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# Prevalence of Contagious Mastitis Pathogens in Bulk Tank Milk from Dairy Farms in Lower Saxony, Germany

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**Abstract:** The aim of this study was to determine the prevalence of *Streptococcus* (*Sc.*) *agalactiae*, *Prototheca* spp., *Staphylococcus* (*S.*) *aureus*, and especially methicillin-resistant *S. aureus* as well as *Mycoplasmopsis* (*M.*) spp. and *M. bovis* in bulk tank milk (BTM) on dairy farms in Lower Saxony, Germany. BTM samples were collected in January 2023 from 208 selected dairy farms. The samples were quantitatively culturally analyzed for *S. aureus* and *Prototheca* spp. Presumptive *S. aureus* colonies were further confirmed by MALDI-TOF. Presumptive *Prototheca* spp. colonies were confirmed by light microscopy. *Sc. agalactiae* and *Mycoplasmopsis* spp. were detected by real-time polymerase chain reaction (rtPCR). *Sc. agalactiae* was detected in two herds (1% (Confidence Interval 95% (CI) 0.3–3.4)). *S. aureus* was confirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) in 38 herds (18.3% (CI 13.6–24.1)), assuming a threshold of >10 cfu/mL milk. A total of 154 isolates identified as *S. aureus* by MALDI-TOF were transferred to agar with added oxacillin for resistance testing, of which 19 isolates (12.3% (CI 8–18.5)) showed growth. The 19 isolates came from eight different farms (3.8% (2–7.4)). *Prototheca* spp. were identified in 13 herds (6.3% (CI 3.7–10.4)). *Mycoplasmopsis* spp. were detected by PCR in 18 herds (8.7% (CI 5.5–13.3)). Of these, *M. bovis* was present in three herds (1.4% (0.5–4.2)). The herd prevalence of *Sc. agalactiae* in BTM appears to be at low levels in the sampled area. The prevalence of *Mycoplasmopsis* spp. in the herds was higher than expected compared to previous studies. It is interesting to note that the percentage of *M. bovis* in the total *Mycoplasmopsis* spp. was only 16.7%.

**Keywords:** mastitis; bulk tank milk; *Streptococcus agalactiae*; *Staphylococcus aureus*; *Mycoplasmopsis*; *Prototheca*; prevalence



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## 1. Introduction

Mastitis is considered the most important production disease in dairy cows, resulting in high economic losses in the dairy industry [1,2]. Mastitis is a multifactorial disease, with the relevance and frequency of infectious factors constantly evolving [3,4]. The role of various "rare" mastitis pathogens is controversially discussed in the literature.

The analysis of bulk tank milk (BTM) is a simple, fast, and inexpensive alternative or adjunct to the analysis of quarter milk samples. The interpretation of the results is particularly useful for qualitative or semi-quantitative tests. The quantitative testing of BTM is useful for pathogens whose increased presence in bulk milk can lead to an increased risk of pathogen spread and increased risk of new infections on the farm. BTM testing can be used primarily for the early detection of infection risks from microorganisms that are transmitted during milking and which, due to their high rate of spread, pose a massive threat to udder health on dairy farms [5]. It can also be useful as an adjunct to herd sanitation, for example, in the case of *Mycoplasmopsis* (*M.*) spp. [6]. BTM is therefore a herd risk assessment test. Ideally, the microorganisms tested should be undetectable. However,

in the case of *Staphylococcus* (*S.*) *aureus*, the risk of infected cows increases with increasing herd size and the associated increase in milkings per cluster. Thus, a limit of more than 10 cfu *S. aureus*/mL of bulk milk is considered to increase the risk of infection [7]. If *Streptococcus* (*Sc.*) *agalactiae* and/or *Mycoplasmopsis* spp. are detected, immediate action is required to achieve pathogen-free status [5]. In the case of *Prototheca* (*P.*) spp., an overview of their distribution on German cattle farms is of particular interest, as few data are available on this subject.

Although there are no longitudinal studies on the herd prevalence of *Sc. agalactiae* in Germany, the prevalence of intramammary infections caused by *Sc. agalactiae* has long been reported to decrease in recent years, especially since it was assumed that adequate therapy with penicillin would eradicate the pathogen [8–10]. In Germany, the treatment of *Sc. agalactiae* mastitis has been recommended for more than seventy years. In particular, the application of penicillin and the systematic drying-off of cows with penicillin on farms with *Sc. agalactiae* has been described since 1953. This resulted in a significant reduction in *Sc. agalactiae* in German dairy farms and a shift in mastitis pathogens towards *S. aureus* and later environmental pathogens, but a complete disappearance of *Sc. agalactiae* was not achieved [11]. In recent years, there have been increasing reports that the prevalence of *Sc. agalactiae* infections is increasing again [12–14]. Those studies are mainly from the Scandinavian countries. The study by Mweu et al. from Denmark, analyzing bacteriological culture data from all BTM samples collected annually as part of the Danish mandatory surveillance program for *Sc. agalactiae*, describes a steady increase in prevalence from 1.2% to 4.7% between 2000 and 2008. This suggests that the eradication of the pathogen has not been successful and that *Sc. agalactiae* may play a greater role in the pathogen spectrum of mastitis pathogens in the future. In a German study of pathogen distribution in microbiologically positive (quarter) milk samples from all laboratories using the German diagnostic standard, a proportion of 2% (0.1–4.3) *Sc. agalactiae* was observed [15]. In general, the presence of *Sc. agalactiae* indicates problems in herd management, as *Sc. agalactiae* is a pathogen that can be eliminated from individual animals with appropriate antibiotic therapy. The elimination of the pathogen is desirable for a long-term udder healthy herd, although this is difficult to achieve on very large farms [7]. Poor teat and udder hygiene, poor environmental hygiene, and a farm with a high number of cows have been described as risk factors [12,16,17]. The entry of the pathogen onto the farm can occur especially in the case of poor internal biosecurity or the purchase of new infected animals [8]. Infection with *Sc. Agalactiae* usually results in subclinical mastitis (SCM), with a sharp increase in cell count. However, it can also cause severe clinical mastitis (CM), with a complete cessation of milk production. *Sc. agalactiae* is an obligate pathogen of the mammary gland, and infection is generally known to be transmitted during milking, with recent studies also describing the possible environmental attachment of the pathogen and potential infection by this route [8,18,19].

*Prototheca* spp. and methicillin-resistant *S. aureus* (MRSA) are described as other "rare" mastitis pathogens that should be considered mainly because of the great difficulty in treating them, as no evidence-based therapies are described [20], and, in the case of MRSA, because of its potential zoonotic risk [21,22]. Recent studies have reported a rise in the number of cases of both MRSA [23,24] and *Prototheca* spp. [25].

*Prototheca* spp. are colorless algae that are well established in the environment. Therefore, both types of transmission—environmental and contagious—are possible. When a *Prototheca* spp. infection occurs in the herd, several animals are usually affected at the same time. Predisposing factors include poor hygiene, wet areas, and the heavy, uncontrolled use of antibiotics [26]. Mastitis caused by *Prototheca* spp. often occurs as a chronic process with very high somatic cell counts (SCC). However, acute CM has also been reported [27,28]. There is no known therapy with good efficacy against the pathogen [29–31]. As it is often a livestock problem, extensive remediation measures are required, resulting in high economic losses. The pathogen is still rarely associated with CM, but an increased incidence has been reported recently [32]. A recent Polish study described *Prototheca* spp. as the third most

important pathogen after staphylococci and streptococci [25]. This raises the question of whether the pathogen should also be classified as rare on German dairy farms. A Canadian study of tank milk found a prevalence of 6% [33].

In general, *S. aureus* is one of the most common mastitis pathogens worldwide. In Lower Saxony, Germany, it represents only a small proportion of clinical cases of mastitis in studies. For example, in a recent study, the percentage of *S. aureus* in CM cases was 3.7% [34]. In 2019, the analysis of pathogen distribution in microbiologically positive (quarter) milk samples revealed a proportion of *S. aureus* of 11.4% in Germany and 5.3% in the sampled region [15]. *S. aureus* is a cow-associated pathogen with numerous pathogenicity factors that make it difficult to control [35]. Since it is almost impossible to eliminate *S. aureus* from the herd, the most important aspect is to improve working standards to prevent new infections.

MRSA shows resistance to ß-lactam antibiotics and further limits treatment options, resulting in increased culling rates [20,24]. The potential zoonotic risk is important from a societal perspective, as there are ongoing discussions about increased antibiotic use and antibiotic resistance in livestock. However, the risk of transmitting MRSA to humans through milk is considered very low if the milk is properly processed and controlled [36]. MRSA infections are associated with both CM and SCM [22]. MRSA transmission in dairy herds is primarily associated with poor milking hygiene practices [19]. Recent studies showed an increasing trend of MRSA in the dairy industry [24]. A study described an increase in prevalence from 4.1% in 2009 to 9.7% in 2014 [23]. A meta-analysis estimating the global prevalence of MRSA isolated from bovine mastitis cases described an average prevalence of 4–30%, with the lowest prevalence described in Europe at 1–18% [37]. In Germany, a comparison of three cross-sectional studies shows that the prevalence of MRSA increased slightly from 2010 to 2014 but decreased again slightly until 2019. Larger conventional farms are more frequently affected than smaller and organic farms [38].

The infections caused by *Mycoplasmopsis* spp., especially *M. bovis*, are characterized by a high infectious potential. The transmission of the pathogen occurs during the milking process. Herd size is estimated to be a significant risk factor [39,40]. It causes many other diseases in cows besides mastitis. Chronically infected animals are the reservoir for the pathogen and the main risk factor for introducing it to a farm is infected purchased animals. In general, *Mycoplasopsis* spp. is a normal colonizer of the upper respiratory, genitourinary, and digestive tracts of cows. Occurrence in the udder (milk) indicates problems. *M. bovis* infections result in subclinical, mild clinical, or severe mastitis [41]. Several quarters are usually affected. In milder forms, cows do not show any general signs of distress. In clinical cases, the affected quarters show atrophy and agalactia [40]. *M. bovis* is characterized by natural resistance to antibiotics that interfere with cell wall synthesis. The pathogen has been described as insensitive to therapeutic intervention. However, the disease is also often self-limiting and disappears some time after the outbreak, sometimes without targeted intervention. In most cases, however, a rapid culling of the cows is advisable to prevent new infections in other cows [40,42]. There are recommendations to cull all cows that test positive for mycoplasma, or to cull only cows with CM that test positive for *Mycoplasmopsis* spp., as it is believed that intramammary mycoplasma infections are self-limiting [6]. The herd prevalence in Germany, in the Weser-Ems region, was 1.46% in 2012 [43]. In other countries, prevalences of 1.5% (Belgium) and 3.4% (Canada) have been described [44,45]. An Israeli longitudinal study reported values of 0–0.68% between 2004 and 2007, 3.77% in 2008, and 0.77–2.77% between 2009 and 2014 [46].

The aim of this study is to obtain information on the current herd prevalence in the collection area of the Central Weser Milk Control Association (CWMCA), Germany, and to gain knowledge on the importance of the pathogens addressed to obtain an up-to-date, sample-based overview of the possible occurrence of the pathogens. To detect increasing trends at an early stage, it is important to closely monitor the "rare" but contagious, zoonotic, and difficult-to-treat pathogens. This enables rapid intervention when numbers

increase, preventing serious economic and animal health problems. The benefits of testing BTM samples can be discussed in this context.

## 2. Materials and Methods

All applicable guidelines for the care and use of animals were followed. The study was approved by the Animal Welfare Committee of the university (University of Veterinary Medicine Hannover, Foundation, Hannover, Germany; file reference: TVO-2022-V-56). The date when ethical approval was obtained was 29 August 2022. An application for a license for animal testing was not required by the local government due to the study design. The study complied with the International Guiding Principles for Biomedical Research Involving Animals (1985).

### *2.1. Milk Samplings—BTM*

For this study, BTM samples were acquired from the CWMCA in Germany. Only farms with a single milk collection volume of more than 4000 L were included in this study. This represents farms with a size of approx. 80 cows or more in lactation, if calculated with a two-day collection and an average production per cow per day of 25 L of milk. The total number of samples was 226 from 208 different farms. Each farm was sampled on Friday, 28 January and Saturday, 29 January 2023. The unpreserved milk samples were then sent refrigerated to the microbiology laboratory of the University of Applied Sciences and Arts (Hannover, Germany) for testing on 30 January 2023.

BTM samples were analyzed by real-time polymerase chain reaction (rtPCR) for *Mycoplasmopsis* spp., *M. bovis*, and *Sc. agalactiae*. The determination of *S. aureus* and *Prototheca* spp. was detected by culturing on selective media. The further differentiation of grown colonies was performed by MALDI TOF analysis. In addition, *S. aureus* isolates were tested for oxacillin resistance.

### *2.2. Data Sampling*

To identify possible risk factors or consequences, further parameters were recorded. The following data were collected: the total number of cows on the farm, cows in lactation at the time of sampling, average milk production in kg per cow per day, milking technique (robot or conventional), cell count from January to May 2023 as a geometric mean, and average SCC at the time of sampling in the milk performance test. To ensure the anonymity of the data, it was only possible to generate data from the farms that were available to the CWMCA. Data were available for the following number of positive farms: for *Sc. agalactiae* in 1 of 2 farms, *Prototheca* spp. in 9 of 13 farms, *S. aureus* in 28 of 38 farms, oxacillin-resistant *S. aureus* in 5 of 8 farms, *Mycoplasmopsis* spp. in 13 of 18 farms, and *M. bovis* in 3 of 3 farms. The mean values obtained were compared with the available data from the annual reports of the CMWCA and the State Control Association of Lower Saxony (SCALS) [47].

### *2.3. DNA Extraction and PCR Analysis*

For chromosomal DNA extraction from milk, the DNeasy Blood and Tissue Kit from Qiagen GmbH, Hilden, Germany, was used and PCR was performed according to previously published methods [48–50]. The primers used and the corresponding nucleotide sequences are shown in Table 1 and the primer conditions during PCR are shown in Table 2.

**Table 1.** Nucleotide sequences of the PCR primers used for detection of *Sc. agalactiae* and *Mycoplasmopsis* spp./*bovis* by in vitro amplification.

| Primer | Primer Sequence (5′-3′) | Specificity |
|---|---|---|
| Sag432 | CGT TGG TAG GAG TGG AAA AT | *Sc. Agalactiae* |
| Sag1018 | CTG CTC CGA AGA GAA AGC CT | *Sc. Agalactiae* |

**Table 1.** *Cont.*

| Primer | Primer Sequence (5′-3′) | Specificity |
|---|---|---|
| MGSO | TGC ACC ATC TGT CAC TCT GTT AAC CTC | *Mycoplasmopsis* spp. |
| GPO1 | ACT CCT ACG GAG GCA GCA GTA | *Mycoplasmopsis* spp. |
| MboF | CCT TTT AGA TTG GGA TAG CGG ATG | *M. bovis* |
| MboR | CCG TCA AGG TAG CAT TTC CTA T- | *M. bovis* |

**Table 2.** Primer conditions during PCR [48–50].

| Forward Primer | Reverse Primer | Annealing Temp. (°C) | Size of Product Amplified |
|---|---|---|---|
| Sag432 | Sag1018 | 65 | 586 |
| GPO1 | MGSO | 60 | 724 |
| MboF | MboR | 60 | 360 |

### *2.4. Determination of Presumptive S. aureus in BTM*

For enumeration to estimate the number of *S. aureus* per mL of tank milk as an expression of the biosecurity of *S. aureus* on the farm, Baird Parker agar supplemented with egg yolk tellurite emulsion was used and evaluated accordingly [51,52]. Presumptive *S. aureus* colonies were examined by MALDI-TOF. Plates with more than one presumptive colony detected in 0.1 mL (≈ >10 cfu/mL) were considered positive. This method can be used to identify farms with an increased risk of spreading *S. aureus*. A value greater than 10 cfu/mL indicates that there are too many infected animals or animals with too much pathogen shedding on the farm [7,53].

All confirmed *S. aureus* isolates underwent an oxacillin resistance screening in accordance with CLSI 2013 VET01-A4. Therefore, the isolates were streaked on Mueller-Hinton agar with 4% NaCl (0.68 mol/L) and 6µg oxacillin/mL and examined for growth.

### *2.5. Determination of Prototheca spp. in BTM*

*Prototheca* spp. were enumerated by inoculating 100 µL and a tenfold dilution of the tank milk sample on Yeast Extract Glucose Chloramphenicol (YGC) agar followed by incubation at 25 °C for 72 h. To distinguish grown *Prototheca* spp. colonies from yeast colonies, Gram staining and microscopy were performed [51].

## 3. Results

In 2023, 546 farms with an average herd size of 143.6 cows were represented in the CWMCA. The average milk on CWMCA farms in 2023 was 10,701 kg per cow per year. In the entire SCALS, the number of farms was 5810 with an average cow number of 122.8 and an average milk yield per cow per year of 10,034 kg in 2023. In the SCALS, the average SCC from October 2022 to September 2023 was 234 × $10^3$ cells/mL. In January 2023, the average SCC was 229 × $10^3$ cells/mL. In 2022, 21.9% of all farms in the SCALS had an automatic milking system. In the CWMCA, the proportion was 29%. The participating farms had more than approximately 80 cows in lactation. A total of 226 samples were analyzed from these 208 herds.

Table 3 shows the herd prevalences and associated 95% confidence intervals for the different pathogens.

*Sc. agalactiae* was detected by PCR in the BTM from two herds (1% (Confidence Interval 95% (CI) 0.3–3.4)).

The PCR for *Mycoplasmopsis* spp. was positive in 19 samples from 18 different herds (8.7% (CI 5.5–13.3)). Of these, *M. bovis* was present in three herds (1.4% (CI 0.5–4.2)). Therefore, *M. bovis* accounted for 16.7% of the total *Mycoplasmopsis* spp. detected in BTM.

**Table 3.** Herd prevalence for the different pathogens.

| Pathogen | Positive Herds | Prevalence | 95% Confidence Interval |
|---|---|---|---|
| *Sc. agalactiae* | 2 | 1% | 0.3–3.4% |
| *Mycoplasmopsis* spp. | 18 | 8.7% | 5.5–13.3% |
| *M. bovis* | 3 | 1.4% | 0.5–4.2% |
| *S. aureus* | 38 | 18.3% | 13.6–24.1% |
| oxacillin-resistant *S. aureus* | 8 | 3.8% | 2–7.4% |
| *Prototheca* spp. | 13 | 6.3% | 3.7–10.4% |

Presumptive *S. aureus* colonies with a threshold of >10 cfu/mL milk were found in 181 of 226 (80.1% (CI 74.4–84.8)) samples cultured on Baird-Parker agar. Of these, *S. aureus* was confirmed by MALDI-TOF in the BTM of 38 herds (18.3% (CI 13.6–24.1)). The other presumptive colonies were mostly non-*aureus* staphylococci (N*a*S). A total of 154 isolates identified as *S. aureus* by MALDI-TOF were transferred to agar with added oxacillin for resistance testing, of which 19 isolates (12.3% (CI 8–18.5)) showed growth. The 19 isolates came from eight different farms and resulted in a herd prevalence of *S. aureus* with an oxacillin resistance of 3.8% (CI 2–7.4).

*Prototheca* spp. were detected on 13 farms (6.3% (CI 3.7–10.4)) by culture on YGC agar and a microscopic identification of BTM.

As shown in Table 4 and Figure 1, the farms that were positive for *Prototheca* spp. (386), *Mycoplasmopsis* spp. (225), and *M. bovis* (290) had on average more animals than the average farm in the SCALS and CWMCA (123/144). The difference for the *Prototheca* spp. positive farms was as much as 242 more cows than on the average farm in the CMWCA.

**Table 4.** Mean values of farms with positive pathogen detection in bulk tank milk (BTM) compared to the average of all farms belonging to the State Control Association of Lower Saxony (SCALS) and the Central Weser Milk Control Association (CWMCA).

| | Number of Cows (SD/CI95) | Average Milk Yield in kg per Cow per Day (SD/CI95) | Farms with an Automatic Milking System in Percent | Average SCC * × 1000 (SD/CI95) |
|---|---|---|---|---|
| *Sc. agalactiae* | 177 | 29.7 | 100 | 188 |
| *S. aureus* | 172 (±94/137–207) | 31.7 (±3.8/30.3–33.1) | 32.1 | 259 (±126/212–306) |
| Oxacillin-resistant *S. aureus* | 150 (±75/84–216) | 30.9 (±4.7/26.8–35) | 0 | 236 (±118/132–340) |
| *Mycoplasmopsis* spp. | 225 (±118/161–289) | 33.6 (±3.8/31.5–35.7) | 16.7 | 290 (±140/214–366) |
| *M. bovis* | 290 (±197/68–512) | 33 (±1.9/31.8–34.2) | 0 | 207 (105/138–276) |
| *Prototheca* spp. | 386 (±227/237–535) | 32.4 (±5/29.1–35.7) | 11.1 | 273 (±123/246–300) |
| Average of the farms in the CWMCA (2023) | 144 | 32.7 | 29 | 245 |
| Average of the farms in the SCALS (2022/2023) | 123 | | 21.9 | 234 |

* SCC = somatic cell count; * SD = standard deviation; * CI95 = 95% Confidence Interval.

The average milk yield per animal per day was highest on *Mycoplasmopsis* spp. positive farms with 33.6 kg and lowest on *Sc. agalactiae* positive farms with 29.7 kg and oxacillin-resistant *S. aureus* positive farms with 30.9 kg. The corresponding comparison of average milk yields is shown in Figure 2.

In the SCALS, 21.9% of all farms had an automatic milking system in 2022. This value was lower for most farms that were positive for a pathogen in the bulk milk (Figure 3). In the case of oxacillin-resistant *S. aureus* and *M. bovis*, the cows on all farms that were positive for a pathogen in bulk milk were even milked conventionally. Only in the case of *S. aureus* (32.1%) and *Sc. agalactiae* (100%) was the proportion of farms with automatic milking systems higher than the average of all farms (Figure 3).

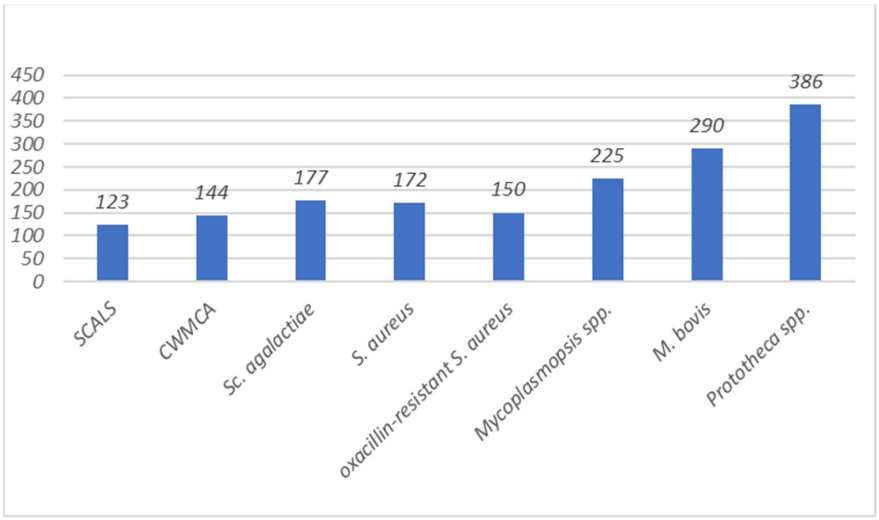


**Figure 1.** Average number of cows in farms positive for each pathogen compared to average number of cows in farms of the State Control Association of Lower Saxony (SCALS) and Central Weser Milk Control Association (CWMCA).

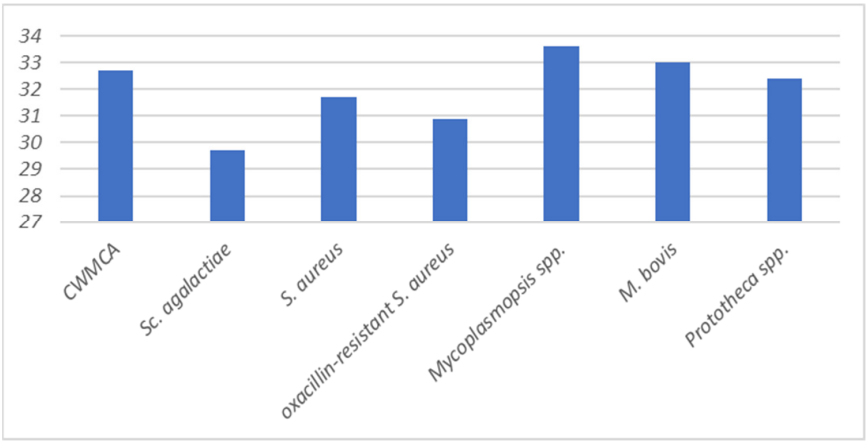


**Figure 2.** Average milk yield per cow per day in farms positive for each pathogen compared to the average in the Central Weser Milk Control Association (CWMCA).

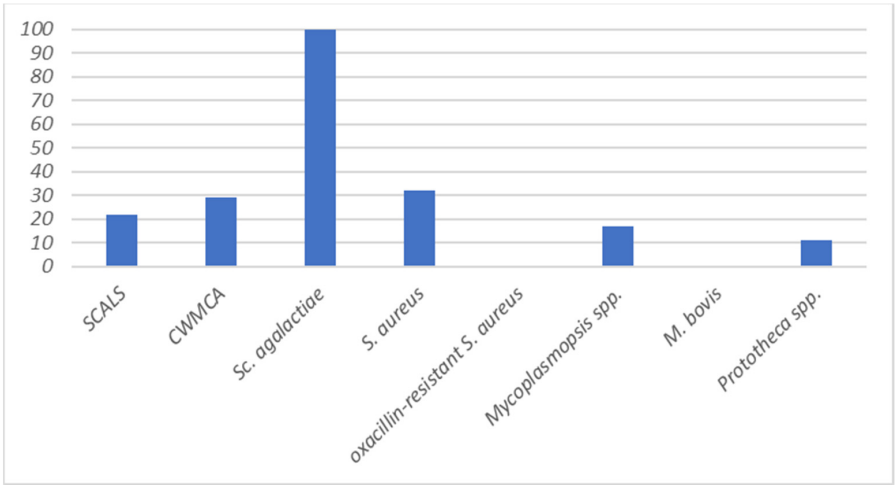


**Figure 3.** Percentage of positive farms with an automatic milking system (AMS) compared to the percentage of farms with AMS in the State Control Associations of Lower Saxony (SCALS) and Central Weser Milk Control Association (CWMCA).

Regarding the SCC at the time of sampling, especially farms with a positive finding for bulk milk for *Mycoplasmopsis* spp. (290 × $10^3$ cells/mL), *Prototheca* spp. (273 × 100 cells/mL) and *S. aureus* (259 × $10^3$ cells/mL) had a higher SCC than the average of all farms belonging to the SCALS and CWMCA (234 × $10^3$ cells/mL/245 × $10^3$ cells/mL). A comparison of the average SCC is shown in Figure 4.

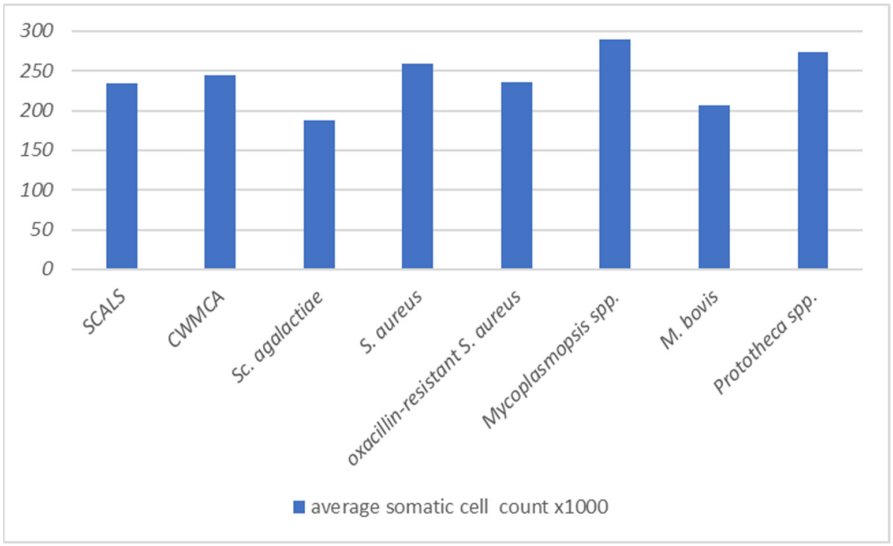


**Figure 4.** Average somatic cell count (SCC) in the bulk tank milk in farms positive for each pathogen compared to the average SCC in farms of the State Control Association of Lower Saxony (SCALS) and Central Weser Milk Control Association (CWMCA).

## 4. Discussion

The quality of bulk milk testing depends on several factors. However, the microbiological testing of BTM is a key benefit for general monitoring to assess the mastitis situation in sanitation programs, for initial or overview testing on farms that do not have up-to-date data, and for periodic testing to assess treatment success on larger farms. A qualitative or semi-quantitative test is therefore most useful for pathogens that should not be detectable on the farm, or at least not in the milk. Examples of such pathogens are *Sc. agalactiae*, *Mycoplasmopsis* spp., and *Prototheca* spp. [5,13]. In the case of *S. aureus*, a quantitative test of the BTM is useful as it gives an indication of the current level of hygiene measures, i.e., the current risk of spreading the pathogen. If more than 10 cfu/mL of *S. aureus* is detected in the BTM, the farm is considered to be at risk of having too many cows infected with *S. aureus* or cows with very high pathogen shedding [7]. It should be noted that there is a greater need for caution when interpreting quantitative results than qualitative or semi-quantitative studies. The contamination of bulk milk must be considered, especially by environmental pathogens and skin colonizers (e.g., N*a*S). [5,51]. It is important to note that not all cows are milked into the tank. BTM contains the milk of lactating cows whose milk is suitable for human consumption. Cows with CM are usually milked separately. Therefore, BTM testing can only be used as a tool to screen for specific pathogens, in order to identify unrecognized carriers or a generally high pathogen potential in the herd, or to detect newly emerging pathogens. In particular, control tests of BTM for contagious pathogens such as *Sc. agalactiae*, *S. aureus*, and *Mycoplasma* spp. are considered useful in long-term control and eradication strategies [51]. Several studies support this viewpoint and conclude that a regular testing of BTM is crucial [44,54,55]. In conclusion, a positive test result provides useful information for the farm, while a negative result does not necessarily mean that the pathogen is not present or does not pose a problem or risk to the herd.

The herd prevalence of *Sc. agalactiae* in BTM was 1%, which is well below the values found in Scandinavian studies [12–14]. Herd prevalence appears to be at a similar level with prevalence in positive quarter milk samples from a previous German study [15]. In a

comparable study from Germany, where 51 herds were tested for *Sc. agalactiae*, only one herd tested positive in BTM and quarter milk samples [54]. A study from Switzerland reported a prevalence of *Sc. agalactiae* of 0.2%/0.5% at quarter/cow level and 2.1% at farm level [56], which is close to the level found in our study. In other European countries, higher prevalences of *Sc. agalactiae* were found in some cases (5.8% in Slovakia and 15.6% in Poland), but these studies examined quarter milk samples from cows with present mastitis [57,58].

However, it is important to note that the farm structure in the sampled area differed significantly from the farm structure found in large parts of Scandinavian farms. Since *Sc. agalactiae* is most problematic on farms with very large numbers of animals [12,16,17], and there were no farms with more than 1000 cows on the sampled farms, this must be considered. In addition, selective drying off and greater restrictions on the use of antibiotics were introduced early on in Scandinavian countries, so it may not have been possible to completely eradicate the pathogen from affected cows. *Sc. agalactiae* is more prevalent on farms with automatic milking systems. Also, in the present study, the only positive finding for which data were available was a farm with an automatic milking system. However, it should be kept in mind that this was an isolated case, so an incidental finding is possible. To identify possible reservoir farms for *Sc. agalactiae*, further work should focus on the investigation of larger farms with high cow numbers. A major risk factor for bringing *Sc. agalactiae* onto the farm is the purchase of infected animals. It is therefore advisable to regularly check the status of *Sc. agalactiae* via BTM, especially if there are large or regular changes in the herd, such as the purchase of new cows [7].

Testing for *Mycoplasmopsis* spp. in BTM showed a higher prevalence than previous studies [43–45], whereas the detected prevalence of *M. bovis* of 1.4% is consistent with the detected prevalence of 1.5% in Belgium [45], but higher than in a comparable study from France, where none of the 345 BTM samples were positive, giving a prevalence of <1% [59]. In a Portuguese study investigating BTM, a prevalence of 3% was described for *Mycoplasmopsis* spp. and 2.4% for *M. bovis* [60]. Thus, in this study, the proportion of *M. bovis* in the total of positive *Mycoplasmopsis* spp. samples is higher than in our study. Herd size has been described as a critical factor [39,40,60]. This can be confirmed by the present study, as it also shows that farms positive for *Mycoplasmopsis* spp. and especially *M. bovis* were above average in terms of herd size compared to the average in the SCALS and CWMCA.

It should be noted that *Mycoplasmopsis* spp. are normal colonizers of various organ systems in cattle. Therefore, the pathogen could also have entered the BTM through contamination. However, if the milking process is clean, the pathogen should not be found in the milk, as this would indicate that the pathogen is transmitted during milking and therefore represents a major health risk. As *Mycoplasmopsis* spp. are pathogens that spread quickly but are also self-limiting, it would be interesting in a further study to confirm whether the same farms are still positive after a few months or whether new farms have *Mycoplasmopsis* spp. in the bulk milk.

As *M. bovis* was found in only three of the 18 farms positive for *Mycoplasmopsis* spp., it must be noted that other species of the genus *Mycoplasmopsis* spp. were probably involved. In Europe, *M. bovis* is described as the predominant species causing mastitis, while other mycoplasmas are rare, but this may be due to a lack of testing for other species. Species such as *M. bovigenitalium*, *M. alkalescens*, *M. canadense*, and *M. californicum* have also been isolated from milk and considered possible mastitis pathogens, but it is not easy to link their presence to disease [40,46,61]. It is also noticeable that the average SCC of farms positive for *Mycoplasmopsis* spp. was significantly higher than the average of all farms belonging to the SCALS. At the same time, however, the average milk yield of the farm per animal per cow did not seem to be affected. This indicates subclinical problems.

When testing for *S. aureus* on Baird-Parker agar, presumptive *S. aureus* colonies were found in many BTM samples (80.1%). Nevertheless, MALDI-TOF revealed that *S. aureus* was present in only 18.3% of cases. Most of the remaining cases were N*a*S. These must also

be considered as potential causative agents of SCM but may also represent contaminants in the BTM. An Italian study of 844 BTM samples found a prevalence of *S. aureus* of 47.2%, but only the results of the modified Baird-Parker agar are shown and no confirmation by MALDI-TOF was performed [62]. However, the fact that 18.3% of farms had *S. aureus* levels > 10 cfu/mL milk in the BTM still shows that the pathogen was clearly present in the sampled area and can lead to potential new *S. aureus* infections. The farms in which the threshold value is exceeded currently appear to have an increased risk for the spread of *S. aureus*. This study confirms that it is almost impossible to eliminate *S. aureus* from the herd. Therefore, the prevention of new infections is the most important means to limit the spread.

The prevalence of oxacillin-resistant *S. aureus* of 3.8% shows that resistance to ß-lactam antibiotics may be a problem in the sampled area. This prevalence is similar to prevalences from previous studies on MRSA. For example, in the Italian study of 844 BTM samples, the prevalence of MRSA was also 3.8% [23,24,37,38,62]. A study in England and Wales reported that methicillin-resistant staphylococci were present in 5% of flocks. However, the proportion of methicillin-resistant *S. aureus* was only 0.83%. The remaining staphylococci were resistant to N*a*S. Thus, the prevalence of resistant *S. aureus* in the United Kingdom appears to be lower than in the German region studied [63].

It will be important to monitor the development of prevalence in the future, as there is an ongoing discussion about limiting the use of antibiotics in veterinary medicine, and the use of critical important substances. An increase in oxacillin-resistant *S. aureus* on dairy farms may intensify this debate. Whether there is a zoonotic potential needs to be determined in future studies. Nonetheless, the risk of the transmission of MRSA to humans via milk is considered to be low [36]. However, the detection of resistant *S. aureus* strains in raw milk confirms the recommendation that it should not be consumed without prior pasteurization [38]. Yet, it is very important to monitor the development and presence of resistant *S. aureus* strains, as the increasing threat of resistance leads to problems in the treatment of animals. Moreover, a significant rise in occurrence brings about not only discussions on animal welfare problems, but also political restrictions on the use of antibiotics.

The available herd milk samples show a clear prevalence (6.3%) of *Prototheca* spp. in the sampled region. It seems that the pathogen is much more widespread than assumed and is therefore a possible risk factor for causing mastitis. However, unlike in Poland [25], Prototheca spp. cannot be considered the third most important mastitis pathogen in the region studied. The prevalence is comparable to the prevalence (6%) found in a Canadian study [33].

Since *Prototheca* spp. infections mean that several animals are usually affected at the same time and chronic processes usually develop [27,28], this has an impact on the SCC in the bulk milk of the herd. In the present study, the average SCC was also higher than the average SCC in the SCALS. The correlation between the presence of *Prototheca* spp. and increased SCC has also been described in previous studies [25,64]. Furthermore, it is noticeable that the number of cows on farms with *Prototheca* spp. in the bulk milk was significantly higher than the average. Therefore, the number of cows on the farm should be considered as an important risk factor for the presence of *Prototheca* spp. In contrast, an automatic milking system does not seem to be a risk factor for the presence of the pathogen. Since *Prototheca* spp. occur in the environment, contamination must be considered, although the contamination of BTM is only possible in the case of serious problems in husbandry and animal hygiene. In the case of a positive result, it must therefore be assumed that infected animals are present on the farm or that there is a high risk of infection so that measures must be taken in any case [5].

## 5. Conclusions

This study confirms the notion that testing BTM for pathogens that a herd should be completely free of, or for which a certain threshold should not be exceeded in tank milk, is

most helpful in assessing the current risk to the farm. However, a negative result in the tank milk does not necessarily indicate that the farm is free of the pathogen in question. It is important to note that not all animals are milked into the bulk milk, and affected animals may shed the pathogen intermittently, so the test may be falsely negative. It is advisable to monitor the BTM several times for a safer control.

Furthermore, this study highlights the significance of monitoring the pathogens under investigation, as they represent a potential threat to the dairy industry. If these pathogens are detected in the BTM, the farm must take action to reduce the pathogen density or eliminate the pathogen. Risk factors such as herd size and the presence of an automatic milking system must be considered. An elevated SCC in the BTM may indicate that the cows being milked into the bulk tank have SCM or represent a reservoir of pathogens that could endanger other cows. It is therefore crucial to educate farmers about the pathogens described so that appropriate measures can be taken in case of an outbreak.

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