Antimicrobial activity of crude extracts from actinomycetes against mastitis pathogens

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ABSTRACT

The emergence of antimicrobial resistance to commonly used antibiotics has necessitated the development of new antimicrobial products. Crude extracts produced by actinomycetes contain antimicrobial metabolites that can inhibit bacterial growth. The objective of our study was to evaluate the antimicrobial activity of crude extracts (Caat1–54 and CaatP5–8) produced by actinomycetes against isolates of *Staphylococcus aureus*, *Staphylococcus chromogenes*, *Streptococcus dysgalactiae*, and *Streptococcus uberis*, which were obtained from the milk of cows affected by mastitis in 23 dairy herds. Twenty isolates of each bacterial species were used to define minimum inhibitory concentrations (MIC) of both crude extracts and ceftiofur (positive control). The MIC₅₀ and MIC₉₀ were defined at the concentration required to inhibit the growth of 50 and 90% of bacterial isolates tested, respectively. The MIC results were evaluated by survival analysis. *Staphylococcus aureus* isolates presented MIC₉₀ of Caat 1–54 ≥ 6.25 $\mu\text{g/mL}$, ceftiofur ≥ 12.5 $\mu\text{g/mL}$, and Caat P5–8 ≥ 100 $\mu\text{g/mL}$. *Streptococcus uberis* presented MIC₉₀ of ceftiofur ≥ 0.39 $\mu\text{g/mL}$, Caat 1–54 ≥ 50 $\mu\text{g/mL}$, and Caat P5–8 ≥ 100 $\mu\text{g/mL}$. *Staphylococcus chromogenes* isolated from subclinical mastitis presented MIC₉₀ of Caat 1–54 ≥ 0.78 $\mu\text{g/mL}$ and ceftiofur and Caat P5–8 of ≥ 6.25 and ≥ 100 $\mu\text{g/mL}$, respectively. *Streptococcus dysgalactiae* isolated from clinical mastitis presented similar MIC₉₀ values between antimicrobials tested (ceftiofur, Caat 1–54, and Caat P5–8), but these values (≥ 100 $\mu\text{g/mL}$) were higher than the values obtained from other pathogens evaluated in the present study. Our results indicate that Caat 1–54 and Caat P5–8 crude extracts present in vitro antimicrobial activity against isolates of *Staph. aureus*, *Staph. chromogenes*, *Strep. dysgalactiae*, and *Strep. uberis* isolated from clinical and subclinical mastitis.

Key words: intramammary infection, mastitis pathogen, antimicrobial, minimum inhibitory concentration

INTRODUCTION

Bovine mastitis is one of the most common infectious diseases in dairy herds, and bacterial infections are the main cause of mastitis (Pol and Ruegg, 2007a). *Staphylococcus* spp. and *Streptococcus* spp. are the most frequently isolated bacterial genera causing IMI (Makovec and Ruegg, 2003). Thus, besides economic losses associated with reduced milk production in cases of subclinical mastitis, some costs are associated with antibiotic treatment and disposal of milk with antibiotic residues in cases of clinical mastitis (Rajala-Schultz et al., 2004; Schukken et al., 2009).

The use of antibiotics for mastitis treatment can increase the risk of antimicrobial resistance and reduce efficacy of antibiotic treatments in affected cows (Barkema et al., 2006; Oliveira et al., 2012; Saini et al., 2012). Furthermore, contact between cattle and humans in livestock and the role of animals as a reservoir and source of antimicrobial-resistant bacteria are also a concern to human health (Pol and Ruegg, 2007b; Fitzgerald, 2012).

Most *Staphylococcus aureus* isolates obtained from bovine mastitis cases were resistant to penicillin, ampicillin, and erythromycin (Jagielski et al., 2014; Liu et al., 2017), whereas *Streptococcus dysgalactiae* isolates have presented high frequency of resistance to kanamycin, sulfonamide, and streptomycin (Zhang et al., 2018). On the other hand, isolates of *Staph. aureus*, CNS, and *Streptococcus* spp. presented low resistance frequency to different classes of antimicrobials commonly used in mastitis treatments (Ruegg et al., 2015; Schmidt et al., 2015). These results were obtained by studies developed in different regions of the world and this divergence may be related to antimicrobial usage. Therefore, periodic monitoring of the susceptibility of mastitis pathogens to commonly used antimicrobials and the effectiveness of new active compounds with

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antimicrobial activity should be done to reduce treatment failures and cross-resistance to human pathogens.

Monitoring antimicrobial resistance can be done by in vitro susceptibility tests such as the disk diffusion method, standard broth dilution (macrodilution and microdilution), and agar dilution techniques (CLSI, 2008). Minimum inhibitory concentration by broth microdilution is a quantitative technique whose results indicate the potential of an antimicrobial to inhibit bacterial growth (Cortinhas et al., 2013).

Most active antimicrobial compounds are produced using biological fermentation. Similarly, the fermentation of actinomycetes, especially *Streptomyces*, produces crude extracts composed of several metabolites that have antimicrobial activity (Taddei et al., 2006; Chater et al., 2010). Previous studies reported antimicrobial activity of metabolites produced by actinomycetes against human pathogens (bacteria, fungi, and viruses; de Oliveira et al., 2010; Rakshanya et al., 2011; Priya et al., 2015). However, few studies evaluated antimicrobial activity of metabolites produced by actinomycetes against bovine mastitis pathogens. Our hypothesis was that crude extracts obtained from actinobacteria could inhibit growth of mastitis pathogens in vitro. Therefore, the objective of our study was to evaluate antimicrobial activity, using MIC, of 2 crude extracts (Caat 1–54 and Caat P5–8) from actinomycetes against *Staphylococcus aureus*, *Staphylococcus chromogenes*, *Streptococcus dysgalactiae*, and *Streptococcus uberis* isolates from clinical and subclinical mastitis.

MATERIALS AND METHODS

Crude Extracts Production and Active Compound Identification

Actinomycetes used in our study were provided from the bacterial strain collection of Brazilian Agricultural Research Corporation (Coleção de Microrganismos de Importância Agrícola e Ambiental, CCMA, EMBRAPA, Brazil). Strains were maintained as suspensions of mycelia fragments in 20% glycerol at -20°C and reactivated onto plates containing potato dextrose agar medium at 30°C . To obtain crude extracts, actinomycetes named Caat 1–54 and Caat P5–8 were cultivated in 500-mL Erlenmeyer flasks containing 100 mL of potato dextrose medium at 30°C under shaking conditions at 150 rpm for 10 d. Both crude extracts, Caat 1–54 and Caat P5–8, were submitted to the same purification process. The isolate Caat 1–54 was inoculated in 4.5 L of yeast extract-malt extract medium (ISP2) and, after 10 d, the broth fermentation was submitted to liquid-liquid extraction with ethyl acetate (Bauermeister et al., 2016). A total of 240 mg of crude extract was puri-

fied using a preparative LC with UV detection with a binary pump (Shimadzu Corp., Kyoto, Japan). The following conditions were used: (1) phase A was a gradient of 0.1% formic acid; (2) phase B was methanol/0.1% formic acid; (3) gradient started with 70% phase B, increased linearly to 95% within 40 min, followed by additional hold for 10 min in 95% phase B with a flow rate of 16 mL/min and detection at 280 and 310 nm; and (4) peak purification was done using a Shim-Pack C18 column (Shimadzu Corp.) 19.0×250 mm, 10 μm .

Both crude extracts were fractionated in preparative HPLC. Each fraction was bioassayed against 2 strain identified biochemically as *Staph. aureus* and analyzed by direct infusion-MS to identify the chemical profile. For this bioassay, 2 mL of each fraction was dried and solubilized in 10 μL of methanol. An aliquot of 5 μL was then applied to a thin-layer chromatography plate that was not subjected to the chromatographic process. The thin-layer chromatography plates were covered with potato dextrose agar medium and, after 24 h, a bacterial suspension at a turbidity of 0.5 McFarland standard was spread over the top of the thin-layer chromatography plates containing the medium. Inhibition zones were verified using MTT (thiazolyl blue tetrazolium bromide; Sigma-Aldrich, Saint Louis, MO) staining after 24 h of bacteria incubation.

Identification of Bacterial Species

Twenty isolates from each type of pathogen (*Staph. aureus*, *Staph. chromogenes*, *Strep. dysgalactiae*, and *Strep. uberis*) were obtained from milk samples of mammary quarters ($n = 80$) of cows affected by mastitis. These cows were distributed in commercial dairy herds ($n = 23$) in São Paulo and Minas Gerais (southeast Brazil). Milk samples were submitted to microbiological procedures as described in the National Mastitis Council guidelines (Hogan et al., 1999). *Staphylococcus aureus* isolates were obtained from cases of clinical ($n = 10$; 10 herds) and subclinical ($n = 10$; 9 herds) mastitis, whereas *Staph. chromogenes* ($n = 20$; 7 herds) were obtained from cases of subclinical mastitis. Furthermore, *Strep. dysgalactiae* ($n = 20$; 8 herds) were obtained from cows affected by clinical mastitis and *Strep. uberis* were isolated from both clinical ($n = 10$; 6 herds) and subclinical ($n = 10$; 5 herds) mastitis. All isolates were identified at the species level using matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF)-MS (Bruker Daltonik GmbH, Bremen, Germany) coupled with Biotyper Real Time Classification 3.0 software (Bruker Daltonik GmbH), as described by Barreiro et al. (2010). Thus, in the present study, only isolates with species identification score ≥ 2 were used (Barreiro et al., 2010). After MS

analysis, isolates were cryopreserved at -80°C in brain heart infusion broth (Becton, Dickinson and Company, Franklin Lakes, NJ) with 20% glycerol.

Evaluation of Antimicrobial Activity of Crude Extracts

Minimum inhibitory concentration of crude extracts (Caat 1–54 and Caat P5–8) was determined by broth microdilution technique. Ceftiofur hydrochloride (Sigma-Aldrich) was used as positive control. A sterile microdilution 96-well plate (Cral, Cotia, Brazil) was used for each pathogen (CLSI, 2008). Cryopreserved isolates were retrieved and plated in agar supplemented with 5% bovine blood. After 24 h of incubation at 37°C , catalase and morphological analysis were performed to check bacterial characteristics. Isolates were then incubated at 37°C for 24 h in brain heart infusion broth (Becton, Dickinson and Company). Afterward, bacterial suspensions were standardized to 0.5 McFarland using a nephelometer (Uniscience, São Paulo, Brazil) in 0.9% saline solution.

In the present study, cation-adjusted Mueller Hinton broth (Becton, Dickinson and Company) had a pH ranging from 7.2 to 7.4 (CLSI, 2008). Additionally, Mueller Hinton broth was supplemented with 5% of equine serum for the growing of *Streptococcus* spp. (CLSI, 2008; Perini et al., 2014). In a 96-well plate, 4 wells were used for broth sterility control and to evaluate growth of isolates. Twenty isolates of each mastitis pathogen (*Staph. aureus*, *Staph. chromogenes*, *Strep. dysgalactiae*, and *Strep. uberis*) were used for MIC determination of crude extracts from actinomycetes and *Staph. aureus* ATCC 29213 was used as quality control. Analyses were performed in duplicate.

In the first column of the plate, 15 μL of each antimicrobial and 135 μL of Mueller Hinton broth were applied to each well, resulting in a final concentration of 100 $\mu\text{g}/\text{mL}$ of each antimicrobial. In the other columns, except for control wells, 75 μL of Mueller Hinton broth was applied. Serial dilutions were done by transferring 75 μL from the first column to the second. After second column homogenization, 75 μL was transferred from this column to the third and so on. Therefore, 12 different serial dilutions of each crude extract (Caat 1–54 and Caat P5–8) and ceftiofur (positive control) were obtained. The resulting concentrations ranged from 0.048 to 100 $\mu\text{g}/\text{mL}$. After antimicrobial dilution, 7.5 μL of each standardized bacterial suspension was applied to wells. The plates were covered with a plastic film and homogenized at 200 rpm for 10 min on a stirring table (Quimis, Diadema, Brazil). The plates were then incubated at 37°C for 24 h under aerobic conditions. Afterward, 20 μL of MTT was added to the

plates, homogenized on a stirring table at 200 rpm for 10 min, and packed in silver foil. Briefly, the MTT was used to better visualize bacterial growth and determine MIC values (Shi et al., 2007). After 3 h of reaction, plates were evaluated by visual inspection and the lowest antimicrobial concentrations that inhibited growth of mastitis pathogens were determined. The ceftiofur breakpoints were used to classify isolates causing bovine mastitis as susceptible ≤ 2 $\mu\text{g}/\text{mL}$, intermediate between >2 and <8 $\mu\text{g}/\text{mL}$, or resistant ≥ 8 $\mu\text{g}/\text{mL}$ in accordance with the guidelines in CLSI document M31-A3 (CLSI, 2008); these breakpoints were also used to classify *Staph. chromogenes* isolates (Cortinhas et al., 2013).

Statistical Analysis

The MIC_{50} and MIC_{90} of Caat 1–54 and Caat P5–8 were determined at the lowest concentration of crude extracts needed to inhibit 50 and 90%, respectively, of tested isolates of each bacterial species. Survival analysis was performed to evaluate the differences between antimicrobials tested against bacterial isolates, in accordance with procedures done by Cortinhas et al. (2013), by PROC LIFETEST (SAS ver. 9.4, SAS Institute Inc., Cary, NC). Thus, antimicrobial concentrations (Caat 1–54, Caat P5–8, and ceftiofur) were used as time variable and inhibition of bacterial growth was used as event. When isolates were not inhibited at the highest concentration of antimicrobial tested (100 $\mu\text{g}/\text{mL}$), they were considered resistant and these isolates were censored by statistical modeling. Antimicrobial concentration was defined as time variable, whereas the null hypothesis of no difference in the survival strata (antimicrobial concentration at inhibition) was tested via log-rank and Wilcoxon tests, in Kaplan-Meier curves. Any P -values <0.05 were considered significant.

RESULTS

The active fractions of Caat 1–54 and Caat P5–8 produced by actinomycetes exhibited notable antimicrobial activity against gram-positive mastitis pathogens after HPLC and spectroscopic analyses. Direct infusion-MS analyses detected the most active fractions (**Fr**) of Caat 1–54, which ranged from Fr32 to Fr35, whereas for Caat P5–8 the range was from Fr13 to Fr14. The Caat 1–54 active fractions were grouped and Lysolipin I was detected (Figure 1a) by high-resolution mass spectrometry, heteronuclear multiple bond correlation, heteronuclear multiple-quantum correlation, and nuclear magnetic resonance analysis. The Caat P5–8 active fractions were grouped and the active compound (Figure 1b) was assigned to kalafungin structure based on nuclear magnetic resonance, heteronuclear multiple

Figure 1. Active compounds identified from crude extracts (Caat 1–54 and Caat P5–8) produced by actinomycetes. Chemical structure of (a) lysolipin I in Caat 1–54 crude extract; and (b) kalafungin in Caat P5–8 crude extract.

bond correlation, heteronuclear single-quantum correlation, and MS/MS fragmentation patterned experiments.

The Caat 1–54 crude extract inhibited 90% of *Staph. aureus* ($\text{MIC}_{90} \geq 6.25 \mu\text{g/mL}$), *Staph. chromogenes* ($\text{MIC}_{90} \geq 0.78 \mu\text{g/mL}$), *Strep. dysgalactiae* ($\text{MIC}_{90} \geq 100 \mu\text{g/mL}$), and *Strep. uberis* ($\text{MIC}_{90} \geq 50 \mu\text{g/mL}$) in different concentrations (Table 1). Heterogeneous Kaplan-Meier survival curves (Figure 2) were observed among these bacterial species ($P < 0.0012$ for log-rank test and $P < 0.0017$ for Wilcoxon test).

The Caat P5–8 inhibited mostly in 3 consecutive serial concentrations ranging from 25 to 100 $\mu\text{g/mL}$ against all evaluated isolates (Table 1). Briefly, *Staph. aureus*, *Staph. chromogenes*, and *Strep. uberis* had $\text{MIC}_{90} \geq 100 \mu\text{g/mL}$, whereas *Strep. dysgalactiae* presented $\text{MIC}_{90} > 100 \mu\text{g/mL}$. Survival curves (Figure 3) were heterogeneous among the 4 species bioassayed ($P < 0.001$ for log-rank and Wilcoxon tests).

Ceftiofur inhibited *Staph. aureus*, *Staph. chromogenes*, and *Strep. uberis* isolates at MIC_{90} concentrations of ≥ 12.5 , ≥ 6.25 , and $\geq 0.39 \mu\text{g/mL}$, respectively (Table 1). For *Strep. dysgalactiae* it was observed that 90% of isolates presented bacterial growth at the highest concentration analyzed; therefore, MIC_{90} was $> 100 \mu\text{g/mL}$ (Table 1). These different proportions of inhibition resulted in heterogeneous (Figure 4) survival curves ($P < 0.001$ for log-rank and Wilcoxon tests).

The quality control strain *Staph. aureus* ATCC 29213 presented a MIC value of $1.56 \mu\text{g/mL}$ for ceftiofur, which is somewhat above the recommended CLSI-acceptable quality control ranges (0.25, 0.50, and $1.0 \mu\text{g/mL}$; CLSI, 2008). Therefore, the MIC value of $1.56 \mu\text{g/mL}$ obtained for ceftiofur in the current study could have been similar if the same breakpoints of CLSI had been adopted.

According to the evaluation of susceptibility of mastitis pathogens to ceftiofur, 80% of *Staph. aureus* isolates were classified as intermediate and 20% were resistant, whereas for *Staph. chromogenes* 97.5% of isolates were classified as intermediate and 2.5% as resistant. In relation to *Streptococcus* spp., 80.5% of *Strep. dysgalactiae* isolates were considered susceptible and 20% were resistant whereas 95% of *Strep. uberis* isolates were susceptible and 5% resistant to ceftiofur.

DISCUSSION

In the present study, we evaluated in vitro antimicrobial activity of crude extracts (Caat 1–54 and Caat P5–8) produced by actinomycetes isolated from the Caatinga biome, which is in northeast Brazil, against mastitis pathogens. Therefore, MIC_{50} and MIC_{90} crude extracts were determined in vitro against *Staph. aureus*, *Staph. chromogenes*, *Strep. dysgalactiae*, and *Strep. uberis* isolated from cows affected by clinical and subclinical mastitis. We observed that Caat 1–54 and Caat P5–8 presented antimicrobial activity due to the active compounds lysolipin I (Drautz et al., 1975) and kalafungin (Kakinuma et al., 1990), respectively, against isolates of *Staph. aureus*, *Staph. chromogenes*, *Strep. dysgalactiae*, and *Strep. uberis*. The Caat 1–54 crude extract presented antimicrobial activity against *Staph. aureus*, *Staph. chromogenes*, *Strep. dysgalactiae*, and *Strep. uberis* at lower concentrations than Caat P5–8 extract.

The Caat 1–54 extract presented lower values of MIC_{50} and MIC_{90} for *Staph. aureus* than ceftiofur and Caat P5–8 extract. Although Caat 1–54 presented higher inhibition of *Staph. aureus*, this extract presented higher variability of MIC values because 8 tested dilutions were necessary to inhibit all isolates of *Staph. aureus* (MIC of Caat 1–54 between ≥ 0.096 and $\geq 12.5 \mu\text{g/mL}$ for clinical and subclinical cases). This variability of MIC values for the inhibition of *Staph. aureus* may be associated with genetic differences among strains, as the isolates were obtained from different herds and some studies have already reported a great diversity in *Staph. aureus* population (Smith et al., 2005; Costa et al., 2012). On the other hand, ceftiofur and Caat P5–8 extract presented higher concentrations than Caat 1–54

Table 1. Percentage of isolates inhibited for each tested concentration and MIC₅₀ and MIC₉₀ values for ceftiofur and crude extracts (Caat 1-54 and Caat P5-8) produced by actinomycetes against mastitis pathogens¹

Antimicrobial	MIC (μg/mL)														P-value			
	<0.048	0.048	0.096	0.19	0.39	0.78	1.56	3.12	6.25	12.5	25	50	100	>100	MIC ₅₀ ²	MIC ₉₀ ²	Log-Rank	Wilcoxon
Caat 1–54																		
<i>Staphylococcus aureus</i>	0	0	2.5	37.5	25	5	5	12.5	2.5	5	0	0	0	5	≥0.39	≥6.25	<0.0012	<0.0017
<i>Staphylococcus chromogenes</i>	0	0	0	0	7.5	82.5	10	0	0	0	0	0	0	0	≥0.78	≥0.78		
<i>Streptococcus dysgalactiae</i>	5	0	0	2.5	20	20	5	7.5	5	2.5	5	2.5	17.5	7.5	≥1.56	≥100		
<i>Streptococcus uberis</i>	5	0	2.5	20	15	7.5	17.5	0	0	2.5	12.5	12.5	5	0	≥0.78	≥50		
Caat P5–8																		
<i>Staph. aureus</i>	0	0	0	0	0	0	0	0	0	0	32.5	47.5	15	5	≥50	≥100	<0.0001	<0.0001
<i>Staph. chromogenes</i>	0	0	0	0	0	0	0	0	0	0	2.5	22.5	75	0	≥100	≥100		
<i>Strep. dysgalactiae</i>	0	0	0	0	0	2.5	0	0	0	2.5	12.5	55	15	12.5	≥50	>100		
<i>Strep. uberis</i>	0	0	0	5	0	0	0	0	0	0	20	55	20	0	≥50	≥100		
Ceftiofur																		
<i>Staph. aureus</i>	0	0	0	0	0	0	0	30	50	20	0	0	0	0	≥6.25	≥12.5	<0.0001	<0.0001
<i>Staph. chromogenes</i>	0	0	0	0	0	0	0	27.5	70	2.5	0	0	0	0	≥6.25	≥6.25		
<i>Strep. dysgalactiae</i>	80	0	0	0	0	0	0	0	0	0	2.5	0	2.5	15	<0.048	>100		
<i>Strep. uberis</i>	15	0	0	45	35	0	0	0	5	0	0	0	0	0	≥0.19	>0.39		

¹In vitro assays considering isolates (n = 80) of *Staphylococcus aureus* (n = 20), *Staphylococcus chromogenes* (n = 20), *Streptococcus dysgalactiae* (n = 20), and *Streptococcus uberis* (n = 20).

²MIC₅₀ and MIC₉₀ = minimum inhibitory concentrations required to inhibit growth of 50% and 90% of tested isolates, respectively.

to inhibit *Staph. aureus*, and only 3 consecutive serial dilutions for inhibition *Staph. aureus* growth (MIC of ceftiofur between ≥ 3.12 and ≥ 12.5 $\mu\text{g/mL}$, and MIC of Caat P5–8 between ≥ 25 and ≥ 100 $\mu\text{g/mL}$, clinical and subclinical cases).

Few studies have evaluated the antimicrobial activity of crude extracts produced by actinomycetes against mastitis pathogens. Active metabolites produced by 2 actinomycetes from Amazonia (*Streptomyces* spp. DPUA 1542 and *Nocardia* spp. DPUA 1571) presented antimicrobial activity against *Staph. aureus* isolated from bovine mastitis (Cunha et al., 2010). However, it was difficult to compare the results obtained by Cunha et al. (2010) with the results obtained in the present study because the former used the disk diffusion method. In the present study, we used the microdilution technique because it provides quantitative results (CLSI, 2008).

Due to the concern regarding bacterial resistance and therapy failures, some studies (Diaz et al., 2010; Perini et al., 2014) performed initial trials to evaluate in vitro antimicrobial activity of antimicrobial compounds against pathogens. Among alternatives, essential oils and plants extract have been evaluated most. Diaz et al. (2010) evaluated antimicrobial activity of extracts

obtained from medicinal plants used in Brazil against 15 *Staph. aureus* isolated from bovine mastitis. Thirty-seven extracts were evaluated and 4 of these extracts were observed to inhibited *Staph. aureus* growth. *Cymbopogon nardus* extract presented MIC value of 500 $\mu\text{g/mL}$ and *Baccharis dracunculifolia* presented MIC value of 800 $\mu\text{g/mL}$. The highest MIC values (1,000 $\mu\text{g/mL}$) were obtained from *Senna macranthera* and *Artemisia absinthium*. These MIC results were higher than MIC₉₀ values against *Staph. aureus* that were observed in the present study (Caat 1–54 ≥ 6.25 $\mu\text{g/mL}$, and Caat P5–8 ≥ 100 $\mu\text{g/mL}$).

Perini et al. (2014) evaluated the antimicrobial activity of 8 essential oils against *Staph. aureus* isolated from cows affected by clinical and subclinical mastitis. *Cinnamomum cassia* oil presented the best antimicrobial activity against *Staph. aureus* isolates (MIC = 0.4 $\mu\text{L/mL}$). Perini et al. (2014) observed that isolates of *Staph. aureus* were inhibited at 10 different dilutions of *Cymbopogon flexuosus*, *Cymbopogon winterianus*, and *Salvia sclarea*. Similarly, in the present study, we observed that *Staph. aureus* isolates were also inhibited at 8 (0.096, 0.19, 0.39, 0.78, 1.56, 3.12, 6.25, and 12.5 $\mu\text{g/mL}$) of the 12 tested concentrations of Caat 1–54 crude extract. In fact, as mentioned herein, this variability of MIC values

Figure 2. Kaplan-Meier survival curves of Caat 1–54 crude extract for *Staphylococcus aureus*, *Staphylococcus chromogenes*, *Streptococcus dysgalactiae*, and *Streptococcus uberis* isolated from clinical and subclinical bovine mastitis cases.

for the inhibition of bacterial growth may be caused by the diversity of *Staph. aureus* strains (Smith et al., 2005; Costa et al., 2012). Furthermore, previous results suggest a bacterial growth inhibition from plants to treat bovine mastitis (Ananda Baskaran et al., 2009). Active compounds, such as *trans*-cinnamaldehyde, eugenol, carvacrol, and thymol, were evaluated against isolates obtained from clinical mastitis. *Trans*-cinnamaldehyde was the most efficient antimicrobial compound because it inhibited the growth of all tested pathogens (*Staph. aureus*, *Escherichia coli*, *Strep. uberis*, *Streptococcus agalactiae*, and *Strep. dysgalactiae*).

Ceftiofur is a third-generation cephalosporin antibiotic with broad-spectrum antimicrobial activity that is frequently used for the treatment of infectious diseases in dairy cows (Rajala-Schultz et al., 2004; Saini et al., 2012). For this reason, ceftiofur was used in the present study as a positive control. Previous studies evaluated the susceptibility of selected mastitis pathogens to ceftiofur using the agar dilution method and reported similar values of MIC₅₀ (0.5 µg/mL) and MIC₉₀ (1.0 µg/mL) for *Staph. aureus* obtained from clinical and subclinical mastitis (Oliveira et al., 2012; Cortinhas et al., 2013). Saini et al. (2012) described similar MIC₅₀ and MIC₉₀ (1 µg/mL) to those reported by Oliveira

et al. (2012) and Cortinhas et al. (2013). Ruegg et al. (2015) studied only *Staph. aureus* isolated from clinical mastitis and described that MIC₅₀ and MIC₉₀ of ceftiofur was 0.25 µg/mL, which was lower than MIC (0.5 to 1.0 µg/mL) described by Oliveira et al. (2012), Saini et al. (2012), and Cortinhas et al. (2013). However, in the present study, MIC₅₀ and MIC₉₀ values of ceftiofur against *Staph. aureus* isolates were higher than the results obtained (0.25 to 1.0 µg/mL) in those studies (Oliveira et al., 2012; Saini et al., 2012; Cortinhas et al., 2013; Ruegg et al., 2015).

In the present study, 80% of *Staph. aureus* and 97.5% of *Staph. chromogenes* isolates were classified according to CLSI (2008) guidelines as intermediate, and we also classified some isolates (20% of *Staph. aureus* and 2.5% of *Staph. chromogenes*) as resistant to ceftiofur. Likewise, in another Brazilian study that evaluated the susceptibility of CNS isolated from bovine mastitis, 75% of isolates were resistant to amoxicillin and 80% were resistant to penicillin (Freitas et al., 2005). In summary, the current higher percentage of ceftiofur resistance could be explained due to the uncontrolled use of antimicrobials for mastitis treatment in Brazilian dairy herds, as antimicrobials could be bought and used without a veterinary prescription.

Figure 3. Kaplan-Meier survival curves of Caat P5-8 crude extract for *Staphylococcus aureus*, *Staphylococcus chromogenes*, *Streptococcus dysgalactiae*, and *Streptococcus uberis* isolated from clinical and subclinical bovine mastitis cases.

The Caat 1–54 extract presented lower values of MIC_{50} and MIC_{90} for *Staph. chromogenes* than ceftiofur and Caat P5–8 extract. Both MIC_{50} and MIC_{90} presented the same values for each evaluated antimicrobial category because the inhibition of growth of isolates occurred more frequently in only 3 concentrations of the serial dilutions tested (ceftiofur = 3.12, 6.25, and 12.5 $\mu\text{g/mL}$; Caat 1–54 = 0.39, 0.78, and 1.56 $\mu\text{g/mL}$; Caat P5–8 = 25, 50, and 100 $\mu\text{g/mL}$). *Staphylococcus chromogenes* is considered a minor pathogen causing subclinical mastitis because its inflammatory response at quarter level is lower (median values of $200,000 < \text{SCC} \leq 500,000$ cells/mL) than that caused by major pathogens (*Staph. aureus*, *Strep. dysgalactiae*, and *Strep. uberis*; Piepers et al., 2013; Tomazi et al., 2015). Nevertheless, it has been described that intramammary infections caused by *Staph. chromogenes* can increase SCC comparable to *Staph. aureus* (Supré et al., 2011), and this is the reason for the evaluation of the susceptibility of *Staph. chromogenes* to crude extracts in the present study.

Ceftiofur presented lower MIC_{50} (<0.048 $\mu\text{g/mL}$) against *Strep. dysgalactiae* than Caat 1–54 (≥ 1.56 $\mu\text{g/mL}$) and Caat P5–8 (≥ 50 $\mu\text{g/mL}$). Furthermore, MIC_{90} of these 3 antimicrobials presented higher values (>100

$\mu\text{g/mL}$ for ceftiofur, Caat 1–54, and Caat P5–8) than MIC_{50} . This result suggests that MIC_{90} of ceftiofur was 14 dilutions higher in relation to MIC_{50} (variation of <0.048 to >100 $\mu\text{g/mL}$). The MIC_{90} of Caat 1–54 was 7 dilutions higher than MIC_{50} (variation of ≥ 1.56 to >100 $\mu\text{g/mL}$) and MIC_{90} of Caat P5–8 was 3 dilutions higher in comparison to MIC_{50} (variation of ≥ 50 to >100 $\mu\text{g/mL}$). In the present study, MIC_{50} of ceftiofur against *Strep. dysgalactiae* was <0.048 $\mu\text{g/mL}$, because bacterial growth inhibition was observed at the lowest concentration of antimicrobial tested. Because of this, it was not possible to precisely determine MIC_{50} for these isolates. Cortinhas et al. (2013) reported a similar value of MIC_{50} (0.03 $\mu\text{g/mL}$), but a different value of MIC_{90} (0.06 $\mu\text{g/mL}$) for ceftiofur against *Strep. dysgalactiae* isolated from bovine mastitis when compared with the results of the present study.

Considering that all evaluated antimicrobials were tested under the same serial dilution, varying from 0.048 to 100 $\mu\text{g/mL}$, ceftiofur presented higher in vitro antimicrobial activity against *Strep. uberis* than Caat 1–54 and Caat P5–8 crude extracts, as a lower concentration of ceftiofur was necessary to inhibit *Strep. uberis* isolates. Caat 1–54 presented higher antimicrobial activity against *Strep. uberis* clinical and subclini-

Figure 4. Kaplan-Meier survival curves of ceftiofur crude extract for *Staphylococcus aureus*, *Staphylococcus chromogenes*, *Streptococcus dysgalactiae*, and *Streptococcus uberis* isolated from clinical and subclinical bovine mastitis cases.

cal mastitis due to lower MIC₉₀ when compared with Caat P5–8 (≥ 50 vs. ≥ 100 $\mu\text{g/mL}$). However, Caat 1–54 extract presented MIC values against *Strep. uberis* isolated from clinical and subclinical mastitis with a wide range between the evaluated dilutions (<0.048 to 100 $\mu\text{g/mL}$). The MIC results of different compounds with antimicrobial activity were not found for *Strep. uberis* for comparison with the results obtained in the present study. The MIC₅₀ of ceftiofur against *Strep. uberis* isolated from clinical (≥ 0.19 $\mu\text{g/mL}$) and subclinical (≥ 0.39 $\mu\text{g/mL}$) mastitis were lower than the values described by Cortinhas et al. (2013; MIC₅₀ of 1 $\mu\text{g/mL}$; MIC₉₀ of 2 $\mu\text{g/mL}$) and by Rajala-Schultz et al. (2004; MIC of 4 $\mu\text{g/mL}$).

In this preliminary study, we observed that Caat 1–54 and Caat P5–8 crude extracts produced by actinomycetes from Caatinga biome presented in vitro antimicrobial activity against *Staph. aureus*, *Staph. chromogenes*, *Strep. dysgalactiae*, and *Strep. uberis* isolated from cases of bovine mastitis. Despite in vitro inhibition of bacterial growth by crude extracts, future studies should be undertaken to evaluate the pharmacokinetics and cytotoxicity of Caat 1–54 and Caat P5–8 on the mammary tissue before the in vivo testing. As Caat 1–54 and Caat P5–8 present desirable antimicrobial characteristics, both crude extracts might be a potential alternative in the developing products against mastitis-causing pathogens.

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