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Research paper

Effect of *Aloe barbadensis Miller* (Aloe vera) associated with beta-lactam antibiotics on the occurrence of resistance in strains of *Staphylococcus aureus* and *Streptococcus uberis*

Oscar Chacón[a], Natalia Forno[a], Lissette Lapierre[b], Rubén Muñoz[a], Marcela Fresno[c], Betty San Martín[a,*]

[a] *Laboratory of Veterinary Pharmacology, Faculty of Veterinary and Animal Sciences, University of Chile, Santa Rosa 11735, Postal code 8820808, La Pintana, Santiago, Chile*

[b] *Department of Animal Preventive Medicine, Faculty of Veterinary and Animal Sciences, University of Chile, Santa Rosa 11735, Postal code 8820808, La Pintana, Santiago, Chile*

[c] *Núcleo de Investigaciones Aplicadas en Ciencias Veterinarias y Agronómicas, Universidad de las Américas, Cinco de Abril 0620, Postal coda 9251454, Maipú, Santiago, Chile*

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ABSTRACT

Introduction: Aloe vera (*Aloe barbadensis Miller*) is a medicinal plant that has been used empirically for thousands of years. Among its biological activities, Aloe vera's antimicrobial capacity makes it a possible alternative in combating the evolution of bacterial resistance.

The objective of this study was to evaluate the appearance of chromosomal resistance in gram-positive bacteria causing bovine mastitis when exposed to Aloe vera, used either individually or in combination with beta-lactam antibiotics.

Methods: The Minimum Inhibitory Concentration (MIC) of Aloe vera, both alone and in combination with cloxacillin or ceftiofur, *versus Staphylococcus aureus* and *Streptococcus uberis* was determined by means of the broth macrodilution technique. Serial multipassage techniques allowed us to assess the development of resistance against the described treatments in these strains.

Results: After 15 serial passages of *Staphylococcus aureus* ATCC 29213 (methicillin-sensitive *S. aureus*; MSSA) and *Staphylococcus aureus* ATCC 43300 (methicillin-resistant *S. aureus*; MRSA) *versus* Aloe vera alone and in combination with cloxacillin or ceftiofur, we did not observe an increase in MIC. However, in serial passages 6 and 14, the MIC for MSSA increased 5.3 and 3.3 fold *versus* cloxacillin and ceftiofur, respectively, and for MRSA in serial passages 6 and 7 the MIC increased 26.6 and 46.6 fold *versus* the indicated antibiotics. *S. uberis* ATCC 29,213 did not register MIC increases after 15 serial passages against Aloe vera alone or in combination with ceftiofur or cloxacillin.

Conclusion: The combination of Aloe vera and ceftiofur or cloxacillin delays the appearance of chromosomal resistance in *S. aureus* strains. Likewise, the synergistic and additive effect of these antibiotics when combined with Aloe vera, would allow for a dose reduction in antibiotics when treating clinical mastitis symptoms of dairy cattle.

1. Introduction

The inappropriate use of antibiotics in animal production is an important cause of increased bacterial resistance [1,2], representing the greatest threat to the efficacy of antibiotic treatment in human and veterinary medicine. In veterinary medicine, bacterial resistance not only has implications for the health of animals, but also for public health, due to the links between excessive antibiotic use, intensification of animal production systems and globalization, increasing the risk of transmission of multi-resistant bacteria between different ecosystems [3–8].

In the dairy industry, the disease that has the greatest economic impact worldwide is bovine mastitis [2]. This disease is caused by a diversity of etiological agents, including *Staphylococcus aureus* and

* Corresponding author.
E-mail address: bsmartin@uchile.cl (B.S. Martín).

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Streptococcus uberis [9]. These pathogens are highly relevant in cases of clinical and sub-clinical mastitis, as they have the capacity to form biofilms, generating high levels of prevalence and chronicity. The ability to form biofilms reduces susceptibility to antibiotics, complicating disease control and eradication [10–12].

Antibiotics from the beta-lactam family are the primary treatment for gram-positive bacteria. In 2007, the USDA reported that in the United States 72% of farms treated all of their animals with drying therapies, principally cephalosporin and penicillin. Within the penicillin family, cloxacillin, the antibiotic of choice for the treatment of *S. aureus* infections that produce penicillinase, is widely used in the treatment and control of resistant gram-positive bacteria. Within the cephalosporin family, ceftiofur is widely used in the treatment of clinical mastitis caused by *S. aureus* and *S. uberis* [13]. Both antibiotic are part of the Critically important antimicrobials for Human Medicine List from the World Health Organization, and both are considered of critically important antibiotics, where ceftiofur is also considered of the highest priority [14].

With few exceptions, the development and discovery of new antibiotics has decreased, while bacterial resistance has steadily increased [15]. In this context, a large variety of plant species with antimicrobial properties have been used in phytopharmacological research [16].

Aloe vera is one of the most studied plants, with around 360 extant species. Only four species present medicinal properties, with *Aloe barbadensis Miller* being the most commonly used [17,18]. The antimicrobial activity of this plant has been demonstrated on strains of *Staphylococcus aureus, Escherichia coli, Shigella flexneri, Helicobacter pylori,* and *Pseudomonas aeruginosa* [19–22].

Additionally, it has been proven that medicinal plants with antimicrobial activity belonging to the genuses *Plumbago, Ocimum, Punica, Vitis*, among others, have a synergistic effect when combined with synthetic antibiotics against strains of methicillin-resistant *S. aureus* (MRSA) [23] and *Acinobacter baumanni* [24].

The inhibitory effect of Aloe vera has been associated with its phenolic compounds and polysaccharides, which could act on different target sites of the bacteria [25] Thus, we hypothesize that a combination of inhibitory components of Aloe vera and cloxacillin or ceftiofur could delay or prevent resistance in gram-positive bacteria, as in order to develop resistance against this drug combination the bacteria would have to undergo various point mutations [15].

Thus, the objective of this study is to evaluate the development of chromosomal resistance in strains of *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* ATCC 43300 and *Streptococcus uberis* ATCC 9927 exposed to Aloe vera, either alone or in combination with beta-lactam antibiotics.

2. Material and methods

2.1. Aloe vera

The experiment described in this paper have used a commercial lyophilized Aloe vera, CAS number 85507-69-3 (Concentrated Aloe Corporation, United States of America). This product consist in lyophilized inner gel from Aloe vera plants that are approximately two years old at the time of harvest, without preservativesor antimicrobials.

2.2. Antibiotics

The antibiotics used was cloxacillin sodium sal hydrate (C_{19} H_{17} Cl N_3 O_5 S Na H_2O), CAS number 7081-44-9 (Dr. Ehrenstorfer, Germany) and Ceftiofur hydrochloride (C_{19} H_{17} N_5 O_7 S_3 Cl H), CAS number 103980-44-5 (Dr. Ehrenstorfer, Germany).

2.3. Extraction of inhibitory compounds from lyophilized Aloe vera

The extraction of inhibitory components from lyophilized Aloe vera (Aloe vera of America, Inc.) was carried out following as a basis the methodology described by Habeeb et al. [21] and modified by San Martín et al. [26]. Methanol was chosen as solvent for the extraction process due to its high polarity and good solubility with phenolic compounds present in plant materials, compared with other solvents like ethyl acetate/water, acetone, hexane and chloroform [21,27]. During the entire extraction process, and subsequent handling, the extract was protected from exposure to light.

Briefly, 25.6 g of lyophilized powder, 400 mL of methanol (Merck, Darmstadt, Germany) and 5 mL of glacial acetic acid (Merck, Darmstadt, Germany) were used; this mix was then stirred, sonicated and centrifuged at 5000 r.p.m. for 10 min. Subsequently, the supernatant was collected and the extraction process was repeated for the remaining fraction of lyophilisate.

The supernatants were dried in a rotary evaporator at 25 °C. Once dry, the extract was reconstituted with 8 mL $NaHCO_3$ and the pH was adjusted to 5.0 ± 0.2 with 6 M NaOH. Mueller Hinton broth was added to the extract to reach a final volume of 20 mL. The stock solution of Aloe vera was 1280 mg/mL and was maintained at −80 °C until use.

2.4. Quantification of phenolic compounds

Total phenolic compounds present in the Aloe vera extract were quantified using the Folin-Ciocalteu method [28,29]. The Folin-Ciocalteu reagent (Merck, Darmstadt, Germany) was diluted 1:10, v/v. The control curve was prepared using gallic acid as standard, achieving concentrations of 0, 20, 40, 60, 80 and 100 mg/mL.

The samples were prepared in a final volume of 4 mL, with 400 µl of extract, 2000 µl diluted Folin reagent and 1600 µl Na_2CO_3. It was incubated for 15 min at 45 °C with shaking; the samples were read on a Pharo 300 spectrophotometer (Spectroquant®) and calibrated at a wavelength of 725 nm. Samples were analyzed in triplicate.

2.5. Bacterial strains

We used the following strains: *Staphylococcus aureus* subsp. *aureus* ATCC® 29213™, *Staphylococcus aureus* subsp. *aureus* ATCC® 43300™ and *Streptococcus uberis* ATCC® 9927™.

2.6. Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The Minimum Inhibitory Concentration (MIC) of cloxacillin, ceftiofur, and Aloe vera against the strains under study were determined using the macrodilution technique in broth [30].

From the stock solutions, dilutions of the inhibitory agents were made considering the recommendations of the CLSI for *in vitro* broth susceptibility testing. This dilutions are recomended for testing the activity of antimicrobial agents against bacterial culture [30].

In the case of cloxacillin and ceftiofur, stock solutions of 1280 µg/mL were prepared; from these solutions we performed decreasing serial dilutions, obtaining concentrations between 0.015 and 128 µg/mL. For Aloe vera, a stock solution of 1280 mg/mL was prepared, from serial dilutions were made, obtaining concentrations between 0.0156 and 128 mg/mL.

Eight mL of adjusted Mueller-Hinton Cation broth (Mueller-Hinton II broth), 1 mL of bacterial inoculum (adjusted to 0.5 McFarland and a final concentration of 5×10^5 CFU) and 1 mL of the inhibitory agent (Aloe vera or antibiotics) were placed in each tube. A tube with 9 mL of Mueller-Hinton II broth and 1 mL of bacterial inoculum (adjusted to 0.5 McFarland) was used as a positive control, and a tube with 10 mL of Mueller-Hinton II broth without bacterial inoculum was used as a negative control. All tubes were incubated at a temperature of 37 ± 2 °C for 18 h. The samples were analyzed in triplicate.

The MIC of each antibiotic and the Aloe vera extract was determined visually by turbidity, comparing each with the negative

control. This was confirmed by absorbance, using a Pharo 300 spectrophotometer (Spectroquant®), calibrated at a wavelength of 625 nm [30]. The MIC was defined as the lowest concentration of the antibiotic that inhibited bacterial growth.

The minimum bactericidal concentration (MBC) was defined by the set of tubes used to define the MIC. They were extracted and seeded in a 100 µL plate count agar from those tubes with concentrations corresponding to 2, 3 and 4 times the MIC. As a positive control, a plaque planted from the inoculum of the tube free from inhibitory agents was used; a plaque with no inoculum and no antibiotic was used as a negative control. Plates were incubated at 37 ± 2 °C for 24 h. After incubation, the MBC was evaluated by counting CFU/mL; the CMB was defined as the antibiotic concentration that inhibited 99.9% of bacterial proliferation.

In order to rule out the possibility that solvents used in the extraction of the inhibitory compounds of the plant could significantly influence the inhibition of bacterial growth, the lyophilized Aloe vera was replaced with a compound without antimicrobial activity, in this case diatomaceous earth, which is a siliceous sedimentary material without antimicrobial activity, and the same extraction process was carried out on this new substrate. Diatomaceous earth extract was tested in concentrations equivalent to ¼ MIC, ½ MIC, and 1 MIC of Aloe vera, and its effect on bacterial proliferation was evaluated. Each concentration of diatomaceous earth extract was compared with the positive control, with no inhibitors, by CFU/mL count quantified after incubation at 37 ± 2 °C for 24 h. The test was performed in triplicate.

2.7. Determination of the concentration of ceftiofur, cloxacillin and Aloe vera to be used in combination

To define the concentrations of ceftiofur and cloxacillin that were used in combination with Aloe vera, curves of bacterial proliferation were performed for each. We followed the recommendations of National Committee for Clinical Laboratory Standards [31], which state that in the case of combining compounds with antimicrobial activity, the inhibitory agent with greater antimicrobial potency should be present in a concentration that does not affect bacterial proliferation.

Bacterial proliferation curves were carried out using the macrodilution method in broth, using decreasing concentrations of antibiotic from the MIC defined for each. The dynamics of bacterial proliferation were evaluated in cultures incubated at 37 ± 2 °C with mechanical agitation at 150 r.p.m.; samples were taken at 0, 4, 8, 10 and 24 h of incubation. At the time of each sampling, sowing was carried out in microdrops, and CFU/ml was quantified after incubation at 37 ± 2 °C for 24 h.

To define antibiotic concentration, comparisons were made between the CFU/mL of each concentration with the positive control curve. The selected concentrations of ceftiofur and cloxacillin were combined with concentrations corresponding to ½ MIC, ¼ MIC and 1/8 MIC for Aloe vera. The lowest concentration that combined with the antibiotic inhibited bacterial proliferation was selected as Aloe vera's concentration.

2.8. Determination of the interaction between inhibitory agents

The interaction type of each combination used against *S. aureus* ATCC 29213, *S. aureus* ATCC 43300 and *S. uberis* ATCC 9927 was defined using bacterial death curves. The interaction was defined as a synergistic interaction when the association generated a reduction ≥2 log10 CFU /mL, and as additive when the reduction was < 2 log10 CFU/mL at 24 h of incubation, as compared to the initial inoculum.

2.9. Development of bacterial resistance

The serial passage methodology described by Farrell et al. [32] was used to evaluate the development of chromosomal bacterial resistance. This test was performed for each inhibitory agent individually (ceftiofur, cloxacillin, and Aloe vera) and for associations (Aloe vera + ceftiofur and Aloe vera + cloxacillin).

For each inhibitory agent, the first passage was started using 3 serial dilutions under the MIC and 3 double serial dilutions over the MIC. Tubes were inoculated with a bacterial concentration of 5 × 105 CFU/mL and incubated at 37 ± 2 °C for 18 h. After incubation, inoculum from the tube containing the concentration of antibiotic closest to MIC and with an equivalent turbidity to the positive control tube was used for the second passage. This procedure was repeated until 15 serial passages were completed. All tests were performed in triplicate. The inoculum selected for each passage was stored in glycerol (15%). We analyzed the strain that generated chromosomal type resistance when its MIC was increased 4 times.

To demonstrate that the strains obtained after the serial passage process were derived from the strains used at the beginning of the study, pulsed-field gel electrophoresis (PFGE) was performed on both the parental and final strains. The samples were processed according to the provisions of *Unified PFGE for Gram-Positive Bacteria* with some modifications [33]. In short, a bacterial suspension with an optical density of 0.6 to 420 nm was generated for all strains. The suspension was subjected to preincubation at 55 °C for 45 min, with 1% proteolytic enzyme lysozyme (Applichem, Glenview, IL, USA) and 0.00125% lysostatin (St. Louis, MO, USA). Subsequently, blocks were prepared with agarose "SeaKem Gold" at 1% (Lonza, Rockland, ME USA) and supplemented with SDS at 1% and 1 mg/ml proteinase K (Invitrogen, Waltham, MA USA). The cells included in the plug were lysed for 3 h at 55 °C; the genome was digested with 30 U of *Sma I* (Thermo-Fisher Scientific, Waltham, MA USA) at 25 °C for 3 h. The agarose blocks were loaded on a 1% "Pulse Field Certified" agarose gel (Bio-Rad, Hercules, CA USA) with 0.5% TBE buffer (45 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA). Electrophoresis was performed in a CHEF-DR III system (Bio-Rad) at 14 °C for 20 h, 6 V/cm with an initial pulse of 5.0 s and a final pulse of 40.0 s. In each gel of 30 wells, 3 molecular weight markers were included, corresponding to *Salmonella* braenderup H9812, in order to normalize each gel. The blocks of Salmonella branderup were treated as mentioned above, without including the preincubation with lysozyme/lysostaphin, while their genome was digested with 50 U of enzyme Xba I for 3 h at 37 °C. The images obtained from each gel were analyzed using the software "Gel Compar II" version 5.10 (Applied Maths, Sint-Martens-Latem, Belgium).

Finally, dendrograms were generated using the Dice similarity coefficient, based on bands and the UPGMA method, with a range of 1.5–2% tolerance in the same position [34–36]. Strains were considered clones when they presented a band pattern similarity of ≥ 95%.

2.10. Statistical analysis

The data were analyzed using the statistical software GraphPad Prism 6.01. To define the antibiotic concentrations used in our combinations, a Kruskal-Wallis non-parametric analysis of variance followed by Dunn's multiple comparison tests with a level of significance of $p \leq 0.05$ were performed.

3. Results

3.1. Quantification of phenolic compounds

The content of total phenols present in the Aloe vera extract was 1.8 ± 0.02 g GAE/L, equivalent to 114.03 mg GAE/100 g of extract.

3.2. Minimum inhibitory concentration and minimum bactericidal concentration

The antimicrobial activity of Aloe vera extract against the bacterial strains studied is shown in Table 1. The MICs of cloxacillin and ceftiofur in the reference strains are within the range established by the Clinical

Table 1
Minimum inhibitory concentration and minimum bactericidal concentration of ceftiofur, cloxacillin and Aloe vera extract against reference strains.

Microorganism	MIC			MBC		
	Cloxacillin µg/mL	Ceftiofur µg/mL	Aloe vera mg/mL	Cloxacillin µg/mL	Ceftiofur µg/mL	Aloe vera mg/mL
S. aureus ATCC 29213	0.25	0.25	40	4	4	120
S. aureus ATCC 43300	2	0.5	40	8	4	120
S. uberis ATCC 9927	0.25	0.125	20	2	2	60

MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration.

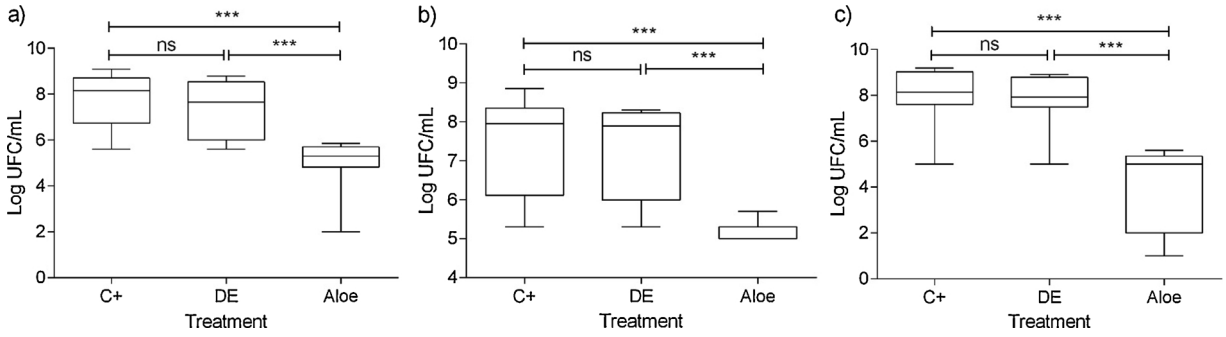

Fig. 1. Comparison between the effect of diatomaceous earth extract (40 mg/mL) and Aloe vera extract (40 mg/mL) on the proliferation of *S. aureus* ATCC 29213 (a), *S. aureus* ATCC 43300 (b) and *S. uberis* ATCC 9927 (c).
ns: Non significant; (***) $p < 0.001$; C+: Positive control; De: Diatomaceous earth; Aloe: Aloe vera extract.

Laboratory Standards Institute [30].

3.3. Effects of solvents contained in the extract

Fig. 1 shows the results of the solvent test on the proliferation of *S. aureus* ATCC 29213, *S. aureus* ATCC 43300 and *S. uberis* ATCC 9927. Solvents used in the extraction process do not significantly influence bacterial proliferation with respect to the positive control ($p > 0.05$).

3.4. Choice of inhibitory agent concentrations

Table 2 shows the lowest antibiotic concentrations that used in combination with Aloe vera were able to achieve inhibition. Five associations achieved an additive effect against the strains studied; the only association that achieved a synergistic effect was Aloe vera and cloxacillin against *S. aureus* ATCC 29213.

3.5. Number of serial passages presenting resistance

The strain *S. aureus* ATCC 29213 needed an average of 8 serial passages to increase MIC 4-fold against cloxacillin (from 0.25 to 1 µg/mL) and an average of 14 serial passages against ceftiofur (from 0.25 to 1 µg/mL) (Table 3 and Figs. 2 and 3). After 15 passages, there was no increase in MIC against Aloe vera alone or in combination with cloxacillin or ceftiofur.

The strain *S. aureus* ATCC 43300 needed an average of 7 serial passages to increase MIC 4-fold against cloxacillin (from 2 to 8 µg/mL) and an average of 6 serial passages against ceftiofur (from 0.5 to 2 µg/mL) (Table 3 and Figs. 4 and 5). After 15 passages, there was no increase in MIC against Aloe vera alone or in combination with cloxacillin and ceftiofur.

The strain *S. uberis* ATCC 9927 did not increase MIC over 15 serial passages compared to any of the previously described treatments.

3.6. Pulsed-field gel electrophoresis

After performing PFGE, the strains obtained at the end of the serial passages had a band pattern that were indistinguishable from their parental strains. Supplementary Figs. 1–3 show the dendrograms of the parental *S. aureus* ATCC 2921, *S. aureus* ATCC 43300 and *S. uberis* ATCC 9927 strains, as well as the strains obtained at the end of the passages.

4. Discussion

Bacterial resistance has rapidly become a problem with global implications; therefore, the search for new therapeutic alternatives for the treatment of infectious diseases of bacterial origin should be one of the priority objectives in its management, as indicated by the World Health

Table 2
Concentrations of the combinations that achieved an inhibitory effect on *S. aureus* ATCC 29213, *S. aureus* ATCC 43300 and *S. uberis* ATCC 9927.

Microorganism	Association concentration	Initial inoculum[a]	Final inoculum[b]	Effect type
S. aureus ATCC 29213	20 mg/mL AV + 0.06 µg/mL CLX	5.56 ± 0.22	3.1 ± 0.17	Synergistic
	20 mg/mL AV + 0.06 µg/mL CEF	5.56 ± 0.22	4.25 ± 0.24	Aditive
S. aureus ATCC 43300	20 mg/mL AV + 0.06 µg/mL CLX	5.35 ± 0.1	4.7 ± 0.24	Aditive
	30 mg/mL AV + 0.125 µg/mL CEF	5.35 ± 0.1	5.29 ± 0.27	Aditive
S. uberis ATCC 9927	10 mg/mL AV + 0.06 µg/mL CLX	5.18 ± 0.12	5.13 ± 0,11	Aditive
	10 mg/mL AV + 0.03 µg/mL CEF	5.18 ± 0.12	5.07 ± 0.17	Aditive

AV: Aloe vera; CLX: Cloxacillin; CEF: Ceftiofur; (*) Quantified after incubation at 37 ± 2 °C for 24 h.
[a] Initial inoculum corresponds to amount of CFU (Log10CFU/mL) before administering the treatment.
[b] Final inoculum corresponds to amount CFU (Log10CFU/mL) after administering the treatment.

Table 3
Changes in antibiotic susceptibility after 15 serial passages for the strains *S. aureus* ATCC 29213, *S. aureus* ATCC 43300 and *S. uberis* ATCC 9927.

Microorganism	Antibiotic	Initial MIC (μg/mL)	N° resistance passages*	Final MIC (μg/mL)	Level of increase CIM
S. aureus ATCC 29213	Cloxacillin	0.25	6	2	5.3
	Ceftiofur	0.25	14	1	3.3
	Aloe vera	40	–	40	0
	Aloe + cloxacillin	(20 mg + 0.06 μg)/mL	–	(20 mg + 0.06 μg)/mL	0
	Aloe + ceftiofur	(20 mg + 0.06 μg)/mL	–	(20 mg + 0.06 μg)/mL	0
S. aureus ATCC 43300	Cloxacillin	2	6	32	26.6
	Ceftiofur	0.5	7	64	46.6
	Aloe vera	40	–	40	0
	Aloe + cloxacillin	(20 mg + 0.6 μg)/mL	–	(20 mg + 0.06 μg)/mL	0
	Aloe + ceftiofur	(30 mg + 0.125 μg)/mL	–	(30 mg + 0.125)/mL μg)/mL	0
S. uberis ATCC 9927	Cloxacillin	0.25	–	0,25	0
	Ceftiofur	0,125	–	0.125	0
	Aloe vera	20	–	20	0
	Aloe + cloxacillin	(10 mg + 0.06 μg)/mL	–	(10 mg + 0.06 μg)/mL	0
	Aloe + ceftiofur	(10 mg + 0.03 μg)/mL	–	(10 mg + 0.03 μg)/mL	0

* Resistance emergency: 4-fold increase in MIC compared to the initial passage. Results in triplicate.

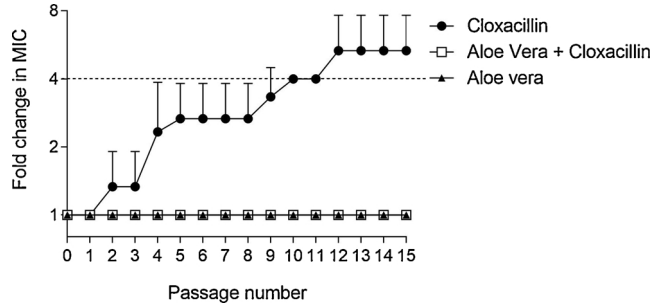

Fig. 2. Level of change with respect to the initial MIC of *S. aureus* ATCC 29213 after 15 serial passages *versus* Aloe vera, cloxacillin and Aloe vera + cloxacillin. In each passage, the reference strain with no prior exposure to antibiotic treatment was used as a control. The dotted line indicates a 4-fold increase in the initial MIC of the strain.

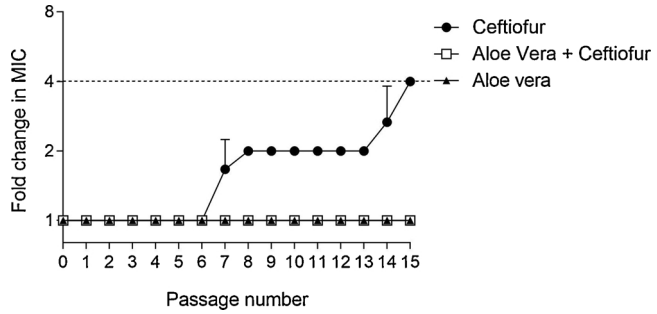

Fig. 3. Level of change with respect to the initial MIC of *S. aureus* ATCC 29213 after 15 serial passages *versus* Aloe vera, ceftiofur and Aloe vera + ceftiofur. In each passage, the reference strain with no prior exposure to antibiotic treatment was used as a control. The dotted line indicates a 4-fold increase in the initial MIC of the strain.

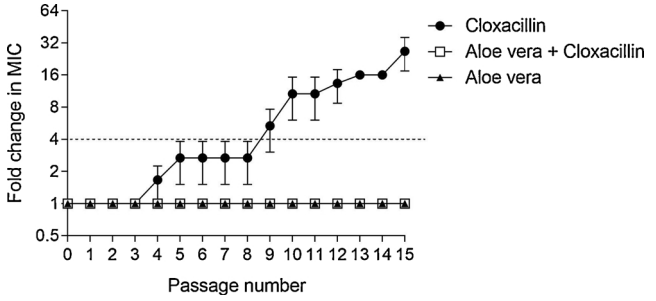

Fig. 4. Level of change with respect to the initial MIC of *S. aureus* ATCC 43300 after 15 serial passages *versus* Aloe vera, cloxacillin and Aloe vera + cloxacillin. In each passage, the reference strain with no prior exposure to antibiotic treatment was used as a control. The dotted line indicates a 4-fold increase in the initial MIC of the strain.

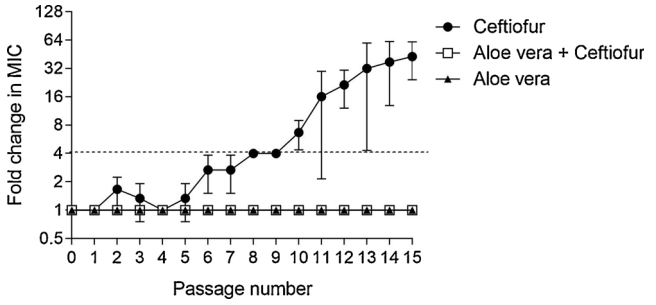

Fig. 5. Level of change with respect to the initial MIC of *S. aureus* ATCC 43300 after 15 serial passages *versus* Aloe vera, ceftiofur and Aloe vera + ceftiofur. In each passage, the reference strain with no prior exposure to antibiotic treatment was used as a control. The dotted line indicates a 4-fold increase in the initial MIC of the strain.

Organization [8]. The antimicrobial activity of Aloe vera against pathogenic bacteria such as *S. aureus*, *Helicobacter pylori*, *Streptococcus mutans, Pseudomonas aeruginosa*, among others, has been described previously in various publications [20,21,24,37]. However, the minimum inhibitory concentration differs between different investigators.

After extracting Aloe vera compounds with antimicrobial activity based on methanol, we determined an MIC value of 40 mg/mL was sufficient against *S. aureus* ATCC 29213 and *S. aureus* ATCC 43300. Our results are consistent with those reported by Habeeb et al. [21], who obtained a MIC of 37.5 mg/mL against strains of methicillin-resistant *S. aureus* using an extraction of *Aloe barbadensis Miller* based on hexane, chloroform, and methanol. Other authors, such as Cataldi et al. [16], recorded a MIC of 800 mg/mL against the strain *S. aureus* ATCC 29213 after performing an extraction based on 2% dimethyl sulfoxide; Pandey and Mishra [38], obtained a MIC of 0.5 mg/mL using an ethanol extract against strains of *S. aureus* isolated from humans.

Although such variations in MIC can be related to the chemical solvents used in the extraction process of inhibitory compounds, research has indicated that variables related to climate (temperature, rainfall, wind patterns), geography (growth orientation, place of harvest), and the plant itself (age) can also influence and modify the concentration and potency of various compounds with biological activity in plants, such as Aloe vera [39].

It is important to highlight that by combining Aloe vera with

cloxacillin or ceftiofur, the necessary concentrations were 4 to 32-fold lower than the MIC defined for each antibiotic alone against *S. aureus* ATCC 29213 (Figs. 2 and 3), *S. aureus* ATCC 43300 (Figs. 4 and 5) and *S. uberis* ATCC 43300. The reduction of the MIC of each antibiotic can be explained by the interactions developed between the components of the combinations. Previous *in vitro* studies indicate that the additive and synergistic effects that occur when combining compounds of natural origin and traditional antibiotics, reduce the MIC values of each component used [23,24].

A second aspect worth highlighting, and the reason we conducted this research, is that Aloe vera used either alone or in combination with ceftiofur and cloxacillin could delay the appearance of chromosomal resistance. Similar results were obtained by Suzuki et al. [40], who used serial passages and subsequent massive sequencing to demonstrate that simultaneous use of enrofloxacin and enoxacin on *Escherichia coli,* suppresses the acquisition of resistance against these antibiotics. In the case of Suzuki et al. [40], two types of antibiotics were used, not a combination of an antibiotic with a natural product, but as in the study conducted by them, there was a delay in the appeareance of chromosomal resistance. The use of massive sequencing techniques has allowed researchers to corroborate that the use of drug combinations and their alternation slow down the evolution of resistance against some of the drugs that make up the combination [41].

It is expected that the evolution of bacterial resistance will be faster in monotherapies than in drug associations with different mechanisms of action, because mutations that confer resistance to an antimicrobial agent should not provide a great advantage in a multidrug environment [41], and at the same time, many more independent mutations would be required in order to become resistant to combination therapy [15].

Aloe vera, when used individually, did not generate the appearance of resistant strains; this effect can be attributed to the fact that this plant possesses more than one mechanism of antimicrobial action. Aloe vera has about 75 active compounds, of which anthraquinones aloin and Aloe emodin have antimicrobial activity [16,20]. Aloin works to destabilize metalloproteinases and decrease the availability of intracellular calcium available in gram-positive bacteria, thus inhibiting protein synthesis, mechanism of action homologous with tetracyclines [42,43]. On the other hand, the ability of Aloin disrupt cell membranes has been proven; this process occurs through weakening of hydrophobic interactions between the carbon chains of the phospholipid bilayer [44].

In this investigation, *S. uberis* did not increase its MIC levels compared to any of the treatments. Although Haenni et al. [45], describes that strains of *S. uberis* may develop resistance after selective pressure caused by synthetic antibiotics, their phylogenetic closeness with *Streptococcus pyogenes* could explain the lack of increased resistance levels. Even though S. *pyogenes* historically has been exposed to the action of beta-lactam, there are practically no isolates of this bacterium, or any other phylogenetically related, resistant to penicillins; likewise, obtaining experimentally resistant strains *in vitro* is equally complex. Thus, the development of resistance can vary within different microorganisms belonging to the same genus [46].

Finally, it should be noted that during serial multipassages, the final strains come from the parental strain used at the beginning of the experiment. Molecular typing techniques such as the pulsed field electrophoresis, used in this work, are useful for monitoring strains for short periods of time. The analysis of dendrograms showed that the R.ACLX2 strain has a band pattern with 97% homology to the parental strain, as well as an additional band. This can be explained due to genetic events such as insertions, deletions, re-arrangements or substitutions that can generate changes in the size of the DNA fragments which, depending on their magnitude and location, may or may not be detected. Mutations in the restriction site of the SmaI enzyme can generate variations in the molecular weight of the DNA fragments obtained by enzymatic digestion [47].

5. Conclusions

The use of Aloe vera, either alone or in combination with synthetic antibiotics, is effective in inhibiting and delaying the onset of resistance in strains of *S. aureus* ATCC 29213 and *S. aureus* ATCC 43300. These results show that compounds of natural origin are a viable alternative to address the current problem of bacterial resistance. Antibiotics administered in combination with Aloe vera make it possible to confront the problem of the excessive synthetic antibiotic use in the treatment of bacterial diseases and contributing to the search for new therapeutic alternatives in order to deal with bacterial resistance. Further research is needed before using this strategy in veterinary practice, like the pharmacokinetic and pharmacodynamics study of this combinations in an animal model, like bovine clinical mastitis, among other studies.

In this study, we confirmed that Aloe vera extract, used alone or in combination with synthetic antibiotics, has an inhibitory effect and delays the selection of resistant strains of bacterial pathogens commonly isolated from bovine clinical mastitis cases.

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.eujim.2019.100996.

References

[1] S. Levy, Reduced antibiotic use in livestock: how Denmark tackled resistance, Environ. Health Perspect. 122 (2014) 160–165.
[2] S.P. Oliver, S.E. Murinda, Antimicrobial resistance of mastitis pathogens, Vet. Clin. North Am. Food Anim. Pract. 28 (2012) 165–185.
[3] G. Gonzalez-Hein, N. Cordero, P. Garcia, G. Figueroa, Molecular analysis of fluoroquinolones and macrolides resistance in *Campylobacter jejuni* isolates from humans, bovine and chicken meat, Rev. Chilena Infectol. 30 (2013) 135–139.
[4] M.A. Leverstein-van Hall, C.M. Dierikx, J. Cohen Stuart, G.M. Voets, M.P. van den Munckhof, A. van Essen-Zandbergen, T. Platteel, A.C. Fluit, N. van de Sande-Bruinsma, J. Scharinga, M.J. Bonten, D.J. Mevius, National, E.s.g, Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains, Clin. Microbiol. Infect. 17 (2011) 873–880.
[5] S. Ramonaite, E. Tamuleviciene, T. Alter, N. Kasnauskyte, M. Malakauskas, MLST genotypes of *Campylobacter jejuni* isolated from broiler products, dairy cattle and human campylobacteriosis cases in Lithuania, BMC Infect. Dis. 17 (2017) 430.
[6] J. Rushton, J.P. Ferreira, K.D.C. Stärk, Antimicrobial Resistance: The Use of Antimicrobials in the Livestock Sector. OECD Food, Agriculture and Fisheries Papers, No. 68, OECD Publishing, Paris, 2014.
[7] R. Schrijver, M. Stijntjes, J. Rodriguez-Bano, E. Tacconelli, N. Babu Rajendran, A. Voss, Review of antimicrobial resistance surveillance programmes in livestock and meat in EU with focus on humans, Clin. Microbiol. Infect. 24 (2018) 577–590.
[8] World Health Organization, Global Action Plan on Antimicrobial Resistance, (2015) http://www.who.int/iris/handle/10665/193736.
[9] A. Lundberg, A.K. Nyman, A. Aspan, S. Borjesson, H.E. Unnerstad, K.P. Waller, Udder infections with Staphylococcus aureus, *Streptococcus dysgalactiae*, and *Streptococcus uberis* at calving in dairy herds with suboptimal udder health, J. Dairy Sci. 99 (2016) 2102–2117.
[10] F. Gomes, M.J. Saavedra, M. Henriques, Bovine mastitis disease/pathogenicity: evidence of the potential role of microbial biofilms, Pathog. Dis. 74 (2016).
[11] A. Raza, G. Muhammad, S. Sharif, A. Atta, Biofilm producing *Staphylococcus aureus* and bovine mastitis: a review, Mol. Microbiol. 3 (2013) 1–18.
[12] R. Seixas, J.P. Santos, R. Bexiga, C.L. Vilela, M. Oliveira, Short communication: antimicrobial resistance and virulence characterization of methicillin-resistant staphylococci isolates from bovine mastitis cases in Portugal, J. Dairy. Sci. 97 (2014) 340–344.
[13] S.P. Oliver, B.E. Gillespie, S.J. Headrick, H. Moorehead, P. Lunn, H.H. Dowlen, D.L. Johnson, K.C. Lamar, S.T. Chester, W.M. Moseley, Efficacy of extended

ceftiofur intramammary therapy for treatment of subclinical mastitis in lactating dairy cows, J. Dairy Sci. 87 (2004) 2393–2400.
[14] World Health Organization, Critically Important Antimicrobials for Human Medicine, 6th Revision. Ranking of Medically Important Antimicrobials for Risk Management of Antimicrobial Resistance Due to Non-human Use, (2019).
[15] T. Bollenbach, Antimicrobial interactions: mechanisms and implications for drug discovery and resistance evolution, Curr. Opin. Microbiol. 27 (2015) 1–9.
[16] V. Cataldi, S. Di Bartolomeo, E. Di Campli, A. Nostro, L. Cellini, M. Di Giulio, *In vitro* activity of Aloe vera inner gel against microorganisms grown in planktonic and sessile phases, Int. J. Immunopathol. Pharmacol. 28 (2015) 595–602.
[17] E. Rodriguez Rodriguez, J. Darias Martin, C. Diaz Romero, Aloe vera as a functional ingredient in foods, Crit. Rev. Food Sci. Nutr. 50 (2010) 305–326.
[18] G.A. Vega, C.N. Ampuero, N.L. Díaz, M.R. Lemus, El aloe vera (*Aloe barbadensis Miller*) como componente de alimentos funcionales, Rev. Chil. Nutr. 32 (2005) 208–214.
[19] K. Ajeigbe, J. Nebedum, E. Nwobodo, C. Uba, O. Adesanya, O. Fadare, D. Ofusori, Comparative study of the ethanolic extracts of four nigerian plants against some pathogenic microorganisms, J. Med. Plant Res. 3 (2009) 23–28.
[20] L. Cellini, S. Di Bartolomeo, E. Di Campli, S. Genovese, M. Locatelli, M. Di Giulio, *In vitro* activity of Aloe vera inner gel against *Helicobacter pylori* strains, Lett. Appl. Microbiol. 59 (2014) 43–48.
[21] F. Habeeb, E. Shakir, F. Bradbury, P. Cameron, M.R. Taravati, A.J. Drummond, A.I. Gray, V.A. Ferro, Screening methods used to determine the anti-microbial properties of Aloe vera inner gel, Methods 42 (2007) 315–320.
[22] S. Gautam, P. Awasthi, Nutrient composition and physio-chemical characteristics of Aloe vera (*Aloe barbadensis*) powder, J. Food Sci. Technol. 44 (2007) 224–225.
[23] A. Vijaya Lakshmi, S. Harasreeramulu, D.V.V. Satyanarayana Raju, Assessment of antibacterial potential of selected medicinal plants and their interactions with antibiotics on MRSA in the health care workers of Visakhapatnam hospitals, J. Pharm. Res. 6 (2013) 589–592.
[24] S. Chusri, T. Siriyong, P. Na-Phatthalung, S.P. Voravuthikunchai, Synergistic effects of ethnomedicinal plants of Apocynaceae family and antibiotics against clinical isolates of *Acinetobacter baumannii*, Asian Pac. J. Trop. Med. 7 (2014) 456–461.
[25] J.H. Hamman, Composition and applications of Aloe vera leaf gel, Molecules 13 (2008) 1599–1616.
[26] B. San Martín, N. Forno, D. García, F. Kuhlmann, D. Iragüen, M. Calderón, Optimización en la extracción de los compuestos con actividad antimicrobiana desde la pulpa de hoja de *Aloe barbadensis Miller,* XXIV congreso Panamericano de ciencias veterinarias, Asociación consejo científico veterinario de Cuba, Cuba, 2014.
[27] C. Proestos, M. Komaitis, Application of microwave-assisted extraction to the fast extraction of plant phenolic compounds, LWT-Food. Sci. Technol 41 (2005) 652–659.
[28] M.P. Kahkonen, A.I. Hopia, H.J. Vuorela, J.P. Rauha, K. Pihlaja, T.S. Kujala, M. Heinonen, Antioxidant activity of plant extracts containing phenolic compounds, J. Agric. Food Chem. 47 (1999) 3954–3962.
[29] V.L. Singleton, J.A. Rossi, Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents, Am. J. Enol. Vitic. 16 (1965) 144.
[30] Clinical Laboratory Standards Institute, Performance Standards for Antimicrobial Susceptibility Testing; Twenty-second Informational Supplement, CLSI, Wayne, PA, 2012.
[31] National Committee for Clinical Laboratory Standards, Standard M26-A. Methods for Determining Bactericidal Activity of Antimicrobial Agents 19 (1999) (18). 29 p.
[32] D. Farrell, M. Robbins, W. Rhys-Williams, W. Love, Investigation of the potential for mutational resistance to XF-73, retapamulin, mupirocin, fusidic acid, daptomycin, and vancomycin in methicillin-resistant *Staphylococcus aureus* isolates during a 55-passage study, Antimicrob. Agents Chemother. 55 (2011) 1177–1181.
[33] Centers for Disease Control and Prevention, Unified PulsedField Gel Electrophoresis (PFGE) Protocol for Gram Positive Bacteria, (2012) https://www.cdc.gov/hai/pdfs/labsettings/unified_pfge_protocol.pdf.
[34] S. Heng, C. Heng, S. Puthucheary, Stacking gels: a method for maximising output for pulsed-field gel electrophoresis, Indian J. Med. Microbiol. 27 (2009) 142–145.
[35] C. Matsumoto, E. Chimara, S. Bombarda, R. Duarte, S.C. Leão, Diversity of pulsed-field gel electrophoresis patterns of Mycobacterium abscessus type 2 clinical isolates, J. Clin. Microbiol. 49 (2011) 62–68.
[36] Z. Bakuła, A. Brzostek, P. Borówka, A. Żaczek, I. Szulc-Kiełbik, A. Podpora, P. Parniewski, D. Strapagiel, J. Dziadek, M. Proboszcz, J. Bielecki, J. Van Ingen, T. Jagielski, Molecular typing of *Mycobacterium kansasii* using pulsed-field gel electrophoresis and a newly designed variable-number tandem repeat analysis, Sci. Rep. 13 (2018) 4462.
[37] P.F. Bertolini, O. Biondi Filho, A. Pomilio, S.L. Pinheiro, M.S. Carvalho, Antimicrobial capacity of Aloe vera and propolis dentifrice against *Streptococcus mutans* strains in toothbrushes: an *in vitro* study, J. Appl. Oral Sci. 20 (2012) 32–37.
[38] R. Pandey, A. Mishra, Antibacterial activities of crude extract of *Aloe barbadensis* to clinically isolated bacterial pathogens, Appl. Biochem. Biotechnol. 160 (2010) 1356–1361.
[39] S. Kumar, A. Yadav, M. Yadav, J.P. Yadav, Effect of climate change on phyto-chemical diversity, total phenolic content and *in vitro* antioxidant activity of *Aloe vera* (L.) Burm.f, BMC Res. Notes 10 (2017) 60.
[40] S. Suzuki, T. Horinouchi, C. Furusawa, Acceleration and suppression of resistance development by antibiotic combinations, BMC Genomics 18 (2017) 328.
[41] S. Kim, T.D. Lieberman, R. Kishony, Alternating antibiotic treatments constrain evolutionary paths to multidrug resistance, Proc. Natl. Acad. Sci. U. S. A 111 (2014) 14494–14499.
[42] E. Barrantes, M. Guinea, Inhibition of collagenase and metalloproteinases by aloins and aloe gel, Life Sci. 72 (2003) 843–850.
[43] H. Levin, R. Hazenfratz, J. Friedman, D. Palevitch, M. Perl, Partial purification and some properties of an antibacterial compound from *Aloe vera*, Phytother. Res. 2 (1988) 67–69.
[44] D.S. Alves, L. Perez-Fons, A. Estepa, V. Micol, Membrane-related effects underlying the biological activity of the anthraquinones emodin and barbaloin, Biochem. Pharmacol. 68 (2004) 549–561.
[45] M. Haenni, L. Galofaro, M. Ythier, M. Giddey, P. Majcherczyk, P. Moreillon, J.Y. Madec, Penicillin-binding protein gene alterations in *Streptococcus uberis* isolates presenting decreased susceptibility to penicillin, Antimicrob. Agents Chemother. 54 (2010) 1140–1145.
[46] M. Haenni, P.A. Majcherczyk, J.L. Barblan, P. Moreillon, Mutational analysis of class A and class B penicillin-binding proteins in *Streptococcus gordonii*, Antimicrob. Agents Chemother. 50 (2006) 4062–4069.
[47] R.V. Goering, Pulsed field gel electrophoresis: a review of application and interpretation in the molecular epidemiology of infectious disease, Infect. Genet. Evol. 10 (2010) 866–875.