https://doi.org/10.52973/rcfcv-e34466 Revista Científica, FCV-LUZ / Vol. XXXIV, rcfcv-e34466

Effect of Intramammary Hypochlorous Acid Administration on Subclinical Mastitis in Dairy Cows

Efecto de la Administración Intramamaria de Ácido Hipocloroso en la Mastitis Subclínica en Vacas Lecheras

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ABSTRACT

Subclinical mastitis is a significant issue in dairy farming due to its impact on milk production and quality, leading to economic losses despite the absence of visible symptoms. The present study aimed to investigate the potential use of intramammary hypochlorous acid (HOCl) treatment as a viable substitute to antibiotics for the treatment of subclinical mastitis (SCM) in dairy cows. A total of 232 Holstein–Friesian dairy cows with 928 udder quarters were subjected to the California Mastitis Test (CMT) to identify the SCM. The results indicated that 141 cows had SCM in at least one udder quarter, with a rate of 60.78% in the herd. Among these cows, 259 udder quarters showed varying degrees of CMT–positive. Before the treatment, somatic cell counts (SCC) were determined, and bacterial cultures were performed on randomly selected 74 CMT–positive udder quarters. The HOCl was administered intramammary to these quarters for 5 days immediately after milking. The CMT, SCC, and bacterial culture were repeated on the 3rd and 5th days of the treatment. An increase in SCC was observed on the 3rd and 5th day of the treatment compared to before treatment ($P<0.001$). The bacterial growth reduced from 64.86% before treatment to 49.95% on the 3rd day and 22.97% on the 5th day of the treatment. *Staphylococcus aureus* was the most prevalent bacterium before the treatment. On the 3rd day of the treatment, bacterial growth rate, particularly in *Candida* spp., decreased compared to before the treatment. However, on the 5th day of the treatment, *S. aureus* and the combination of *S. aureus* with *Candida* spp. continued to show high growth rates. In conclusion, this study underscores that HOCl is a potential alternative to antibiotics for treating SCM in dairy cows. Further research covering both clinical and subclinical mastitis is recommended, along with studies aiming to prolong the presence of HOCl in the udder, determine its ideal dose, and increase its impact on more cells.

Key words: Dairy cow; hypochlorous acid; subclinical mastitis; treatment

RESUMEN

La mastitis subclínica es un problema significativo en la producción lechera debido a su impacto en la producción y calidad de la leche, lo que lleva a pérdidas económicas a pesar de la ausencia de síntomas visibles. El presente estudio tuvo como objetivo investigar el uso potencial del tratamiento intramamario con ácido hipocloroso (HOCl) como un sustituto viable a los antibióticos para el tratamiento de la mastitis subclínica (MSC) en vacas lecheras. Un total de 232 vacas lecheras Holstein–Friesian con 928 cuartos mamarios fueron sometidas a la prueba de mastitis de California (CMT) para identificar la MSC. Los resultados indicaron que 141 vacas tenían MSC en al menos un cuarto de la ubre, con una tasa del 60.78% en el rebaño. Entre estas vacas, 259 cuartos mamarios mostraron diversos grados de CMT positivo. Antes del tratamiento, se determinaron los conteos de células somáticas (SCC) y se realizaron cultivos bacterianos en 74 cuartos mamarios CMT positivos seleccionados al azar. El HOCl se administró por vía intramamaria a estos cuartos durante 5 días inmediatamente después del ordeño. El CMT, el SCC y el cultivo bacteriano se repitieron en el tercer y quinto día del tratamiento. Se observó un aumento en el SCC en el tercer y quinto día del tratamiento en comparación con antes del tratamiento ($P<0.001$). El crecimiento bacteriano se redujo del 64.86% antes del tratamiento al 49,95% en el tercer día y al 22,97% en el quinto día del tratamiento. *Staphylococcus aureus* fue la bacteria más prevalente antes del tratamiento. En el tercer día del tratamiento, la tasa de crecimiento bacteriano, particularmente en *Candida* spp., disminuyó en comparación con antes del tratamiento. Sin embargo, en el quinto día del tratamiento, *S. aureus* y la combinación de *S. aureus* con *Candida* spp. continuaron mostrando altas tasas de crecimiento. En conclusión, este estudio subraya que el HOCl es una alternativa potencial a los antibióticos para tratar la MSC en vacas lecheras. Se recomienda realizar más investigaciones que abarquen tanto la mastitis clínica como la subclínica, junto con estudios que apunten a prolongar la presencia de HOCl en la ubre, determinar su dosis ideal y aumentar su impacto en más células.

Palabras clave: Ácido hipocloroso; mastitis subclínica; tratamiento; vaca lechera

Received: 05/06/2024 Accepted: 22/07/2024 Published: 24/09/2024

INTRODUCTION

Mastitis is an inflammatory condition of mammary tissue and the surrounding connective structures. It is one of the most important economic problems in dairy cattle farming [1]. Due to its highly complex aetiology, it is impossible to eradicate under current conditions. Mastitis leads to economic losses, including reduced milk yield, milk loss, decreased milk quality, treatment costs, decreased value of the cow, culling of the mastitic cows, and costs of protection and control measures [2]. Clinical mastitis (CM) is typically identifiable through conspicuous symptoms like udder swelling and milk clot formation, but subclinical mastitis (SCM) manifests without visible changes in the udder or milk [3]. The SCM is reported to be 15–40 times more prevalent than its clinical counterpart [4]. This subtle form, often unnoticed by farmers, has the potential to infect healthy animals within the herd and cause greater economic losses than the clinical form [5]. It is reported that 70–80% of the financial losses due to mastitis are caused by the SCM [1]. From a public health perspective, there is a risk of pathogenic microorganisms in mastitic milk and dairy products being transmitted to humans. Additionally, the presence of antibiotic residues in milk due to the treatment of mastitis poses another public health threat and diminishes the milk quality [6]. Microorganisms in milk can lead to food toxicities in humans, while antibiotic residues can cause allergic reactions. The use of antibiotics may also contribute to the development of resistant bacterial strains in milk, and consuming such milk may result in antibiotic resistance [7, 8].

Subclinical mastitis can be detected by tests such as the California Mastitis Test (CMT) or Somatic Cell Count (SCC) measurement [3]. The SCC is a crucial parameter used in assessing milk quality and diagnosing SCM [9, 10, 11, 12]. Somatic cells are defined as epithelial cells that pass from the mammary tissue and inflammatory cells that pass from the systemic bloodstream into the milk. Most somatic cells are macrophages, neutrophils, and leukocytes [13]. High SCC in milk results, indicating intramammary infection primarily, also leads to decreased industrial efficiency and shorter shelf life of dairy products [14, 15, 16, 17]. The CMT is a practical method used to interpret the somatic cells in udder quarters, and an increase in CMT score indicates high SCC in the milk [18].

It needs to devise a strategy that detects mastitis at earlier stages and can be treated with the lowest-cost alternative medicines regardless of severity. Such measures can increase the milking life of dairy animals and help fight antibiotic resistance, which is an important factor threatening human health. For this purpose, research is being conducted on alternative mastitis treatment approaches that do not leave residues in milk.

Hypochlorous acid (HOCl), a naturally produced compound by neutrophils, has exhibited antimicrobial and wound-healing properties without cytotoxicity for the last 15 years [19]. It is considered a physiological substance that is non-flammable and non-irritating, and compatible with the tissues and blood pH. It can be applied to all mucous membranes and enter the systemic circulation. It has been reported that it does not cause any toxic or adverse reactions and provides asepsis of infected areas. It has been used in various surgical procedures, such as perforated appendicitis, sternotomy, mediastinitis, and peritoneal lavage [20, 21]. Hypochlorous acid, reported to have a broad spectrum of antimicrobial effects, is effective in as short as 12 seconds and has antibacterial, antiviral, antifungal, antiparasitic, and anti-inflammatory effects [21, 22, 23, 24]. Due to its ability to reduce bacterial load on the periocular skin surface, it has been used in the treatment of blepharitis [25], cleaning biofilm-contaminated implant surfaces [26], intraperitoneal treatment [27], open wound treatment [21, 28], and as a potent antiseptic [29]. It has also been reported to be effective against viruses [30, 31].

This study aimed to investigate the safety and efficacy of HOCl, a promising alternative to antibiotics with no known side effects that can be used safely in human medicine to treat SCM in dairy cows.

MATERIALS AND METHODS

Animal material

The study was carried out on 232 Holstein-Friesian dairy cows on a dairy farm with integrated facility conditions (shared ration and maintenance conditions).

Clinical procedures

The sequence of procedures on udders with the SCM included the CMT test, SCC measurement, milk sampling for bacterial isolation, milking, and HOCl administration. The experimental design is presented in TABLE I.

TABLE I
Experimental design

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
		1. milking	**3. milking**	**5. milking**	**7. milking**	**9. milking**
		HOCl	HOCl	HOCl	HOCl	HOCl
12 h		**2. milking**	**4. milking**	**6. milking**	**8. milking**	**10. milking**
	1. CMT 2. SCC 3. Sampling 4. Milking	HOCl	HOCl	1. CMT 2. SCC 3. Sampling 4. Milking 5. HOCl	HOCl	1. CMT 2. SCC 3. Sampling 4. Milking

HOCl: Hypochlorous Acid, CMT: California Mastitis Test, SCC: Somatic Cell Count, Sampling: Sample collection for bacterial culture

California mastitis test (CMT)

To detect the SCM, the CMT was performed on all quarters after cleaning and following standard farm procedures. The first milk was discarded from each quarter, and the next 2–3 mL milk sample was placed in four sections of the CMT test cup. The test cup was tilted at a 45° angle to ensure that an equal amount of milk (2 mL) remained in each test area, and 2 mL of CMT solution (Bovivet CMT Liquid, Kruuse, Denmark) was added to each area. The test cup was circularly rotated for 10 seconds. The presence and severity of the SCM were evaluated based on the reaction observed in the test cup's sections, according to the method described by Roy *et al.* [32]. The reactions were scored from 0 to 3. If the mixture showed a homogeneous distribution (score 0) or slight thickening (trace), it was considered healthy. If slime or gel formed, it was recorded as SCM positive and scored based on severity (scores 1–3) [33].

Collection of milk samples

In the presented study, 74 of 141 cows with a positive CMT result in at least one udder lobe at different degrees were randomly determined, and one udder lobe of these cows (74 udder lobes) was selected for HOCl treatment. The selected cows were aged between 3 to 6 years old, had at least one calving, did not have any local/systemic infections, and had not received intramammary or systemic antibiotic treatment within the last week. Milk samples were taken from all quarters included in the study on days 0, 3, and 5 before evening milking to determine the SCC and perform bacteriological isolation. The teats were cleaned with 70% alcohol and were allowed to dry. The initial milk, at risk of high SCC and bacterial count, was discarded, and 5 mL of milk was aseptically collected in sterile tubes from each quarter. In cases where SCM was observed in more than one-quarter of the same cow, the rear quarters were preferred for microbiological sampling. However, HOCl treatment was applied to all subclinical mastitic quarters.

The milk samples designated for bacteriological isolation were promptly refrigerated at +4°C (RT59QBPN, Samsung, South Korea) delivered to the laboratory, and subjected to isolation procedures without delay.

Determination of somatic cell count

Separate milk samples were also taken before the microbiological sampling on days 0, 3, and 5 to measure the SCC. Rapid SCC measurements were conducted using a portable automatic somatic cell counting device [DeLaval (DCC), Tumba, Sweden].

Intramammary hypochlorous acid administration

Intramammary HOCl was administered for 5 days immediately after the morning and evening milkings. The milk in the relevant quarter was completely removed before infusion of the HOCl [0.02% Hypochlorous Acid, Crystalin Animal Health, NHP, Turkey]. A total of 100 mL HOCl was applied to each quarter using modified syringes for the teat channel. In our previous preliminary study [34], we determined that success in the treatment of SCM could be achieved with 3 applications of 100 ml HOCl at 12-hour intervals. The solution was distributed to the upper tissue parts of the quarter via careful massaging.

Bacteriological culture and isolation

For bacteriological isolation, the frozen milk samples were brought to room temperature and gently inverted 10–12 times for homogenization. Initially, 10 µL of each sample was streaked onto Müller Hinton Agar. Subsequently, to identify the microorganisms thriving under aerobic conditions both general media (sheep blood agar) and selective media (MacConkey Agar, Mannitol Salt Agar, BBL Bile Esculin Agar, Candida Selective Agar), along with Sabouraud Dextrose Agar (SDA) for fungi cultivation were used. The bacterial and yeast cultures were incubated (Nuve EN 400, Nuve, Turkey) under aerobic conditions at 37°C for 24–48 hours for identification. After establishing the Gram morphology of the isolated microorganisms, cultural and biochemical properties were characterised [35]. The cultivated SDA plates were incubated for one month to evaluate fungal growth. Growth status and macroscopic colony morphologies were assessed.

Statistical analyses

Statistical analysis of the study was performed using the SPSS 22.0 packet program (SPSS, IBM SPSS Statistics, Chicago, IL, USA). We employed a Paired Samples T–Test to compare the mean SHS values. Additionally, McNemar's test was applied to evaluate the isolation results. Graphs were created using GraphPad Software, Inc 8.0. Statistical significance was indicated as follows: * for $P<0.05$, ** for $P<0.01$, and *** for $P<0.001$.

RESULTS AND DISCUSSION

California mastitis test

As a result of the CMT conducted on 232 Holstein–Friesian dairy cows on the farm, 141 cows had subclinical infection in at least one-quarter, and the SCM percentage was 60.78%. The proportion of healthy cows was 39.22% (91 of 232) (FIG. 1A). At the quarter level, 27.91% (259 of 928) were positive for varying degrees of SCM. The proportion of CMT–negative healthy and blind quarters was 69.94% (649 of 928) and 2.15% (20 of 928), respectively. Six of the blind quarters were in healthy cows, and 14 were in cows diagnosed with SCM (FIG. 1B).

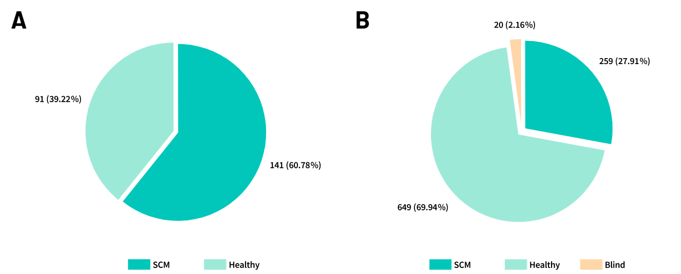

FIGURE 1. The distribution of cows (A) and udder quarters (B) diagnosed with subclinical mastitis (SCM) on the farm

The number of affected udder quarters among the 141 cows with SCM is presented in TABLE II. The 141 cows with SCM were found to have 259 infected udder quarters, 14 blind udder quarters, and 291 CMT–negative udder quarters.

When the CMT results were evaluated according to udder quarter localisation, the distribution of infected quarters was 69 (26.64%), 72 (27.80%), 57 (22.01%), and 61 (23.55%) in the left frontal, right

TABLE II
The distribution of affected udder quarters in cows with subclinical mastitis (SCM)

Udder quarter	Number of cows	Total quarters (cow / %)	Blind quarters (cow/quarter)
1 quarter	72	72 / 27.79%	6 / 7
2 quarters	36	72 / 27.79%	4 / 5
3 quarters	17	51 / 19.69%	2 / 2
All quarters	16	64 / 24.71%	–
Total	141	259 / 100.00%	12 / 14

frontal, left rear and right rear quarters, respectively (TABLE III). When categorizing front and rear quarters in total, it was observed that 54.44% (141) were in the front quarters, and 45.56% (118) were in the rear quarters (TABLE III).

TABLE III
The distribution of affected udder quarter localization and California Mastitis Test (CMT) results in cows with subclinical mastitis (SCM)

Udder quarter	CMT (+)	CMT (++)	CMT (+++)	CMT (Total quarter / %)	CMT (–)	Blind	Total
Right front	50	16	6	72 / 27.80%	157	3	232
Right rear	39	17	5	61 / 23.55%	166	5	232
Left front	39	27	3	69 / 26.64%	160	3	232
Left rear	28	21	8	57 / 22.01%	166	9	232
Total number	156	81	22	259 / 100.00%	649	20	928
Total %	60.23%	31.27%	8.49%	27.91%	69.93%	2.15%	100.00%

CMT: California Mastitis Test

Somatic cell count

The 74 udder quarters included in the study, initially showed varying degrees of SCM based on CMT results with an average SCC of 629.279 ± 68.555 before HOCl administration. The average SCC on the 3rd (before 6th milking) and 5th (before 10th milking) days of treatment were determined as 1.136,864 ± 91.009 and 1.180,000 ± 72.427, respectively. The mean SCC of cows was significantly higher following the treatment when compared to before HOCl treatment (P<0.001). The average SCC values measured in the milk samples taken on days 0, 3, and 5 in the study are shown in FIG. 2.

Bacteriological Results

In the present study, 74 random samples were taken for microbiological culture among the 259 CMT-positive udder quarters (representing 27.91% of the total). Before the intramammary HOCl treatment, 64.86% (48) of the cows exhibited microbial growth, while there was no microbial growth in 26 samples (35.14%). On the 3rd day of the HOCl treatment (before the 6th milking), the percentage reduced to 45.95%. On the 5th day of the treatment (before the 10th milking), microbial growth was further reduced to 22.97%. The bacterial isolation responses of milk samples obtained from udder quarters with SCM on days 0, 3, and 5 in the study are presented in FIG. 3.

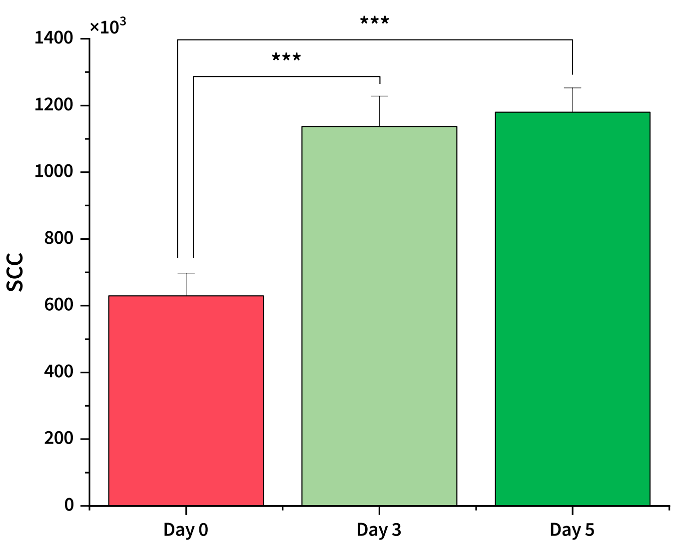

FIGURE 2. The mean Somatic Cell Count (SCC) values before Hypochlorous Acid (HOCl) administration (Day 0), on the 3rd (Day 3), and 5th days (Day 5) of HOCl treatment. Shape (***) indicates the statistical difference between groups, with *P*<0.001

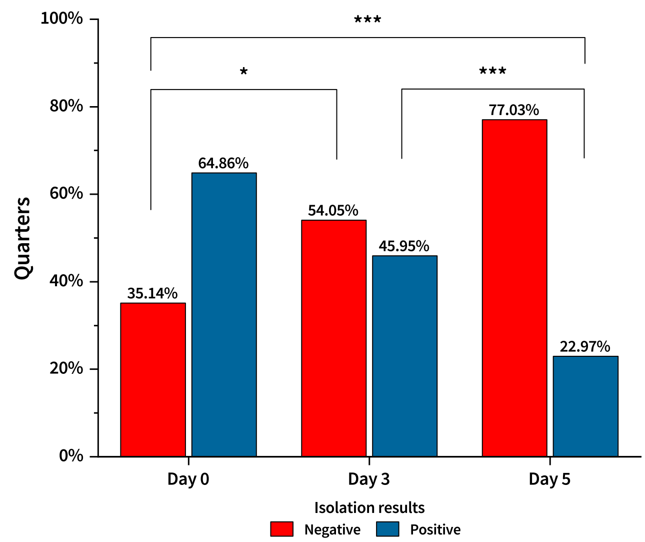

FIGURE 3. Distribution of bacterial isolation responses of cows included in the study. Day 0: Before intramammary Hypochlorous Acid (HOCl) treatment, Day 3: 3rd day of HOCl treatment, Day 5: 5th day of HOCl treatment. Statistical analyses were conducted using SPSS followed by the McNemar test. Different numbers of shapes (*) between columns indicate statistical differences between days of bacterial isolation, *P* values *; *P*<0.05, **; *P*<0.01, *; *P*<0.001**

The percentage of isolated microorganisms was sporadic. Before intramammary HOCl treatment, 35.14% (26 samples) of the milk samples showed no bacterial growth. By the 3rd day of treatment, this increased to 54.05% (40 samples), and by the 5th day, 77.03%

(57 samples) of the samples showed no bacterial growth. Before the intramammary HOCl treatment, the bacterial growth in milk samples was as follows: *Staphylococcus aureus* (*S. aureus*) was present in 17.57% (13 samples), Coagulase–Negative *Staphylococci* (CNS) in 12.16% (9 samples), *Corynebacterium* spp. in 9.46% (7 samples), *Candida* spp. in 2.7% (2 samples), and *Enterococcus* spp. in 1.35% (1 sample). Mixed cultures included *S. aureus* with *Candida* spp. in 12.16% (9 samples), CNS with *Candida* spp., and CNS with *S. aureus* in 2.70% (2 samples each), and CNS with *Corynebacterium* spp., CNS with *Micrococcus* spp., *Candida* spp. with *Corynebacterium* spp., and *S. aureus* with *Enterococcus faecalis* in 1.35% (1 sample each). The isolation results before the HOCl treatment are presented in FIG. 4A. On the 3rd day of intramammary HOCl treatment, *S. aureus* was found in 9.46% (7 samples), CNS in 8.11% (6 samples), *Candida* spp. in 4.05% (3 samples), and *Streptococcus* spp. in 1.35% (1 sample). Mixed cultures were observed as follows: *S. aureus* and *Candida* spp. in 14.86% (11 samples), *Candida* spp. and *Corynebacterium* spp. in 2.70% (2 samples), and CNS and *Corynebacterium* spp., CNS and *S. aureus*, CNS and *Candida* spp. in 1.35% (1 sample each). The isolation results on the 3rd day of the HOCl treatment are presented in FIG. 4B. On the 5th day of intramammary HOCl treatment, *S. aureus* was isolated in 9.46% (7 samples) and CNS in 1.35% (1 sample). Mixed cultures of *S. aureus* and *Candida* spp. were found in 12.16% (9 samples). The bacterial isolation results on the 5th day of the HOCl treatment are presented in FIG. 4C.

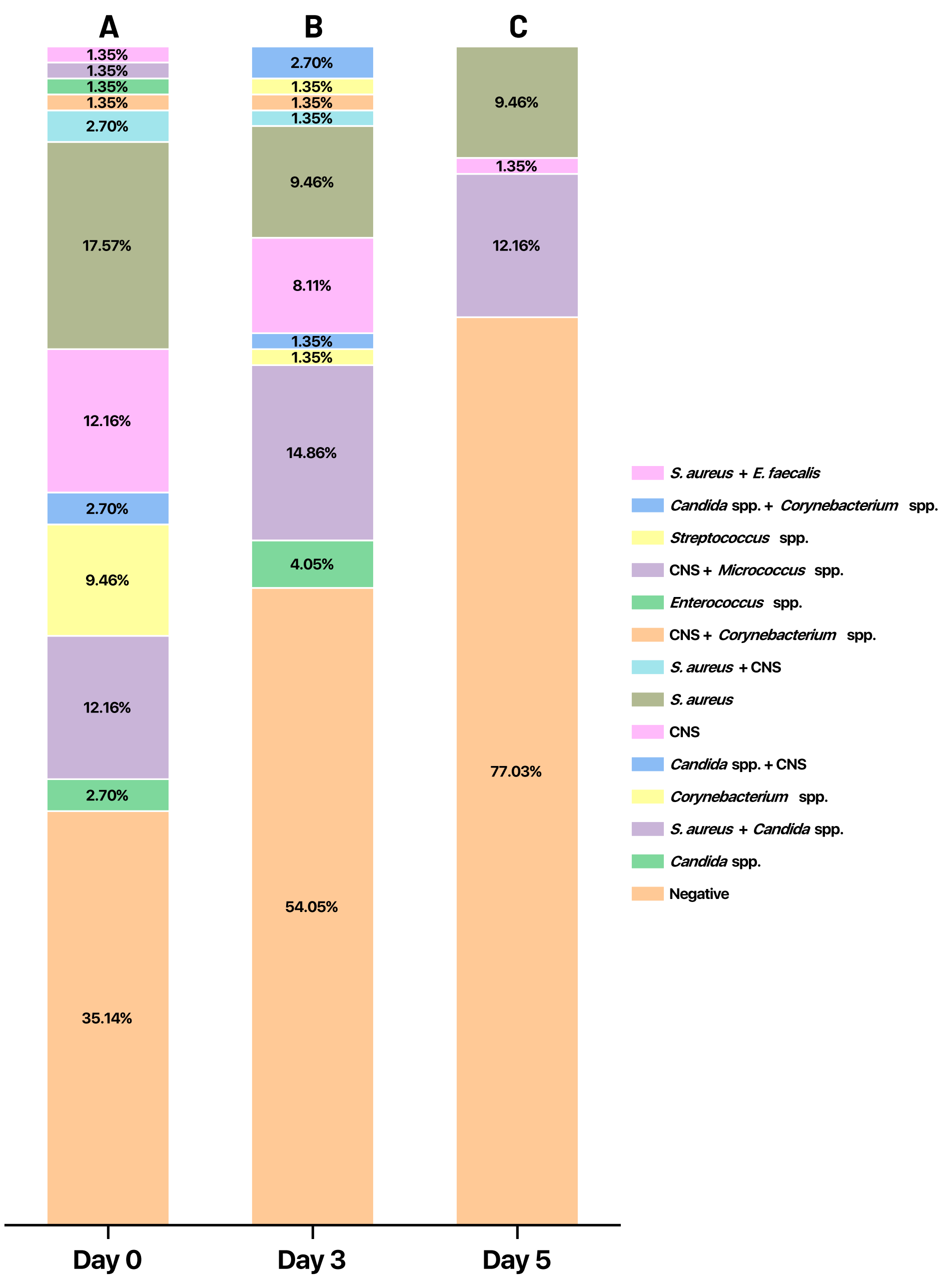

FIGURE 4. Bacteriological isolation results before the intramammary Hypochlorous Acid (HOCl) treatment (A), 3rd (B), and 5th (C) day of HOCl treatment. (CNS: Coagulase–Negative, *S. aureus: Staphylococcus aureus, E. faecalis: Enterococcus faecalis*)

In milk-producing operations, the most critical factor determining the milk quality is the animal's and udder's health. Inflammation of the mammary gland is referred to as mastitis, regardless of its cause [36]. In the subclinical form of mastitis, even though the animal's and udder's health may appear normal, the decrease in milk yield and quality is often unnoticed by the breeder, leading to underestimated economic losses it causes or will cause [37, 38]. The SCM can also spread to healthy cows within the herd if left untreated. The incidence of SCM in cows has been reported to range between 19.20% and 85% [3, 39]. This poses a significant concern for the global dairy industry and represents a more widespread problem than clinical mastitis [40]. The present study found an SCM rate of 60.78% (FIG. 1A). This is consistent with prior research, which reported a higher SCM among dairy cows [10, 11, 12]. The results obtained in this study support the conclusion that SCM is one of the significant problems for dairy farms worldwide. The high ratio of this disease in dairy cows can lead to substantial financial losses if it is not addressed.

The dairy industry must prioritize the early diagnosis and treatment of SCM to mitigate its effects and reduce its occurrence. Determination of the SCC is a critical point in evaluating milk quality, detecting issues affecting udder health, and especially in diagnosing the SCM. In this study, the CMT emerged as a valuable tool for evaluating the SCC in milk, with the total incidence of mastitis at the udder quarter level determined as 27.91% based on the positive CMT in this study (FIG. 1B), aligning with the findings of Sanford *et al.* [18]. Mammary somatic cells consist of epithelial cells from secretory tissue and leukocytes involved in infection response, as outlined by Harmon [13]. Notably, an elevated CMT score correlates with increased milk SCC levels, indicating its utility in diagnosing mastitis.

The study was extended to understand the distribution patterns of SCM within the udder quarters. This may be important for the development of targeted diagnostic and treatment strategies. Some research has suggested that mastitis is more common in the rear udder quarters [41, 42, 43], while others have reported it to be more frequent in the front quarters [44]. In the current study, 54.44% of SCM cases were found in the front udder lobes, while 45.56% were in the rear udder lobes. Additionally, the right front and left front quarters were the most affected, with positivity rates of 27.80% and 26.64%, respectively. The results indicate that there is no significant difference in the localization of SCM in the udder lobes (TABLE III). In terms of the number of infected udder lobes, the number of cows with SCM in one, two, three, and four udder quarters was 72, 36, 17, and 16, respectively (TABLE II). Various factors, including those related to the animal, herd management, and the farmer, as well as the clinical type of mastitis, the severity of SCM, and the diagnostic tool used, may have led to different results between studies. It is recommended to take these factors into account when evaluating the occurrence and distribution of mastitis. Implementing distinctive interventions targeting mastitis distribution patterns in udder quarters may be suggested to prevent the progression of SCM to more severe clinical stages.

Subclinical mastitis can progress to CM if left untreated. If not treated correctly, CM can lead to loss of milk production and udder atrophy [45]. In various studies, the rate of blind udder quarters has been reported to range from 3.83% to 4.84% [46, 47]. The presence of blind udder quarters in this study accounts for 2.15% at the herd level (FIG. 1B), along with the previous research reports.

In addition to SCM not being noticed by breeders, another important problem is the widespread use of antibiotics for treatment [48, 49]. In field conditions, antibiotics are generally used in the treatment of mastitis with a trial-and-error approach. The overuse of antibiotics is a significant concern, leading to the selection of antibiotic-resistant pathogens and posing challenges for treatment as well as potential public health problems. The presence of antibiotic residues in milk due to the use of antibiotics to treat mastitis can also negatively affect milk quality and human health [48, 49, 50]. To overcome this problem, there is a growing interest in exploring alternative approaches to antibiotics for the control and treatment of mastitis, crucial for the safety and quality of milk. In our preliminary study [34], which was previously conducted on cows with SCM in two different dairy farms, post-milking intramammary HOCl infusion was tested for 3 milkings at 12-hour intervals. No adverse effects related to the treatment were observed, and a reduction in bacterial growth in the udder quarters was detected at the end of the treatment. Therefore, we suggested that it could be an alternative to antibiotic use. In the presented study, the effectiveness of intramammary administration of HOCl, a naturally occurring substance produced by neutrophils [19] and safe in human medicine, was investigated over a 5-day period with 12-hour intervals to treat SCM with closely monitoring changes in somatic cell count (SCC). Before the administration of HOCl, the mean SCC measured in milk samples with positive CMT was 629.279 ± 68.555. It was observed that the mean SCC increased on the 3rd and 5th days of the treatment and was 1.136,864 ± 91.009 and 1.180,000 ± 72.427, respectively. Wellnitz *et al*. [51] mentioned an increase in milk SCC treated with lipopolysaccharide (LPS) alone or prednisolone and LPS combination, observed for up to 36 hours. In our previous preliminary study [34], it was determined that HOCl application increased SCC and decreased after 1 week of treatment. The increase in SCC following intramammary treatment in the current study is believed to be a result of the activation of favourable inflammatory response to microorganisms and immune cell migration to the mammary gland.

Mastitis can be caused by bacteria, fungi, viruses, chemicals, and physical factors [52]. Some studies have shown that bacterial growth was absent in 25% to 39.20% of CMT-positive milk samples from cows with SCM [46, 53]. In this study, 35.14% of samples (a total of 74 quarters) tested negative for bacteria before the HOCl treatment. This rate falls within the range reported in other studies.

The pre-treatment microbiological culture results showed that 64.86% of microbiological samples tested positive for bacteria. Following treatment with HOCl, this percentage was reduced to 45.95% and 22.97% on day 3rd and 5th of treatment, respectively.

The rise in the number of samples with no bacterial growth following HOCl treatment is consistent with the known antimicrobial properties of HOCl. It is effective at eliminating a wide range of pathogens, including both bacteria and fungi [21]. The observed increase in the proportion of samples showing no bacterial growth over time highlights HOCl's effectiveness in progressively lowering the microbial load.

In the study, bacterial isolation results from milk samples before HOCl treatment revealed the presence of pathogens such as *S. aureus*, *Candida* spp., *Corynebacterium* spp., *Enterococcus* spp., and CNS. *S. aureus* emerged as the most prevalent bacterium in the investigation and is considered a major infectious pathogen causing bovine mastitis. These bacteria can spread from an infected cow to others through various routes, such as contaminated milking equipment or the hands of farmers. *S. aureus* infection becomes a significant issue in the dairy farming industry, particularly in cases of bovine SCM. These bacteria can produce staphylococcal toxins and form biofilms, which help protect them from the immune system and treatment methods. CNS were previously thought to be harmless components of the normal skin flora. However, they are now recognized as significant opportunistic pathogens causing mastitis. *Corynebacterium* spp., commonly found as a commensal bacterium in bovine mammary glands, may provide protection against intramammary infections caused by other mastitis pathogens. Initially thought to colonize only the teat canal, *Corynebacterium* spp. has also been observed in the teat cistern, gland cistern, and mammary parenchyma, emphasizing its role as an indicator of milking hygiene practices [54].

On the 3rd day of treatment, a reduction in the prevalence of *S. aureus* and CNS were noted, with *Candida* spp. and *Streptococcus* spp. also present but at lower rates. The mixed cultures showed some persistence. By the 5th day, the prevalence of *S. aureus* remained at 9.46%, while CNS dropped to 1.35%. Mixed cultures of *S. aureus* and *Candida* spp. were still present. This persistence of certain bacteria and mixed cultures suggests that while HOCl effectively reduces the overall microbial load, some bacteria, particularly *S. aureus* may survive or adapt to the treatment, leading to a partial resurgence of mixed cultures. This pattern of microbial reduction and stabilization aligns with the understanding of HOCl's antimicrobial properties. HOCl is known to disrupt microbial communities by targeting a broad spectrum of pathogens, which explains the initial decrease in bacterial presence. However, the survival or adaptation of certain bacteria, such as *S. aureus* highlights the challenge of completely eradicating all strains, which may lead to a partial return of mixed cultures over time. A study conducted on a dairy farm in the same region as the present study identified *Staphylococcus* spp. as the most common bacterial species, with an occurrence of 24.5%, and observed fungi as one of the most common pathogens, which supports the current study findings [55]. Excessive and indiscriminate use of antibiotics are the primary factors contributing to the increased frequency of fungal infections [56]. In the present study, it was noted that *Candida* spp. could be found in milk samples before HOCl treatment, either in combination with other pathogens or as a single agent.

CONCLUSION

The study emphasized the need for a holistic approach to maintaining milk production, milk quality, and the health of humans and dairy animals against SCM. In conclusion, the study demonstrated that bacterial growth was suppressed and the number of udder quarters, in which microorganisms were not isolated, increased on the control days after intramammary HOCl treatment. This suggests that HOCl can be used as an alternative to antibiotics for treating SCM in cows. Further research is recommended to develop more practical devices for intramammary use, optimize HOCl delivery, determine the ideal doses, and penetrate more effectively into secretory cells. The bacteriological results, in particular, could be encouraging for targeting new research.

Ethics approval

This study was conducted with the approval and guidelines of the Animal Research Local Ethics Committee of Aydin Adnan Menderes University (64583101/2023/113).

Conflict of interest

The authors declare that they have no conflict of interest.

ACKNOWLEDGEMENT

The authors thanks to Prof. Dr. Süheyla Turkyilmaz for providing support in microbiological analysis.

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