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Biofilm-producing ability and efficiency of sanitizing agents against *Prototheca zopfii* isolates from bovine subclinical mastitis

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ABSTRACT

The objectives of the present study were to evaluate (1) the capacity of the microalga *Prototheca zopfii* isolated from subclinical bovine mastitis cases to form biofilms; and (2) the resistance of these isolates to sanitizing agents. Ten isolates of *P. zopfii* from cows with subclinical mastitis (somatic cell count $>200 \times 10^3$ cells/mL), distributed in 5 dairy farms, were evaluated for their capacity to form biofilms in polystyrene microplate assays and stainless steel coupons, at 25°C and 37°C ± 1°C. *Prototheca zopfii* were isolated from milk samples via microbiological culture and analyzed by 18S rRNA gene sequencing. Biofilm formation on the coupons was observed by scanning electron microscopy. The resistance to sanitizing agents was assessed using the biofilm-forming *P. zopfii* isolates in stainless steel coupon assays, which were subjected to 3 sanitizers: peracetic acid, sodium hypochlorite, and iodine solution. To evaluate resistance to the sanitizers, the minimum inhibitory concentration (MIC) technique was performed using decreasing concentrations of the sanitizing agents (20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, and 0.019 g/L). After inoculating the isolates, all concentrations were evaluated at 3 distinct incubation periods (24, 48, and 72 h) to assess the effect of incubation time on the MIC. Using the polystyrene microplate assays, 1 isolate showed weak biofilm production, 5 moderate, and 4 strong, when incubated at 25°C ± 1. For isolates incubated at 37°C ± 1, 6 showed weak biofilm production and 4 moderate. All *P. zopfii* isolates (n = 10) had the capacity to form biofilms on stainless steel coupons. The longer the incubation period of the *P. zopfii* isolates at different dilutions, the greater the concentrations of sanitizer needed to prevent growth of the microalgae under the tested conditions. We detected a significant effect of sanitizer and time of incubation (24, 48, and 72 h) on MIC values against *P. zopfii* isolates. The isolates were sensitive in vitro to peracetic acid ($MIC_{90} \geq 0.019$ g/L), sodium hypochlorite ($MIC_{90} \geq 0.312$ g/L), and iodine solution ($MIC_{90} \geq 0.625$ g/L), after 24 h of incubation (where MIC_{90} = concentration needed to inhibit 90% of isolates). Of the tested sanitizers, peracetic acid had the greatest efficiency against *P. zopfii.* We conclude that *P. zopfii* isolates are capable of biofilm production, which may contribute to their persistence in a milking and dairy environment.

Key words: subclinical mastitis, biofilm-producing isolate, sanitizer, *Prototheca zopfii*

INTRODUCTION

The occurrence of mastitis caused by the microalga *Prototheca zopfii* has been described in several countries (Corbellini et al., 2001; Möller et al., 2007; Osumi et al., 2008; Marques et al., 2010b; Ricchi et al., 2010; Pieper et al., 2012). The frequency of bovine protothecal mastitis caused by *P. zopfii* has been increasing worldwide, which may represent a serious problem due to the inherent resistance to routine therapy of these microalgae (Cunha et al., 2010; Pieper et al., 2012; Ricchi et al., 2010). This resistance is associated with the capacity of the microalgae to infect and survive in macrophages and to invade mammary tissue, making them responsible for a persistent infection with intermittent shedding of *P. zopfii* in milk (Marques et al., 2006).

The treatment of mastitis caused by *Prototheca* spp. with antimicrobials produces only temporary improvement of clinical signs due to the low rate of cure in vivo, and because of this, the causative agent is not eliminated (Costa et al., 1996). Therefore, culling cows infected with *P. zopfii* is one of the recommended control measures to reduce the disease (Jánosi et al., 2001).

The main risk factors associated with mastitis caused by *P. zopfii* in dairy herds are transmission between

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infected and healthy cows during milking (Jánosi et al., 2001), the use of previous or intensive treatment with antibiotics (Corbellini et al., 2001; Pieper et al., 2012), and deficiencies in hygiene during premilking preparation of dairy cows. Furthermore, *P. zopfii* survive in feces and are able to contaminate practically every environment that comes in contact with bovine feces. In the same manner, feces of calves fed milk contaminated with *P. zopfii* may be an important source of environmental contamination (Jánosi et al., 2001).

Prototheca zopfii shows high resistance to heat treatment (Melville et al., 1999; Lassa et al., 2011). Such resistance represents a risk to the consumption of milk and dairy products, because heat treatment is used to eliminate many pathogenic agents (Melville et al., 1999). Additionally, cases of human protothecosis caused by *P. zopfii*, *Prototheca blaschkeae*, and *Prototheca wickerhamii* isolated from cows with subclinical mastitis have been reported (Melville et al., 1999; Marques et al., 2006; Lass-Flörl and Mayr, 2007).

In the milking and dairy environments, surfaces such as stainless steel, glass, rubber, and polypropylene can be contaminated by microorganisms. Subsequently, the microorganisms may multiply and produce biofilms (Pompermayer and Gaylarde, 2000; Davies, 2003). The time required for multiplication is an important factor in biofilm production and is associated with the frequency of equipment cleaning (Gibson et al., 1999). Marques et al. (2010b) suggested that microalgae form cellular clusters and, in this manner, have resistance to heat treatment. Davies (2003) reported that cases of chronic infection are generally due to resistance of biofilm-producing bacteria to conventional antimicrobial treatments.

Considering the difficulty of treating mastitis caused by *Prototheca* spp., the hypothesis of the present study is that the *P. zopfii* isolates, with biofilm production capacity, are more resistant to sanitizers such as peracetic acid, sodium hypochlorite, and iodine solution commonly used in predipping. To our knowledge, no previous studies have evaluated the ability of *P. zopfii* isolated from bovine mastitis to produce biofilms. The objectives of the present study were to evaluate (1) the capacity of *P. zopfii* isolates from subclinical mastitis cases to produce biofilm; and (2) the resistance of *P. zopfii* isolates to sanitizing agents.

MATERIALS AND METHODS

Sampling and Isolation of Prototheca

A total of 285 dairy cows, distributed in 21 dairy farms from Pirassununga (São Paulo State, Brazil) were enrolled in this study. Mammary quarter milk samples (n = 1,140) were subjected to screening for subclinical mastitis cases caused by *Prototheca* spp. Subclinical protothecal mastitis was defined as a lack of clinical signs, isolation of *Prototheca* spp., and SCC $>200 \times 10^3$ cells/mL. On the other hand, mammary quarters were considered healthy when they had no isolation of microorganisms following a 72-h incubation after milk sampling and SCC $<200 \times 10^3$ cells/mL (Bradley and Green, 2005). The SCC was determined by flow cytometry using a Fossomatic FC (Fossomatic FC, Rellingen, Germany).

Prototheca spp. isolates were identified according to Oliver et al. (2004). Briefly, 0.01 mL of milk was spread onto a quadrant of a blood agar plate (Becton, Dickinson and Co., Sparks, MD) and incubated aerobically at 37°C ± 1°C for up to 48 h. After growth on blood agar, the isolates were streaked on a plate of Sabouraud dextrose agar (Oxoid Ltd., Basingstoke, UK) and incubated at 37°C ± 1°C for up to 72 h. After the incubation period, bacterial colonies were classified according to their morphological features (color, appearance, size, and presence of hemolysis), and counts (cfu/mL) of *Prototheca* spp. were determined. Colonies grown in blood agar and Sabouraud dextrose that had creamy and white or greyish-white characteristics were identified as *Prototheca* spp. by light microscopy. *Prototheca* spp. identification was based on colony morphology and on smears by Gram staining and methylene blue technique (Oliver et al., 2004). The isolates of *Prototheca* spp. were cryopreserved in triplicate at −20°C in tryptone soy broth (**TSB**; Oxoid Ltd.) supplemented with 0.6% yeast extract (HiMedia Laboratories, Mumbai, India) and 20% sterile glycerol (Sigma Aldrich, St. Louis, MO), for further analysis.

DNA Extraction and PCR

Before DNA extraction, all *Prototheca* spp. isolates were cultured overnight on TSB supplemented with 0.6% yeast extract (HiMedia Laboratories) at 37°C under aerobic conditions to evaluate the purity of the colonies. Subsequently, a single *Prototheca* spp. colony was resuspended in 1 mL of autoclaved Milli-Q water (Millipore Corp., Bedford, MA); a 0.5-mL aliquot from the autoclaved water volume was further used during the extraction process. The aliquot was heat shocked twice (99°C × 10 min) and then rapidly frozen. The microbial DNA was extracted by using the phenol-chloroform method (Sambrook et al., 1989). Briefly, 500 μL of the aliquot was mixed with an equal amount of phenol-chloroform (Sigma Aldrich, São Paulo, Brazil) and then centrifuged at 14,000 × *g* for 10 min at

4°C. After centrifugation, the supernatant was removed and the pellet was again mixed with an equal volume of chloroform. The aliquot was re-centrifuged under the same conditions. Sodium acetate at 1 *M* (1/10 of the collected volume) was added to a new sterile tube with supernatant and cold isopropanol (equal volumes; Merck, Sharp & Dohme, São Paulo, SP, Brazil) in order, and centrifuged at 14,000 × *g* for 10 min at 4°C. All of the supernatant was carefully discarded so as to not disrupt the pellet, and the tube was left to dry on clean paper for 5 min. The pellet was then washed with 70% ethanol (Merck, Sharp & Dohme, São Paulo, SP, Brazil) and centrifuged at 14,000 × *g* for 10 min at 4°C. The supernatant was discarded and the tube left to dry on paper. Finally, the extracted DNA was hydrated in 50 μL of DNase-free water and incubated at 65°C for 1 h, and stored at −20°C until further use. The DNA was quantified using a spectrophotometer (Thermo Scientific, Toronto, ON, Canada) and its integrity observed [in the 0.6 % agarose gel (TAE 1×) for the presence of a single band of DNA; up to 5 μL of gDNA was applied in each well and ran at 120 V for 30 min; Promega, Madison, WI].

The amplification reactions of DNA were done with final volumes of 25 μL containing 12.5 μL of 2× Master Mix (Promega), 1 μL of each primer (10 μ*M*), 0.5 μL of $MgCl_2$ (1.5 m*M*), 1 μL of deoxynucleotide triphosphates (dNTP, 0.2 m*M*; Invitrogen, São Paulo, SP, Brazil), 0.2 μL of GoTaq (Invitrogen), 50 ng of DNA, and a final volume of Ultrapure water (Sigma-Aldrich, São Paulo, Brazil). The primers were the same as those designed by Huss et al. (1999) for the 18S rDNA gene (forward: 5′-WACCTGGTTGATCCTGCCAGT-3′; reverse: 5′-GATCCTTCYGCAGGTTCACCTAC-3′; W and Y represent the possibility of nitrogen bases A or T and C or T in the sequencing of *Prototheca* spp., respectively). The amplification reaction was performed in a thermal cycler (Master Cycler, Eppendorf, Hamburg, Germany), programmed for 1 cycle at 95°C (4 min), followed by 35 cycles at 95°C (1 min), 54°C (1 min), and 72°C (2.5min), and 1 final cycle at 72°C (5 min), according to the standardized protocol in Marques et al. (2008).

Prothoteca zopfii ATCC 16533 was used as a positive control and Ultrapure water as a negative control. The PCR product underwent electrophoresis at 110 V for 30 min on a PowerPac Basic (BioRad, Hercules, CA), using 1% agarose gel (Promega) prepared using Tris-acetate-EDTA buffer, and stained with SYBR Safe DNA gel stain (Invitrogen). The 100-bp DNA Ladder (Life Technologies, Gaithersburg, MD) marker was used as a molecular weight indicator. Gels were photographed with the Gel Documentation System BioSens SC750 (Bio-Tech Co. Ltd., Shanghai, China) using the GeneScope V1.73 program.

Sequencing Reactions and Precipitation Protocols

Specific amplification bands (about 1,800 bp) were cut from the gel and purified using the GFX TM PCR DNA and Gel Band Purification kit (GE Healthcare, Munich, Germany), according to the manufacturer's specifications. The sequencing reactions were performed on a Veriti thermal cycler (Applied Biosystems, Paisley, UK), using the following cycle conditions: 95°C for 4 min, and 25 cycles at 95°C for 20 s, 50°C for 15 s, and 60°C for 4 min. The PCR products were precipitated using absolute ethanol, EDTA, and sodium acetate. Formamide was added to separate the double helix before sequencing. The sequencing reaction was obtained using the Big-Dye Terminator v3.1 Cycle Sequencing kit on the ABI3500 Genetic Analyzer (Applied Biosystems, Foster City, CA), according to the manufacturer's specifications.

The electrospherograms obtained from the sequencing reaction on the ABI3500 platform were edited using the Genious R6 software (Biomatters Ltd., Auckland, New Zealand), manually removing the low quality initial and final bases [high-quality (HQ) reads <30], as well as the undefined bases due to background throughout the sequence. The edited sequences were then aligned, when possible, using the identity criteria. The final consensus sequences generated by this process were compared against the National Center for Biotechnology Information nucleotide database, using the BLAST tool and the Megablast algorithm, for species identification (http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi).

Prototheca spp. Biofilm Production Assays

Polystyrene Microplates. The capacity of the *Prototheca* spp. isolates (n = 10) to produce biofilm was determined according to the procedure described by Stepanović et al. (2003). Each isolate was briefly resuspended in TSB supplemented with 0.6% yeast extract (HiMedia Laboratories), incubated at 37°C ± 1°C for 24 h, and diluted to 10^8 cells/mL (0.5 on MacFarland scale).

Aliquots of 200 μL of each isolate, in triplicate, were transferred to 96-well flat-bottomed sterile polystyrene microplates and incubated simultaneously at 25°C and 37°C ± 1°C for 24 h. After incubation, the microplates were agitated, fixed, stained with crystal violet for 5

min, dried, and resolubilized with 33% (vol/vol) glacial acetic acid. The biofilm production on the microplates was measured using an ELISA reader (Labsystems Inc., Helsinki, Finland) fixed at 620 nm, and the results were expressed as optical density (**OD**) values. The negative control triplicates contained only sterile TSB, were prepared as described by Stepanović et al. (2003), and were used as reference to determine the capacity of the *Prototheca* spp. isolates to produce biofilm. The mean values of the negative controls ($\mathbf{OD_{NC}}$; *Staphylococcus epidermidis* ATCC 12228) were 0.1246 ± 0.0063 and 0.1754 ± 0.0061 for samples incubated at 25°C and 37°C ± 1°C, respectively. Isolates of *Prototheca* spp. were considered biofilm producers when the OD values were 3 times greater than the standard deviation (**SD**) of the OD_{NC} mean value. A positive control was also included (*Staphylococcus epidermidis* ATCC 35983). The mean values of the positive controls ($\mathbf{OD_{PC}}$) were 0.3873 ± 0.0436 and 0.4483 ± 0.0188, which were incubated at 25°C and 37°C ± 1, respectively. Briefly, the cutoff OD was defined as 3 SD above the mean OD of the negative control. In addition, the capacity of the isolates to produce biofilm was classified as weak ($OD_{NC} < OD \leq 2 \times OD_{NC}$), moderate ($2 \times OD_{NC} < OD \leq 4 \times OD_{NC}$), or strong ($OD > 4 \times OD_{NC}$). Differences in the degree of biofilm formation by the triplicates were examined using the Friedman test, followed by the Wilcoxon-signed rank test. *P*-values of 0.05 were considered significant (Stepanović et al., 2003).

Scanning Electron Microscopy. The capacity to produce biofilm on the stainles steel was evaluated by scanning electron microscopy, according to protocols described by Chandra et al. (2008) and Kadouri and O'Toole (2005). Two stainless steel coupons (1.0 × 1.0 cm), one for each isolate, were introduced into the inferior portion of the wells of a flat-bottomed sterile polystyrene microplate (24 wells). Then, 2 mL of suspension of each isolate was added, in duplicate, to the wells and incubated at 25°C and 37°C ± 1°C for 48 h without agitation. After incubation, the stainless steel coupons were washed with phosphate buffer to remove nonadherent cells and fixed in modified Karnovsky glutaraldehyde solution for at least 3 h. Next, they were washed with 0.05 *M* cacodylate buffer. The coupons were subsequently washed with autoclaved distilled water and submitted to dehydration using increasing concentrations of acetone (25, 50, 75, and 100%), remaining in each concentration for 10 min. After fixation and dehydration, the acetone evaporated and the coupons were refrigerated (4°C ± 1) until visualization using scanning electron microscopy (SEM-Hitachi TM300, Hitachi, São Paulo, Brazil).

Profile of Resistance to Sanitizers and MIC

The resistance profile to sanitizing agents was determined on *Prototheca* spp. isolates that were shown to produce biofilm in the stainless steel coupon assays (n = 10). Three sanitizers were evaluated: peracetic acid solution (77240, Sigma-Aldrich Co., Steinheim, Germany), iodine solution (38060, Sigma-Aldrich Co.), and sodium hypochlorite (425044, Sigma-Aldrich Co., St. Louis, MO).

The MIC technique was used to determine the resistance profile of *Prototheca* spp. isolates to the sanitizers. This technique quantitatively measures the in vitro activity of an antimicrobial agent against a certain microorganism (NCCLS, 2000). To perform the test, 12 tubes were prepared with culture medium (TSB supplemented with yeast extract at 0.6%), to which decreasing concentrations of the sanitizing agents (20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, and 0.019 g/L) were added. The tubes were then inoculated with a standard suspension of 100 μL of each tested isolate. After incubation at 30°C ± 1°C, 3 readings (at 24, 48, and 72 h) were taken by visual comparison of the tubes. All isolates were evaluated, in duplicate, to determine in vitro resistance to sanitizers, totalling 720 assays. Furthermore, for each tested isolate, 1 negative control (culture medium and sanitizing agent) and 1 positive control (strain ATCC 16533 of *Prothoteca pzofii* and TSB) were included. The MIC was determined as the lowest concentration of a sanitizing agent that prevented visible growth (without turbidity) of the microalgae (NCCLS, 2000). To confirm the dilutions that prevented growth of *Prototheca* spp., 100 μL of each dilution was inoculated in Sabouraud dextrose agar according to Marques et al. (2006). Dilutions that did not show turbidity (no growth) in vitro were considered inhibitors (Marques et al., 2010a).

Results for peracetic acid, sodium hypochlorite, and iodine solutions were summarized by calculating the MIC_{50} and MIC_{90} as described by Cortinhas et al. (2013), where MIC_{50} and MIC_{90} indicate MIC required to inhibit growth of 50% and 90% of the isolates, respectively. Survival analysis was used to determine whether the sanitizers had different MIC. The range of sanitizer concentrations tested was used as the time variable in the survival analysis. Inhibition of *Prototheca* spp. growth was used as the event, and isolates that had growth at the highest concentration tested were defined as not inhibited. Kaplan-Meier survival curves were based on 3 different incubation times (24, 48 and 72 h) of peracetic acid, sodium hypochlorite, and iodine solution concentrations, and the null hypothesis

Table 1. Biofilm production of *Prototheca zopfii* isolated from bovine subclinical mastitis[1]

	Weak		Moderate		Strong		Total	
Temperature and incubation time	No. (%)	OD	No. (%)	OD	No. (%)	OD	No. (%)	OD
25°C, 48 h	1 (10)	0.2236 ± 0.0381	5 (50)	0.4713 ± 0.1680	4 (40)	1.0476 ± 0.5731	10 (100)	0.6771 ± 0.3170
37°C, 48 h	6 (60)	0.2964 ± 0.0526	4 (40)	0.6405 ± 0.2689	—	—	10 (100)	0.4341 ± 0.1391

[1]Capacity of the isolates to produce biofilms was classified according to Stepanović et al. (2003). The average optical density (OD) values of the negative controls (OD_{NC}) were 0.1246 ± 0.0063 (mean ± SD) and 0.1754 ± 0.0061, for incubations at 25°C and 37°C ± 1°C, respectively. The average values of the positive controls (OD_{PC}) were 0.3873 ± 0.0436 and 0.4483 ± 0.0188, for incubations at 25°C and 37°C ± 1°C, respectively. The isolates were classified as weak ($OD_{NC} < OD \leq 2 \times OD_{NC}$), moderate ($2 \times OD_{NC} < OD \leq 4 \times OD_{NC}$), or strong ($OD > 4 \times OD_{NC}$).

of no difference in the survival strata (concentration at inhibition) was tested via log-rank and Wilcoxon tests.

RESULTS AND DISCUSSION

All *Prototheca* spp. isolates (n = 10) were confirmed by 18S rRNA gene sequencing to be *P. zopfii.* Mammary quarters infected with *P. zopfii* had average lnSCC of 7.77 ± 2.14 × 10^3 cells/mL and >10 cfu/0.01 mL of milk spread on blood agar.

In the present study, 5 of 21 dairy farms had positive diagnoses of *P. zopfii.* The number of farms on which *P. zopfii* was detected (23.8%) was less than that (75%) reported by Ricchi et al. (2010) in Italy. The frequency of subclinical mastitis caused by *P. zopfii* found in the present study at the quarter level (0.9%) was less than that reported in Brazil (18.5% at the quarter level) by Costa et al. (1997), probably because none of the farms enrolled in the present study had reported outbreaks caused by *P. zopfii.* Frequency of IMI caused by *P. zopfii* depends on control of risk factors, the environment, and the immune response of the animal; however, complete elimination of microalgae from the mammary gland is difficult, because *P. zopfii* causes chronic and persistent mastitis with intermittent elimination of this pathogen (Roesler and Hensel, 2003).

Polystyrene Microplate Assay for Biofilm Production

In the present study, all *P. zopfii* isolates (n = 10) had the capacity to produce biofilm as assessed by the polystyrene microplate assay method. Of isolates that were incubated at 25°C ± 1 (n = 10), 1 showed weak biofilm production, 5 moderate, and 4 strong ($P < 0.02$; Table 1). Of those isolates incubated at 37°C ± 1°C (n = 10), 6 showed weak biofilm production and 4 moderate ($P < 0.01$; Table 1). Our results suggest that biofilm production capacity may vary according to incubation temperature, because at 25°C, 4 isolates had strong biofilm production, whereas none showed strong biofilm production when incubated at 37°C ± 1°C. To our knowledge, no previous study has evaluated the relationship between biofilm production capacity of bacteria versus microalgae associated with subclinical mastitis.

The clustering behavior of microorganisms in biofilms depends on the environmental conditions and the growth-promoting genes of the microorganism. Gerstel and Romling (2001) reported that the multi-cellular expression of the *agfD* gene of *Salmonella typhimurium* leads to production of polymers, thin agglomerates of fimbria and cellulose that form the extracellular matrix. Furthermore, Knobloch et al. (2001) reported that intercellular polysaccharide adhesin, originally from the cluster gene *icaADBC*, is essential in biofilm production by *Staphylococcus epidermidis* multi-cells. Thus, different microorganisms express specific genes, which lead to the production of extracellular matrix-forming substances that are directly related to the production of biofilm.

Although *P. zopfii* is a microalga, some comparisons of the present study were made with bacterial models used for assessing biofilm production. This is because microalgae produce biofilms in a similar manner to bacteria; initially, they agglomerate and then produce the extracellular matrix. Therefore, assessment of biofilm production by algae may be similar to that used for other bacterial agents (Stepanović et al., 2003; Nagai et al., 2013; Lee et al., 2014).

The results of the present study indicate the capacity to produce biofilm by *P. zopfii*; however, further studies on gene expression are needed to identify the specific genes associated with biofilm production by *P. zopfii.*

Scanning Electron Microscopy

All *P. zopfii* isolates that produced biofilm on the stainless steel coupons were submitted to scanning electron microscopy. The coupons incubated in microplates at 25°C ± 1°C (n = 10) showed greater microalgae clustering, with different sizes of endospores (Figure 1A and 1B). Similarly, *P. zopfii* isolates were able to

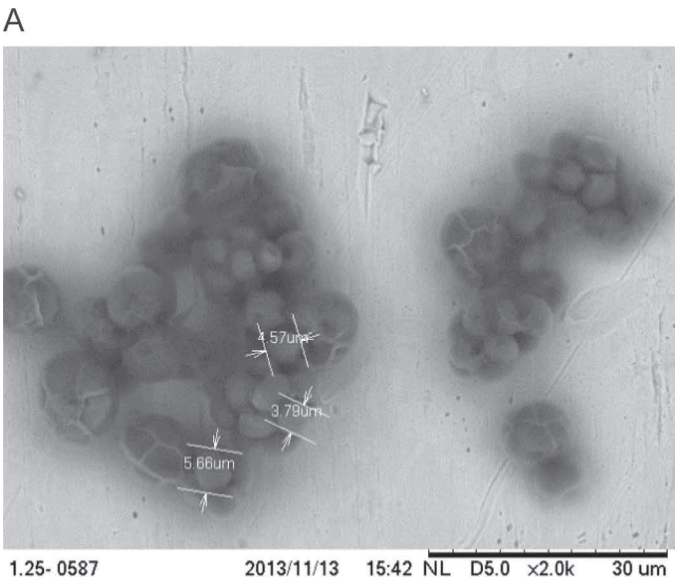

Figure 1. Biofilm production in stainless steel coupons by *Prototheca zopfii* (isolate 1) at 25°C, showing release of endospores after rupture and endospores ranging from 3.79 to 5.66 μm (A), and larger endospores (11.8 μm; B; scanning electron microscopy).

produce biofilm at 37°C ± 1°C (n = 10), although the microalgae cells were surrounded by a dark structure, similar to the lipidic layer of the cellular wall (Figure 2). This structure was similar to sporangia (enclosures in which spores form) and might have prevented greater clustering of the microalgae on the surface of the stainless steel coupons when incubated at 37°C.

Microalgae reproduce asexually by internal septation (endosporulation). *Prototheca* spp. are organized in a cell of oval or spherical shape called the mother cell (sporangium), and a viable mother cell may give rise to 2 to 16 daughter cells, known as endospores (Janosi et al., 2001; Buzzini et al., 2004; Camboim et al., 2010). The endospores remain inside the sporangium, surrounded by a trilaminar capsule of sporopolenine, which, when ruptured, releases the cells and a new reproductive cycle begins. In this study, the release of the endospores was observed in isolates incubated at the lower incubation temperature (25°C ± 1°C; Figure 1). Once the sporangium ruptures, the endospores need to cluster to initiate a new reproductive cycle. This clustering period is the initial phase of biofilm production (Davies, 2003).

The use of stainless steel coupons was described by Lee et al. (2014) to evaluate biofilm production of *Staphylococcus aureus* from mastitis cases and in milking environments. Considering that stainless steel is a widely used material, it simulates the biofilm production that can occur in milking and dairy environments with deficient sanitizing equipment and utensils. Furthermore, Osumi et al. (2008) reported that an infection route of *P. zopfii* might be contamination of milking equipment. Our results on the capacity of *P. zopfii* to produce biofilm agree with the findings of Marques et al. (2010b), that *P. zopfii* form cellular clusters and thus have resistance to heat treatment.

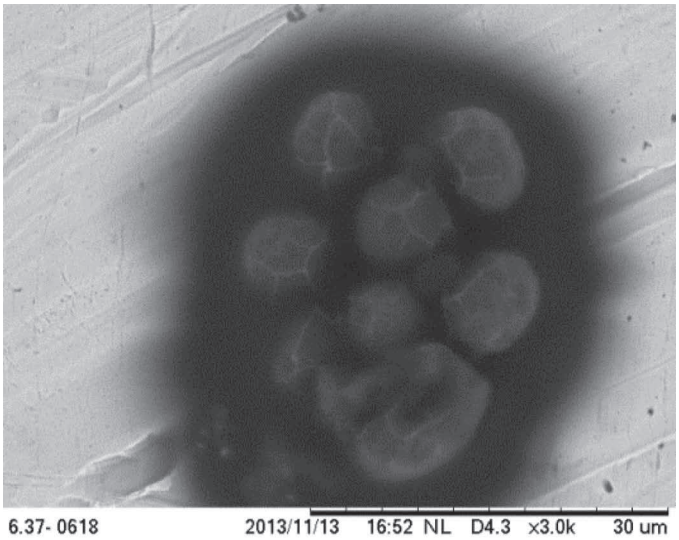

Figure 2. Biofilm production in stainless steel coupons by *Prototheca zopfii* at 37°C. All *Prototheca zopfii* isolates were surrounded by a dark structure, similar to the lipidic layer of the cellular wall (scanning electron microscopy).

Table 2. Number of *Prototheca zopfii* isolated for different MIC (NCCLS, 2000) of peracetic acid, sodium hypochlorite, and iodine solution used in vitro as sanitizers against *P. zopfii*[1]

Incubation time/sanitizer	MIC (g/L)													
	0.019	0.039	0.078	0.156	0.312	0.625	1.25	2.5	5	10	20	MIC_{50}[2]	MIC_{90}[2]	*P*-value[3]
24 h														
Peracetic acid	10	0	0	0	0	0	0	0	0	0	0	0.019	0.019	<0.001
Sodium hypochlorite	0	0	1	6	2	1	0	0	0	0	0	0.156	0.312	
Iodine solution	1	0	2	4	1	2	0	0	0	0	0	0.156	0.625	
48 h														
Peracetic acid	8	0	2	0	0	0	0	0	0	0	0	0.019	0.078	<0.001
Sodium hypochlorite	0	0	0	1	7	2	0	0	0	0	0	0.312	0.625	
Iodine solution	0	0	0	4	4	2	0	0	0	0	0	0.312	0.625	
72 h														
Peracetic acid	2	0	8	0	0	0	0	0	0	0	0	0.078	0.078	<0.001
Sodium hypochlorite	0	0	0	0	4	5	1	0	0	0	0	0.625	0.625	
Iodine solution	0	0	0	0	7	3	0	0	0	0	0	0.312	0.625	

[1]In vitro assays considering isolates (n = 10) of *P. zopfii.*

[2]MIC_{50} and MIC_{90} refer to MIC required to inhibit growth of 50% and 90%, respectively, of *P. zopfii* isolates tested.

[3]Difference in the survival strata (concentration at inhibition) was tested via log-rank and Wilcoxon tests.

Our scanning electron microscopy results were similar to those of Costa et al. (2004). However, in the images shown by Costa et al. (2004), cellular clustering of *P. zopfii* was was originated from an isolate from a clinical case of bovine mastitis, suggesting that, regardless of the type of mastitis, microalgae may produce biofilms.

Davies (2003) reported that conventional antimicrobial treatments are generally incapable of eliminating biofilms, which can result in chronic infection caused by bacteria. Based on results described by Costa et al. (1996), mastitis caused by *Prototheca* spp. is not responsive to treatment, control of this disease should focus on prevention strategies.

Resistance Profile of Prototheca spp. to Sanitizers Determined by MIC

We detected a significant effect of sanitizer and incubation time (24, 48, and 72 h) on MIC values against *P. zopfii* isolates (Table 2). The longer the incubation period, the higher the concentration of sanitizer needed to prevent reproduction of the *P. zopfii* isolates. Lassa et al. (2011) evaluated the effectiveness of 3 sanitizers at preventing reproduction of *P. zopfii*; however, in contrast to the present study, they reported that the efficiency of the sanitizer depends more on the concentration used than on the incubation period.

All *P. zopfii* isolates were sensitive to the 3 sanitizers at 24, 48, and 72 h of incubation. After 24 h of incubation, *P. zopfii* isolates were sensitive in vitro to peracetic acid (MIC_{90} ≥0.019 g/L), sodium hypochlorite (MIC_{90} ≥0.312 g/L), and iodine solution (MIC_{90} ≥0.625 g/L). Similar to the present study, Lassa et al. (2011) assessed the efficiency of teat sanitizers in vitro (iodine, didecyldimethylammonium, and dodecylbenzenesulfonic acid) on reproduction of *P. zopfii*. In that study, of the 3 sanitizers, iodine had the greatest efficiency for prevention of mastitis caused by *P. zopfii*. However, the concentration of iodine (0.1 g/L) that prevented multiplication of *P. zopfii* after 12 h of incubation was less than the MIC_{90} described in the present study (0.625 g/L), after 24 h of incubation.

Studies evaluating the use of sanitizers to reduce contamination of utensils and milking and dairy farm equipment or to prevent the IMI caused by *P. zopfii* are scarce (Cunha et al., 2010; Marques et al., 2010a; Lassa et al., 2011). Marques et al. (2010a) assessed effects of different salt concentrations and pH on *Prototheca* spp. resistance, and observed that *P. zopfii* had reproduction capacity in all of the pH buffers except to acetic acid. In the present study, the sanitizer that had the greater efficiency (lowest MIC) was peracetic acid. The higher efficiency of peracetic acid may be due to its high oxidating potential and molecular weight, which is similar to that of acetic acid. Indole-3 acetic acid combined with horseradish peroxidase (IAA/HRP) had cytotoxic effects on *P. zopfii* (Cunha et al., 2010). Furthermore, in response to exposure to the IAA/HRP, cellular viability and colony production of *P. zopfii* were reduced. In another study, the use of teat sanitizers was evaluated and, in contrast to the present study, iodine was the most efficient sanitizer (Lassa et al., 2011).

CONCLUSIONS

Prototheca zopfii isolates are capable of producing biofilms that may contribute to persistence of this species in milking environments and the milk-processing

industries. *Prototheca zopfii* is sensitive in vitro to peracetic acid (MIC_{90} ≥0.019 g/L), sodium hypochlorite (MIC_{90} ≥0.312 g/L), and iodine (MIC_{90} ≥0.625 g/L) after 24 h of incubation. However, the longer the incubation period, the higher the concentration of sanitizer needed to prevent reproduction of the *P. zopfii* isolates. Among the tested sanitizers, peracetic acid was more effective than sodium hypochlorite or iodine.

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