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Molecular typing and differences in biofilm formation and antibiotic susceptibilities among *Prototheca* strains isolated in Italy and Brazil

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ABSTRACT

Bovine mastitis caused by *Prototheca* is a serious and complex problem that accounts for high economic losses in the dairy industry. The main objective of this study was to identify and characterize at genetic level different *Prototheca* strains and provide the most complete data about protothecal antibiotic resistance. The study involves 46 isolates from Italian (13 strains) and Brazilian (33 strains) mastitic milk. These strains were identified by multiplex PCR and single strand conformation polymorphism analysis and characterized by randomly amplified polymorphic DNA (RAPD)-PCR. Moreover, biofilm production and antibiotic susceptibility were evaluated. Forty-two strains resulted as *Prototheca zopfii* genotype 2, whereas 4 isolates could belong to a potential new *Prototheca* species. The RAPD-PCR, performed with 3 primers (M13, OPA-4, and OPA-18), showed a notable heterogeneity among isolates and grouped the strains according to the species and geographical origin. Biofilm production was species-dependent and *P. zopfii* genotype 2 strains were classified as strong biofilm producers. In vitro antibiotic susceptibility tests indicated that *Prototheca* strains were susceptible to antibacterial drugs belonging to aminoglycosides group; the highest activity against *Prototheca* strains was observed in the case of colistin sulfate, gentamicin, and netilmicin (100% of susceptible strains). It is interesting to note that all the Italian *P. zopfii* genotype 2 strains showed lower minimum inhibitory concentration values than the Brazilian ones. Nisin showed more efficacy than lysozyme and potassium sorbate, inhibiting 31% of the strains. Results obtained in this study confirmed that RAPD-PCR is a rapid, inexpensive, and highly discriminating tool for *Prototheca* strains characterization and could give a good scientific contribution for better understanding the protothecal mastitis in dairy herd.

Key words: *Prototheca*, randomly amplified polymorphic DNA (RAPD)-PCR, biofilm production, antibiotic susceptibility, netilmicin

INTRODUCTION

The genus *Prototheca* includes unicellular achlorophyllous yeast-like microalgae that are spherical, oval, or even kidney-shaped, with dimensions ranging from 3 to 30 μm in diameter. They reproduce by formation of a variable number of sporangiospores within a sporangium (Di Persio, 2001). To date, 6 species are well described for the genus *Prototheca*: *Prototheca zopfii*, *Prototheca wickerhamii*, *Prototheca stagnora*, *Prototheca ulmea*, *Prototheca blaschkeae*, and *Prototheca cutis* (Marques et al., 2015). *Prototheca zopfii* is classified into 2 genotypes (genotype 1 and 2) based on biochemical, serological, and genetic assays (Roesler et al., 2006). Among *Prototheca* species, *P. wickerhamii* and *P. cutis* have been associated mainly with human diseases (Lass-Flörl and Mayr, 2007; Satoh et al., 2010), whereas *P. zopfii* genotype 2, *P. wickerhamii*, and *P. blaschkeae* have been mostly related to bovine mastitis (Marques et al., 2006; Capra et al., 2014). Bovine mammary protothecosis results in substantial decrease in milk production and increase in somatic cell count. It may even lead to cow culling, causing high financial losses (Wawron et al., 2013).

Protothecal bovine infection is slowly progressive and occasionally subclinical, making it difficult to be recognized early. The frequency of bovine protothecal mastitis has been increasing worldwide, which may represent a serious problem due to the inherent resistance of these microalgae to different drugs (Capra et al., 2014). Several reports showed that strains of *Prototheca* were resistant to conventional antibiotics

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used in antimycotic and antibacterial therapies (Lopes et al., 2008; Gao et al., 2012; Wawron et al., 2013). This resistance is associated with the capacity of the microalgae to infect, evade macrophage response, and invade the mammary tissue, making them responsible for persistent infections (Marques et al., 2006). Moreover, the high resistance of *Prototheca* to conventional pharmacologic treatment could be linked to the ability of these microalgae to produce biofilm. Biofilms are matrix-enclosed microbial masses that adhere to biological or nonbiological surfaces (Hall-Stoodley et al., 2004). They represent a mode of growth protection that allows survival of microorganisms in a hostile environment. At present, only one study has evaluated the biofilm production by *Prototheca* strains isolated from bovine mastitis (Gonçalves et al., 2015).

The purpose of the current study was to investigate and describe the genotypic diversity of the population of *Prototheca* strains isolated from bovine mastitic milk samples collected in Italy and Brazil. Two well-known typing methods [randomly amplified polymorphic DNA (**RAPD**) analysis and inter-simple sequence repeat (**ISSR**) analysis] were used for characterizing these strains. Further information was also gained studying the biofilm-producing ability and the in vitro antibiotic-antibacterial susceptibility of *Prototheca* strains. We aimed to provide the most complete data about protothecal antibiotic resistance; for that reason, we tested 28 antibiotics and 3 antimicrobial agents (nisin, lysozyme, and potassium sorbate) against *Prototheca* isolates. Moreover, another objective of our study was to highlight eventual differences linked to *Prototheca* species or geographical origin of the strains.

MATERIALS AND METHODS

Prototheca Strains

A total of 46 *Prototheca* spp. strains were used in this study. The isolates were originally retrieved from milk samples of cows affected by clinical and subclinical mastitis from different dairy herds in Italy and Brazil. The 13 Italian strains came from 20 milk samples, originating from 10 dairy farms located in Lombardia (north Italy), whereas the Brazilian ones (33 strains) were collected from 270 milk samples originating from 18 farms located in 3 states of Brazil (São Paulo, 25 isolates; Rio Grande do Sul, 5 strains; and Minas Gerais, 3 isolates). The isolation of *Prototheca* strains was performed by plating 0.1 mL of milk samples onto Sabouraud agar SB (Biolife, Milan, Italy) plates. The plates were incubated aerobically at 37°C for 48 h. The colonies with the typical aspect were randomly picked and streaked out 3 times on SB agar to check for purity. After purification and microscopic examination, the isolates were stored at −18°C in Litmus Milk (Biolife). The following reference strains were also included in the study: *P. zopfii* genotype 2 SAG2021^{T}, *P. zopfii* genotype 1 SAG2063^{T}, and *P. blaschkeae* SAG2064^{T} [deposited in the Culture Collection of Algae (SAG) at University of Göttingen, Göttingen, Germany]. All strains were routinely grown in Sabouraud broth SB (Biolife) at 37°C for 48 h under aerobic conditions.

DNA Extraction and Identification of Prototheca strains

Genomic DNA was isolated as previously described by Cremonesi et al. (2012) and the strains identification was performed by multiplex PCR according to Capra et al. (2014).

PCR-Single Strand Conformation Polymorphism

To further confirm the identity of the strains, all *Prototheca* isolates were subjected to single strand conformation polymorphism (**SSCP**)-PCR analysis as previously described by Cremonesi et al. (2012). This technique is based on the simultaneous analysis of 2 different regions of 18S rDNA gene, allowing the identification of the *Prototheca* species.

RAPD Analysis

The RAPD-PCR was used to explore the genetic diversity of the 49 *Prototheca* strains. The DNA of *P. stagnora* ATCC 16528 (STAG) and *P. ulmea* ATCC 50112 (ULM) were included in the RAPD-PCR analysis. The RAPD-PCR assay was developed by screening 10 primers: M13 (5′-GAGGGTGGCGGTTCT-3′); OPAA10 (5′-GGACTACCAGGGTATCTAAT-3′; as described by Decimo et al., 2014); D11344 (5′-AGTGAATTCGCGGTCAGATGCCA-3′), D8635 (5′-GAGCGGCCAAAGGGAGCAGAC-3′; according to Morandi et al., 2013); 208 (5′-ACGGCCGACC-3′), 272 (5′-AGCGGGCCAA-3′; as reported by Saitou et al., 2010); HLWL85 (5′-ACAACTGCTC-3′; according to Wulff et al., 2006); OPA-4 (5′-AATCGGGCTG-3′); OPA-13 (5′-CAGCACCCAC-3′); and OPA-18 (5′-GAGAGCCAAC-3′; Gómez and González, 2001). The amplification with OPA-4, OPA-13, and OPA-18 started with an initial denaturation at 94°C for 2 min, followed by 55 cycles at 94°C for 1 min, 40°C for 2 min, 72°C for 2 min, and final extension at 72°C for 5 min. Grouping of the RAPD-PCR profiles was obtained with the BioNumeric 5.1 software package (Applied Maths, Kortrjik, Belgium) using the unweighted pair group method using arithmetic averages (UPGMA) cluster analysis.

The reproducibility value of the RAPD-PCR assay, calculated from 2 repetitions of independent amplification of *Prototheca* type strains, was higher than 90%.

ISSR Analysis

The ISSR-PCR patterns were used to analyze the genetic diversity of *Prototheca* strains. Profiles of ISSR-PCR for *Prototheca* strains were obtained using the 5′-anchored $(CAG)_4$ primer (5′-ARRTYCAGCAG-CAGCAG-3′), where R (A or G) and Y (C or T) indicate degenerate sites (Gallardo et al., 2014). Amplification was performed with an initial denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, annealing temperature of 50°C for 1 min, elongation at 72°C for 2 min, and final extension at 72°C for 5 min. Grouping of the ISSR-PCR profiles was obtained as described for RAPD-PCR. The reproducibility value of the ISSR-PCR assay, calculated from 2 repetitions of independent amplification of type strains, was higher than 90%.

Discriminatory Power

The discriminatory power of the typing methods (RAPD- and ISSR-PCR) was calculated based on Simpson's index of diversity (D). Ideally, the index, which is based on the testing of a large number of unrelated isolates, should be equal to 1.0. (Hunter, 1990).

Biofilm Formation

A modification of the microwell assay (Kwiecinski, 2015) was used. *Prototheca* cell suspension in Sabouraud broth SB (Biolife) with 6% glucose was prepared and incubated for 48 h at 37°C (Shin et al. 2002). Next, a 96-well, flat-bottom cell culture plate was filled with 200 μL of *Prototheca* cultures diluted 1:9 in SB + glucose. Each strain was tested in triplicate. Wells with negative controls contained only SB + glucose. Plates were incubated at 37°C, without agitation, for 24 h. Afterward the medium was aspirated and wells were washed with PBS, dried at 45°C for 3 h, stained with 200 μL of 0.4% wt/vol safranin for 5 min, rinsed with sterile water, and dried overnight at room temperature. The stain that was bound to the biofilm was solubilized by addition of 200 μL of 33% acetic acid. Absorbance of 100 μL of this solution at 450 nm (OD_{450}) was measured with an Infinite F200 PRO microplate reader (Tecan, Männedorf, Switzerland; Schlag et al. 2007). Results were expressed as optical density (**OD**) values. Negative control triplicates containing only sterile SB + glucose were used as reference to determine the capacity of the *Prototheca* strains to produce biofilms (Stepanović et al. 2003). 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 ($OD > 4 \times OD_{NC}$), where OD_{NC} is the optical density of the negative control (Stepanović et al. 2003).

Antibiotic Susceptibility

To provide the most complete data about protothecal antibiotic resistance 28 different drugs were tested against *Prototheca* strains. The antibiotic susceptibility was determined by the disc diffusion method performed on Mueller Hinton agar (Biolife) according to the Clinical and Laboratory Standards Institute (CLSI, 2007). The following antimicrobial drugs (Oxoid, Basingstoke, UK) were used: amikacin (30 μg), ampicillin (10 μg), aztreonam (30 μg), cefepime (30 μg), ceftazidime, (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), colistin sulfate (10 μg), erythromycin (15 μg), phosphomycin (50 μg), gentamicin (10 μg), imipenem (10 μg), kanamycin (30 μg), levofloxacin (5 μg), meropenem (10 μg), mupirocin (200 μg), netilmicin (10 μg), nitrofurantoin (300 μg), oxacillin (1 μg), penicillin G (10 units), piperacillin (100 μg), quinupristin/dalfopristin (15 μg), rifamycin (30 μg), streptomycin (10 μg), tetracycline (30 μg), ticarcillin/clavulanic acid (85 μg), tobramycin (10 μg), and vancomycin (30 μg). Plates were incubated for 48 h at 37°C and diameter of growth inhibition zones was measured (mm).

No universally accepted guidelines specific for *Prototheca* spp. applicable in the interpretation of drug susceptibility testing were available. According to the size of inhibition zone, the strains were divided into 3 categories: susceptible (≥9 mm), intermediate (3–8 mm), and resistant (≤2 mm). Reference strain *Staphylococcus aureus* ATCC 19095 was used as a quality control.

The antimicrobial agents that gave positive reaction against *Prototheca* strains were further analyzed for MIC detection using E-test strips (bioMerieux, Marcy l'Etoile, France). The E-test strips contained increasing concentration gradients of the antimicrobials (from 0.016 to 256 mg/L) for all drugs tested. The strips were stored at 4°C before use. The MIC results were expressed as MIC_{50} and MIC_{90} (MIC inhibiting 50 and 90% of the isolates of the species tested, respectively).

Susceptibility to Nisin, Lysozyme, and Potassium Sorbate

Stock solutions of nisin (Sigma Aldrich, St. Louis, MO; 2.5% pure nisin, potency of 10^6 IU/g) were prepared at concentrations of 0.5 g in 10 mL of 0.02 *N* HCl (Ávila et al., 2014). Lysozyme (Sacco Srl, Cadorago, Italy) was dissolved in distilled water to yield a stock

solution of 800 mg/kg (Ávila et al., 2014). A potassium sorbate stock solution of 60 mg/mL was also prepared (Wan Norhana et al., 2012). All solutions were filter-sterilized (0.20 μm) and freshly prepared before use. For the determination of antialgal activity, a volume of 100 μL of each stock solution was placed in duplicate into wells (5 mm diameter) made in plates of Mueller Hinton agar inoculated with 16 h culture of *Prototheca* strains (~10^6 cfu/mL). After incubation at 37°C for 24 to 48 h, the diameter of growth inhibition zones was measured and antialgal activity was expressed in millimeters. The antimicrobial agents that gave positive reaction against *Prototheca* strains were further analyzed for MIC. Antimicrobial agents were diluted in (1:2) opportune solvents at concentration ranging from 195.3 to 50,000 IU/g for nisin, 25 to 800 mg/kg for lysozyme, and 3.75 to 60 mg/mL for potassium sorbate. A volume of 100 μL from each concentration was placed in duplicate into wells (5 mm diameter) made in plates of Mueller Hinton agar, as described above. Formation of a growth inhibition zone was used to determine the MIC. As described for antibiotic susceptibility, MIC results were expressed as MIC_{50} and MIC_{90}.

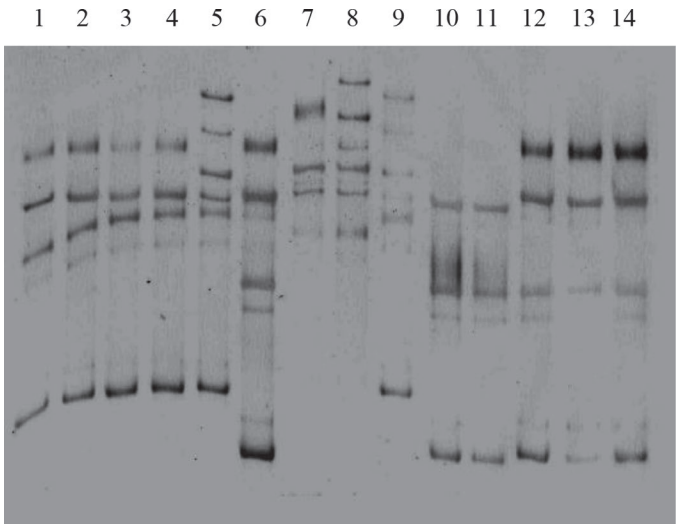

Figure 1. Polymerase chain reaction-single strand conformation polymorphism (SSCP-PCR) analysis of the two 18S ribosomal DNA fragments amplified. Lanes 1–4 = strains belonging to the potential new species (PR21, PR22, PR23, and PR24); lanes 5 and 9 = *Prototheca blaschkeae* SAG2064^T; lane 6 = *Prototheca zopfii* genotype 2 SAG2021^T; lane 7 = *Prototheca ulmea* ATCC50112; lane 8 = *Prototheca stagnora* ATCC16528; lanes 10 and 11 = *P. zopfii* genotype 1 SAG2063^T; lanes 12–14 = *P. zopfii* genotype 2 isolated from field samples.

RESULTS AND DISCUSSION

Identification and Characterization of Prototheca Strains

For the identification of isolates, a multistep approach was used. First, the multiplex-PCR assay was performed on the 46 isolates, and then the results were confirmed by the SSCP-PCR methodology. Forty-two strains (91.3%), 13 from Italy and 29 from Brazil, were identified as *P. zopfii* genotype 2. This finding is in agreement with previous reports that showed the predominance of *P. zopfii* genotype 2 in bovine intramammary infections detected in Italy and Brazil (Salerno et al., 2010; Capra et al., 2014). The absence of *P. zopfii* genotype 1 among isolates corresponded to previous observations, showing that genotype 1 strains play no role in bovine mastitis, and for that reason it is considered nonpathogenic and probably an environmental milk contaminant (Jagielski et al., 2011). Four strains collected in Brazil (3 from Minas Gerais and 1 from Rio Grande do Sul) previously identified as *P. blaschkeae* by multiplex-PCR assay showed a partial SSCP-PCR matching pattern between the reference *P. blaschkeae* SAG 2064^T strain and *P. zopfii* genotype 2 SAG 2021^T strain (Figure 1). This technique, which was previously reported as an accurate and a highly suitable method for the identification of *Prototheca* spp. (Cremonesi et al., 2012), amplified 2 different portions of 18S rRNA gene. These strains showed a shared and unique SSCP-PCR pattern compared with other *Prototheca* strains, potentially ascribable to a new protothecal species.

RAPD-PCR and ISSR-PCR Analysis

Both RAPD- and ISSR-PCR were carried out to explore the genetic diversity of *Prototheca* strains. With regard to RAPD-PCR, from the 10 primers tested, 3 (M13, OPA-4, and OPA-18) revealed a clear and reproducible amplification pattern and were consequently used for genotypic characterization of wild and reference strains. The RAPD-PCR reactions were highly reproducible; identical banding patterns were produced when DNA from independent cultures of the same strain were analyzed. Figure 2 shows the dendrogram derived from the combination of amplification profiles obtained with 3 primers. Grouping of the RAPD-PCR patterns was performed by the unweighted pair group method using arithmetic averages (UPGMA) cluster analysis. Strains with a similarity coefficient equal to or higher than 90% may be considered to be extremely close genotypically, and perhaps even identical. All the strains were grouped according to species, and a notable genotypic heterogeneity among *Prototheca* strains was evident. Considering the *P. zopfii* genotype 2, the coefficient of similarity was 55.3% and the genetic polymorphism detected allowed the identification of 28 different RAPD genotypes among 42 analyzed strains,

highlighting an adequate degree of variability. With exception of PR1 strain, the RAPD dendrogram successfully grouped all the *P. zopfii* genotype 2 strains based on their geographical origins. The reference strain SAG 2021^{T} fell in the middle of the cluster. The 4 strains identified as potential new species of *Prototheca* were clustered together and showed a coefficient of similarity of 62.9%. Moreover, it is interesting to note that RAPD clearly separated the potential new species from other *Prototheca* species, showing 41.9% homology with *P. zopfii* genotype 2, and 12.5 and 18.7% with *P. zopfii* genotype 1 and *P. blaschkeae*, respectively.

Smaller differences were found among the *Prototheca* strains using ISSR-PCR. Considering the 42 *P. zopfii* genotype 2, 81% of strains (11 from Italy and 23 from Brazil) showed a coefficient of similarity equal to 79.1%. The remainder 8 strains and the reference strain SAG 2021^{T} presented different band patterns of low similarity. Nevertheless, using the $(CAG)_4$ primer it was not possible to discriminate among the Italian and Brazilian strains and distinguish *P. zopfii* genotype 2 from the potential new species (data not shown).

The discriminatory power of the 2 techniques was calculated by means of the Simpson′s index of diversity (D). The values of the Simpson's index for the single primers tested in RAPD-PCR analysis were 0.94, 0.92, and 0.85 for M13, OPA-4, and OPA-18, respectively, whereas the Simpson′s index obtained combining the 3 RAPD primers was 0.98. The $(CAG)_4$ primer showed a Simpson′s index of 0.91. As reported in several studies, RAPD-PCR showed to be a useful tool in the characterization of prokaryotic and eukaryotic organisms (Gómez and González, 2001; Morandi et al., 2013). Our data confirmed that RAPD-PCR gives reproducible band patterns that can be used for characterizing *Prototheca* strains at the genetic level. In the present study, the use of M13, OPA-4, and OPA-18 primers allowed us to reach reliable conclusions, and only the dendrogram generated from the combination of 3 out of 10 primers was able to separate the strains according to the species and geographical origin. In addition, the advantages of this technique include efficiency, low cost, and quickness (Kumari and Thakur, 2014). Furthermore, the combined use of molecular techniques (RAPD-PCR, SSCP-PCR) allowed us to detect a possible new species. To the best of our knowledge, this is the first study where RAPD-PCR was used to investigate the genetic diversity among *Prototheca* strains. Currently, it is possible to find some reports that used different genotyping techniques, such as 18S rDNA sequence analysis, PCR-RFLP (Jagielski et al., 2011), and internal transcribed spacer (ITS)-PCR (Marques et al., 2015) to identify or determine the composition of *Prototheca* population causing bovine mastitis.

Biofilm Formation

The polystyrene microplate assay method was used to assess the ability of *Prototheca* strains to produce biofilm (Table 1). Different levels of biofilm production were observed among *P. zopfii* genotype 2 strains: 37 of them showed strong biofilm production (10 strains form Italy and 27 from Brazil), 4 showed moderate production (2 from Italy and 2 from Brazil), and 1 displayed a weak biofilm producer (collected in an Italian sample). All strains belonging to the new protothecal species were classified as weak producers. The level of biofilm production did not show a correlation with the geographical origin of the strains. Considering the references strains, biofilm production was strong in *P. zopfii* genotype 2 SAG 2021^{T} and weak in *P. zopfii* genotype 1 SAG 2063^{T} and in *P. blaschkeae* SAG 2064^{T}. Data presented in Table 1 suggest that ability to produce biofilm is linked to the species, but is also strain-dependent. In fact, a large variability in biofilm production was detected inside the *P. zopfii* genotype 2 species (from 0.126 ± 0.023 to 1.104 ± 0.264 OD). Moreover, our results highlighted that the majority of the strains were able to produce biofilm at 37°C. To the best of our knowledge, biofilm production by *Prototheca* strains isolated from bovine mastitis has only been evaluated in one study (Gonçalves et al., 2015). Our findings are partially different from that obtained by Gonçalves et al. (2015), who found 10 *P. zopfii* that did not show strong biofilm production at 37°C. Those authors did not specify the genotype of *P. zopfii* tested and, according to our results, they probably studied strains that belonged to genotype 1.

The ability to form biofilms could influence the major frequency of *P. zopfii* genotype 2 strains in bovine infections. Biofilm formation was frequently associated with mastitis (Melchior et al., 2006), but the exact relation between the biofilm-forming microorganisms and clinical outcomes is intricate and only partly understood (Akers et al., 2015). Our data provided new information about biofilm production by *Prototheca* strains, *P. zopfii* genotype 2, in particular, was a potent biofilm producer; this finding could influence the persistence of this species in milking environments and in IMI. As suggested by Kwiecinski (2015), further studies are necessary to determine mechanisms of action and effective treatments in protothecosis.

Antibiotic Susceptibility

The in vitro antibiotic susceptibility of all 46 *Prototheca* strains was examined using agar disc diffusion method with 28 different agents (Table 2). All strains were in vitro resistant to ampicillin, aztreonam, ce-

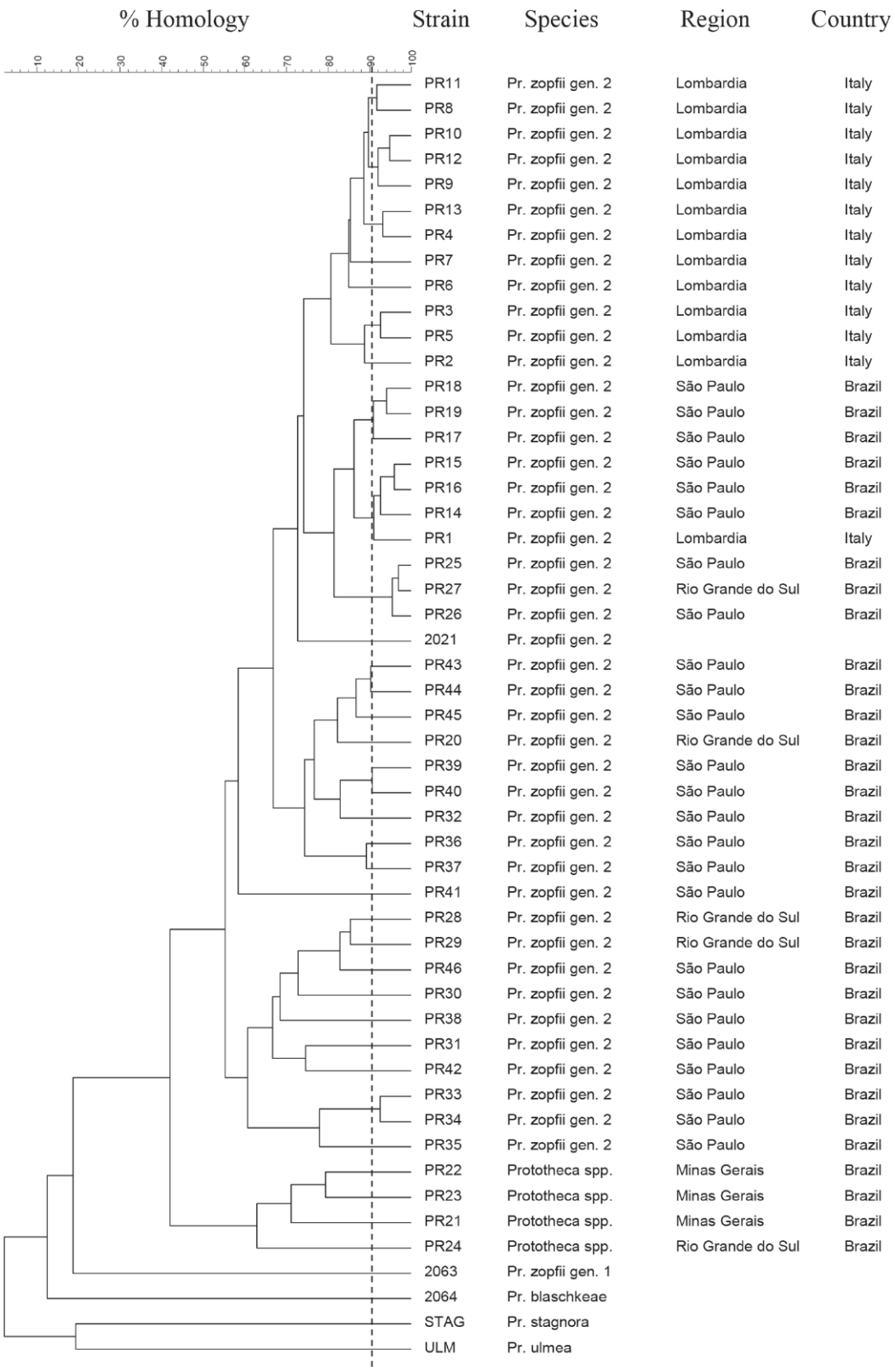

Figure 2. Unweighted pair-group method with arithmetic averages (UPGMA)-based dendrogram derived from the combined randomly amplified polymorphic DNA-PCR patterns generated with primers M13, OPA-4, and OPA-18 of the *Prototheca* strains considered in this study. The reference strains used in this analysis were: *Prototheca zopfii* genotype 2 SAG2021^{T} (2021), *P. zopfii* genotype 1 SAG2063^{T} (2063), *Prototheca blaschkeae* SAG2064^{T} (2064), *Prototheca stagnora* ATCC16528 (STAG), and *Prototheca ulmea* ATCC50112 (ULM).

Table 1. Biofilm production of *Prototheca* strains considered in this study [results expressed in optical density (OD) at 450 nm ± SD]

Biofilm		*Prototheca zopfii* gen. 2	*Prototheca* spp.	*P. zopfii* gen. 2 (SAG2021^T)	*P. zopfii* gen. 1 (SAG2063^T)	*Prototheca blaschkeae* (SAG2064^T)
Weak	No. of strains	1	4		1	1
	OD minimum	0.126 ± 0.023	0.095 ± 0.017		0.122 ± 0.019	0.125 ± 0.016
	OD maximum		0.206 ± 0.067			
Moderate	No. of strains	4				
	OD minimum	0.355 ± 0.025				
	OD maximum	0.431 ± 0.067				
Strong	No. of strains	37		1		
	OD minimum	0.452 ± 0.136		0.620 ± 0.116		
	OD maximum	1.104 ± 0.264				

[1]The average OD values of the negative control were 0.109 ± 0.030.

fepime, ceftazidime, chloramphenicol, ciprofloxacin, erythromycin, phosphomycin, mupirocin, nitrofurantoin, oxacillin, penicillin G, and piperacillin (diameter of inhibition zone ≤2 mm). Moreover, resistance to imipenem, meropenem (97.8% of resistant strains), levofloxacin and vancomycin (95.6%), quinupristin/dalfopristin, rifamycin, and ticarcillin (93.5% of resistant strains) was observed. The highest activity was demonstrated by streptomycin (45.6% of susceptible strains), tobramycin (21.8% intermediate, 32.6% susceptible), amikacin (16.9% intermediate, 71.7% susceptible), kanamycin (21.7% intermediate, 69.6% susceptible), tetracycline (97.8% susceptible), colistin sulfate, gentamicin, and netilmicin (100.0% of susceptible strains). The results of disc diffusion test and previously published data (Lopes et al., 2008; Gao et al., 2012; Wawron et al., 2013; Shahid et al., 2016) showed that all *Prototheca* strains were resistant to a wide range of antibacterial agents. Our findings indicated that the drugs belonging to aminoglycosides group (amikacin, gentamicin, kanamycin, netilmicin, streptomycin, and tobramycin) were more effective than other antibiotics. The effectiveness of gentamicin and kanamycin was previously confirmed by other authors (Lopes et al., 2008; Sobukawa et al., 2011; Gao et al., 2012; Wawron et al., 2013),

Antimicrobial agents that were able to inhibit at least the 50% of the *P. zopfii* genotype 2 strains were further analyzed for MIC detection using E-test strips (Table 3). Different values of MIC_{50} and MIC_{90} were observed. The best activity against *Prototheca* was demonstrated by netilmicin (MIC_{50} and MIC_{90} 12 and 24 μg/mL, respectively) and gentamicin (MIC_{50} and MIC_{90} 8 and 32 μg/mL, respectively) that were able to inhibit all 49 strains tested. Also colistin sulfate inhibited all *Prototheca* strains but at a drug concentration >24 μg/mL (MIC_{50} and MIC_{90} 24 and 128 μg/mL, respectively). The in vitro effectiveness of gentamicin and colistin against *Prototheca* were observed by different authors (Sobukawa et al., 2011; Gao et al., 2012; Wawron et al., 2013), but, currently, no data are available for the anti-*Prototheca* activity of netilmicin. Netilmicin is a derivative of gentamicin and is able to inhibit growth of pathogenic bacteria implicated in bovine mastitis, as well as gentamicin-resistant isolates. Recently, Olivares-Pérez et al. (2015) suggested the use of this antimicrobial agent combined with improve milking hygiene for the mastitis prevention.

Considering the geographical origin (Table 4), it is possible to note that *P. zopfii* genotype 2 isolated from Italy showed lower MIC values than strains from Brazil. These differences were particularly evident in the case of tetracycline (32.0 vs. >256.0 μg/mL), colistin sulfate (4.9 vs. 56.0 μg/mL), and kanamycin (10.3 vs. 30.9 μg/mL). Minimum inhibitory concentrations of kanamycin in Brazilian strains was also higher than MIC detected in *P. zopfii* strains isolated in Japan (18.5 μg/mL; Sobukawa et al., 2011). Mean value of netilmicin MIC was identical for all strains (about 12.0 μg/mL), confirming the effectiveness of this antibiotic. Strains belonging to the potential new species were less resistant than *P. zopfii* genotype 2 from Brazil, in particular with regard to colistin sulfate, kanamycin, and tetracycline. As observed by Jagielski et al. (2012), differences in MIC values against *Prototheca* strains originating from different countries may be the consequence of epidemiological, environmental, and technological factors (e.g., circulation of several epidemic *Prototheca* clones in the different countries, different climatic and ecological conditions, and heterogeneous prophylaxis and therapy protocols applied in dairy herds for milk sampling and so on), which could have affected the phenotype of strains. For this reason, it is very important to monitor the dissemination of antibiotic-resistant strains responsible for mastitis or serious therapeutic problems. In fact, the responsible use of antimicrobials in domestic animals is an emergent concern in the One Health concept, to conserve these drugs for human therapy approaches (Ribeiro et al., 2015).

Table 2. In vitro susceptibility of the *Prototheca* strains to antibiotics and food additives

Test agent	Total (46 strains)	*Prototheca zopfii* gen. 2 (42 strains)	*Prototheca* spp. (4 strains)
Amikacin	42	38	4
Ampicillin	R[1]	R	R
Aztreonam	R	R	R
Cefepime	R	R	R
Ceftazidime	R	R	R
Chloramphenicol	R	R	R
Ciprofloxacin	R	R	R
Colistin sulfate	46	42	4
Erythromycin	R	R	R
Phosphomycin	R	R	R
Gentamicin	46	42	4
Imipenem	1	1	R
Kanamycin	42	38	4
Levofloxacin	2	2	R
Meropenem	1	1	R
Mupirocin	R	R	R
Netilmicin	46	42	4
Nitrofurantoin	R	R	R
Oxacillin	R	R	R
Penicillin G	R	R	R
Piperacillin	R	R	R
Quinupristin/dalfopristin	3	2	1
Rifamycin	3	2	1
Streptomycin	21	18	3
Tetracycline	45	41	4
Ticarcillin/clavulanic acid	3	2	1
Tobramycin	25	21	4
Vancomycin	2	1	1
Nisin	38	38	R
Lysozyme	R	R	R
Potassium sorbate	R	R	R

[1]R = resistant strain.

Susceptibility to Nisin, Lysozyme, and Potassium Sorbate

All the tested strains were resistant to lysozyme and potassium sorbate. Thirty-one *P. zopfii* genotype 2 isolates (73.8%) displayed in vitro susceptibility to nisin, whereas strains belonging to the potential new species and *P. blaschkeae* were resistant (Tables 3–4). In the last decade, the high level of antibiotic resistance observed in *Prototheca* isolates led many authors to study new algaecide compounds against *Prototheca* strains. Lopes et al. (2008), Salerno et al. (2010), and Gonçalves

Table 3. Minimum inhibitory concentration ranges and concentrations inhibiting 50 and 90% (MIC_{50} and MIC_{90}, respectively) of antibiotics and food additive used in this study[1]

	Prototheca zopfii gen. 2 (42 strains)				*P. zopfii* gen. 2 ($SAG2021^T$)	*P. zopfii* gen. 1 ($SAG2063^T$)	*Prototheca blaschkeae* ($SAG2064^T$)
Test agent	SS[2]	MIC range	MIC_{50}	MIC_{90}	MIC	MIC	MIC
Amikacin	38	6–32	32	32	>32	16	4
Colistin sulfate	42	4–128	24	128	6	4	8
Gentamicin	42	6–32	8	32	12	4	4
Kanamycin	38	4–32	32	32	8	4	16
Netilmicin	42	4–24	12	24	24	3	3
Tetracycline	41	32–>256	>256	>256	128	64	8
Tobramycin	21	8–16	>256	>256	8	3	16
Nisin	31	12,500–50,000	25,000	50,000	12,500	25,000	R[3]

[1]MIC results expressed in μg/mL (antibiotics) and IU/g (nisin).

[2]SS = susceptible strains (number).

[3]R = resistant strain.

Table 4. Minimum inhibitory concentration geometric means (gm) of antibiotics and food additive used in this study

	Italy		Brazil			
	Prototheca zopfii gen. 2 (13 strains)		*P. zopfii* gen. 2 (29 strains)		*Prototheca* spp. (4 strains)	
Test agent	SS[1]	MIC gm[2]	SS	MIC gm	SS	MIC gm
Amikacin	10	16.6	28	29.4	4	11.7
Colistin sulfate	13	4.9	29	56.0	4	12.9
Gentamicin	13	9.9	29	12.3	4	13.0
Kanamycin	13	10.3	25	30.9	4	10.0
Netilmicin	13	12.0	29	12.3	4	14.1
Tetracycline	13	32.0	28	>256.0	4	14.3
Tobramycin	4	9.5	17	15.2	4	10.2
Nisin	11	26,794.3	20	18,574.9	—	—

[1]SS = susceptible strains.

[2]MIC gm expressed in μg/mL (antibiotics) and IU/g (nisin).

et al. (2015) demonstrated the in vitro effectiveness of some disinfectants and peracetic acid-, sodium hypochlorite-, and iodine-based antiseptics in the control of mammary protothecosis in dairy herds. Other authors (Bouari et al., 2011; Morandi et al., 2015) showed the algaecidal effect of the main components of essential oils (carvacrol, cinnamaldehyde, and thymol) against these microalgae. To our knowledge, the present study is the first one to test nisin against *Prototheca* species involved in animal disease. Nisin (E234) is a bacteriocin produced by some strains of *Lactococcus lactis* that exhibits wide-spectrum antimicrobial action. Toxicological studies showed that the intake of this bacteriocin does not cause any toxic effect in humans. Because of this, nisin is the only bacteriocin approved for food use and was included as a biopreservative ingredient in the European food additive list (Balciunas et al., 2013). Our results showed that a large percentage of *P. zopfii* genotype 2 involved in bovine protothecal mastitis were sensitive to different concentrations of nisin. *Lactococcus lactis* strains are widely used in the production of cheese, and several of these strains are able to synthesize high quantities of nisin (from 4,000 to 119,000 IU/mL) in different conditions (Jiang et al., 2015; Zhao et al., 2015). These observations and our results will lead us to explore the use of the nisin-producing *Lc. lactis*, alone or in combination with other lactic acid bacteria, to suppress the growth of *P. zopfii*.

CONCLUSIONS

This study shows that RAPD-PCR, which is a rapid, inexpensive, and highly discriminating tool (D = 0.98) can be successfully used to characterize *Prototheca* strains and could give a good scientific contribution for better understanding the protothecal mastitis in dairy herd. The importantly high activity of netilmicin against *Prototheca* spp. validates its potential use as a therapeutic agent for bovine protothecosis. Moreover, in vitro results presented herein indicate by first time a high percentage of *P. zopfii* genotype 2 isolates inhibited by nisin. Nevertheless, further studies are needed to assess more accurately the in vitro potency of netilmicin and nisin to determine their clinical efficiency. We think that additional investigations on biofilm development and antibiotic efficacy will yield insights for the prevention of protothecal infections.

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