ABSTRACT

Prototheca spp. is a frequent cause of bovine mastitis and is highly resistant to commonly used disinfectants. This study aimed to: (1) evaluate the antimicrobial activity of polyhexamethylene biguanide (PHMB) against mastitis-causing Prototheca spp., and (2) evaluate the biofilm production ability of Prototheca spp. A total of 85 Prototheca bovis and 2 Prototheca blaschkeae isolates from bovine mastitis cases were submitted to biofilm production assays and antimicrobial susceptibility tests against PHMB and disinfectants commonly used in dairy herds (chlorhexidine digluconate, povidone-iodine, sodium dichloroisocyanurate, and sodium hypochlorite). The minimal inhibitory concentration (MIC) and minimal algicidal concentration (MAC) were determined by microdilution assays. We observed that PHMB (MIC90: ≥2 µg/mL and MAC90: ≥4 µg/mL) and chlorhexidine gluconate (MIC 90 and MAC 90: ≥2 µg/mL) presented the highest antimicrobial activity against P. bovis isolates, followed by sodium dichloroisocyanurate (MIC90 and MAC90: ≥1,400 µg/mL), sodium hypochlorite (MIC90 and MAC90: ≥2,800 µg/mL), and povidone-iodine (MIC90 and MAC90: ≥3,200 µg/mL). Concerning P. blaschkeae isolates, PHMB (MIC and MAC ≥1 µg/mL) and chlorhexidine gluconate (MIC and MAC ≥1 µg/mL) were the disinfectants that presented the lowest concentration values required to inhibit the isolates. Regarding biofilms formation, 63.5% (n = 54/85) of the P. bovis isolates were classified as strong, 28.3% (n = 24/85) moderate, and 8.2% (n = 7/85) weak biofilm producers. In contrast, the P. blaschkeae isolates were classified as weak and moderate biofilm producers. These findings suggest that PHMB has the potential to be used for teat and milking-equipment disinfection for the prevention of mastitis-causing Prototheca spp. in dairy herds.

Key words: mastitis, disinfectants, microalgae, prevention, antimicrobial activity

INTRODUCTION

Prototheca spp. are a chlorophyllous unicellular microalgae that cause opportunistic infections in humans and animals (Ahrholdt et al., 2012). Prototheca spp. are widely distributed in the environment, especially in areas with the presence of organic matter (Jagielski and Lagneau, 2007; Shave et al., 2021). During the last 2 decades, bovine mastitis caused by Prototheca spp. has increased in herds globally (Pieper et al., 2012; Jagielski et al., 2019b; Park et al., 2019). Protothecal mastitis is commonly persistent and causes irreversible damage to the mammary alveolus and parenchyma (Shahid et al., 2020), which results in significant milk losses, culling of infected cows, and high costs for treatment and prevention (Jagielski et al., 2019a; Kano, 2019).

Considering the increasing importance of bovine mastitis caused by Prototheca spp., the economic losses, and the lack of effective treatment protocols, it is necessary to search for new compounds to control Prototheca spp. in the dairy herds, as well as to identify virulent factors, such as the biofilm production ability. The first evidence of Prototheca spp. producing biofilm was presented in a previous study (Gonçalves et al., 2015). Prototheca spp. biofilms are composed of different lineage cells (e.g., young-small to sporangia-containing progeny cells) bound together by DNA and polysaccharides (Kwiecinski, 2015). The Prototheca spp. antimicrobial resistance may be associated with biofilm production capacity (Ely et al., 2019; Libisch et al., 2022). Therefore, it is well-known that biofilm production can reduce the chance of mastitis cure (Degen et al., 2015).

Antimicrobial efficacy against Prototheca bovis is certainly important for developing control and preven-
tion measures in dairy herds. Previous studies reported sodium hypochlorite, iodine (Salerno et al., 2010; Gonçalves et al., 2015), silver nanoparticles (Jagielski et al., 2018), and antimicrobial peptides (Sperotto et al., 2021) presented high antimicrobial efficacy against Prototheca spp. isolates by in vitro assays. Polyhexamethylene biguanide (PHMB) is a cationic polymer that presents antimicrobial activity against gram-positive and gram-negative bacteria, fungi, and protozoa (Kamaruzzaman et al., 2017; Sowlati-Hashjin et al., 2020). This broad spectrum of antimicrobial activity is associated with 2 primary mechanisms of action: perturbation of the microorganism’s phospholipid bilayer (Sowlati-Hashjin et al., 2020), and the condensation of the selective bacterial chromosomes (Chindera et al., 2016). Polyhexamethylene biguanide has been evaluated against bacteria causing mastitis, such as Staphylococcus aureus (Kamaruzzaman et al., 2016; Leite et al., 2021). Despite the evidence of broad-spectrum antimicrobial activities, including activity against fungi (Sanada et al., 2014), parasites (Firdessa et al., 2015), and green algae (Poštulková and Kopp, 2016), the antimicrobial activity of PHMB against mastitis-causing Prototheca spp. isolates has not been evaluated.

The hypothesis was that PHMB can inhibit and inactive biofilm-producing P. bovis isolated from bovine mastitis at lower concentrations than other disinfectants commonly used for teat disinfection. The current study aimed to evaluate the antimicrobial activity of polyhexamethylene biguanide (PHMB) and disinfectants (chlorhexidine digluconate, povidone-iodine, sodium hexamethylene biguanide (PHMB) and disinfectants aimed to evaluate the antimicrobial activity of polyhexamethylene biguanide (PHMB) against the common used for teat disinfection. The current study mastitis at lower concentrations than other disinfectants isolated from bovine P. bovis activate biofilm-producing mastitis-causing (2016), the antimicrobial activity of PHMB against fungi (Sanada et al., 2014), parasites (Firdessa et al., 2015), and green algae (Poštulková and Kopp, 2016), the antimicrobial activity of PHMB against mastitis-causing Prototheca spp. isolates has not been evaluated.

The hypothesis was that PHMB can inhibit and inactive biofilm-producing P. bovis isolated from bovine mastitis at lower concentrations than other disinfectants commonly used for teat disinfection. The current study aimed to evaluate the antimicrobial activity of polyhexamethylene biguanide (PHMB) and disinfectants (chlorhexidine digluconate, povidone-iodine, sodium dichloroisocyanurate, sodium hypochlorite) against the biofilm-producer P. bovis causing bovine mastitis.

**MATERIALS AND METHODS**

No human or animal subjects were used, so this analysis did not require approval by an Institutional Animal Care and Use Committee or Institutional Review Board.

**Sample Selection**

Prototheca spp. isolates (n = 87) were obtained from 20 dairy herds located in the South and Southeast regions of Brazil. A total of 28 mammary quarters (38%) and 59 composite milk samples (68%), from 34 clinical (39%) and 53 subclinical mastitis cases (61%), were enrolled in this study. It was considered a clinical mastitis case when it was observed that there was an abnormality in milk characteristics (clots, blood, or inflammation signs in the mammary quarter). A subclinical mastitis case was considered when the cow had a somatic cell count >200,000 cells/mL without visual abnormality on milk, mammary quarter, or cow. The screening of Prototheca spp. isolates were based on nonprobabilistic convenience sampling, which was obtained from milk samples submitted to the microbiological identification routine at the Milk Quality Research Laboratory (Qualileite), Pirassununga, University of São Paulo.

The microorganism isolation was performed according to NMC (2017) guidelines. Briefly, 10 µL of milk samples were streaked onto plates containing agar supplemented with 5% of bovine blood. The plates were incubated at 37°C for 24–48 h under aerobic conditions. Colonies that presented a grayish-white color, opaque appearance, an average of 1 to 2 mm in diameter, irregular borders, and oval cells in microscopy were identified as Prototheca spp. The isolates were maintained, refrigerated (4°C), in Sabouraud dextrose media (Kasvi) for further analysis.

**Reference Strains**

Reference strains of Prototheca ciferrii (RZI-3), P. bovis (LZ-5), and Prototheca blaschkeae (RZIII-3) were kindly provided by Tomasz Jagielski at the Institute of Microbiology at the University of Warsaw in Poland for positive control to MALDI-TOF MS identification, molecular identification by conventional PCR, biofilm formation capacity, and in vitro antimicrobial susceptibility to disinfectants.

**MALDI-TOF MS Identification of Prototheca spp.**

The Prototheca spp. identification by MALDI-TOF MS was performed as described by Fidelis et al. (2021). Briefly, Prototheca spp. fresh colonies and control strains were dissolved in 300 µL of autoclaved Milli-Q water, followed by the addition of 900 µL of absolute ethanol. After incubation for 10 min at room temperature, the Prototheca suspension was centrifuged twice (Mikro 200r, Hettich) at 16,089 × g for 2 min. Then, the supernatant was discarded, and the pellet dried at room temperature for 10 min. The resulting pellet was suspended in 50 µL of formic acid (70%), followed by the addition of 50 µL of acetonitrile (100%). This solution was transferred to an Eppendorf containing 3 units of sterile zirconia beads (1.0 mm). The suspension was bead-beaten (Loccus; L-Beader 3 Biotech) in 5 cycles of 1 min at 4,000 rpm. Then, the supernatant was discarded, and the pellet dried at room temperature. The spectra of isolates and control strains were sub
mitted to identification by MALDI-TOF MS Biotype 4.1.70, supplemented with a previously local library that contains spectra of mastitis-causing *Prototheca* spp. species (Fidelis et al., 2021). Scores ≥1.7 and <2.0 were considered dependable for genus identification, whereas scores ≥2.0 were considered dependable for species identification.

**PCR Identification of Prototheca spp.**

For DNA extraction, the isolates were previously submitted to a cell disruption step. Briefly, 3 fresh distinct colonies from each plate were dissolved in 1 mL of Milli-Q water autoclaved with 3 units of sterile zirconia beads (1.0 mm). The solution was submitted to cell disruption for 5 cycles of 1 min at 4,000 rpm in a cell disruptor (Locus). The supernatant, except for the beads, was transferred to a new microtube and centrifuged at 16,089 × g for 2 min. The supernatant was discarded and the resulting pellet was suspended in 250 µL of ethylenediamine tetra acetic acid (Accuris Quicksilver) solution and submitted to DNA using the Wizard Genomic DNA Purification Kit (Promega) following the manufacturer's recommendations. The extracted DNA was kept frozen (−20°C) for further analysis.

Molecular identification of *Prototheca* isolates was performed by PCR using specific primers for 3 *Prototheca* spp. (*P. ciferrii*, *P. bovis*, and *P. blaschkeae*), which had been previously described by Roesler et al. (2006). The PCR was performed in a 25-µL final volume solution containing 5 µL of target DNA (approximately 10 ng/µL), 12.5 µL of GoTaq Green Master Mix 2X (Promega), 0.5 µL of each primer (10 µM), and 6 µL of nuclease-free water. *Prototheca ciferrii* was identified when the 450-bp fragments of the internal amplification control (Proto 18–4f and Proto 18–4f) and *P. ciferrii*-specific amplicon (150 bp; Proto 18–4f and PZGT 1/r) were detected. Similarly, *P. bovis* was identified by the amplification of the fragment control (450-bp) and *P. bovis*-specific amplicon (165 bp; Proto 18–4f and PZGT 2/r). Finally, the *P. blaschkeae*-specific amplicon (126 pb; PZGT 3-IK/ and PZGT 3/r; Roesler et al., 2006).

**Biofilm Formation Phenotypes**

The *Prototheca* spp. biofilm formation capacity was evaluated according to Morandi et al. (2016) with some modifications. The *Prototheca* isolates were inoculated in Tryptic (Trypticase) Soy Broth (TSB; Kasvi) supplemented with 0.6% of yeast extract (Kasvi) and incubated at 37°C for 48 h. The isolates were standardized to a 0.5 McFarland scale in 0.9% sterile saline solution. Afterward, 200-µL aliquots of each isolate were transferred in triplicate to sterile 96-well flat-bottomed polyethylene microplates, followed by incubation at 37°C for 24 h without agitation. The TSB broth plus 0.6% yeast extract without inoculum was used as sterility control. *Staphylococcus aureus* strain ATCC 25923 and *Prototheca* spp. control strains were used as a positive control.

After incubation, the culture medium was aspirated, and the microplates were washed twice with 200 µL of sterile Milli-Q water. The biofilm was heat-fixed at 60°C for 2 h and stained with 250 µL of crystal violet (0.1%) for 15 min, and the violet content was removed by aspiration. Then, the stained biofilm was immediately washed twice using 200 µL of sterile Milli-Q water, and the plates were allowed to dry at room temperature for 1 h. The stain that was bound to the biofilm was solubilized by the addition of 200 µL of 33% acetic acid.

The biofilm mass was measured using a spectrophotometer at 620 nm. Results were expressed in optical density (OD). The mean OD of the isolate was compared with the mean OD of the negative control (ODNC) to determine the capacity of the isolate of *Prototheca* spp. to produce biofilm. The isolates were classified as nonproducer biofilm OD ≤ × ODNC, weak (ODNC < OD ≤2 × ODNC), moderate (2 × ODNC < OD ≤4 × ODNC), and strong biofilm producer (OD >4 × ODNC) according to Stepanović et al. (2003).

**PHMB and Disinfectant Susceptibility Assays**

**Minimal Inhibitory Concentration.** Antimicrobial susceptibility analysis of povidone-iodine (PVP-I; Sigma-Aldrich), chlorhexidine gluconate (CHG; Polyorganic Tecnologia), sodium hypochlorite (NaClO; Rioquimica), sodium dichloroisocyanurate (NaDCC; Merck Sharp & Dohme), and PHMB (Polyorganic Tecnologia) were performed against all 87 *Prototheca* spp. isolates using the broth microdilution assay, according to the CLSI (2017) guidelines (document M27-A4).

To perform the MIC analyses, 96-well microplates were prepared with the PHMB or those above-mentioned disinfectants by manual evaluation. Briefly, a solution with a final volume of 200 µL containing brain heart infusion broth (BHI) and disinfectants was added to the first column of each plate. Then the PHMB and disinfectants were subjected to serial dilution in BHI broth, at the following range of concentrations: (a) PVP-I: 3,200–3.12 µg/mL; (b) CHG: 32–0.03 µg/mL; (c) NaClO: 2,800–2.73 µg/mL; (d) NaDCC: 1,400–1.36 µg/mL; (e) PHMB: 32–0.031 µg/mL.
After incubation of Prototheca spp. in BHI broth for 48 h, the isolates were standardized at 0.5 McFarland (1.0–5.0 × 10⁶ cfu/mL) in a 0.9% sterile saline and then diluted at a ratio of 1:50, and again at a ratio of 1:20, in BHI broth. Then, 100-µL aliquots of each isolate were applied to each well of microplates previously prepared with the PHMB or disinfectants, resulting in a 1:1 ratio of inoculum and disinfectant. The microplates were homogenized for 10 min at 200 rpm at room temperature, followed by incubation at 37°C for 48 h without agitation. The negative control was formed by wells containing only BHI broth, and the reference strains, used as isolate+BHI (1:1) without disinfectant, were considered the positive control. All analyses were performed in duplicate. When the duplicate presented divergent values, the evaluations were repeated. The MIC values were recorded by visual inspection by a single evaluator. For PHMB or each disinfectant, MIC₅₀ and MIC₉₀ values were determined at the concentration required to inhibit the visible growth of 50 and 90% of the isolates, respectively.

**Minimal Algicidal Concentration.** The minimal algicidal concentration (MAC) was determined after obtaining the MIC results. Thus, 20 µL were aspirated from the wells without visible growth, as well as the last well with a visible growth, and applied onto Sabouraud agar, followed by incubation at 37°C for 48 h. By visual inspection, MAC was shown to be the lowest concentration that completely wiped out the isolated growth in Sabouraud media. The MAC₅₀ and MAC₉₀ values were determined, which corresponded to the concentration necessary to inactivate 50 and 90% of the isolates, respectively.

**Statistical Analysis**

The effect of PHMB or disinfectant on MIC and MAC values was evaluated using an ANOVA model, and differences of least squares means (Leite et al., 2021). All disinfectants or all PHMB were compared among each other (all against all). An evaluation was also performed considering groups of disinfectants based on the main active component (i) biguanides (PHMB and CHG), (ii) chlorinated compounds (NaClO and NaDCC), and (iii) iodophors (PVP-I). In addition, a Kaplan-Meier survival analysis was conducted to determine the antimicrobial effect of PHMB or disinfectants against Prototheca isolates. For this, the antimicrobial concentration was used as a time variable, and inhibition of bacterial growth was used as an event. When isolates were not inhibited at the MIC₅₀ and MAC₉₀ concentrations, they were considered resistant and censored by the statistical test. The Kaplan-Meier survival curves were compared by the log-rank test. The statistical analysis was evaluated by RStudio software 1.4.1717 (RStudio), using survival, dplyr, and psych packages. All differences were considered significant for \( P < 0.05 \) values.

**RESULTS**

**MALDI-TOF MS and PCR Identification**

The MALDI-TOF MS allowed the identification of all 87 isolates of *Prototheca* spp. (100% at the genus level and 86% at the species level). Furthermore, PCR permitted to confirm the identification of 97.7% (n = 85) as *P. bovis* and 2.3% (n = 2) as *P. blaschkeae*. No mastitis-causing isolates were identified as *P. ciferrii* when using both methods of identification.

**Biofilm Formation Phenotypes**

High OD variability was observed in *P. bovis* biofilms: 63.5% of the isolates (n = 54/85) were classified as strong (OD: 0.572 ± 0.122), 28.3% (n = 24/85) as moderate (OD: 0.276 ± 0.082), and 8.2% (n = 7/85) as weak (OD: 0.139 ± 0.037) biofilm producers (Table 1). The reference strain of *P. bovis* (LZ-5) was classified as a strong (OD: 0.242 ± 0.057) biofilm producer, whereas *P. blaschkeae* (RZIII-3) and *P. ciferrii* (RZI-3) reference strains were both classified as weak biofilm producers (OD: 0.094 ± 0.004 and 0.142 ± 0.029, respectively), concerning the negative control. Only one *P. blaschkeae* isolate was classified as a moderate biofilm producer and the other as a weak biofilm producer.

**Antimicrobial Activity of PHMB and Disinfectants Against *P. bovis* Isolates**

**Minimal Inhibitory Concentration.** The PHMB (MIC₅₀ ≥1.0 µg/mL; MIC₉₀ ≥2.0 µg/mL) and the CHG (MIC₅₀ and MIC₉₀ ≥2.0 µg/mL) values presented the highest antimicrobial activity against *P. bovis* isolates (n = 85). The MIC₅₀ and MIC₉₀ values obtained for PHMB were lower (\( P < 0.0001 \)) when compared with NaDCC (MIC₅₀ ≥700 µg/mL and MIC₉₀ ≥1,400 µg/mL) and NaClO (MIC₅₀ and MIC₉₀ ≥2,800 µg/mL). In addition, PHMB presented higher antimicrobial activity (\( P < 0.0001 \)) than PVP-I (MIC₅₀ and MIC₉₀ ≥3,200). However, no difference was observed between antimicrobial concentration values for CHG and PHMB (\( P = 0.9988 \); Table 2).

Kaplan-Meier survival curves of PHMB and CHG were heterogeneous when compared with the other dis-
infectants \((P < 0.0001)\) for the log-rank test. However, Kaplan-Meier survival curves obtained for PHMB and CHG were homogeneous \((P < 0.0001)\) for the log-rank test; Figure 1).

**Minimal Algicidal Concentrations.** The MAC analysis showed that PHMB \((\text{MAC}_{50} \geq 2.0 \, \mu g/mL; \text{MAC}_{90} \geq 4.0 \, \mu g/mL)\) and CHG \((\text{MAC}_{50} \text{ and MAC}_{90} \geq 2.0 \, \mu g/mL)\) can inactivate the \(P. \text{bovis}\) isolates at low concentrations. Sodium dichloroisocyanurate \((\text{MAC}_{50} \geq 700 \, \mu g/mL; \text{MAC}_{90} \geq 1,400 \, \mu g/mL)\) and \(\text{NaClO} \) \((\text{MAC}_{50} \text{ and MAC}_{90} \geq 2,800 \, \mu g/mL)\) present high antimicrobial values \((P < 0.0001)\) when compared with PHMB. Similar to the MIC analysis, PHMB showed higher antimicrobial activity \((P < 0.0001)\) than PVP-I \((\text{MAC}_{50} \text{ and MAC}_{90} \geq 3,200 \, \mu g/mL)\). No difference \((P = 0.9967)\) was observed between the antimicrobial concentrations of PHMB and CHG (Table 3).

Polyhexamethylene biguanide and CHG presented heterogeneous survival curves \((P < 0.0001)\) for the log-rank test when compared with the other disinfectants, whereas PHMB and CHG survival curves were homogeneous \((P < 0.0001)\) for the log-rank test; Figure 2).

**Evaluation of Antimicrobial Activity by Disinfectant Groups.** Concerning the evaluation based on the groups of disinfectants, biguanides (CHG and PHMB) presented lower MIC\(_{90}\) \((\geq 2.0 \, \mu g/mL)\) and MAC\(_{90}\) \((\geq 2.0 \, \mu g/mL)\) than the chlorinated group \((\text{NaDCC and NaClO}; \text{MIC}_{90} \text{ and MAC}_{90} \geq 1,400 \, \mu g/mL; P < 0.0001)\), and iodophors group \((\text{PVP-I}; \text{MIC}_{90} \text{ and MAC}_{90} \geq 3,200 \, \mu g/mL; P < 0.00001)\). The MIC\(_{90}\) and MAC\(_{90}\) values obtained for the chlorinated group were lower than those observed for the iodophors group \((P < 0.0001)\) for the log-rank test. The survival curves of the disinfectant groups were heterogeneous \((P < 0.0001)\) for the log-rank test).

**Antimicrobial Activity of PHMB and Disinfectants Against \(P. \text{blaschkeae}\) Isolates.** For \(P. \text{blaschkeae}\) isolates \((n = 2)\), the MIC results showed that PHMB \((\geq 0.5 \, \mu g/mL)\) and CHG \((\geq 0.5 \, \mu g/mL)\) presented lower concentrations than the other disinfectants: NaDCC \((\geq 350 \, \mu g/mL)\), NaClO \((\geq 1,400 \, \mu g/mL)\), and PVP-I \((\geq 800 \, \mu g/mL)\). Similar to the MIC results, PHMB \((\geq 1 \, \mu g/mL)\) and CHG \((\geq 1 \, \mu g/mL)\) presented the lowest MAC values against \(P. \text{blaschkeae}\) isolates.

**DISCUSSION**

The present study aimed to evaluate the in vitro antimicrobial activity of PHMB against \(P. \text{bovis}\) biofilm producer causing mastitis. To our knowledge, this is the first study that evaluated the antimicrobial activity of PHMB against \(P. \text{bovis}\). We observed that \(P. \text{bovis}\) isolates were classified as strong biofilm producers. In addition, PHMB and CHG showed high antimicrobial activity against \(P. \text{bovis}\) isolates, with lower MIC and MAC values when compared with the common disinfectants used for pre- and post-dipping, such as PVP-I, NaClO, and NaDCC.

All mastitis-causing \(P. \text{bovis}\) isolates displayed biofilm production capacity. Most \(P. \text{bovis}\) isolates \((63.5\%)\) were classified as strong biofilm producers. In addition, PHMB and CHG showed high antimicrobial activity against \(P. \text{bovis}\) isolates, with lower MIC and MAC values when compared with the common disinfectants used for pre- and post-dipping, such as PVP-I, NaClO, and NaDCC.

**Table 1.** Biofilm formation phenotype scores for \(Prototheca \text{bovis}\) and \(Prototheca \text{blaschkeae}\) isolates from clinical and subclinical mastitis cases in dairy cows

<table>
<thead>
<tr>
<th>Biofilm production</th>
<th>(Prototheca \text{bovis})</th>
<th>(Prototheca \text{blaschkeae})</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>%</td>
<td>OD</td>
</tr>
<tr>
<td>Weak</td>
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</tr>
<tr>
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<td>3.5</td>
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<tr>
<td>Moderate</td>
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<td></td>
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<tr>
<td>Total</td>
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<tr>
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<tr>
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<tr>
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</tr>
<tr>
<td>CM</td>
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<tr>
<td>SCM</td>
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<td>41.2</td>
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</table>

\(^1\)Biofilm production evaluation of \(P. \text{bovis}\) \((n = 85)\) and \(P. \text{blaschkeae}\) \((n = 2)\) isolates from clinical and subclinical mastitis cases. The biofilm production capacity of the isolates was classified according to Stepanović et al. (2003). The isolates were classified as weak \((\text{OD}_{NC} < \text{OD} \leq 2 \times \text{OD}_{NC})\), moderate \((2 \times \text{OD} < \text{OD} \leq 4 \times \text{OD}_{NC})\), or strong \((\text{OD} > 4 \times \text{OD}_{NC})\) biofilm producers.

\(^2\)OD = the average of optical density values observed for each isolate in triplicate.

\(^3\)CM = clinical mastitis.

\(^4\)SCM = subclinical mastitis.
is similar to the results reported (88.0%; 37/42) by Morandi et al. (2016). Another study that evaluated the biofilm production ability of 10 strains of *P. bovis* showed that the isolates were moderate (40%, *n* = 4) and weak (60%, *n* = 6) biofilm producers (Gonçalves et al., 2015). Thus, it might be inferred that *P. bovis* present a high biofilm production ability, which may allow the permanence of this microorganism in the environment or in milking equipment (Libisch et al., 2022). Neither of the *P. blaschkeae* isolates were considered strong biofilm producers. However, because of the small number of *P. blaschkeae* isolates evaluated in our study, it was not possible to evaluate the differences in biofilm formation capacity between the 2 *Prototheca* species.

In the present study, the high variability of OD results obtained from the biofilms produced by *P. bovis* suggest that the type of strain can influence the biofilm production (Morandi et al., 2016).

Chlorhexidine and PHMB displayed the highest antimicrobial activities (low MIC and MAC values). This is the first report of PHMB activity against *P. bovis*. Chlorhexidine is a disinfectant widely used in dairy herds as a teat disinfectant (Schwenker et al., 2022). Our results showed that CHG can inhibit and inactivate *Prototheca* spp. isolates at the lowest concentrations evaluated in this study (MIC<sub>90</sub> and MAC<sub>90</sub> ≥2 µg/mL). These concentrations were lower than the minimal microbicide concentration values reported by Melville et al. (2002; ≥100 µg/mL) and by Krukowski et al. (2013; 480–195 µg/mL). Nevertheless, Sobukawa et al. (2011) reported lower MAC<sub>90</sub> values (≥0.003 µg/mL) to inactivate *Prototheca* spp. than the results reported in this study. This powerful antimicrobial activity can be attributed to the CHG’s ability to rapidly degrade the organelles present in the *Prototheca* spp. cytoplasm (Melville et al., 2002). The low antimicrobial concentrations of CHG observed in our study suggest that it could be used as an option for teat disinfection for protothecal mastitis control, although low susceptibility or resistance to CHG has been reported (de Frutos et al., 2017).

In our study, PHMB inhibited and inactivated *P. bovis* isolates at lower concentrations (MIC<sub>90</sub> ≥2 µg/mL and MAC<sub>90</sub> ≥4 µg/mL). Low PHMB concentrations against mastitis-causing *Staph. aureus* were reported in 2 previous studies (Kamaruzzaman et al., 2016; Leite et al., 2021). Despite *Staph. aureus* being a completely different pathogen from *Prototheca*, both studies described the antimicrobial activity of PHMB against *Staph. aureus* isolates that present biofilm formation capacity. For example, Leite et al. (2021) observed MIC<sub>90</sub> values of PHMB ≥0.5 µg/mL, and Kamaruzzaman et al. (2016) MIC<sub>90</sub> values of PHMB ≥4 µg/mL against mastitis-causing *Staph. aureus*. Our algicidal concen-
Concentrations were lower than the MAC values reported for guanidine (≥10 µg/mL), a compound that has a similar structure to PHMB (Alves et al., 2017), but is not a polymer, suggesting that the polymer structure aids potency.

The PHMB groups can interact with the phospholipid head groups in the membrane of the microorganism, allowing the PHMB to enter the cell. Polyhexamethylene biguanide selectively binds to DNA and condenses the chromosomes, which blocks the DNA replication process (Chindera et al., 2016). Although we did not evaluate the direct effect of PHMB on the biofilm produced by *Prototheca* spp. isolates, theoretically, the PHMB antibiofilm activity could explain the higher antimicro-

**Figure 1.** Kaplan-Meier survival curves of minimal inhibitory concentration results obtained for mastitis-causing *Prototheca bovis* isolates against (a) polyhexamethylene biguanide; (b) chlorhexidine gluconate; (c) sodium hypochlorite; (d) sodium dichloroisocyanurate; (e) iodine-polyvinylpyrrolidone; and (f) disinfectants groups.
bial activity against biofilm-producing *P. bovis* isolates observed in our study, as reported by Kamaruzzaman et al. (2017). In this instance, PHMB could serve as an option for disinfecting milking equipment and for pre- and post-teat dipping.

Our results showed that NaDCC presented lower MIC and MAC values than those observed for NaClO. The lower MIC values obtained for NaDCC may be related to the further release of HClO because NaDCC acts as a HClO-reservoir, maintaining the antimicrobial activity for a longer period (Chan et al., 2020). Finally, PVP-I presented the least potent antimicrobial activity (MIC<sub>90</sub> and MAC<sub>90</sub> ≥3,200 µg/mL) against *Prototheca* spp. isolates. In contrast, Hsieh et al. (2020) reported that lower concentrations of PVP-I (MAC<sub>90</sub> = 48.83–390.63 µg/mL) can inactivate *Prototheca* spp. isolates. These discrepant results can be attributed to the different origins of the isolates. In this case, the environment, climatic conditions, and epidemiology can influence on the antimicrobial resistance (Morandi et al., 2016). Additionally, it is difficult to precisely measure the amount of bound and free iodine on PVP-I by in vitro studies, which hinder the interpretation of the results (Lepelletier et al., 2020).

The major limitation of this study was the lack of a standard method for the evaluation of *Prototheca* spp. susceptibility against disinfectants. The antimicrobial susceptibility assays for *Prototheca* spp. are usually performed to determine the minimal microbicidal concentration, using the macro dilution method (Salerno et al., 2010; Sobukawa et al., 2011; Gonçalves et al., 2015). However, the methodologies in the literature present some divergences, such as inoculum and antimicrobial preparation. For the present study, we selected the broth microdilution method following the CLSI (2017) document M27-A4 for yeasts, as conducted by Jagielski et al. (2018). The broth microdilution method allows the MIC and MAC values to be determined, although differences in methodologies (e.g., inoculum preparation) can influence the results, and direct comparisons between studies become limited.

Furthermore, because PHMB has been used for skin wound treatment (de Mattos et al., 2019), further in vivo studies should be performed to evaluate the PHMB efficacy for teat disinfection and prevention of bovine mastitis caused by *Prototheca* spp.

**CONCLUSIONS**

*Prototheca bovis* isolates showed a high ability to produce biofilm, which may contribute to the majority presence of this species in bovine mastitis cases. Also, we observed that PHMB and CHG can inhibit and inactivate *P. bovis* isolates at lower concentrations than
other disinfectants. This result suggests that PHMB can be a potential alternative for use as a teat disinfectant and a milking equipment disinfection. However, further studies regarding toxicity, residues in milk, and in vivo antimicrobial effect should provide useful information to verify the benefits and safety of PHMB use in dairy herds.

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