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Resistance of *Corynebacterium pseudotuberculosis* in the Brazilian semiarid environment[1]

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ABSTRACT.- Sá M.C.A., Oliveira S.A.S., Dantas Jr E.M., Gouveia G.V., Gouveia J.J.S., Veschi J.L.A. & Costa M.M. 2018. **Resistance of *Corynebacterium pseudotuberculosis* in the Brazilian semiarid environment**. *Pesquisa Veterinária Brasileira 38(6):1091-1096.* Universidade Federal do Vale do São Francisco, Campus de Ciências Agrárias, Rodovia BR-407 Km 12, Lote 543, Projeto de Irrigação Nilo Coelho, C1, Zona Rural, Petrolina, PE 56300-000, Brazil. E-mail: gisele.veneroni@univasf.edu.br

The semiarid northeast of Brazil contains a unique biome known as caatinga, with a maximum temperature of 40 ºC and a relativity humidity of 56%. The caatinga is characterized by a variety of plants, including *Cereus jamacaru* Dc (mandacaru), *Poincianella microphylla* Mart. ex G. Don (catingueira), *Pilosocereus gounellei* FAC Weber (xique-xique) and *Mimosa tenuiflora* (Willd.) Poir (jurema preta). Sheep and goat industries are economically strong in that region, despite the fact that caseous lymphadenitis is highly prevalent. The aim of the present study was to assess the survival and biofilm production of *Corynebacterium pseudotuberculosis* isolates in the environment and under controlled temperatures (28°C, 37°C and 42°C) under different surfaces (plants, soil, wood, wire and thorns). In addition, we investigated the effects of applying the disinfectants chlorhexidine, hypochlorite and quaternary ammonia in soil, tiles, wood and vegetation cover. Four strains of *C. pseudotuberculosis* were selected (two from goats and two from sheep) for inoculation according to their *in vitro* biofilm production. Adherence to microplates was used to assess the biofilm-forming ability of the bacteria. Lower survival rates were observed when isolates of *C. pseudotuberculosis* were subjected to a temperature of 42°C. In terms of caatinga biome plants, contamination of jurema-preta plants resulted in the lowest survival rates. The disinfectant quaternary ammonia promoted a lower inoculum survival in all surfaces. The disinfectants and the higher temperature contributed to the reduction of biofilm production in isolates of *C. pseudotuberculosis*. knowledge of these patterns is important for the establishment of disease control measures, given the questionable efficacy of the treatment and the immuno-prophylaxis of caseous lymphadenitis.

INDEX TERMS: Resistance, *Corynebacterium pseudotuberculosis,* Brazilian semiarid, survival, biofilm, caseous lymphadenitis, disease, bacterioses.

RESUMO.- [**Resistência de *Corynebacterium pesudotuberculosis* no ambiente do semiárido brasileiro.**] O semiárido nordestino do Brasil possui um bioma exclusivo, a caatinga, que apresenta temperatura máxima de 40ºC e uma umidade relativa do ar de 56%. A caatinga é caracterizada por uma diversidade de plantas, entre elas *Cereus jamacaru* DC. (mandacaru), *Poincianella microphylla* Mart ex G. Don (catingueira), *Pilosocereus gounellei* F.A.C. Weber (xique-xique) e *Mimosa tenuiflora* (Willd.) Poir (jurema preta). A produção de ovinos e caprinos está em franca expansão, porém a linfadenite caseosa é uma enfermidade de alta prevalência na região. Objetiva-se com o presente estudo, verificar a sobrevivência e produção de biofilme em isolados de *Corynebacterium pseudotuberculosis* em temperaturas de 28ºC, 37ºC e 42ºC, quando inoculado em superfícies de solo, madeira, arame e espinho e em plantas nas condições ambientais da caatinga. Além disto, foi verificado o efeito da aplicação dos desinfetantes clorexidine,

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hipoclorito e amônia quaternária sobre o solo, piso (lajota), madeira e vegetação de cobertura do solo. Foram selecionadas quatro amostras de *C. pseudotuberculosis* (dois caprinos e dois ovinos) para inoculação de acordo com a sua produção *in vitro* de biofilme. A adesão a microplacas foi utilizado para avaliar a capacidade de formação de biofilme das bactérias. As menores taxas de sobrevivência foram observadas quando isolados de *C. pseudotuberculosis* foram submetidos a uma temperatura de 42°C. Com relação as plantas do bioma caatinga, a contaminação na planta jurema-preta apresentou menores índices de sobrevivência. O desinfetante amônia quartenária promoveu uma menor sobrevivência do inóculo em todas as superfícies. Os desinfetantes e temperatura contribuíram para a redução na produção de biofilme nos isolados de *Corynebacterium pseudotuberculosis*. O conhecimento destes padrões é importante para o estabelecimento de medidas de controle da enfermidade, dada a eficiência questionável do tratamento e imunoprofilaxia da linfadenite caseosa.

TERMOS DE INDEXAÇÃO: Resistência, *Corynebacterium pesudotuberculosis,* semiárido brasileiro, sobrevivência, biofilme, linfadenite caseosa, doença, bacterioses.

INTRODUCTION

The northeast of Brazil, particularly the semiarid region, is an extremely important area for sheep and goat production industries (Sampaio et al. 2009). This region contains an exclusive biome known as the caatinga, which is characterized by the presence of cactus and deciduous species, including *Cereus jamacaru.* Dc (mandacaru), *Poincianella microphylla* Mart ex G. Don (catingueira), *Pilosocereus gounellei* F.A.C. Weber (xique-xique) and *Mimosa tenuiflora* (Willd.) Poir (jurema-preta). These species are eaten by goats and sheep that graze in the area in extensive and semi-extensive production systems. These plants are very important for animal grazing, mainly during the dry season, when they are essential for animal survival (Alcoforado-Filho et al. 2003).

Due the high density under pasture, the caatinga contains large quantities of animal waste, which can become a source of contamination linked to several illnesses. Plants may retain pathogens responsible for spreading a number of diseases on their stems, leaves and thorns (Riet-Correa et al. 2011). In the semiarid region, the spines of cacti can cause injuries in sheep and goat, contributing to pathogen entry (Riet-Correa et al. 2011). Soil and plants are important in the transmission of diseases to humans and animals, such as that caused by *Pantoea agglomerans* (Cruz et al. 2007).

The agent of caseous lymphadenitis is the bacterium *Corynebacterium pseudotuberculosis*, which has several virulence factors similar to phospholipase D, an enzyme with aglycoprotein exotoxin whose action favors survival (McNamara et al. 1995). Among the proteins forming the cell wall, it is mainly those that are covalently bound to peptidoglycans that are associated with biofilm formation, which contributes to an increase in pathogenicity (Merino et al. 2009) and is associated with the bacterium's ability to adapt its infectivity to different environments (Clutterbuck et al. 2007). This ability of micro-organisms could be related to biofilm formation (Merino et al. 2009).

The aims of the present study were to assess the survival and biofilm production of *C. pseudotuberculosis* isolates stored in plants from caatinga biome and to compare different surfaces from animal production systems, which were tested at various temperatures. The effectiveness of different disinfectants under material contaminated with *C. pseudotuberculosis* isolates was also evaluated.

MATERIALS AND METHODS

Ethics Committee

This study is part of a project entitled "Phenotypic and genotypic aspects related to the virulence and survival in the environment of *Corynebacterium pseudotuberculosis* aimed at implementing measures to control caseous lymphadenitis" and it was approved by the ethics committee on studies and research at the Federal University of the Valley San Francisco under no. 0005/110414.

Corynebacterium pseudotuberculosis isolates

All experiments were performed using four isolates of *C. pseudotuberculosis* with the following characteristics: two were negative for biofilm formation (one from sheep and one from goats), and two were positive for biofilm formation (from sheep and goats). The sheep isolate positive for biofilm formation was classified as a moderate biofilm producer, and the goat isolate positive for biofilm formation was classified as a strong biofilm producer.

Survival of *Corynebacterium pseudotuberculosis* in caatinga plants

Plants. Four native species from caatinga were used: *Cereus jamacaru* DC. (mandacaru), *Poincianella microphylla* Mart ex G. Don (catingueira), *Pilosocereus gounellei* F.A.C. Weber (xique-xique) and *Mimosa tenuiflora* (Willd.) Poir (jurema-preta). These plants were located at Embrapa Semiarido experimental station (Latitude: 09º04'S, Longitude: 40°19'W) in an area of 900m^2. The experiment was performed in the open caatinga environment form September 27, 2013 to November 27, 2013. In this period, the average temperature was 34ºC; the relativity humidity was 56%; and the average rainfall was 0.66mm.

Inoculum. Inocula of the selected *C. pseudotuberculosis* isolates were prepared in 5mL of brain heart infusion (BHI) broth to obtain a suspension with a turbidity of 0.5 on the McFarland scale (10^6 Colony Forming Units). The inocula were applied to pool of lymph node secretions that had previously been autoclaved (120ºC for 15 minutes).

Plant contamination. Three specimens of each plant species were randomly selected for contamination. The contamination procedure was as follows: for each isolate, contaminated swabs were applied to the stalks and spines of the plants. At each application location, five grams of caseous material with *C. pseudotuberculosis* inoculum was spread.

Survival analysis. After applying the inoculum to the plants, samples were collected every 48 h from the contamination site using swabs to analyze the bacteria survival. The swabs with the inoculum were streaked onto sheep blood agar (8%) and incubated for 48h at 37ºC. The relativity humidity reproduced the open caatinga conditions (56%). Compatible colonies were determined based on morphological, biochemical and staining analyses (Quinn et al. 2005). The survival time (in days) was the subject of the analysis.

Survival of *C. pseudotuberculosis* on different substrates and at different temperatures. The survival of *C. pseudotuberculosis* was examined in soil with animal residues and on *Pinus* wood (10 cm x 3 cm), mandacaru (*Ceres jamacaru*) spines and galvanized wire (5cm x 5cm) under incubation at several different temperatures (28ºC, 37ºC and 42ºC). The materials were obtained from sheep and

goat farms autoclaved and placed separately in individual Petri dish (100mm x 15mm).

Subsequently, inocula were generated, and contamination was executed according to the above descriptions. The contaminated materials were transferred to incubators at 28ºC, 37ºC and 42ºC, mimicking the caatinga environment. The average relativity humidity was 64% at 28ºC, 41% at 37ºC and 42ºC. Samples were collected every 48 h to assess the bacteria survival. For this purpose, swabs containing the inocula were seeded on sheep blood agar (8%) and incubated for 48 h at 37ºC. Compatible colonies were determined based on morphological, biochemical and staining analyses (Quinn et al. 2005). The survival time (in days) was also examined.

Survival of *C. pseudotuberculosis* on contaminated surfaces treated with disinfectants. Soil samples were collected from sheep and goat production areas and areas of vegetation cover. Wood and tile samples were also collected from the properties which these animals were kept. All of these materials were sterilized via autoclaving. Each type of material was placed in an individual Petri dish (100mm x 15mm). Subsequently, inocula prepared according to the above descriptions were spread on the materials (soil, wood and tile), which were then held at the environmental temperature (28º) for 48 h, after which the disinfectants doses were applied during this period. These procedures were performed in triplicate at 15-day intervals. The disinfectants used in the experiments were chlorhexidine (0.5%), hypochlorite (0.125%) and quaternary ammonia (15%). One substrate with 1mL of the disinfectant was placed in each Petri dish. The disinfectants were applied individually in the surface. Swab samples were collected every 48 h and streaked on sheep blood (8%) agar plates. These plates were incubated for 48 h at 37ºC. Compatible colonies were determined based on morphological, biochemical and staining analyses (Quinn et al. 2005). The survival time (in days) was also evaluated.

Biofilm detection test. Following experimental contamination, *C. pseudotuberculosis* isolates were collected the plants, soils, vegetation cover and floor material from goat and sheep production facilities. After collection, the isolates were seeded on BHI agar and incubated at 37ºC for 48h. Subsequently, the isolated colonies were inoculated in to 3mL of tryptone soy broth (TSB), followed by incubation at 37°C for a further 48 h. Next, a 100µL aliquot was inoculated onto a microplate containing 100µL of TSB broth, followed by incubation at 37°C for 24 h. The microplates were then washed three times with 200µL of distilled water and dried at room temperature. Subsequently, they were stained with 100µL of 0.25% violet crystal for 5 minutes at room temperature. They were then washed a further six times with distilled water. To dissolve the dye, 200µL of alcohol-acetone was used (80:20). The absorbance was measured using a microplate reader at 595nm (adapted from Merino et al. 2009).

Statistical analysis. For each experiment, the survival time after contamination was compared using the non-parametric Kruskal-Wallis test (Conover 1999) and the agricolae package (Mendiburu 2014) (R v.3.0.2) (R Core Team 2013).

RESULTS

With regard to the *Corynebacterium pseudotuberculosis* survival on the plants, the first collection of caseous material was performed on the third day of observations, confirming that growth of *C. pseudotuberculosis* had occurred on all of the plants (Table 1). However, in the case of jurema-preta, only the sheep isolate grew on one of the three plants, and biofilm production was moderate. The second collection of material revealed growth on the catingueira and xique-xique plants. After ten days of observations, samples were collected from other surface areas on the contaminated plants. Despite the lack of humidity, the isolates were still surviving at this point. The survival rates decreased over time. After thirty days, no growth was observed on the surface of any of the evaluated plants. For greater control, collections were performed for a further 15 days.

No significant difference was found between the isolates deposited on the catingueira, xique-xique and mandacaru plants. Furthermore, no significant difference was observed between the isolates deposited on the mandacaru and jurema-preta plants. However, a difference in the survival of isolates deposited on catingueira and xique-xique plants was recorded in relation to those deposited on jurema-preta plants (Table 2).

The non-parametric statistical analysis displayed in Table 2 demonstrates the effects of temperature on the period survival of *C. pseudotuberculosis* isolates. No significant difference was observed between 28°C and 37°C. However, survival between 28°C and 37°C was significantly different from survival at 42ºC, where fewer isolates survived.

Table 2 displays the results of the non-parametric analysis testing the effects of the surface on the period of survival. No significant differences were recorded between wire and wood, or between wood and soil, or between soil and thorns. However, significant differences were found between the wire and soil growth surfaces and between wire and thorns. *C. pseudotuberculosis* isolates survived longer on the wire surface than on the thorn surface.

With regard to the efficacy of the disinfectants on the studied surfaces, the results confirmed that the isolates survived for a shorter period when they were exposed to quaternary ammonia. Under treatment with hypochlorite and

Table 1. Survival time in days of sheep and goat *Corynebacterium pseudotuberculosis* isolates on Caatinga plants

Isolate	Caatinga Plants											
	Mandacaru			Caatingueira			Xique-Xique			Jurema-preta		
	*P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3
G. biofilm positive	31**	23	10	31	31	16	31	31	31	3	0	25
G. biofilm negative	03	31	03	10	31	31	31	03	03	25	16	23
S. biofilm positive	10	03	16	0	31	31	25	16	16	03	03	0
S. biofilm negative	31	23	3	8	31	03	25	25	25	25	25	0

G = Goat, S = Sheep, *P = Plant, **Number of days of *Corynebacterium pseudotuberculosis* survival on Caatinga plants, *Cereus jamacaru* DC.c (mandacaru), *Caesalpinia pyramidalis* Tul. (caatingueira), *Pilosocereus gounellei* F.A.C. Weber (xique-xique) and *Mimosa hostilius* Benth. (jurema-preta).

Table 2. Non-parametric analysis of the effects of contamination on thesurvival of *Corynebacterium pseudotuberculosis*

Plants	Mean*
Catingueira	29.62[a]
Xique-xique	29.08[a]
Mandacaru	22.29[ab]
Jurema-preta	17.00[b]
Temperature	Mean*
37°C	82.52[a]
28°C	79.62[a]
42°C	55.35[b]
Surface	Mean*
Wire	93.01[a]
Wood	78.62[ab]
Soil	63.86 [bc]
Thorn	54.50 [c]
Type of isolate	Mean*
Sheep isolate with negative biofilm	87.22 [a]
Goat isolate with strong biofilm	79.18 [ab]
Goat isolate with negative biofilm	64.56 [bc]
Sheep isolate with moderate biofilm	59.02 [c]

* Days after contamination.

chlorhexidine, the inocula showed resistance for approximately 40 days, with lower survival rates being observed on the plant surface. In the control group, only plant material was used, without adding disinfectants. In this group, the highest survival rates were observed at 56 days, which were recorded on three surfaces (wood, soil and plant) for the sheep isolate, with moderate biofilm production.

When exposed to hypochlorite and chlorhexidine, the isolates that exhibited strong biofilm production survived for up to 44 days, regardless of the contamination surface. The same result was recorded for the biofilm-negative isolates. The biofilm-negative sheep isolate survived for 18 days after adding chlorhexidine to each of the tested surfaces.

The adherence to microplates test was used to analyze the biofilm production of *C. pseudotuberculosis* isolates after exposure to the disinfectants as well as following the exposure of the isolates to the caatinga environment. The results confirmed that the isolates exhibited decreased biofilm production.

With regard to the different periods of survival observed among the different types of *C. pseudotuberculosis* isolates, the biofilm-negative sheep isolates provided a distinct, statistically significant result compared with the biofilm-negative goat isolates and the moderate biofilm sheep isolate. Furthermore, the strong biofilm goat isolate was significantly different from the moderate biofilm sheep isolate. No significant differences were recorded for the other isolates (Table 2).

DISCUSSION

Studies addressing the survival of *Corynebacterium pseudotuberculosis* are essential to gain an understanding of the epidemiology of caseous lymphadenitis. For this purpose, contamination of materials containing purulent exudates is more effective than using a cellular suspension, because it more accurately mimics the conditions that occur on rural properties (Augustine & Renshaw 1986). With regard to the contamination of caatinga plants with *C. pseudotuberculosis*, jurema-preta showed a shorter survival period than the other plants, due to its antimicrobial potential (Pereira et al. 2009). When the isolates were placed on the stem, the antimicrobial substances produced by jurema-preta may have reduced the survival rates of the isolates. Previous studies have shown that jurema-preta exhibits antimicrobial activity in tests using isolates of *Staphylococcus aureus* from cattle with mastitis (Bezerra et al. 2009). This plant was also found to exhibit antifungal activity when a bark extract was used in antimicrobial assessments (Davet et al. 2009).

With regard to the survival of the isolates on mandacaru, contamination was introduced to both the stem and thorns. The fact that the stem of this plant has a smooth surface may have increased the exposure of the isolates to the sun, which favored their desiccation. A rapid loss of humidity is associated with a reduction in the survival rates of *C. pseudotuberculosis* (Augustine & Renshaw 1986). In addition, the antibacterial activity of crude extracts of mandacaru has previously been demonstrated against the following bacteria: S*taphylococcus epidermidis; Staphylococcus aureus; Pseudomonas aeruginosa* and *Escherichia coli* (Davet et al. 2009). This activity may explain the lower survival rates of the isolates on this plant.

Although the survival period of the inocula on mandacaru was relatively short, this plant is very important in the dissemination of this agent during animal infections, given that the animals eat these cacti on a daily basis. Mandacaru is one of the most common plants in the semiarid northeast of Brazil. It has a long trunk covered with thorns, which may be a significant contamination vector. When these thorns cause any type of skin trauma or contact any mucous membrane, they enable the penetration of *C. pseudotuberculosis* into the animal's body, leading to the development of caseous lymphadenitis (Cruz et al. 2007).

The isolates that were placed on catingueira survived longer than those applied to jurema-preta. The difference in these results could be associated with the roughness of the stem. The stem of the jurema-preta plant is surrounded by a rough skin, whereas the stem itself exhibits roughness in the case of catingueira plants. The rougher the surface, the longer *C. pseudotuberculosis* will survive, probably due to the formation of grooves, which tend to retain humidity and nutrients (Augustine & Renshaw 1986).

In relation to the survival rates observed at different temperatures and on different surfaces, none of the isolates placed on thorns or in the soil showed significant differences at 42°C, where the lowest survival rates were recorded. In other bacteria, such as *Escherichia coli*, when the cells suffer thermal stress, they reduce their replication in an attempt to adapt to the environmental alterations (Jozefczuk et al. 2010).

Temperature can affect gene expression in *C. pseudotuberculosis.* At a temperature of 43°C, the *pld* gene expression is significantly affected, as the temperature modifies the phospholipid structure of the cell wall (McKean et al. 2007). This could explain the lower survival rates of the isolates recorded at 42°C. The response of the *pld* gene to thermal shock has been previously associated with the remodeling of the membrane and the elimination of nutrients, which are essential in the initial phases of pathogenic infections (McKean et al. 2007).

Further studies should be conducted to determine whether there is an association between the virulence and survival of *C. pseudotuberculosis* in the environment, particularly in the caatinga, where temperatures regularly reach 42 ºC (or more) in the dry season (Kavamura et al. 2013).

C. pseudotuberculosis survives for long periods in wet soil. A previous study confirmed its survival for eight months at a temperature of 37 °C (Augustine & Renshaw 1986). In the present study, the caseous material was placed in dry soil and only survived for 39 days. This difference could be associated to different humidity conditions in the applied methods. With regard to the survival of *C. pseudotuberculosis* at different temperatures, low temperatures (4°C) have been associated with longer survival (55 days) of the bacteria (Augustine & Renshaw 1986). In a previous study involving *C. pseudotuberculosis* biovar *equi* and wet soils, a survival period of longer than 19 weeks was recorded. The addition of organic material has also been associated with the multiplication of bacteria in soil (Spier et al. 2014).

About the efficacy of the disinfectants, the results of the present study differed from those reported a study based on micro-dilution in plates (Sá et al. 2013). Regarding the efficiency of the disinfectants that were used in the currently research, when using quartenary ammonia and chlorhexidine on surfaces (soil, wood, tile and plants), quaternary ammonia was more efficient than chlorhexidine. Nevertheless in similar study Sá et al. (2013) found that chlorhexidine was more efficient than quaternary ammonia. This difference could be due to the controlled laboratory conditions employed or the interference of the organic material used in the substrates. This organic material reduces antibacterial activity in disinfectants based on organic acids, which are biodegradable (Jaenischi et al. 2010). In the absence of organic material, sodium hypochlorite, quaternary ammonia and peracetic acid reduce the number of colony forming units of *P. aeruginosa* by 100%, thereby exhibiting the greatest efficacy rates in terms of controlling this bacterium (Scur et al. 2014).

Materials contaminated with feces have been associated with greater survival of bacteria, due to higher humidity levels and a greater quantity of organic material (Augustine & Renshaw 1986). The same authors reported that several factors are involved in the transmission of caseous lymphadenitis, regardless of the animals that are contaminated, and cited the need to minimize direct and indirect contact with the agent responsible for this disease. As a preventative measure, disinfectants are used to clean facilities (Scur et al. 2014).

Previous studies have noted that further research on the different reactions to disinfectants is required. This research will help to reduce the survival rates of isolates, given that the chemical interaction between a disinfectant and biofilm-producing bacteria depends on enzymes that modulate the microenvironment to produce antimicrobial effects (Augustin et al. 2004). Thus, the exposure of the isolates to several surfaces may have caused adhesion and protected the isolates from chemical interactions with the disinfectants. A previous study used chlorhexidine to reduce biofilm production by *Streptococcus mutans* showed that biofilm consolidation was reduced by 21.38% (Liu et al. 2012). The present study corroborates these results. When chlorhexidine was applied to the isolate that was a strong producer of biofilm, its ability was weakened on tiles, wood and vegetation (Sá et al. 2013). No biofilm was formed by the *C. pseudotuberculosis* isolates in soil.

In relation to biofilm formation and the survival of *C. pseudotuberculosis*, all of the isolates exhibited similar behavior, surviving for approximately 42 days. However, most of the isolates lost the ability to produce biofilm, becoming either weak producers or non-producers. This decrease in biofilm formation could be associated with stress exerted on the bacteria by the adverse conditions of the environment and the disinfectants applied to the isolates. These factors may have modified the structure of the membrane or the quantity of lipopolysaccharides present. Upon the analysis of biofilm formation in *Burkholderia cepacia*, it became clear that biofilm formation decreased when the bacterium was exposed to thermal stress and ultra-violet radiation (Ferreira et al. 2013). When *C. pseudotuberculosis* was subjected to stress in the caatinga environment. It was more sensitive in the jurema-preta plant in the catingueira and xique-xique plants.

CONCLUSIONS

Corynebacterium pseudotuberculosis was more sensitive to jurema preta when submitted to stress in the Caatinga environment.

The survival of the isolates was lower when the temperature was 42°C. Also, when isolates of *C. pseudotuberculosis* were submitted to caatinga environmental conditions at different temperatures, with disinfectant application, the production of biofilm decreased. Then, they were classified as weak biofilm producers or non-producers.

Conflict of interest statement.- The authors have no competing interests.

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