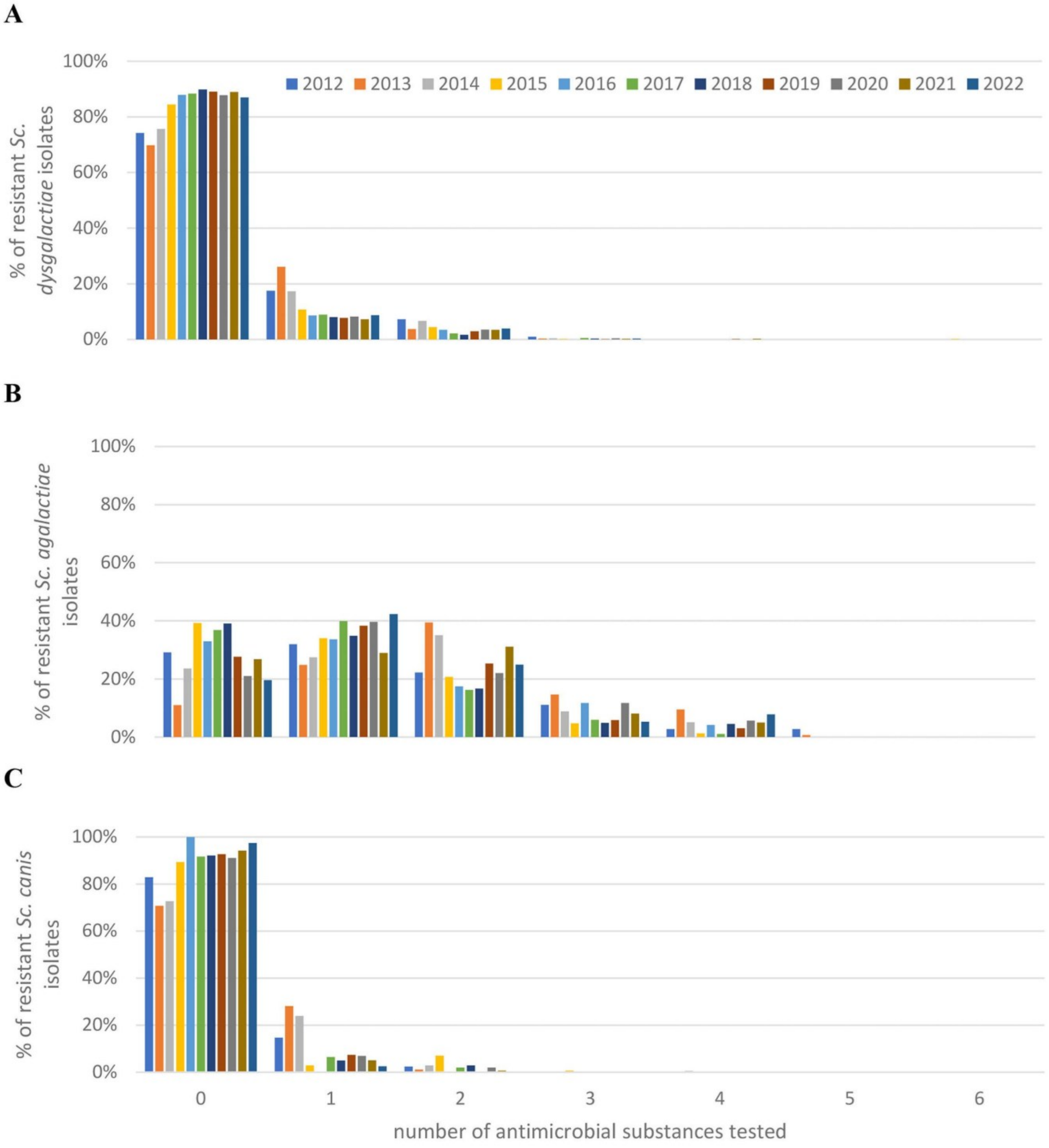

Figure 2. Number of antimicrobial agents to which *Streptococcus dysgalactiae* (A), *Streptococcus agalactiae* (B), and *Streptococcus canis* (C) isolates were tested in vitro resistant by year.

romycin. Unfortunately, to our knowledge, Europe has no monitoring programs that include *Strep. canis*. This could be due to the fact that *Strep. canis* is known to be quite rare in mastitis in dairy cows (Król et al., 2015) and therefore may not be as much of a focus in monitoring programs. In their study Groh et al. (2023) found *Strep. canis* isolates in only 0.3% of all QMS they examined in Bavaria, or 16.5% in clinical mastitis samples and 0.5% in subclinical mastitis samples. In contrast to Groh et al. (2023), in our study the most *Strep. canis* isolates were detected in cases of subclinical mastitis. This could be due to the increased number of samples from subclinically affected quarters in comparison to clinically affected quarters. Another study reported *Strep. canis* to be in less than 1% of all tested samples in Canada (Levison et al., 2016), but comprehensive data are limited. The relatively high frequency of *Strep. canis* isolates in our study could be due to the fact that our samples were largely from herd screenings that also included cows without mastitis, plus all QMS with detected *Strep. canis* were considered. However, Chaffer et al. (2005) reported a MIC50 and MIC90 at 0.06 µg/mL for erythromycin in 18 out of 55 *Strep. canis* isolates. This was similar to our results, as resistance was also overall low and the MIC90 shifted toward ≤0.125 µg/mL. This was the lowest concentration we tested and likely included isolates that were inhibited at 0.06 µg/mL already.

The second highest AMR for *Strep. dysgalactiae* and *Strep. canis* was observed against pirlimycin (6% and 2%, respectively). Both species showed a MIC90 at the lowest tested MIC. Cameron et al. (2016) reported similar results as 100% of their *Strep. dysgalactiae* isolates were inhibited by pirlimycin at the lowest MIC (0.25 µg/mL). Antimicrobial resistance monitoring conducted from 2002 to 2008 in the Netherlands (MARAN, 2009) showed an MIC90 at 4 µg/mL. This was higher than the clinical breakpoint and our observations. However, the Dutch study was conducted before our study, and they observed a decrease in resistant isolates until 2008. Therefore, one could assume that the MIC90 decreased further after 2008.

Similar to *Strep. dysgalactiae* and *Strep. canis*, *Strep. agalactiae* showed an overall decrease of resistant isolates against erythromycin over the study period, whereas for *Strep. agalactiae* MIC90 remained at the highest tested MIC (≥4 µg/mL). The same was observable for pirlimycin, where MIC90 also remained at the highest MIC (≥4 µg/mL). This is consistent with a study from Tomazi et al. (2018) in São Paulo, where *Strep. agalactiae* isolates from clinical mastitis were tested for in vitro resistance. For erythromycin and pirlimycin, they found similar resistance percentage and MIC50 and MIC90 values compared with our study. Also, El Garch et al. (2020) as well as BVL (2021) found similar resistance percentage for erythromycin and pirlimycin. What all 3 species had in common was that the peak of resistance to erythromycin occurred in 2013, followed by a sudden decline in subsequent years. This may be explained by the 16th law amending the German Medicinal Products Act (16th AMG amendment) that was introduced 2014 in Germany, which aimed to significantly minimize the use of antibiotics in livestock farming. Furthermore, a legislative change in 2018 for antimicrobial drug use in veterinary medicine was introduced, which aimed to minimalize critically important antimicrobials (Verordnung über tierärztliche Hausapotheken, TÄHAV; Bundestierärztekammer, 2018). In fact, sales have changed and a decline in sales of antimicrobials in Germany was notable (Sander et al., 2022)—especially for macrolide antibiotics, such as erythromycin, the sales (across all species) declined by 70% between 2011 and 2015. This stark decline in macrolide antibiotic sales was mirrored by the concurrently decreasing percentages of resistance to erythromycin in the 3 here discussed streptococcal species.

Eibl et al. (2021) described previously the occurrence of resistance for *Strep. canis* against macrolides, lincosamides, and streptogramin. In our study only approximately one-third of the *Strep. dysgalactiae*, *Strep. agalactiae*, and *Strep. canis* isolates showed resistance to both erythromycin and pirlimycin. This is surprising, as usually cross-resistance against macrolides, lincosamides, and streptogramin B are present in the MLSb phenotype (Pinto et al., 2013; Haenni et al., 2018). The MLSb phenotype is caused by a methylase, that is coded by the erm (erythromycin ribosome methylation) gene family (Haenni et al., 2018) and Rato et al. (2013) reported that almost all erythromycin-pirlimycin resistant isolates had *ermB*. Isolates that are only resistant to erythromycin but not to pirlimycin may have resistance genes that are only inducibly expressed (EMA, 2011). A possibility to test the inducible lincosamide resistance would be a simple disk diffusion D-test (Foster-Nyarko et al., 2017). Unfortunately, we did not investigate the resistance genotypes or D-test, so we were only able to speculate at this point.

A noteworthy difference of *Strep. agalactiae* from *Strep. dysgalactiae* and *Strep. canis* is its resistance against cefalexin/kanamycin and marbofloxacin. Although *Strep. dysgalactiae* and *Strep. canis* were predominantly susceptible, *Strep. agalactiae* showed not just the highest percentage of resistance to these antimicrobial agents, but also an overall increase in resistance. Similar to a study from Bolte et al. (2020), *Strep. agalactiae* isolates showed the highest MIC for cefalexin/kanamycin. Şahan Yapicier et al. (2021) described a high prevalence of resistance to fluoroquinolones in group B streptococci in Turkey that is comparable to the high resistance of *Strep.*

agalactiae to marbofloxacin in our study. Haenni et al. (2018) also noted a low-level intrinsic resistance against fluoroquinolones. In addition, Carra et al. (2021) found common resistance against aminoglycosides in group B streptococci and concluded that these streptococci have an intrinsic resistance against aminoglycosides. This may explain the high resistance percentage against marbofloxacin and kanamycin in our study but unfortunately not against cefalexin/kanamycin, because in this combination isolates should still be susceptible to cefalexin, a β-lactam. Hu et al. (2018) found in their study in central and northeast China 129 *Strep. agalactiae* isolates to be resistant against penicillin and mostly against cefalexin due to mutations in penicillin-binding proteins (**PBP**). A possible explanation for the high resistance rate against cefalexin in our study may be found in the PBP and their different mutations and expressions. However, because the *Strep. agalactiae* isolates in our study were predominantly susceptible to penicillin and we did not investigate resistance genes, we cannot find a satisfactory explanation for the high resistance to cefalexin/kanamycin.

A possible explanation for the increasing resistance to cefalexin/kanamycin could in turn be found in the changing sales of various antimicrobial agents. Sales of aminoglycoside antibiotics and first-generation cephalosporins, such as kanamycin and cefalexin respectively, have increased after 2015 (Sander et al., 2022). These increasing sales of certain antibiotics could be due to the repeated shortages of single antimicrobial agents around the world. For instance, a report by the Access to Medicine Foundation (2018) found that penicillin had a shortage in 39 countries, including Germany, in 2018, which could explain the use of other antimicrobials that effect the development of resistances. Unfortunately, we do not have treatment records of our individual cows and up to 2022 only the weight of antibiotic sales to German veterinary practices were available (Sander et al., 2022). This limits the inferences that could be drawn with the given data, and we cannot fully clarify why *Strep. agalactiae* behaves differently in its resistance development than *Strep. dysgalactiae* and *Strep. cani*s. Regardless of this Alves-Barroco et al. (2021) and Richards et al. (2012) stated that *Strep. dysgalactiae* and *Strep. canis* seem to be very closely related and Kabelitz et al. (2021) also reported that *Strep. agalactiae* was more likely to be resistant than *Strep. dysgalactiae*.

As for resistance associated with the mastitis status of the quarter, this observation was not entirely clear in our data, in contrast to Sorge et al. (2021) who reported for other streptococcal mastitis pathogens that isolates from clinically affected quarters were less likely to be resistant in vitro than isolates from healthy or subclinically affected quarters. In this context it must be considered, that we included more isolates from clinically affected quarters for resistance testing, whereas the majority of isolates in this study stemmed from subclinically affected quarters. This may be considered as a source of selection bias.

Because all streptococcal isolates were still very susceptible to penicillin, it remains the antimicrobial of first choice. Although in vitro susceptibility is not an indication of treatment success, as a variety of factors can influence the success of a treatment, experience in the field strongly support the use of penicillin against these pathogens. These anecdotal observations are strengthened by a meta-analysis by Nobrega et al. (2020), who also stated that critical antimicrobials do not add benefit to the treatment of mild to moderate mastitis cases. In addition, broad-spectrum antimicrobials increase selection pressure and thus have a greater effect on the development of resistance than narrow-spectrum antimicrobials (Barbosa and Levy, 2000). Therefore, the European Commission (2015) recommends a prudent use of antimicrobials with a rational and targeted use.

CONCLUSIONS

Overall, the percentage of in vitro resistance of *Strep. dysgalactiae* and *Strep. canis* isolates from bovine QMS was generally low and decreased over the whole study period. *Streptococcus agalactiae* showed the highest percentages of resistance, particularly to cefalexin/kanamycin, marbofloxacin, and erythromycin, and also showed an increase in resistant isolates. A decline in resistance to erythromycin was observed in all 3 species, which concurred with the marked decline in sales of macrolide antibiotics in Germany after 2011. However, all 3 species showed almost no resistance to β-lactams, except for *Strep. agalactiae* toward cefalexin/kanamycin. In this context, penicillin should remain the treatment of first choice against streptococcal mastitis in Bavaria and beyond Southern Germany, as other studies are consistent with our findings that streptococci are still predominantly sensitive to penicillin.

NOTES

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Nonstandard abbreviations used: AMR = antimicrobial resistance; AMX/CLV = amoxicillin-clavulanate; CAMP = Christie, Atkins, Munch-Peterson; CEP = cefoperazone; CEQ = cefquinome; CEZ = cefazolin; CMT = California Mastitis Test; ERY = erythromycin; KAN/CEF = kanamycin-cefalexin; MAR = marbofloxacin; MDR = multidrug resistance; OXA = oxacillin; PBP = penicillin-binding proteins; PEN = penicillin; PIR = pirlimycin; QMS = quarter milk samples; TGD = Bavarian Animal Health Service (Tiergesundheitsdienst).

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IV. DISCUSSION

1. General aspects

The objectives of this work were to investigate the distribution of mastitis pathogens in Bavaria from QMS submitted to the laboratory of the TGD between 2014 and 2023 a) in general, b) in relation to the clinical status of the quarters, and c) to determine seasonal differences in the detection risk of mastitis pathogens. Furthermore, the objective was to investigate changes in antimicrobial resistances for *Sc. dysgalactiae*, *Sc. agalactiae*, and *Sc. canis* between 2012 and 2022. As a result, this study provided valuable insights into the dynamic nature of mastitis pathogen distribution influenced by mastitis status and seasonality. Additionally, an overview of *in vitro* resistance trends for three major streptococcal pathogens (*Sc. dysgalactiae, Sc. agalactiae, and Sc. canis*) to commonly used antimicrobials could be obtained.

In 2014, MALDI-TOF MS (matrix-assisted laser desorption ionisation, time of flight mass spectrometry) analysis was introduced in the TGD milk laboratory which made it possible to identify the isolated pathogens more precisely. Before this methodological change, esculin-positive streptococci and gram-negative pathogen species could often not be fully identified. For example, *Sc. uberis* has only been classified as an independent species since 2014, whereas it was previously grouped with other esculin-positive streptococci. To eliminate this potential bias due to misidentification and improve the validity of statistical tests, we selected the period from 2014 to 2023 for Publication I (section III.1). Since this methodological change did not impact esculin-negative streptococci, the study period for Publication II (section III.2) remained from 2012 to 2022.

As for the sampling, a large proportion of QMS in both publications originated from herd screenings, which included samplings from healthy cows. Consequently, a large number of QMS were collected from healthy and subclinically affected quarters, with only a small proportion coming from clinically affected quarters. This may introduce a selection bias, which should be considered when comparing our findings with those of other studies. Furthermore, in Publication I, 81% of the QMS showed no bacterial growth, since, as stated above, a large proportion of QMS stemmed from healthy or subclinically affected quarters. Due to the low pathogen detection rates when including culture-negative samples, percentages often fell below 1%, making the graphical presentation challenging. Therefore, the analysis focused on culture-positive samples to better assess distribution trends. Furthermore, the inclusion of herds or the submission of individual samples was based on voluntary information and not on a random sample. Hence, statements about the prevalence and prevalence within herds in this region should be avoided and comparisons with prevalence data from other studies should be made with caution.

Nevertheless, the strength of this research was that a large amount of QMS from a large number of herds and cows over a long period was analyzed in a single dairy laboratory. This ensured a consistency in sample types across years as well as laboratory methods. Therefore, this data set enabled the identification of long-term trends in both mastitis pathogen distribution and AMR of *Sc. dysgalactiae, Sc. agalactiae,* and *Sc. canis* in Bavaria.

2. Study region Bavaria

Bavaria is a key state for Germany's dairy industry, housing 30% of the country’s dairy cows (BAYERISCHE LANDESAMT FÜR STATISTIK, 2023) and producing approximately a quarter of the nation’s annual milk volume (BITTLMAYER, 2015). The region is predominantly characterized by Simmental cattle, with an average herd size of 44 cows per herd (HÖFLER; BAYERISCHE LANDESAMT FÜR STATISTIK, 2023). This distinguishes Bavaria from other German states, such as those in eastern Germany, where Holstein-Friesian is the primary breed and the average herd size is 197 cows per herd (TERGAST et al., 2022). Consequently, while this study provides valuable insights into one of Germany’s most significant dairy regions, its findings may not be directly applicable to other parts of the country due to regional differences. However, the smaller herd structure observed in Bavaria is also present in neighboring Alpine countries like Austria (KALCHER, 2022), making the results also relevant in regions beyond Bavaria.

3. Distribution of mastitis pathogens

A factor to keep in mind when interpreting the results of Publication I is that the duration of infection influences the pathogen detection (ZADOKS &

FITZPATRICK, 2009). Pathogens such as *E. coli* (GOULART & MELLATA, 2022) are at a lower detection risk due to their short infection duration, making them more difficult to detect when samples are not taken during the acute phase of mastitis. This likely explains the higher proportion of these pathogens in samples from clinically affected quarters and individual submissions, as veterinarians and farmers often submit QMS from acute cases to choose treatment options. In herd screenings sampling was often carried out at some point and pathogens with a short infection duration were therefore more likely to go undetected, leading to a result in the "no-growth" category. This context is important for the interpretation of data from herd screenings with a high number of QMS from CMT-negative and subclinically affected quarters.

Overall, in our study, a decreasing trend of contagious pathogens, as *S. aureus*, and an increase of environmental pathogens as *Sc. uberis* and *E. coli* was observed. This decline in *S. aureus* isolates aligns with the national trends in Germany, where *S. aureus* showed a decrease from 15% in 2015 (DVG, 2015) to 5% in 2022 (DVG, 2024). Globally, trends for *S. aureus* IMI vary. In China a decreasing trend was reported (WANG et al., 2022), while in Norway the proportion was relatively stable between 2000 and 2020 (SMISTAD et al., 2023). In Ontario, Canada, it even increased between 2008 and 2017 (ACHARYA et al., 2021). Nevertheless, KARELL et al. (2024) found also a decrease in AMR for *S. aureus* in Bavaria and concluded that one explanation may be the successfully implemented mastitis control measures. Variations in these measures across countries may explain the different trends, such as for example the inconsistent use of post-milking teat disinfection in Norway (SMISTAD et al., 2023). Another important observation was the frequent detection of *S. aureus* in QMS from CMT-negative and subclinically affected quarters, indicating that cows with undetected infections serve as a reservoir for the transmission of *S. aureus* within herds. This highlights the importance of including healthy and subclinically infected cows in herd screenings, since the main focus in the prevention of *S. aureus* infections, as for other contagious pathogens, should be on reducing the transmission from infected to non-infected quarters – i.e., preventing new infections (ROSSI et al., 2019; WOUDSTRA et al., 2023).

Furthermore, a different pathogen distribution could be found when including mastitis status into the analysis. In QMS from clinically affected quarters, *Sc. uberis* was the most frequently detected pathogen, which is consistent with findings from other studies in Germany (SCHMENGER & KRÖMKER, 2020), Belgium (VERBEKE et al., 2014), and New Zealand (PETROVSKI et al., 2009). *Sc. uberis* showed an overall increasing incidence throughout the study period and an increased detection during the warmer summer month, similar to other environmental pathogens like *E. coli.* Control measures such as post-milking teat disinfection and dry cow therapy are less effective against environmental pathogens like *Sc. uberis* (COBIRKA et al., 2020), which may explain the simultaneous decrease in contagious pathogens and increase in environmental pathogens. The higher incidence of environmental pathogens during warmer months has been reported previously (KOIVULA et al., 2007; OLDE RIEKERINK et al., 2007), with factors such as housing conditions (KOIVULA et al., 2007; OLDE RIEKERINK et al., 2007; ERICSSON UNNERSTAD et al., 2009), higher shedding rates due to a high THI (HAMEL et al., 2021), and heat-related immunosuppression (RAKIB et al., 2020; BOKHARAEIAN et al., 2023) being suggested as contributing factors. Unfortunately, in this study, we could only report the observed differences, and speculate on possible explanations as data on climate conditions and other risk factors, such as farming practices, were not available for all submissions.

4. Resistance trends

When comparing AMR between different studies, it is important to consider the different methods (e.g. disk diffusion, agar diffusion and broth microdilution (BMD)) and differences in the guidelines used, which can make direct comparisons difficult (HAENNI et al., 2018). In Publication II we used BMD as susceptibility testing and the methodology was mostly in accordance with the (at the time) current recommendations of the Clinical and Laboratory Standards Institute (CLSI) (e.g. CLSI, 2023). However, official breakpoints from CLSI were not available for all antimicrobial agents and the indication of streptococcal mastitis in cattle. Further development of these breakpoints would improve laboratory methods and comparability between different studies.

In our study, resistance rates for *Sc. dysgalactiae* and *Sc. canis* were generally low and decreased further throughout the study period, particularly against erythromycin, pirlimycin, and marbofloxacin, with a notable drop in erythromycin resistance after 2013. These findings align with several other studies reporting similarly low resistance rates for *Sc. dysgalactiae* in Germany (BVL, 2021) and Europe (EL GARCH et al., 2020), and for *Sc. canis* in Germany (HASSAN et al., 2005) and Israel (CHAFFER et al., 2005). In *Sc. agalactiae*, a decrease in resistance to erythromycin was also observed, while a key difference noted in this study was the increasing resistance of *Sc. agalactiae* to cefalexin/kanamycin and marbofloxacin. High resistance to cefalexin/kanamycin was similarly reported in a study from northern Germany (BOLTE et al., 2020) and to fluoroquinolones in Turkey (ŞAHAN YAPICIER et al., 2021). The observed decline in resistance to erythromycin corresponds with the decreasing sale of antimicrobials in Germany, particularly macrolide antibiotics such as erythromycin, which sales dropped by approximately 70% from 2011 to 2015 (SANDER et al., 2022). This decline is probably due to the 16th law amending the German Medicinal Products Act, that was introduced in 2014 and aimed to minimize antimicrobial use in livestock farming. Furthermore, in 2018, a legislative change was introduced, aiming to minimize the use of critically important antimicrobials in veterinary medicine (BUNDESTIERÄRZTEKAMMER, 2018). This suggests that policy changes can have a positive impact on antimicrobial sales and therefore resistance development. This aspect underlines the need for ongoing prudent antimicrobial use. In contrast, sales of aminoglycoside antibiotics and first-generation cephalosporins, such as kanamycin and cefalexin, have increased since 2015 (SANDER et al., 2022). This observation may explain the increasing resistance to cefalexin/kanamycin in *Sc. agalactiae.* Unfortunately, a limitation of this study was the lack of detailed treatment records for each cow, which limits the ability to directly correlate antibiotic usage with resistance patterns. Additionally, the study did not investigate the genetic mechanisms underlying the observed resistance, which could provide deeper insights into the AMR trends, particularly the rising resistance in *Sc. agalactiae.*

Overall, the results confirmed that penicillin remains highly effective *in vitro* against all three streptococcal species, as several other studies report (BOLTE et al., 2020; EL GARCH et al., 2020; HERNANDEZ et al., 2021; KABELITZ et al., 2021), supporting its continued recommendation as the first-line treatment for streptococcal mastitis due to its narrow spectrum and low resistance rates.

5. Conclusion and perspective

This study obtained a good overview for the long-term trend development of mastitis pathogen distribution and AMR in *Sc. dysgalactiae, Sc. agalactiae* and *Sc. canis* in Bavaria. Despite progress in the control of contagious mastitis, the increase in pathogens from the environment and their seasonal fluctuations represent a constant challenge for mastitis management and environmental factors continue to play an important role in udder health. These results emphasize the need to continue and improve management practices, especially against environmental pathogens, and to further monitor the spread of mastitis pathogens to track future trends.

For *Sc. dysgalactiae* and *Sc. canis*, quite positive developments could be observed with an overall decreasing resistance in these pathogens. In contrast, *Sc. agalactiae* showed an increasing resistance trend to cefalexin/kanamycin and marbofloxacin. However, all three streptococcal species were almost fully susceptible to β-lactams, and penicillin should remain the first-choice treatment for streptococcal mastitis.

In conclusion, this work underlines the importance of mastitis control measures and in this context the value of antimicrobial agents, but at the same time shows the conflict of antimicrobial usage and the development of resistance. Consequently, continuous monitoring of pathogen distribution trends, resistance trends, and prudent antibiotic use is necessary, to adapt targeted prevention measures, track AMR development in bovine mastitis pathogens, and preserve the effectiveness of existing treatments. While this study provides a good overview of the current trends in Bavaria, subsequent studies should include data about climatic conditions and farming management to further identify risk factors. With regard to AMR testing, future research would benefit from including treatment protocols from the cows sampled, to directly correlate antibiotic usage with resistance pattern.

V. SUMMARY

Bovine mastitis is one of the most important diseases in the dairy industry worldwide because of its economic impact and in terms of animal welfare. Furthermore, it raises public health concerns due to the use of antibiotics, that may promote the development of antimicrobial resistances. The objectives of this study were to investigate the distribution of mastitis pathogens in quarter milk samples (QMS) submitted to the laboratory of the Bavarian Animal Health Services (TGD) between 2014 and 2023 a) in general, b) in relation to the clinical status of the quarters, and c) to determine seasonal differences in the detection risk of mastitis pathogens. Furthermore, the objective was to analyze the *in vitro* antimicrobial resistances for *Streptococcus (Sc.) dysgalactiae*, *Sc. agalactiae*, and *Sc. canis* between 2012 and 2022.

In the respective study period, all QMS sent to the TGD were analyzed and tested using California-Mastitis-Test (CMT). Based on the CMT results, samples were classified as CMT-negative, subclinical, or clinical if milk abnormalities were observed. The samples stemmed from whole herd screenings or individual submissions by farmers and veterinarians and came from over 630,000 cows and over 15,600 herds. Mastitis pathogens were detected in 19% of the samples, resulting in the inclusion of 729,459 isolates in this study. From 2012 to 2022, 65,750 *Sc. dysgalactiae*, 30,486 *Sc. agalactiae*, and 11,336 *Sc. canis* isolates were identified. From those, a subset of isolates per herd, as well as isolates from cows showing subclinical or clinical mastitis, from pretreated quarters or when the client specifically requested it, underwent antimicrobial susceptibility testing via broth microdilution (BMD). The isolates were classified as either susceptible or resistant according to the official breakpoints at the time of this analysis.

Among all culture-positive QMS non-aureus staphylococci (NAS), were the most common isolated pathogens from CMT-negative and subclinically affected quarters (44% and 27%, respectively), followed by *Staphylococcus (S.) aureus* (25% and 17%, respectively) and *Sc. uberis* (8% and 22%, respectively). In contrast, in culture-positive QMS from clinically affected quarters the most frequently isolated pathogens were *Sc. uberis* (32%), *S. aureus* (13%), *Sc. dysgalactiae* (11%), and *Escherichia (E.) coli* (11%). Over the study period, the detection of NAS and

Sc. uberis increased, while that of *S. aureus* declined. Additionally, during the warmer month (June to October) QMS from subclinically affected quarters increased and environmental pathogens, such as *Sc. uberis,* were detected more frequently.

The analysis of *in vitro* resistance of *Sc. dysgalactiae*, *Sc. agalactiae*, and *Sc. canis* revealed that all three pathogens exhibited the highest resistance to erythromycin, marbofloxacin, pirlimycin, and cefalexin/kanamycin, although the degree of resistance varied. Despite this, all three species remained largely susceptible to the remaining antimicrobial agents (penicillin, amoxicillin-clavulanate, oxacillin, cefazolin, cefoperazone, cefquinome). Over the study period, resistance in *Sc. dysgalactiae* and *Sc. canis* decreased, while resistance in *Sc. agalactiae* increased, particularly to cefalexin/kanamycin and marbofloxacin. *Sc. agalactiae* showed also the highest percentage of multidrug resistance (36%). However, all three streptococcal species showed almost no resistance to β-lactams, except for *Sc. agalactiae* toward cefalexin/kanamycin.

This study offered valuable insights into the long-term trends in mastitis pathogen distribution and AMR in *Sc. dysgalactiae, Sc. agalactiae* and *Sc. canis* in Bavaria. Our results confirmed an increased isolation of environmental pathogens and a concurrent decrease in contagious pathogens, which once again highlighted the need for continuous adaptation of mastitis control measures. Furthermore, it showed that the investigated streptococcal species are still highly susceptible to β-lactams and therefore, penicillin should remain the first-choice treatment against streptococcal mastitis.

VI. ZUSAMMENFASSUNG

Die bovine Mastitis ist aufgrund ihrer wirtschaftlichen Bedeutung und im Hinblick auf das Tierwohl eine der bedeutendsten Krankheiten in der weltweiten Milchwirtschaft. Darüber hinaus wirft sie aufgrund ihrer häufigen Behandlung mit Antibiotika, welche die Entwicklung von Antibiotikaresistenzen fördern kann, Fragen bezüglich ihres Einflusses auf die öffentliche Gesundheit auf. Ziel dieser Studie war es, die Verbreitung von Mastitiserregern aus Viertelgemelksproben (VGP), die zwischen 2014 und 2023 an das Labor des Bayerischen Tiergesundheitsdienstes (TGD) gesendet wurden, a) allgemein zu untersuchen, b) in Bezug auf den klinischen Status der Viertel zu setzen und c) saisonale Unterschiede im Nachweisrisiko von Mastitiserregern zu ermitteln. Darüber hinaus war es das Ziel, die *in vitro* Antibiotikaresistenzen für *Streptococcus (Sc.) dysgalactiae*, *Sc. agalactiae* und *Sc. canis* zwischen 2012 und 2022 zu analysieren.

Im jeweiligen Untersuchungszeitraum wurden alle an den TGD gesendeten VGP analysiert und mit dem California-Mastitis-Test (CMT) untersucht. Anhand der CMT-Ergebnisse wurden die Proben als CMT-negativ, subklinisch oder klinisch eingestuft, wenn Milchanomalien festgestellt wurden. Die Proben kamen aus Untersuchungen ganzer Herden oder individuellen Einsendungen von Landwirten und Tierärzten und stammten von über 630.000 Kühen und über 15.600 Herden. Mastitiserreger wurden in 19% der Proben nachgewiesen, was zur Aufnahme von 729.459 Isolaten in die Studie führte. Von 2012 bis 2022 wurden 65.750 *Sc. dysgalactiae*, 30.486 *Sc. agalactiae* und 11.336 *Sc. canis* Isolate identifiziert. Von diesen wurde eine Auswahl an Isolaten pro Herde sowie Isolate von Kühen mit subklinischer oder klinischer Mastitis, aus vorbehandelten Vierteln oder auf ausdrücklichen Wunsch des Kunden, einem antimikrobiellen Empfindlichkeitstest mittels Bouillon-Mikrodilution unterzogen. Die Isolate wurden gemäß den offiziellen Breakpoints zur Zeit der Analyse entweder als empfindlich oder als resistent eingestuft.

Unter allen kulturpositiven VGP waren nicht-aureus Staphylokokken (NAS) die häufigsten isolierten Erreger aus CMT-negativen und subklinisch betroffenen Vierteln (44% bzw. 27%), gefolgt von *Staphylococcus (S.) aureus* (25% bzw. 17%)

und *Sc. uberis* (8% bzw. 22%). Im Gegensatz dazu waren in Kultur-positiven VGP aus klinisch betroffenen Vierteln die am häufigsten isolierten Erreger *Sc. uberis* (32%), *S. aureus* (13%), *Sc. dysgalactiae* (11%) und *Escherichia (E.) coli* (11%). Im Laufe des Untersuchungszeitraums nahm der Nachweis von NAS und *Sc. uberis* zu, während der von *S. aureus* zurückging. Zusätzlich nahm während der wärmeren Monate (Juni bis Oktober) die Anzahl der VGP von subklinisch betroffenen Vierteln zu und Umwelterreger, wie *Sc. uberis*, wurden häufiger nachgewiesen.

Die Analyse der *In-vitro*-Resistenz von *Sc. dysgalactiae*, *Sc. agalactiae* und *Sc. canis* ergab, dass alle drei Erreger die höchste Resistenz gegenüber Erythromycin, Marbofloxacin, Pirlimycin und Cefalexin/Kanamycin aufwiesen, wenngleich der Grad der Resistenz variierte. Trotzdem blieben alle drei Arten weitestgehend empfindlich gegenüber den übrigen Antibiotika (Penicillin, Amoxicillin-Clavulanat, Oxacillin, Cefazolin, Cefoperazon, Cefquinom). Während des Untersuchungszeitraums nahm die Resistenz bei *Sc. dysgalactiae* und *Sc. canis* ab, während die Resistenz bei *Sc. agalactiae* zunahm, insbesondere gegenüber Cefalexin/Kanamycin und Marbofloxacin. *Sc. agalactiae* wies zudem den höchsten Prozentsatz an Multiresistenzen auf (36%). Alle drei Streptokokkenarten zeigten jedoch fast keine Resistenz gegen β-Laktame, mit Ausnahme von *Sc. agalactiae* gegenüber Cefalexin/Kanamycin.

Diese Studie bot wertvolle Einblicke in die langfristigen Trends der Verteilung von Mastitiserregern und der Antibiotikaresistenzen bei *Sc. dysgalactiae*, *Sc. agalactiae* und *Sc. canis* in Bayern. Unsere Ergebnisse bestätigten eine Zunahme der umweltbedingten Erreger bei gleichzeitigem Rückgang der kuhassoziierten Erreger, was einmal mehr die Notwendigkeit einer kontinuierlichen Anpassung der Mastitisbekämpfungsmaßnahmen unterstreicht. Darüber hinaus zeigte sich, dass die untersuchten Streptokokkenarten nach wie vor hochgradig sensibel gegenüber β-Laktamen sind und Penicillin daher weiterhin die erste Wahl bei der Behandlung von Streptokokken-Mastitis sein sollte.

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