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Mastitis challenges in Serbian dairy farming: a study on somatic cell counts and pathogen distribution

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Abstract

Mastitis is pressing concern for dairy herds due to its economic impact and potential health risks. Somatic cell counts (SCC), reflecting udder health, plays a crucial role in mastitis diagnosis. Current research explores the distribution of SCC and its correlation with various mastitis-causing pathogens in dairy farms in Serbia. The study analyzed 194 individual cow milk samples and microbiological testing was conducted under aseptic conditions to isolate and identify mastitis pathogens. The microscopic reference method was employed for assessing SCC in the milk samples. Among mastitis-associated isolates, bacteria were present in 28.87 %, yeast in 12.38 %, while in 5.15 % of milk samples, both bacteria and yeast were present. The relationship between SCC in various sample types (negative, bacteria-positive, yeast-positive, and samples with both bacteria and yeast) was noted. Importantly, samples with both bacteria and yeast presence had the highest SCC. While SCC is a valuable tool for monitoring udder health and the effectiveness of mastitis control programs, its response to specific pathogens is complex and doesn't allow differentiation between pathogen types easily. This research highlights the challenges in distinguishing pathogen types based solely on SCC.

Keywords: bacteria; cows; mastitis; somatic cell count; yeast

Introduction

Mastitis significantly affects dairy herds, leading to economic losses due to high treatment costs, reduced milk production and quality, increased labor and premature culling (Magaš et al., 2013; Benić et al., 2018; Poljak et al., 2022). It also raises concerns about antimicrobial use, leading to resistant strains and antimicrobial residues in dairy products, posing public health risk (Turk et al., 2017). Therefore, monitoring mastitis in dairy cows is crucial for maintaining herd health, milk quality and public health (Maletić et al., 2017; Knežević et al., 2021).

Somatic cell count (SCC) reflects the udder health and is a quantitative method for diagnosing mastitis by enumerating different cell types in milk (leucocytes, including neutrophils, macrophages and lymphocytes) (Darbaz et al., 2023). SCC exceeding 200,000 cells/mL is typically associated with bacterial infection (Sharma et al., 2011). Both, clinical and subclinical mastitis can disrupt the SCC pattern (De Haas et al., 2004). In general, the major mastitis pathogens (*Streptococcus uberis*, *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus dysgalactiae* and *Streptococcus agalactiae*) elicit greater somatic cell response (Bradley and Green, 2005), than the minor pathogens (*Corynebacterium* species and coagulase-negative staphylococci) (Sharma et al., 2011). Interestingly, infections involving the major pathogens are more likely to result in SCC levels over 200,000 cells/mL, whereas minor pathogens typically maintain SCC levels in the range from 50,000 to 150,000 cells/mL (Bradley and Green, 2005). Therefore, it's essential to keep these factors in mind when using SCCs as marker for making decisions regarding health status in dairy herds (Haxhiaj et al., 2022). Significantly, SCC has been linked to intramammary infection resolution, indicating its potential role in antibiotic treatment decisions (Williamson et al., 2022; De Jong et al., 2023).

Hence, this study was conducted to observe the distribution of SCCs and potential correlation to the different mastitis associated pathogens in the selected dairy farms in Serbia. Additionally, the prevalence of pathogens in the milk samples obtained from cows affected by mastitis was assessed.

Material and methods

Isolation and identification of mastitis associated pathogens

The Animal Ethics Committee of the Ministry of Agriculture, Forestry, and Water Management-Veterinary Directorate granted the approval for the experimental protocol (Approval No. 9000-689/2, dated 06 July 2020). This study was conducted from June to December 2021 on two dairy farms located in the Vojvodina Province, Serbia. A total of 194 individual cow milk samples were collected from cows with clinical and subclinical mastitis. Farm veterinarians examined the udders and milk to check for clinical mastitis, while the California Mastitis Test was used to confirm subclinical mastitis by analyzing the milk's SCC. Clinical signs of udder inflammation included swelling, pain and redness, while changes of interest in the first jets of milk were clots, color change and density. The milk samples for microbiological testing were obtained under aseptic conditions. After teat cleaning, drying and disinfection, the first jets of milk were discarded, and a 10 mL sample was collected in sterile tubes. These samples were stored in an ice container at 4 °C during transport to the Laboratory for Milk Hygiene at the Department of Veterinary Medicine, Faculty of Agriculture, University of Novi Sad. The samples were inoculated on the 2 % blood agar, and standard bacteriological diagnostic techniques, as previously described by Kovačević et al. (2021a), were employed for the isolation, identification and determination of mastitis pathogens. Yeast strains were cultured on Sabouraud dextrose agar plates, which were then incubated at 30 °C for 48 hours. Isolates were subsequently identified, using the "API 20 C AUX" (bio Meraux, France).

Determination of the somatic cell count

The SCC in the milk samples was assessed following the microscopic reference method as per the Institute for Standardization of Serbia (SRPS EN ISO 13366-1:2010) (ISO, 2010). To determine the SCC, 0.01 mL of mixed milk from each sample was spread across a 1 cm^2 area on a glass slide. These slides were then air-dried, stained with the Newman-Lampert stain, and examined under a microscope. The SCC result below 200,000 cells/mL was considered low, indicating a healthy mammary gland (Piccinini et al., 2005). In contrast, SCC exceeding 200,000 cells/mL was classified as high, signifying the presence of an intramammary infection.

Statistical analysis

The obtained data were summarized by application of Microsoft Office Excel and statistically processed by Tibco Statistica (v. 13.5). Data were analysed by descriptive statistical methods, while differences between evaluated groups in terms of number of somatic cells were assessed by application of ANOVA.

Results and discussion

Prevalence of mastitis-associated pathogens

Based on laboratory results, 104 (53.60 %) milk samples were negative for both yeast and bacteria (group 1), while the rest of samples (46.40 %) were positive on pathogens presence. Of those, 56 (28.87 %) were positive for bacteria (group 2), 24 (12.38 %) for yeast (group 3), and 10 (5.15 %) were positive for yeast and bacteria (group 4).

Table 1. *Prevalence of mastitis-associated pathogens in the milk samples*

Milk samples	Group 1 negative	Group 2 positive for bacteria	Group 3 positive for yeast	Group 4 positive for bacteria and yeast
No.	104	56	24	10
%	53.60	28.87	12.38	5.15

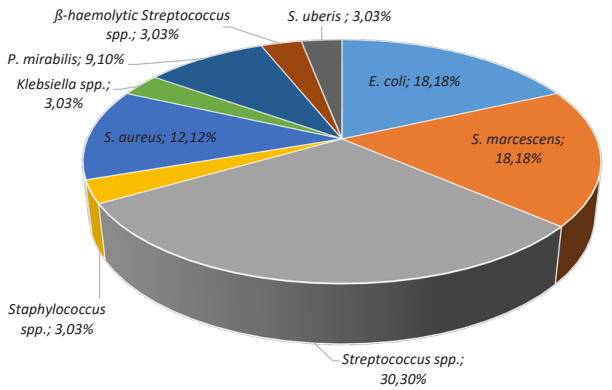

Figure 1. *Frequency of the mastitis-associated bacteria isolates in the tested samples*

Of the isolated bacteriological causes of mastitis, *Streptococcus* spp. was isolated in 20 cases (30.30 %), *E. coli* and *S. marcescens* were present in 12 samples (18.18 %) each, followed by 8 cases (12.12 %) of *S. aureus*, while *Proteus mirabilis* was isolated in 6 cases (9.10 %). *Klebsiella* spp., *Staphylococcus* spp., *S. uberis* and ß-haemolytic *Streptococcus* spp. were isolated in 2 samples, each (3.03 %). With regard to yeast, we isolated *Candida* spp. in 28 samples (82.35 %) of total samples positive for yeast, and *C. albicans* in 6 cases (17.64 %).

Bovine mastitis involves range of microorganisms with changing prevalence linked to factors like sample timing and region-specific variations in infection patterns (Malinowski et al., 2006). Since this disease has gained great attention, mastitis causative agents are well described in the studies conducted in Serbia (Milanov et al., 2014; Kovačević et al., 2021a, 2021b; Kovačević et al., 2022) and worldwide (Janosi and Baltay, 2004; Malinowski et al., 2006; Tenhagen et al., 2006; Bi et al., 2016; Abed et al., 2021). While some researchers explored the relationship between the SCC and mastitis-associated pathogens (Moretti et al., 1998; De Haas et al., 2004; Huang and Kusaba, 2022), to our knowledge no similar studies have been performed yet in Serbia.

Our findings indicate that *Streptococcus* spp. were prevalent in 30.30 % of evaluated samples, which is consistent with study in Italy (33.84 %) (Ceniti et al., 2017). These bacteria are globally recognized as major mastitis pathogens (Kaczorek et al., 2017) with the prevalence of 50 % in Australia, followed by Europe (38 %) (Kabelitz et al., 2021). Smistad et al. (2023) suggests that strategies to control infectious pathogens, like the five-point and 10-point plans, reduced contagious mastitis but had limited impact on environmental pathogens like *Streptococcus* spp. and coliform bacteria. Our findings show a 12.12 % prevalence of *S. aureus*, despite being considered as one of the most common causes of mastitis (Liu et al., 2020). In addition, lower prevalence of *S. aureus* was also reported in Croatia, being isolated in 4.48 % of udder quarter samples (Cvetnić et al., 2021). According to McDougall et al. (2022) prevalence of *S. aureus* can vary with the age being more prevalent and of greater duration in older animals due to its contagious nature.

Coliform-associated mastitis (*E. coli* and *S. marcescens*) in our study had a relatively high prevalence (18.18 %) being comparable to study in Norway (14.50 %) (Smistad et al., 2023), but lower than 21.9 % prevalence in North West Cameroon, where it was significantly associated with factors such as lactation stage, cow breed, history of mastitis and contaminated environment (Abegewi et al., 2022). Furthermore, *E. coli* is recognized as the most common gram-negative coliform bacteria in intensive milk production systems on dairy farms (Morales-Ubaldo et al., 2023). Apart from *E. coli, S. uberis* and *K. oxytoca* were also found, supporting the increasing presence of environmental pathogens (Cervinkova et al., 2013).

The prevalence of *S. uberis* in our study (3.03 %) aligns with other reports ranging from 2 % to 9 % (Bi et al., 2016; Smistad et al., 2023). This pathogen's ability to produce biofilms, capsules, invade mammary gland cells and resist phagocytosis makes controlling *S. uberis* mastitis challenging, even in case of a low farm-level presence (Wente et al., 2019). Furthermore, *Klebsiella* spp., particularly *K. oxytoca*, plays a significant role in bovine mastitis (Song et al., 2023), primarily originating from environmental sources. Improved hygiene practices can reduce transmission (Song et al., 2023). In our study, *K. oxytoca* was found in 3.03 % of samples, but it's less significant in milk samples when compared to *S. aureus* and *E. coli*, as noted by other authors (Song et al., 2023).

Yeast, although infrequently implicated, have been linked to mastitis in dairy cattle. While data on yeast prevalence in Serbian dairy farms is limited, our study's results (12.38 %) are relatively comparable with those from (Milanov et al., 2014), who reported yeast isolation in 6.02 % of milk samples. Candida is a significant pathogen in mycotic mastitis among dairy cows, particularly non-albicans species, as supported by the literature (Zhou et al., 2013). Our study found all yeast to be *Candida* spp., including *C. albicans*. In contrast, Milanov et al. (2014) pointed out that among all the yeast species recovered from cow's milk, *C. albicans* rarely takes dominant role. However, it's important to note that extensive production systems, environmental temperatures and disease duration are significant risk factors contributing to the prevalence of mycotic mastitis (Zhou et al., 2013).

Somatic cell count in relation to type of pathogens

The application of ANOVA has demonstrated a negative statistically significant differences in SCC of milk samples

(Figure 2), while positive on bacteria, positive on yeast and positive on bacteria and yeast ($F(3, 98)=10.895$, $p=0.00$). Moreover, the recorded difference was a result of high SCC in milk samples being positive both for bacteria and yeast.

SCC levels and bacteriological examinations serve as different methods for evaluating the mammary gland's condition (Schwarz et al., 2010; Tommasoni et al., 2023). Besides, using the test-day SCC records helps identify deviations from the usual SCC pattern, indicating potential mastitis-causing pathogens (De Haas et al., 2004). Numerical increase in lactations associated to isolated pathogens, compared with unaffected cows have been reported worldwide (De Haas et al., 2004; Skrzypek et al., 2004; Malinowski et al., 2006; Lopes Júnior et al., 2012; Sumon et al., 2020). Yet, comparing milk SCC in relation to specific bacterial species in the literature is challenging due to variations in methodologies, including the analysis of different milk types by different authors. Typically, the major pathogens are associated to the most significant increase in SCC, while infection by minor pathogens leads to a notably lower SCC rise and rarely to the clinical manifestation of the mastitis (Supré et al., 2011). Our study results indicated that there was a statistically significant difference between the SCC observed among all tested samples (negative, positive for bacteria, positive for yeast and positive both for bacteria and yeast) (Figure 2). Notably, the highest SCC was recorded in milk samples that tested positive for both bacteria and yeast.

Milk samples with SCC levels below 200,000 cells/mL were mostly cultured negative (75 %). In contrast to these results, some of the mastitis cases without detectable bacterial growth exhibited an increase in SCC. The majority of samples showing no bacterial growth indicated SCC levels in the range from 200,000 to 500,000 cells/mL. Besides, samples where SCC levels exceeded 1,000,000 cells/mL were primarily associated with infections caused by *E. coli, S. marcescens* and *S. aureus*. Furthermore, notably elevated SCC levels were linked to infections attributed to both yeast and bacteria, whereas yeast-only infections resulted in a comparatively smaller increase in SCC, typically measuring less than 1 million cells per milliliter.

Passing over the limit of 200,000 cells/mL indicates the transition from health to disease (Skrzypek et al., 2004). Our

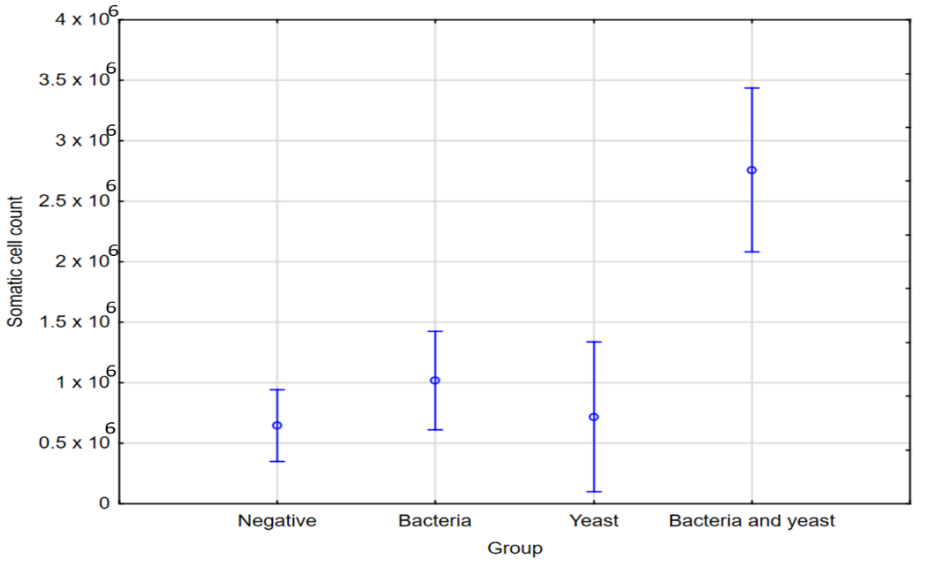

Figure 2. ANOVA - SCC in relation to type of pathogens

Table 2. Microorganisms isolated from milk samples in relation to SCC (x 10^3/mL)

Species	N	<200	200-500	500-1000	1000-5000	5000-10000
		%	%	%	%	%
Streptococcus spp.	18	0.00	9.10	8.56	13.04	0.00
E. coli	10	0.00	4.54	2.12	13.04	0.00
S. marcescens	10	0.00	4.54	2.12	13.04	0.00
S. aureus	6	0.00	0.00	0.00	13.04	0.00
Proteus mirabilis	6	0.00	0.00	2.12	8.70	0.00
Klebsiella spp.	2	0.00	0.00	2.12	0.00	0.00
Staphylococcus spp.	2	0.00	0.00	2.12	0.00	0.00
S. uberis	2	0.00	0.00	2.12	0.00	0.00
Yeast	24	25	9.10	14.90	8.70	0.00
Bacteria+yeast	10	0.00	0.00	2.12	13.04	100
Negative	104	75	72.72	61.70	17.40	0.00
Total	194	100	100	100	100	100

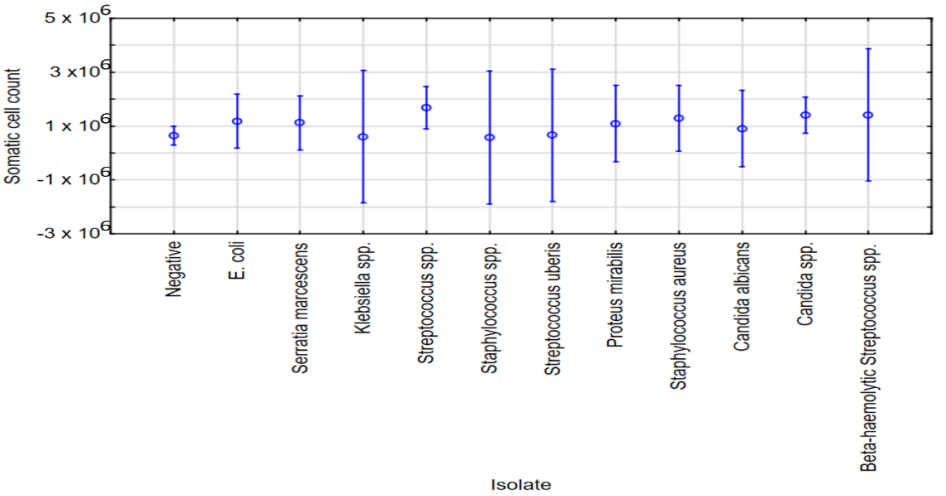

Figure 3. ANOVA - SCC in relation to specific isolated pathogens

study revealed that milk samples with SCC below 200,000 cells/mL were mainly culture negative, consistent with other research (Malinowski et al., 2006). We have also observed higher SCC in cases of mastitis with no bacterial growth, aligning with previous findings suggesting that high SCCs may not always indicate the presence of mastitis pathogens (Souza et al., 2016; Sumon et al., 2020). According to McDougall et al. (2001), the presence of high SCC in milk samples, even in the absence of microorganisms, does not necessarily indicate the udder's health. Nonetheless, even though we couldn't detect any pathogens in those quarters, they could still be infected. Elevated SCC in cases without detected bacteria could result from undetectable bacterial levels during sampling or effective immune elimination (Campos et al., 2022). Negative bacteriological findings may also be influenced by sporadic pathogen shedding, antimicrobials, growth-inhibiting substances, or intracellular survival (Schwarz et al., 2010; Kandeel et al., 2018). It must be pointed out, that SCC can also be affected by non-infectious factors, such as animal's age, stage of lactation, the time of year, milking frequency and nutrition (Bradley and Green, 2005). Our study confirmed mastitis pathogens presence in cows with lower SCC, emphasizing the need for bacteriological culture even when SCC suggests lower likelihood of subclinical mastitis (Katsande et al., 2013; Huang and Kusaba, 2022). It is important to note that SCC and bacteriology may not always produce matching results, as infected udders may not consistently release pathogens, leading to negative test outcomes (De Haas, 2005).

In the group of cows with SCC between 200,000 and 500,000 per milliliter, a substantial number did not test positive for any of pathogens. This pattern is consistent with other authors (Janosi and Baltay, 2004; Souza et al., 2016). Samples with SCC exceeding 1,000,000/mL were primarily associated with *E. coli, S. marcescens,* and *S. aureus* infections. Janosi and Baltay (2004) reported that nearly 50 % of cows infected by coliform bacteria had milk SCC exceeding 400,000/mL. In Serbian study, milk samples had SCC of over 2,000,000 cells per milliliter due to *S. aureus* infection (Radinović et al., 2008). This SCC elevation was also observed in other countries with *S. aureus* infections (Souza et al., 2016; Karzis et al., 2017) De Haas et al. (2004) found that different pathogens affect lactation SCC differently. For instance, *S. aureus* mastitis leads to a long-lasting SCC increase, while *E. coli* mastitis results in a short-term elevation. Infections early in lactation, particularly if caused by persistent pathogens such as *S. aureus*, have a significant impact on lactation SCC (De Haas et al., 2004).

Our study found that infections attributed to both bacteria and yeast were associated with significantly elevated SCC, which is consistent with other studies (Malinowski et al., 2006; Lopes Júnior et al., 2012; Sumon et al., 2017). This increased response may result of interaction between these pathogens as the immune system simultaneously combats both, compared to single-pathogen infections. SCC increases depend on bacteria pathogenicity and affected udder tissue (Pyörälä, 2003). Variation in susceptibility to different bacteria is linked to individual immune responses and distinct infection mechanisms (Campos et al., 2022). Based on findings by Safak et al. (2022), it was determined that maintaining strong cellular immunity can be beneficial in preventing *E. coli*-induced mastitis, while strong humoral immunity is advantageous in reducing the occurrence of *S. aureus* and *S. agalactiae*-induced mastitis. Additionally, SCC response to significant pathogens varies among individual cows, making it impractical to identify pathogen types based solely on SCC (Sharma et al., 2011). As Hariharan et al. (2004) stated, there is weak correlation between SCC and bacteriological results.

Somatic cell count in relation to specific isolated mastitis associated pathogens

The application of ANOVA did not show statistically significant differences in SCC in the milk samples when the specific isolated pathogens, as well as samples being negative were taken into account (Figure 3, $F(11, 90)=.87$, $p=0.57$).

Conclusion

In conclusion, mastitis is significant challenge in the dairy industry. Monitoring udder health using SCC is crucial and analyzing SCC records in the mastitis control programs is effective. However, distinguishing specific pathogens based on SCC is challenging. Current study offers insights into the complex management of the mastitis in the dairy herds.

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Izazovi povezani sa mastitisom u mliječnom govedarstvu u Srbiji: analiza broja somatskih stanica i distribucije patogena

Sažetak

Mastitis predstavlja veliki problem za mliječna stada zbog svog ekonomskog utjecaja i potencijalnih zdravstvenih rizika. Broj somatskih stanica, koji odražava zdravlje vimena, igra ključnu ulogu u dijagnozi mastitisa. Cilj ovog istraživanja je određivanje distribucije somatskih stanica i korelacije s različitim uzročnicima mastitisa na mliječnim farmama u Srbiji. Analizirana su 194 pojedinačna uzorka kravljeg mlijeka. Mikrobiološka ispitivanja provedena su u aseptičnim uvjetima kako bi se izolirali i identificirali uzročnici mastitisa. Za procjenu broja somatskih stanica u uzorcima mlijeka korištena je mikroskopska referentna metoda. Kao uzročnici mastitisa, bakterije su bile prisutne u 28,87 %, gljivice u 12,38 %, dok su u 5,15 % uzoraka mlijeka bile prisutne i bakterije i gljivice. Uočena je veza između broja somatskih stanica u različitim tipovima uzoraka (negativni uzorci, uzorci sa bakterijama, uzorci s gljivicama i uzorci s bakterijama i gljivicama). Naime, uzorci s bakterijama i gljivicama imali su najviši broj somatskih stanica. Iako je broj somatskih stanica vrijedan alat za praćenje zdravlja vimena i učinkovitosti programa kontrole mastitisa, njegov odgovor na specifične patogene je složen i ne dopušta lako razlikovanje između tipova patogena. Ovo istraživanje naglašava izazove u razlikovanju tipova patogena isključivo na temelju broja somatskih stanica.

Ključne riječi: bakterije; krave; mastitis; broj somatskih stanica; gljivice

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