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Archives of Veterinary Science
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Antimicrobial effect of ozonized physiological solution on bacteria causing cystitis in dogs

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***Submitted**: 06/01/2024*
***Accepted**: 23/04/2024*

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Abstract: Bacterial cystitis is a common clinical problem among dogs and is one of the reasons for the empiric administration of antimicrobials. This practice facilitates the selection of bacteria that are multidrug-resistant to antibiotics. In this context, it is urgent to understand and validate therapeutic modalities that complement antimicrobial treatment in cystitis cases. Ozone therapy has been proposed by scientists both in human and animal medicine. Thus, the objective of this study was to evaluate the *in vitro* antimicrobial activity of ozonized saline solution over standard strains and resistant or multi-drug resistant isolates associated with cystitis in dogs. The plating method was used to evaluate the antimicrobial activity of the exposure of 1 mL of phosphate buffer solution containing 10^8 colony forming units (CFU) mL^{-1} for 60 s to 4 mL of ozonized saline solution at 78 µg mL^{-1} over the standard strains (*Staphylococcus aureus* and *Escherichia coli*) and resistant or multi-resistant isolates (*Proteus mirabilis*, *Klebsiella* sp. e *Enterococcus* sp.), and the broth microdilution test to determine the minimum inhibitory and bactericidal concentration for S. aureus, E. coli and P. aeruginosa strains from the same solution. The plating was the only assay in which treatment with ozonized saline resulted in bactericidal activity, promoting a reduction of more than 99% of colony-forming units for most of the tested strains, except for *P. mirabilis*. These results are promising, as bacterial infections are the most common lower urinary tract disorder in dogs, with limited allopathic therapy due to increased drug resistance.
Keywords: NaCl 0.9% ozonized solution; urinary tract infection; ozone therapy; bacterial resistance; integrative therapy.

1. Introduction

The urinary tract infection (UTI) of bacterial etiology is a common disease in dogs and is among the main reasons for the use of antimicrobials (Weese et al. 2019). However, in most cases, these drugs are administered empirically, without performing a urine culture and antibiogram, necessary both for the diagnosis and to guide treatment decisions for this condition (Bartges 2004; Ball et al. 2008). Empirical administration of antimicrobials is of concern as it may result in the selection of multidrug-resistant (MDR) microorganisms or alter the microbiota, predisposing them to further infections (Wilcox 2009). Also, in selecting resistant bacterial populations, inappropriate use of antimicrobials can cause adverse effects on the patient (Wong et al. 2015), significant morbidity to animals, and financial cost (Johnstone 2019). Pressure from guardians to resolve the patient's discomfort and clinical condition has been identified as a possible reason for adherence to empirical treatments in cases of UTI (Sørensen et al. 2018).
The rate at which the resistance of microorganisms to conventional antimicrobial therapies is alarming (Frieri et al. 2017). According to the World Health Organization (WHO), 700,000 patients die each year worldwide due to antimicrobial resistance (AMR). This number of deaths is estimated to increase to 10 million by 2050 (WHO 2019). Thus, the development of new treatment options and alternative antimicrobial therapies is a major challenge.

Medical ozone (O_3) therapy has been proposed and studied as a potential adjuvant or alternative for cases of MDR/AMR infection in human and veterinary medicine (Rowen 2019; Sciorsci et al. 2020). Ozone is a powerful non-antibiotic biocidal gas, easily dissolved in water (Hems et al. 2005). It is widely used as a sterilant for microorganisms present in drinking water (Ngwenya et al. 2013). The bactericidal effect of O_3 is based on the direct attack on microorganisms by oxidation of their biological membranes (Thanomsub et al. 2002). Once added to aqueous solutions, O_3 rapidly decomposes, generating reactive oxygen species (ROS) - superoxide ($\bullet O_2^-$), hydroperoxide radicals ($HO_2\bullet$), and hydroxyl radicals ($OH\bullet$) (Tomiysu et al. 2015). These formed ROS attack the bacterial cell surface oxidizing in mainly two groups: polyunsaturated fatty acids and amino acids from peptides, enzymes, or proteins. Once damaged by oxidation, these free radicals penetrate through the microbial cell membrane, causing damage to DNA (deoxyribonucleic acid) or intracellular proteins, which impacts repair and transcription, and can thus result in cell lysis or death (Patil et al., 2011). Furthermore, unlike antibiotics, bacteria cannot develop resistance to the O_3 mode of action (Rowen 2019), thus preventing the MDR and the spread of AMR (Sciorsci et al. 2020).

In cases of cystitis, case reports in both Wistar rats and humans have revealed antibacterial effects of ozonized saline for some cystitis-causing agents in these species (Tasdemir et al. 2013; Vilela 2015; Logan 2018). We hypothesize that treatment with medicinal ozone will act against bacteria that cause cystitis in companion animals. Thus, this study aimed to evaluate the *in vitro* antimicrobial activity of ozonized saline on standard strains and bacterial isolates with a resistant or multidrug resistance profile associated with cystitis in dogs.

2. Materials and Methods

2.1. Experimental space, materials, and supplies

To evaluate the bactericidal activity of the ozonized saline solution, antimicrobial tests were carried out at the Bacteriology Laboratory of the Federal University of Santa Maria (LABAC/UFSM). The culture media used in this study (MacConkey Agar, Blood Agar, Mueller-Hinton Agar, and Plate Count Agar [PCA]) were purchased from LaborClin (Pinhais, Brazil) and the ozonized 0.9% sodium chloride solution (Ozonized NaCl 0.9% - Sanobiol) was generated in an ozone generator (O_3) device model OeL1.5RM) (Ozone and Life, São José dos Campos, Brazil), coupled to medical oxygen (O_2) cylinder.

2.2. Strains and isolates

To carry out this study, the standard strains *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 25853) were used, and the bacterial isolates *Klebsiella* sp. (SBP 60/21), *Enterococcus* sp. (SBP 65/21) and *Proteus mirabilis* (SBP 65/18). *Klebsiella* sp. and *Enterococcus* sp. were recovered from the urine of two dogs with cystitis and *P. mirabilis* from a canine otitis case. The antimicrobial susceptibility test was performed by the disc diffusion method (CLSI 2013), and all isolates were considered resistant or MDR according to the criteria proposed by Magiorakos et al. (2012). The following antimicrobial susceptibility profiles are demonstrated in Table 1. Strains and isolates were kept lyophilized and stored at -20ºC and before antibacterial assays, the isolates were seeded on Muller-Hinton Agar and incubated at 37°C for 24 h.

Isolates	Antimicrobial susceptibility profiles		
	Sensitive to	**Intermediate sensitivity to**	**Resistant to**
P. mirabilis (SBP 65/18)	Amikacin (30 µg) Ampicillin (2 µg) Ciprofloxacin (5 µg) Florfenicol (30 µg) Gentamicin (10 µg) Marbofloxacin (5 µg) Neomycin (30 µg) Sulfamethoxazole + trimethoprim (25 µg)	-	Amoxicillin + clavulanic acid (30 µg) Cefadroxil (30 µg) Cephalexin (30 µg) Cephalothin (30 µg) Doxycycline (30 µg) Enrofloxacin (5 µg)
Klebsiella sp. (SBP 60/21)	Amikacin (30 µg) Cefepime (30 µg) Ciprofloxacin (5 µg) Doxycycline (30 µg) Gentamicin (10 µg) Neomycin (30 µg) Sulfamethoxazole + trimethoprim (25 µg)	-	Amoxicillin + clavulanic acid (30 µg) Azithromycin (15 µg) Cefovecin (30 µg) Ceftiofur (30 µg) Ceftriaxone (30 µg) Enrofloxacin (5 µg)
Enterococcus sp. (SBP 65/21)	Norfloxacin (10 µg)	Amoxicillin + clavulanic acid (30 µg) Enrofloxacin (5 µg)	Amikacin (30 µg) Cephalexin (30 µg) Cephalothin (30 µg) Marbofloxacin (5 µg) Nitrofurantoin (300 µg) Sulfamethoxazole + trimethoprim (25 µg)

Table 1 - Antimicrobial susceptibility profiles observed for *P. mirabilis* (SBP 65/18), *Klebsiella* sp. (SBP 60/21) and *Enterococcus* sp. (SBP 65/21) isolates of canines.

2.3. Antibacterial activity of ozonized 0.9% NaCl solution (SFO_3) by the plating method

In the first stage of this study, two standard strains were used: *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922) to evaluate the effectiveness of the ozonized saline solution against Gram-positive and negative bacteria. Next, assays were performed with isolates of MDR bacteria commonly identified in cases of cystitis in dogs (*Enterococcus* sp., *Klebsiella* sp., and *P. mirabilis*). All strains and isolates were submitted to three different treatments: pure 0.9% NaCl solution (SS); 0.9% NaCl solution saturated with medical oxygen (SSO_2) and ozonized 0.9% NaCl solution (SSO_3).

To obtain the SSO_3, a volume of 500 mL of 0.9% NaCl solution was ozonized for 10 min at a concentration of 78 µg mL^{-1} in a continuous flow of ¼ L min^{-1} of medicinal O_2 in a device that generated O_3 (Ozone and Life, model OeL1.5RM, São José dos

Campos, Brazil). To generate the physiological solution saturated with oxygen (SSO_2), a volume of 500 mL of 0.9% NaCl solution was used, which was kept in contact with oxygen gas for 10 min, at a flow of ¼ L min^{-1} in the device that generates O_3, but with the batcher turned off, so there is only the output of O_2 gas.

For the test, 1.0 mL of the bacterial solution, containing approximately 10^8 colony-forming units (CFU) mL-1, was mixed for 20 min with 4.0 mL of SSO_3, SSO_2 or SS. After the initial incubation, the solution was exposed to vortexing for one minute (60 s) and 0.5 mL of this solution was added to 4.5 mL of phosphate buffer solution – (PBS), as described by Song et al. (2018). Then, 50 µl of this solution was seeded, in triplicate, in Petri dishes and incubated at 37˚C for 24 h. After this period, the CFU count of each plate was performed and corrected for CFU mL^{-1}.

2.4. Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

Based on the results of the SSO_3 antimicrobial activity tests carried out by using the the plating method, it was decided to carry out the broth microdilution methodology to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of this solution. This assay was performed using two treatments: SSO_3 and its respective negative control (SF), according to the M07-A9 microdilution protocol defined by CLSI (CLSI 2012). Strains of *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 25853) were used. For this test, the saline solution initially ozonized at a concentration of 78 µg mL^{-1} for 10 min was subjected to dilutions from 1:2 to 1:64, thus obtaining an O_3 concentration in this solution that varied between 39 to 1.22 µg mL-1, respectively. The technique was performed within 20 min. after ozonation of the 0.9% NaCl solution. For CBM determination, 10 µL of each SSO_3 sample corresponding to each strain, equal to or greater than the MIC value, was transferred to Muller-Hinton Agar plates, followed by incubation at 37º C for 24 h. After this period, it was observed if there was a growth of CFU on the plates.

2.5. Statistical analysis

Data were analyzed using the IBM SPSS Statistics 26 software. The average of CFU mL^{-1} between the different groups was compared using the Analysis of Variance (ANOVA). The Tukey test was subsequently performed to identify the different treatments, adopting a significance level of 5%.

3. Results

Regarding the antibacterial assays, SSO_3 demonstrated bactericidal activity for the standard strains of *E. coli* and *S. aureus*, and the isolates of *Klebsiella* sp. and *Enterococcus* sp., when compared to control solutions (SS and SSO_2), presenting a $p < 0.05$ for each genus and/or species of bacteria (Table 2). Regarding the CFU mL^{-1} counts, it is possible to observe that for the *Klebsiella* sp. and *Enterococcus* sp. SSO^3 zeroed this count, while for *E. coli* and *S. aureus* strains, there was a reduction of 99.06% and 99.72%, respectively (Figure 1, 2, 3, and 4). On the other hand, for the *P. mirabilis* isolate, treatment with SSO^3 did not show antibacterial activity ($p = 0.949$) compared to control solutions (SS and SSO_2) (Fig. 5).

When comparing the antibacterial activity between the control solutions, there was no statistical difference between the treatments with SS and SSO_2 for *E. coli, S. aureus, Klebsiella* sp., and *P. mirabilis*. For the isolate *Enterococcus* sp., there was a greater proliferation in the treatment with SSO_2, on average 74.44% higher than that of SF. In the MIC and MBC assays, no inhibitory and/or bactericidal activity was observed in any of the dilutions against the standard strains of *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 25853), both for the SS solution (negative control) and for the SSO_3.

Genus/Specie	SS		SSO_2		SSO_3		*P value*
	Mean	SD	Mean	SD	Mean	SD	
S. aureus	$2.48 \times 10^{7\,a}$	6.55×10^{6}	$2.64 \times 10^{7\,a}$	8.39×10^{5}	$2.33 \times 10^{5\,b}$	2.08×10^{5}	0,000
E. coli	$9.68 \times 10^{6\,a}$	2.82×10^{6}	$1.18 \times 10^{7\,a}$	4.16×10^{6}	$2.67 \times 10^{4\,b}$	1.15×10^{5}	0,005
Enterococcus sp.	$4.74 \times 10^{6\,b}$	2.09×10^{6}	$8.28 \times 10^{6\,a}$	4.39×10^{6}	$0^{\,c}$	0	0,001
Klebsiella sp.	$5.2 \times 10^{6\,b}$	9.23×10^{5}	$6.27 \times 10^{6\,a}$	8.93×10^{5}	$0^{\,c}$	0	0,000
P. mirabilis	$1.44 \times 10^{7\,a}$	9.02×10^{5}	$1.44 \times 10^{7\,a}$	2.41×10^{6}	$1.50 \times 10^{7\,a}$	3.70×10^{6}	0,949

Table 2 - Average number of colony-forming units (CFU) mL^{-1} after each treatment and bacteria gender/species. Data expressed as mean and standard deviation (SD). Values of $p < 0.05$ on the same line indicate a significant difference between the groups. Different letters superscript on the same line indicates a significant difference between the methodologies according to Tukey's test ($p < 0.05$). CFU: colony forming units; SD: standard deviation; SF: pure 0.9% NaCl solution; SSO_2 0.9% NaCl solution saturated with oxygen; SSO_3 ozonized 0.9% NaCl solution; *S. aureus: Staphylococcus aureus; E. coli: Escherichia coli; P. mirabilis: Proteus mirabilis.*

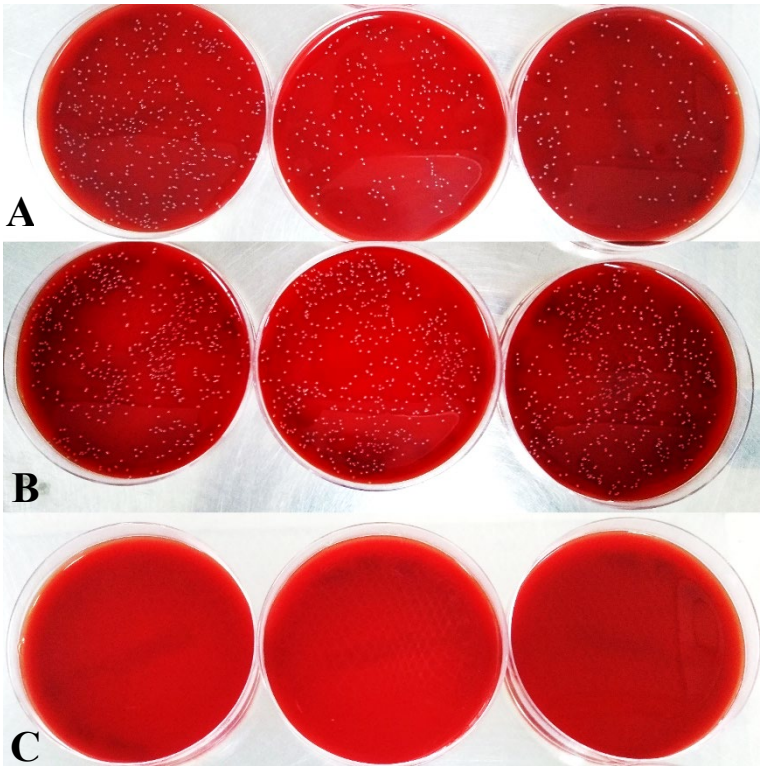

Figure 1 – *Enterococcus sp.* colony-forming units (CFU) mL^{-1} count in agar blood after treatment at 10^{-3} dilution: A: 0.9% NaCl solution (SS); B: 0.9% NaCl solution saturated with oxygen (SSO_2); and C: 0.9% NaCl solution ozonated at 78 µg mL^{-1} (SSO_3). Note that SSO_3 zeroed the CFU counts on the three plates tested (C).

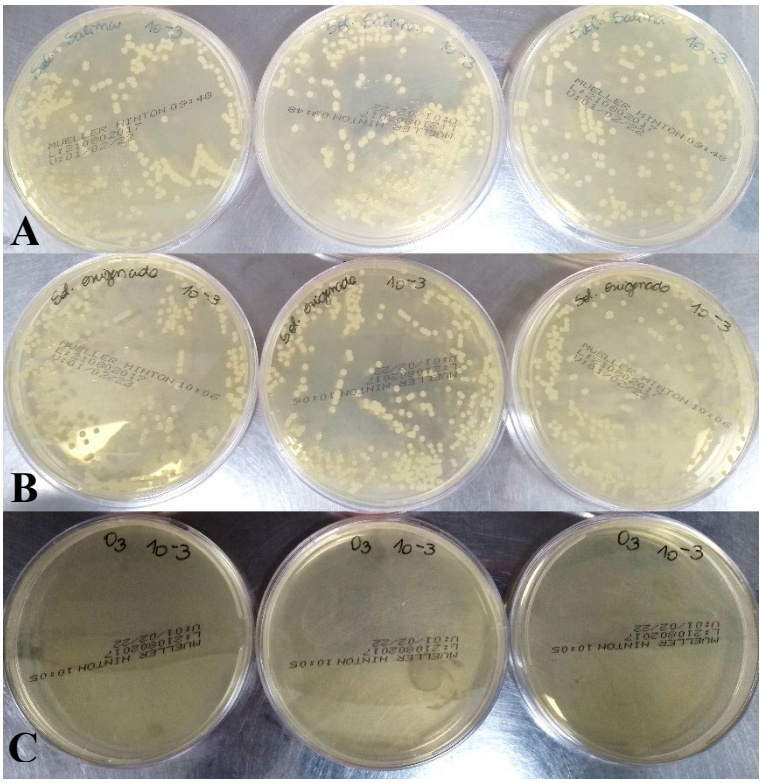

Figure 2 – *Klebsiella* sp. colony-forming units (CFU) mL^{-1} count in Mueller-Hinton agar after treatment at 10^{-3} dilution: A – pure 0.9% NaCl solution (SS); B – 0.9% NaCl solution saturated with oxygen (SSO_2) and C – 0.9% NaCl solution ozonized at 78 µg mL^{-1} (SSO_3). Note that SSO_3 zeroed the CFU counts on the three plates tested (C).

http://dx.doi.org/10.5380/avs.v29i2.94005

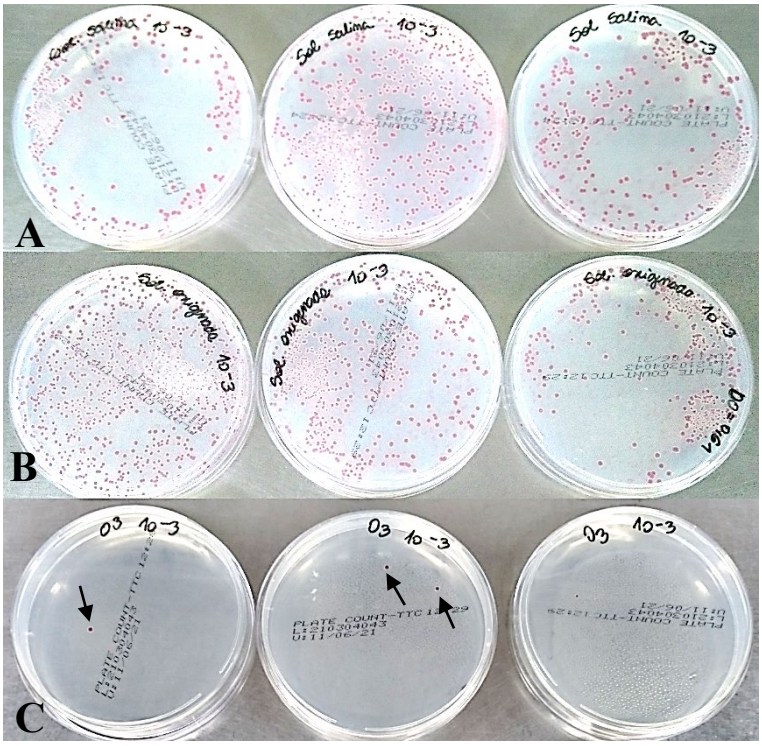

Figure 3 – Escherichia coli colony-forming units (CFU) mL-1 count in Plate Count agar after treatment at 10-3 dilution: A – pure 0.9% NaCl solution (SS); B – 0.9% NaCl solution saturated with oxygen (SSO2) and C – 0.9% NaCl solution ozonized at 78 µg mL-1 (SSO3). Note the marked difference in the number of CFUs (arrows) between SSO3 and the others (SSO2 and SS).

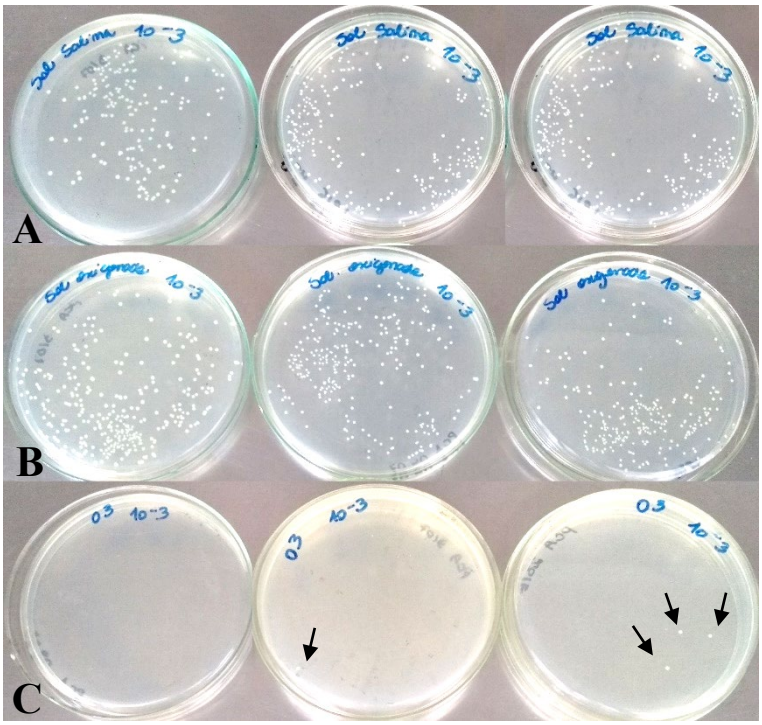

Figure 4 – *Staphylococcus aureus* colony-forming units (CFU) mL^{-1} count in Plate Count agar after treatment at 10^{-3} dilution: A – 0.9% pure NaCl solution (SS); B – 0.9% NaCl solution saturated with oxygen (SSO_2) and C – 0.9% NaCl solution ozonized at 78 µg mL^{-1} (SSO_3). Note the marked difference in the number of CFUs (arrows) between SSO_3 and the others (SSO_2 and SS).

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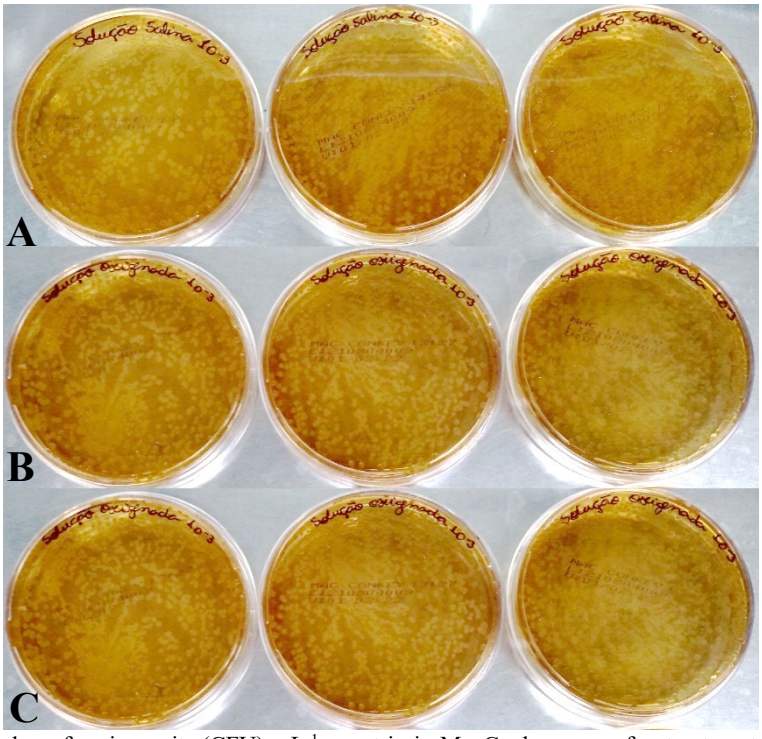

Figure 5 – *Proteus mirabilis* colony-forming units (CFU) mL^{-1} count in in MacConkey agar after treatment at 10^{-3} dilution: A – 0.9% pure NaCl solution (SS); B – 0.9% NaCl solution saturated with oxygen (SSO_2) and C – 0.9% NaCl solution ozonized at 78 µg mL^{-1} (SSO_3). Observe the absence of SSO_3 antibacterial activity compared to the others (SS and SSO_2).

4. Discussion

The emergence of bacterial resistance to antimicrobials, both in human and veterinary medicine, the treatment options for UTIs have become increasingly limited, especially in those individuals who have coexisting conditions that compromise the storage and voiding function of the lower urinary tract (Rafatpanah et al. 2017). Studies in both guinea pigs and humans have shown promising results from the application of O_3 dissolved in water as an alternative to antimicrobial treatment due to its high reactivity and potent oxidizing effect (Tasdemir et al. 2013; Vilela 2015; Logan 2018).

In this work, we demonstrated that 60 s of exposure to SSO_3 was able to reduce more than 99% of the CFU mL^{-1} count of *S. aureus* and *E. coli*, and 100% of the counts of *Klebsiella* sp. and from *Enterococcus* sp. Concerning ozonized water, *in vitro* studies in medicine and dentistry have shown that relatively short exposure times to this solution (around 60 s) can reduce the count of Gram-positive and Gram-negative bacteria, as well as *Candida albicans* (Huth et al. 2009; Johansson et al. 2009; Sadatullah et al. 2012; Song et al. 2018; Tonon et al. 2021). Similar results were also observed in other *in vitro* studies that used ozonized water for sanitizing dental instruments for *S. aureus*, *E. coli*, and *Enterococcus* sp., for antisepsis of root canals and skin wounds, but some with a time of exposure to ozonized solution greater than 60 s (Nagayoshi et al. 2004; César et al., 2012; Song et al. 2018).

The choice of ozone concentration in the ozonized solution (78 µg mL-1) was based on the highest concentration that the generator is capable of producing, to achieve maximum performance, considering that the greater the dissociation of oxygen, the greater the formation of ozone (Hems et al. 2005). In addition, the bubbling time of SS to the oxygen-ozone (O_2/O_3) gas mixture was established based on the study carried out by Santos de Paula et al. (2021), in which the volume of 500 mL of this solution reaches the maximum saturation concentration with O_3 after 10 min of exposure to the O_2/O_3 mixture. The maximum time of 20 min for adding the bacteria solution to SSO_3 was established based on previous studies that demonstrated that O_3 dissolved in water maintains its effect for approximately 30 min (Bialoszewski et al. 2011; Burke et al. 2012).

Despite using the maximum concentration that the generator is capable of producing and respecting the 20 min., limit of the bactericidal action of the ozonated saline solution, ozone did not demonstrate effective activity against all types of bacteria tested in this study. The lack of efficacy of SSO_3 on the *P. mirabilis* isolates observed in this research suggests that the antibacterial activity of this solution may be linked to the profile of the infecting strain or also to the exposure time of the microorganism to the solution, which was 60 s. To date, based on the literature, no study describes the antibacterial activity of SSO_3 against these bacteria, much less with the MDR profile presented by the tested isolates.

The absence of inhibitory and/or bactericidal activity of SSO_3 observed in the MIC and MBC assays was also reported in another study. However, the concentration of ozone injected in the saline solution was 8 µg mL^{-1} (Borges et al. 2017), significantly lower than that used in our experiment (78 µg mL^{-1}). The fact that SSO_3 was subjected to different dilutions and, therefore, to lower concentrations, could be one of the factors that impair or prevent the effect of dissolved O_3 in the solution and the ROS generated by it on the plasma membrane of bacterial cells.

Considering that AMR is an emerging public health problem and that the transmission of resistance genes between pathogens originating from pets and humans has already been documented (Damborg et al. 2016; Pitout and Devinney 2017), the results of this study are promising in terms of public and animal health. Our results may encourage *in vivo* research that evaluates the effectiveness and protocols of bladder lavage with SSO_3 in cases of bacterial cystitis in companion animals, until obtaining the results of bacterial culture and antibiogram, or even be an alternative treatment depending on the type of bacteria involved.

5. Conclusion

The present study demonstrated that SSO_3 (78 µg mL^{-1}) act against bacterial agents causing cystitis and even skin infections in dogs caused by *E. coli*, *S. aureus*, *Klebsiella* sp., and *Enterococcus* sp. In this study, we did not find the antibacterial activity of this solution against the MDR-isolate *P. mirabilis*, which suggests that the effect of SSO^3 may be related to the genus/species of bacteria, as well as the exposure time of the microorganism to it. Therefore, based on our results, further studies that test different times of exposure to SSO^3 are necessary to evaluate the antibacterial activity of this solution on these and other genera/species of bacteria.

Acknowledgments: The authors thank the Federal University of Santa Maria for the opportunity to develop this research, the Coordination for the Improvement of Higher Education Personnel (CAPES – funding code 001), and the National Council for Scientific and Technological Development (CNPq - funding code 870589/1997-0) for its funding.

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