Dairy cow mastitis associated with microalgae of the genus *Prototheca* has been reported worldwide. This alga is extremely resistant to most antimicrobials commonly used in mastitis therapy. In milk processing, different thermal treatments are generally efficient at inactivating and eliminating microorganisms. Until recently, no reports on *Prototheca blaschkeae* susceptibility to heat treatment have been described. Thus, considering the potential zoonotic risk that *Prototheca* may represent, the objective of this study was to test the susceptibility of *P. blaschkeae* field isolates retrieved from bovine mastitis to different temperature/time ratios that are generally used in the milk processing industry: 62°C/15 min and 30 min; 70°C/20 s, 15 min, and 30 min; 75°C/20 s; 90°C/1 s; and 100°C/1 s. The results showed a growth reduction of all isolates after the heat treatments, but only at 100°C was a total growth inhibition observed.

**Key words:** *Prototheca blaschkeae*, *Prototheca zopfii*, bovine mastitis, temperature susceptibility

Green algae of the genus *Prototheca* are one of the few plant-like organisms that cause infections in humans and animals (Matsuda and Matsumoto, 1992; Pore, 1998; Möller et al., 2007). This genus consists of microscopic, unicellular, achlorophyllic algae with asexual reproduction by formation of variable numbers of sporangiospores within a sporangium (DiPersio, 2001; Malinowski et al., 2002). *Prototheca* are ubiquitous and generally saprophytic, being isolated from a variety of environmental sources such as plants, soil, drinking water, sludge, marine water, swimming pools, feces of domestic or wild animals, barn floors, and meat products (Pore et al., 1983; Anderson and Walker, 1988; Lass-Flörl and Mayr, 2007). However, some species in the genus, *Prototheca zopfii*, *Prototheca wickerhamii*, and *Prototheca blaschkeae*, may turn into unusual opportunists and cause pathology in hosts with impaired immunological defenses or when other predisposing factors occur (Pore, 1998; Schultze et al., 1998; Jánosi et al., 2001; Roesler and Hensel, 2003; Marques et al., 2006; Roesler et al., 2006; Marques et al., 2008; Thompson et al., 2009). In humans, *P. wickerhamii* is usually associated with pathology that is expressed essentially by cutaneous or subcutaneous lesions as well as in generalized infections (Zaitz et al., 2006; Hightower and Messina, 2007; Lass-Flörl and Mayr, 2007; Narita et al., 2008). On the other hand, animal infections, mainly bovine mastitis, have been associated with *P. zopfii* (Roesler et al., 2003; Buzzini et al., 2004; Möller et al., 2007). Recently *P. blaschkeae* was also associated with bovine mastitis (Marques et al., 2008), although it was first isolated and described from a human onychomycosis (Roesler et al., 2006). Nevertheless, these 2 species are capable of causing infections in humans as well (Roesler et al., 2006; Lass-Flörl and Mayr, 2007), suggesting a potential zoonotic involvement. In the past, only sporadic reports of cases of *Prototheca* associated with bovine mastitis were described in dairy herds in Europe and in the American continents (Lerche, 1952; Anderson and Walker, 1988; Da Costa et al., 1996). Nowadays, cases of acute to chronic mastitis are increasingly recognized to be endemic worldwide and are gaining economic and public health importance (Santos and Flor, 2000; Bexiga et al., 2003; Roesler and Hensel, 2003; Buzzini et al., 2004; Marques et al., 2006; Marques et al., 2008; Osumi et al., 2008; Thompson et al., 2009). The most prevalent form of protothecosis in animals is bovine mastitis, which generally occurs in a chronic subclinical or a mild clinical inflammatory process in the udder and affects cows that do not respond to routine therapy (Melville et al., 1999; Malinowski et al., 2002; Roesler and Hensel, 2003). Therefore, the implementation of control measures should be the main objective for all dairy herds in order to avoid the spreading of this organism in the parlor environment and to reduce animal culling and, consequently, decrease all the associated economic losses. As previously reported by others (Costa et al., 1998; Melville et al., 1999), milk and dairy products contaminated with *Prototheca* spp. may represent one of the means of transmission of this pathogen to humans.
Generally, different thermal treatments of milk are efficient for inactivating and eliminating microorganisms. Milk pasteurization and ultrapasteurization are the procedures most used nowadays and consist of the appropriate use of heat to destroy pathogenic microorganisms without changing the organoleptic characteristics and their physical and chemical constitution. Several studies reported that *Prototheca* have been isolated from a great variety of pH values, from water treated with chloride, and from pasteurized milk (Pore, 1998; Melville et al., 1999; Jánosi et al., 2001; Marques et al., 2009). However, the reports evaluating the sensitivity of these algae to pasteurization refer only to a single study with *P. zopfii*, and no other published studies analyze the susceptibility of other species in the genus, in particular *P. blaschkeae*, which has recently been described as being associated with bovine mastitis. Considering the increasing importance of *Prototheca* spp. on bovine mastitis (Melville et al., 1999; Roesler and Hensel, 2003; Roesler et al., 2006; Marques et al., 2008, 2009) and the potential to be transmitted to humans, the purpose of this study was to test the susceptibility of 14 *P. blaschkeae* strains isolated from mastitic milk obtained from 11 dairy herds in the northern region of Portugal to different temperature/time ratios. Seventeen strains of *P. zopfii* genotype 2, also retrieved from mastitic milk (16 dairy herds from the same region), were evaluated as well.

The 31 field isolates of *Prototheca* used in this study belonged to the milk pathogens collection of the Laboratory of Infectious Diseases of Veterinary Medicine from Porto University (Portugal). The molecular characterization for the 18S rDNA region (Marques et al., 2008) demonstrated that the isolates were all genetically similar within each species. Two *Prototheca* spp. reference strains were kindly provided by Uwe Roesler (University of Leipzig, Germany). These strains, *P. zopfii* genotype 2 SAG 2021 (accession number AY940456) and *P. blaschkeae* SAG 2064 (accession number AY973041), were isolated from a bovine mastitis milk and from a human onychomycosis, respectively. Cultures were maintained on Sabouraud dextrose agar medium (Merck Laboratories, Darmstadt, Germany) during the study.

The susceptibility tests considered different temperature/time ratios for the 31 *Prototheca* isolates were adapted according to the methodology described previously by Melville et al. (1999). For all evaluations, *Prototheca* cultures grown in Sabouraud dextrose agar with 48 h of incubation were used. Briefly, *Prototheca* suspensions were prepared in sterile sodium chloride solutions at physiologic concentrations (0.85%) at approximately 1 × 10⁶ cells/mL, corresponding to tube 3 of the McFarland scale. From this suspension, one dilution step was performed in sterile milk to achieve the working suspension of 1 × 10⁵ cells/mL, which is in agreement with that defined in the European directives (Hillerton and Berry, 2004) for the acceptable concentration of microorganism present in raw milk for production. Positive and negative controls were always included for all the treatments testing. The positive controls consisted of *Prototheca* suspensions in sterile milk, and the negative controls consisted of sodium chloride solutions and milk. All samples were tested in duplicate and subjected to different temperature/time ratios (62°C/15 min and 30 min; 70°C/20 s, 15 min, and 30 min; 75°C/20 s; 90°C/1 s; and 100°C/1 s) and were immediately placed in ice. The controls were incubated at 37°C during all tested times. Following the heat treatments, 100 μL of each sample, reference strains and controls, was spread on Sabouraud dextrose agar plates and incubated for 48 h at 37°C. The treatment effect was determined by means of the inhibition of growth by counting the number of colony-forming units observed in each plate.

To evaluate the effects on the growth inhibition of *Prototheca* using the different temperature/time ratios, 1-way ANOVA using the logarithmic transformation of colony-forming units (to normalize the data) as the dependent variable was performed using SAS software (SAS Institute, 1989). The linear model included species and time within temperature as main effects. Estimable linear contrasts of least squares means were computed to make inferences and evaluate differences between levels of main effects.

The effect of 8 temperature/time ratios on the counts of *P. zopfii* and *P. blaschkeae* measured in log colony-forming units per milliliter are shown in Table 1. *Prototheca* spp. grew without restrictions in all positive controls (average of 6.3 log cfu/mL) and were significantly different (*P* < 0.001) from all treated samples. On the other hand, no growth was present on the negative controls for each susceptibility test. Both *Prototheca* spp. were affected by the increment of the temperature/time ratios, as shown by the progressive inhibition (% kill) in Table 1. Total growth inhibition was achieved only at 100°C/1 s treatment, indicating that ultrapasteurization is the only industrial procedure that ensures that the milk from endemic regions is free of these agents. A significant difference was found between the 2 species (*P* < 0.01) after adjusting for the time within temperatures effect in the model. This study suggests that *P. blaschkeae* may be more resistant to the heat treatment than *P. zopfii*, with an adjusted log colony-forming units per milliliter mean 1.3 times bigger than the latter (1.33 and 0.97 log cfu/mL, respectively).

No significant differences were found between the field and reference *Prototheca* strains for all temperature/time ratio treatments (Figure 1). Only at 70°C/20
s (Figure 1B) did \textit{P. zopfii} SAG 2021 show an apparent greater resistance than the other strains, but without statistical significance.

Members of the genus \textit{Prototheca} are widespread in many different environments worldwide but are most frequently found in environments with high humidity and content of OM. In these conditions, its dissemination and perpetuation in the environment are enhanced (Jánosi et al., 2001; Tsuji et al., 2006; Zaitz et al., 2006). Mastitic infections by \textit{Prototheca} in dairy herds are generally maintained by clinically healthy shedders (Jánosi et al., 2001; Roesler and Hensel, 2003) and reflect poor dairy cow management and hygiene, especially by defective premilking cleansing and disinfection of the teats (Da Costa et al., 1996; Baumgärtner, 1997; Pore, 1998). Also, these ubiquitous algae are extremely resistant to chemical and physical agents because of the sporopollenin present in the cell wall, which allows recontamination of the environment and promotes its dissemination and perpetuation in the environment and also potentiates the pathogenicity (Costa et al., 1997; Malinowski et al., 2002; Marques et al., 2009). A previous study described the occurrence of human enteritis associated with the consumption of cheese contaminated with \textit{P. zopfii} (Costa et al., 1998). Although direct human infection through dairy products was never confirmed, this finding urges the necessity to implement effective control methods in the dairy farm and active action at the level of milk products quality control. That can be achieved using effective thermal treatments of milk (ultrapasteurization) for human consumption. Melville et al. (1999) also found that \textit{P. zopfii} strains isolated from mastitic milk were resistant in at least 1 of the thermal treatment tests (62–65°C/30 min, 72–75°C/15 s, and 72–75°C/20 s). The temperature/time ratios selected for this study consisted of those recommended for milk pasteurization (Sun, 2005; FDA, 2007). Additional tests were used to expand the temperature/time ratios to further understand the possible effects of time, or temperature, or both, on the growth inhibition of these 2 species. As in the present study, Melville et al. (1999) found a high variability in the susceptibility to temperature/time ratios from all isolates. At pasteurization temperatures (62 and 70°C), the increased exposure time from 15 to 30 min did not significantly increase the growth inhibition in both species (Table 1). One possible explanation is the tendency of these microalgae to form cell clumps, as

Table 1. Effects of temperature/time treatments on counts of \textit{Prototheca zopfii} and \textit{Prototheca blaschkeae}

<table>
<thead>
<tr>
<th>Temperature, °C</th>
<th>Time</th>
<th>\textit{Prototheca zopfii} genotype 2</th>
<th>\textit{Prototheca blaschkeae}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Count, log cfu/mL</td>
<td>Mean</td>
</tr>
<tr>
<td>62</td>
<td>15 min</td>
<td>34</td>
<td>1.476</td>
</tr>
<tr>
<td></td>
<td>30 min</td>
<td>34</td>
<td>1.387</td>
</tr>
<tr>
<td>70</td>
<td>20 s</td>
<td>34</td>
<td>1.258</td>
</tr>
<tr>
<td></td>
<td>15 min</td>
<td>34</td>
<td>0.598</td>
</tr>
<tr>
<td></td>
<td>30 min</td>
<td>34</td>
<td>0.585</td>
</tr>
<tr>
<td>75</td>
<td>20 s</td>
<td>34</td>
<td>0.745</td>
</tr>
<tr>
<td>90</td>
<td>1 s</td>
<td>34</td>
<td>0.912</td>
</tr>
<tr>
<td>100</td>
<td>1 s</td>
<td>34</td>
<td>0.000</td>
</tr>
</tbody>
</table>
described for other pathogens by Grant et al. (2005), preventing a complete exposure to temperature of those in the center. Homogenization of the milk during the treatment (not tested) may contribute to a better inhibition process. Only recently P. blaschkeae was found to also be associated with mastitis (Marques et al., 2008) and study of its susceptibility to different temperature/time ratios treatments was warranted. These results stress the need for the implementation of more efficient quality control measures at both milk production and milk processing in order to reduce mastitis and milk contamination by this potentially zoonotic alga.

ACKNOWLEDGMENTS

This work was supported by Fundação para a Ciência e Tecnologia, Portugal, grant SFRH/BD/28892/2006.

REFERENCES


Baumgartner, B. 1997. Vorkommen und Bekämpfung der Protothecalmilk processing in order to reduce mastitis and milk quality control measures at both milk production and study of its susceptibility to different temperature/time ratios treatments was warranted. These results stress the need for the implementation of more efficient quality control measures at both milk production and milk processing in order to reduce mastitis and milk contamination by this potentially zoonotic alga.

ACKNOWLEDGMENTS

This work was supported by Fundação para a Ciência e Tecnologia, Portugal, grant SFRH/BD/28892/2006.

REFERENCES


Baumgartner, B. 1997. Vorkommen und Bekämpfung der Protothecalmilk processing in order to reduce mastitis and milk quality control measures at both milk production and study of its susceptibility to different temperature/time ratios treatments was warranted. These results stress the need for the implementation of more efficient quality control measures at both milk production and milk processing in order to reduce mastitis and milk contamination by this potentially zoonotic alga.

ACKNOWLEDGMENTS

This work was supported by Fundação para a Ciência e Tecnologia, Portugal, grant SFRH/BD/28892/2006.

REFERENCES