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In vitro efficiency of teat disinfectants with organic matter against *Staphylococcus aureus* isolated from cows with mastitis

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ABSTRACT. Four strains of *Staphylococcus aureus* isolated from cows with mastitis were assessed as to *in vitro* sensitivity to four commercial disinfectants (chlorine, polvidone-iodine, chlorhexidine, and lactic acid) used in teat pre- and post-dipping, in the presence and absence of organic matter. The concentration of the disinfectants was determined in accordance with the Brazilian Pharmacopoeia. Four isolates identified as *S. aureus* from cows diagnosed with subclinical mastitis were subjected to susceptibility testing against disinfectants through an absorbance evaluation at 600 nm on a spectrophotometer. Exposure times (15", 30", 60" and 90") were clocked, and streaking was performed in Brain Heart Infusion (BHI) medium. The iodine sample presented a lower concentration (1.9%) than that given by the manufacturer (2.5%). There was greater ($p < 0.05$) *in vitro* disinfectant activity for chlorhexidine and chlorine, with and without organic matter. However, inhibition by organic matter occurred more intensely in the iodine disinfectant (99.0%). The inhibition percentage of *S. aureus* against iodine and lactic acid at 15, 30, 60 and 90 s was numerically lower than against chlorhexidine and chlorine, regardless of organic matter. Therefore, chlorhexidine and chlorine have greater inhibitory activity against *S. aureus*, both with and without organic matter.

Keywords: dipping; dirt; mastitis; sensitivity; teat.

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Introduction

Mastitis is an inflammation of the mammary gland characterized by physical, chemical and organoleptic changes both in the milk and in the glandular tissue. With a high prevalence in dairy herds, this disease reduces production, affects milk quality, and is responsible for large economic losses. It has a multifactorial nature, being caused by different pathogens and influenced by the environment and factors inherent to the animal (Cunha et al., 2016; Biscotto, Amaral, & Cunha, 2022).

One of the main microorganisms that causes mastitis is *Staphylococcus aureus*, a highly contagious bacterium that is transmitted from one animal to another through contaminated milking material and milkers' hands, and may be present on the skin of the udder. Losses in dairy farming caused by this microorganism are great, resulting in high Somatic Cell Count (SCC), exacerbated losses in milk production and quality, and expenses with medication (Cunha et al., 2015; Rainard et al., 2018).

The appropriate use of disinfecting agents on teats during cow milking aims to reduce the population of pathogenic microorganisms and prevent a potential spread of infectious agents such as *S. aureus* among animals (Ramalho et al., 2012). During milk extraction, the teat sphincter is opened, which increases the risk of microorganisms entering. Furthermore, the same teat cups are used in several cows, which can transmit contagious pathogens from one animal to another. Therefore, disinfecting the teats before (pre-dipping) and after (post-dipping) reduces the entry of microorganisms into the mammary gland (Santos et al., 2016; Ordoñez et al., 2022).

Disinfection by pre-dipping is performed after dirt is removed from the teats, and the first jets of milk are removed for clinical mastitis to be detected in the animal. Disinfection aims to prevent microorganisms that cause environmental mastitis and must be carried out by covering the entire surface of the teat with the disinfectant solution, allowing it to act for 30 s. Then, the teats are dried with paper towels, and the milking sets are attached for milk extraction. Disinfection by post-dipping is performed after the animal has been

completely milked, with a more concentrated disinfectant solution, in order to prevent contagious microorganisms (Medeiros et al., 2009; Massote, Zanateli, Alves, Gonçalves, & Guedes, 2019).

Disinfectants are used as a routine practice in dairy production, as they are low-cost, highly efficient tools that contribute to improving the quality of the milk produced by the herd. However, in most properties, disinfecting agents are chosen based on usage habits, ease of application, or price. The practicalities and limitations of each disinfectant must be reassessed, as their inappropriate use can cause natural selection of resistant strains in a microbial population (Santos, Silva, Oliveira, Oliveira, & Martins, 2018; Bach et al., 2019).

Disinfectants used as dipping differ in terms of their active ingredients. Conventional antiseptic solutions present a sharp drop in efficiency or are even deactivated upon contact with organic matter, which is likely to happen on the teat, or with the product being contaminated through the environment (Coutinho, Medeiros, Silveira, Silva, & Mota, 2012; Ramalho et al., 2012).

A periodic evaluation of disinfectants used on properties is important, as many may not be effective in controlling the most prevalent microorganisms that cause mastitis, especially *S. aureus*. Inefficiency can be further intensified if the solution is contaminated with organic matter (Ramalho et al., 2012; Santos et al., 2016). Therefore, the objective of this study was to evaluate the efficiency of teat disinfectants, with and without organic matter, in eliminating *S. aureus* that causes mastitis in dairy cows.

Material and methods

Using herd cows from Minas Gerais, milk samples were collected to identify animals with subclinical mastitis. After the mug test was conducted to detect clinical mastitis (Cunha et al., 2016), 50 mL milk samples were collected from their mammary quarters into sterile bottles containing Bronopol preservative (Brito, Souza, Faria, & Moraes, 2007) for SCC determination.

SCC determination was carried out by means of flow cytometry, using Bentley Combi System 2300® equipment, owned by Bentley Instruments Incorporated Chaska, United States of America (Bentley Instruments Inc., 1997; 1998). The quarters with SCC greater than 200,000 cells mL^{-1} were considered to have subclinical mastitis (Souza et al., 2016).

Milk samples from all mammary quarters were collected again after asepsis with 70% alcohol from the teats into sterile bottles. Then, the bottles were sent to the laboratory under refrigeration for bacteriological examination to confirm the occurrence of subclinical mastitis, in accordance with the inclusion criteria described by Andersen, Dohoo, Riekerink, and Stryhn (2010) and Souza et al. (2016).

The samples were plated in bacteriological agar added with 5% defibrinated sheep blood. Then, the plates were incubated in aerobiosis at 37°C so that readings could be performed in 24 and 48 hours. The biochemical identification of microorganisms, including *S. aureus*, was carried out in accordance with the methodology established by the National Mastitis Council (Oliver, González, Hogan, Jayarao, & Owens, 2004). Thus, four isolates of *S. aureus* (994, 783, 997 and 837) were used to verify the sensitivity of these microorganisms to disinfectants with or without organic matter added.

To check the efficiency of the disinfectants, solutions commonly used as pre- and post-dipping (chlorine, povidone-iodine, lactic acid, and chlorhexidine digluconate) were purchased in a commercial establishment and sent to a physical-chemical analysis laboratory. Thus, the concentrations of the active ingredients were determined, in triplicate, in accordance with the methodologies set by the Brazilian Pharmacopoeia (Brasil, 2010).

In order for the commercial action of the disinfectants to be assessed, they had the concentrations at which they were purchased maintained, being diluted when necessary and in accordance with the manufacturer. Therefore, the effectiveness of the disinfectants was determined in triplicate, based on the methodology used by Ramalho et al. (2012). For analysis, homogeneous bacterial suspensions were prepared, in sterile saline solution corresponding to McFarland scale 1, using the four samples of *S. aureus* isolated from animals with subclinical mastitis.

Furthermore, 1 L of organic matter mixture was prepared with 10 bovine urine, 10 feces, 10 milk, and 10% soil. This preparation was sterilized at 120°C for 15 min. Then, 50 µL of the organic matter mixture was added to tubes containing 4 mL of Brain Heart Infusion (BHI) broth and 2 mL of the disinfectant solution diluted at 1.5%. Tubes without organic matter were used as a negative control for organic matter.

Subsequently, 2 mL of bacterial suspension were added to the tubes containing the disinfectant solution and organic matter so that the exposure times (15, 30, 60 and 90 s) were clocked. At each time, streaks in BHI medium were performed, and the plates were incubated at 36°C for a qualitative verification of growth through the emergence of colonies.

Then, the mixtures contained in the tubes were incubated at 36°C for 24 hours, and the turbidity of the medium, in addition to the formation of a thin layer on the surface or formation of precipitate at the bottom of the tubes, were observed. Before and after the growth of microorganisms in the tubes, samples were subjected to absorbance determination at 600 nm in a SP220 visible light spectrophotometer (Biospectro Ltda., Curitiba State, Brazil), in accordance with methodology adapted from Stepanović, Vuković, Dakić, Savić, and Švabić-Vlahović (2000).

The absorbance results before and after the growth of *S. aureus* in the BHI medium added with disinfectants with and without organic matter were analyzed descriptively and subjected to comparison of means using Student's t test. The increase in absorbance during the times of exposure to the disinfectants would indicate the growth of *S. aureus* and, therefore, the resistance of the microorganism to the disinfectant in the presence or absence of organic matter.

The inhibition of organic matter on the disinfectants was determined by calculating the difference between the absorbance found in the growth of *S. aureus* in disinfectants with and without organic matter. The qualitative results for the growth of *S. aureus* in the plates were used to obtain the percentage effectiveness of the disinfectants at the exposure times used, with and without organic matter.

All data were analyzed using SigmaPlot 12.0 software (Systat Software Inc., San Jose, USA), at a 5% significance level. The research was approved by the Research and Extension Center [*Núcleo de Pesquisa e Extensão*] (Nupex) of the University Center of Viçosa [*Centro Universitário de Viçosa*] (Univiçosa), under protocol No. 207/2016-I.

Results and discussion

According to the disinfectant labels, the concentrations of the active ingredients were 5.0 chlorine, 2.5 povidone-iodine, and 1.5% chlorhexidine digluconate. The concentration of lactic acid was not on the product's label. After analysis, the concentrations of disinfectants found were 5.35, 1.9, 1.9, and 22.79% chlorine, povidone-iodine, chlorhexidine digluconate, and lactic acid, respectively.

The chlorine and chlorhexidine disinfectants had concentrations higher than those proposed by the manufacturers. The iodine sample, in its turn, had a lower concentration than that given by the manufacturer. If the concentration found in the product is not the one described on the label, this will result in consumer disregard and compromise the expected effectiveness of the product against pathogens that cause mastitis.

There was greater *in vitro* disinfectant activity for chlorhexidine and chlorine with and without organic matter, as the growth of *S. aureus* given in absorbance was lower (Table 1). Evaluating the effectiveness of commercial disinfectants used in pre- and post-dipping against microorganisms isolated from animals with bovine mastitis, Medeiros et al. (2009) observed that iodine and chlorhexidine had greater disinfectant activity against *S. aureus*, and iodine had better efficacy against coagulase-negative Staphylococcus.

Table 1. Absorbance results for *Staphylococcus aureus* samples isolated from animals with mastitis before and after the action of disinfectants with and without organic matter.

Disinfectant	Bacteria	Without organic matter			With organic matter		
		Before	After	Growth	Before	After	Growth
Chlorhexidine	994	1.759	1.845	0.086	1.953	2.015	0.062
	783	1.919	2.007	0.088	1.846	1.919	0.072
	997	1.845	1.933	0.088	2.154	2.192	0.038
	837	1.985	2.058	0.073	2.115	2.160	0.045
Iodine	994	0.151	0.316	0.164	0.430	0.674	0.244
	783	0.391	0.437	0.046	0.183	0.765	0.582
	997	0.181	0.582	0.401	0.172	0.899	0.727
	837	0.098	0.544	0.446	0.182	0.734	0.552
Chlorine	994	0.023	0.041	0.018	0.089	0.159	0.070
	783	0.028	0.050	0.022	0.111	0.148	0.037
	997	0.032	0.051	0.019	0.114	0.127	0.013
	837	0.024	0.044	0.020	0.091	0.093	0.002
Lactic acid	994	0.114	0.433	0.319	0.432	0.547	0.115
	783	0.110	0.499	0.390	0.637	1.037	0.400
	997	0.204	0.451	0.248	0.207	0.660	0.453
	837	0.149	0.350	0.201	0.201	0.493	0.293

Evaluating the action of disinfectants against *S. aureus*, Santos et al. (2016) found greater effectiveness for quaternary ammonium and chlorhexidine compounds, followed by iodine and chlorine. Lactic acid showed the lowest antiseptic activity, the same as what was observed in the present study. The authors also suggested periodic evaluation to reduce the rate of new intramammary infections in the herd due to the variation in the susceptibility profile of *S. aureus* to disinfectants.

Better post-dipping and pre-dipping inhibitions against *S. aureus* microorganisms that cause mastitis have been reported when solutions based on iodine, chlorhexidine and sodium hypochlorite were used (Medeiros et al., 2009; Coutinho et al., 2012; Santos et al., 2016; Santos et al., 2018). In both cases, the teats must be completely immersed, and the concentration of the active ingredient of the disinfectant must be checked (Bach et al., 2019). In the present study, the actual concentration of commercial povidone-iodine (iodine) was lower than that informed on the label, which may have influenced the efficiency of such disinfectant.

Ramalho et al. (2012) also found greater inhibitory action for chlorhexidine, followed by chlorine, iodine, and quaternary ammonium. However, Azizoglu, Lyman, and Anderson (2013) observed low action for chlorhexidine against *S. aureus*, while iodine showed greater inhibition. The authors reported that the susceptibility profile is due to the different genotypes of *S. aureus* isolated from cows with mastitis, which may explain the results of the present study.

The best action of chlorhexidine and chlorine on *S. aureus*, with and without organic matter, was statistically observed (Table 2). Through the growth given in absorbance, *S. aureus* showed greater ($p < 0.05$) average growth compared to the iodine and lactic acid disinfectants, and, therefore, the latter were less efficient than chlorhexidine and chlorine, with and without organic matter.

Table 2. Average absorbance-determined growth of *Staphylococcus aureus* isolated from animals with mastitis, comparing disinfectants with and without organic matter.

Disinfectant	Growth		Organic matter inhibition (%)
	Without organic matter	With organic matter	
Chlorhexidine	0.084Ba	0.054Ca	-35.4
Iodine	0.265Ab	0.526Aa	99.0
Chlorine	0.020Ba	0.031Ca	54.4
Lactic acid	0.289Aa	0.315Ba	8.9

Means followed by different upper and lower case letters between rows and columns respectively differ statistically ($p < 0.05$).

Inhibition by organic matter occurred more intensely in the iodine disinfectant (99.0%). The average growth of *S. aureus* with the use of disinfectant was significantly higher ($p < 0.05$) with organic matter than without organic matter. The lower effect of the organic matter on the disinfectant was a result of lactic acid (8.9%); however, the latter is not the one that showed the greatest *S. aureus* growth inhibition. As previously reported, chlorine and chlorhexidine were the disinfectants that most inhibited *S. aureus*. Therefore, the effect of the organic matter on lactic acid may have been masked by the low inhibition of the disinfectant on *S. aureus*.

Organic matter can interfere with the antimicrobial activity of disinfectants, as it deactivates its active molecule or because it is a physical barrier to protect microorganisms during chemical attack (Coutinho et al., 2012). Thus, adequate management of milking and the environment where the animals are housed is necessary so that their udders are kept clean, especially in summer, when dirt on the teats is most likely to occur (Massote et al., 2019).

The qualitative results showed that the inhibition percentage of *S. aureus* against iodine and lactic acid at 15, 30, 60 and 90 s was numerically lower than against chlorhexidine and chlorine, both with and without organic matter (Table 3). Chlorhexidine inhibited 100% of the samples at 60 and 90 s, with and without organic matter.

The inhibitory action of the disinfectant occurred more efficiently as the time of exposure to it increased, except for chlorine in the absence of organic matter, which did not inhibit *S. aureus* after 60 s of exposure. Peixoto et al. (2019) also observed that contact time is a factor that can interfere with the action of the disinfectant. Therefore, the recommended minimum action time for disinfectants used in pre- and post-dipping during animal milking must be complied with.

At most exposure times of *S. aureus* to all disinfectants, lower percentage inhibitions were observed when the disinfectants were in contact with organic matter. Only at 15 s, the percentage inhibition was equal or lower for lactic acid and chlorine in the absence of organic matter, respectively. It was therefore possible to verify that disinfectants must be used in teats free of organic matter in order to provide better disinfectant action.

Table 3. Inhibition percentage of *Staphylococcus aureus* samples isolated from animals with mastitis at different times of exposure to disinfectants with and without organic matter.

Disinfectant	Organic matter	N	Time (s)			
			15	30	60	90
Chlorhexidine	Absent	12	75.0	91.7	100.0	100.0
	Present	12	66.7	83.3	100.0	100.0
Iodine	Absent	12	8.3	8.3	25.0	33.3
	Present	12	0	0	8.3	16.7
Chlorine	Absent	12	16.7	41.7	58.3	41.7
	Present	12	25.0	33.3	41.7	41.7
Lactic acid	Absent	12	0	8.3	8.3	8.3
	Present	12	0	0	0	0

The chlorhexidine solution has considerable disinfectant action against *S. aureus*, with a high inhibition potential with or without organic matter. Chlorhexidine is widely used to treat superficial teat infections in cows due to its cumulative and continuous effect, remaining on the skin for at least six hours. Furthermore, this disinfectant works in the presence of organic matter, is easy to apply, and cost-effective (Coutinho et al., 2012; Santos et al., 2016; Santos et al., 2018).

The use of chlorine, a disinfectant that also showed good effectiveness in the present study, is a common practice on dairy farms in Brazil, since the product has a low cost. However, according to Amaral, Isa, Dias, Rossi Jr, and Nader Filho (2004), the lower stability of chlorine is a disadvantage, especially when the recommendations and criteria for use of the product by producers are not observed.

Iodine presented a disinfectant action inferior to that of chlorhexidine and chlorine, in addition to being sensitive to organic matter. Iodine solutions should be used to soak teats in low concentrations, as solutions above 1% may leave residues in the milk and affect the integrity of the teats (Medeiros et al., 2009; Bach et al., 2019). It is worth stressing that the concentration of commercial povidone-iodine in the present study was below that informed on the label.

As for lactic acid, the results revealed resistance from *S. aureus*, both with and without organic matter. Evaluating the efficiency of lactic acid without organic matter, Peixoto et al. (2019) found low *in vitro* efficacy of the disinfectant against Staphylococcus spp. (20,7%). However, Medeiros et al. (2009) found that the disinfectant was 100% effective in inhibiting *Staphylococcus* spp. The low susceptibility of *S. aureus* in the absence (8.3%) and presence (0%) of organic matter evidences the inefficiency of the disinfectant against the agent.

Due to the variations in the sensitivity and resistance profile found, it is necessary to regularly evaluate the efficiency of disinfectants used on properties in order to verify the effectiveness of the product and thus contribute to the control of mastitis in the herd. Knowing that iodine is one of the most used disinfectants in practice, its indiscriminate use may be selecting pathogens resistant to the disinfectant, such as *S. aureus* (Martins, Silva, Nakazato, Dutra, & Almeida Filho, 2010; Oliveira et al., 2011; Santos et al., 2016).

In addition to evaluating disinfectants, it is also important to check the profile of microorganisms that cause mastitis in the herd. The formulation of new disinfectant solutions to be used in pre- and post-dipping must be based on the susceptibility of the microorganism to the active ingredient (Silva Junior et al., 2019).

Conclusion

Chlorhexidine and chlorine have greater inhibitory activity against *S. aureus*, both in the presence and absence of organic matter. Organic matter mainly inhibits the antimicrobial activity of iodine against *S. aureus*.

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