J Vet Sci. 2024 Jan;25(1):e6
https://doi.org/10.4142/jvs.23245
pISSN 1229-845X·eISSN 1976-555X

Journal of Veterinary Science

Original Article
Pharmacology

Check for updates

Intramammary preparation of enrofloxacin hydrochloride-dihydrate for bovine mastitis (biofilm-forming *Staphylococcus aureus*)

Diana Córdova-González [1], Edgar Alfonseca-Silva [2], Lilia Gutiérrez [1], Graciela Tapia-Pérez [3], Héctor Sumano [1,*]

[1]Departamento de Fisiología y Farmacología, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, México City 04510, México
[2]Departamento de Microbiología e Inmunología, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, México City 04510, México
[3]Departamento de Genética y Bioestadística, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, México City 04510, México

OPEN ACCESS

Received: Oct 5, 2023
Revised: Nov 7, 2023
Accepted: Nov 14, 2023
Published online: Dec 19, 2023

***Corresponding author:**
Héctor Sumano
Departamento de Fisiología y Farmacología, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, Ciudad de México, Avenida Universidad 3000, Coyoacan, México City 04510, México.
Email: sumano@unam.mx
https://orcid.org/0000-0002-8802-5274

ABSTRACT

Background: Chronic bovine mastitis is linked to biofilm-producing *Staphylococcus aureus* (bp-Sa) or *Staphylococcus* coagulase-negative (bp-Scn).
Objectives: Bp-Sa and bp-Scn were treated with intramammary preparations of either enrofloxacin HCl·2H_2O-dimethyl-sulfoxide-chitosan (enro-C/DMSO/chitosan) or enro-C alone. Their potential to inhibit and degrade biofilm formation *in vitro* was also assessed.
Methods: Milk samples were obtained from the affected quarters in a herd. Phenotypical and genotypical identifications as biofilm-producing *Staphylococcus* species were carried out. Enro-C/DMSO/chitosan and enro-C alone were assessed to determine their *in vitro* efficacy in interfering with biofilm formation and their bactericidal effects. A prolonged eight-day treatment with a twice-daily intramammary insertion of 10 mL of enro-C/DMSO/chitosan or enro-C alone was set to evaluate the clinical and bacteriological cures on day 10 in 15 cows per group and the biofilm-inhibiting ability.
Results: Fifty-seven percent of the isolates were identified as *Staphylococcus* spp., of which 50% were bp-Sa, 46% bp-Scn, and 4% *Staphylococcus pseudintermedius*. One hundred percent of the *S. aureus* isolated and 77% of *Staphylococcus* coagulase-negative were biofilm producers. In both groups, the *icaA* and *icaD* biofilm-producing genes were identified. The experimental preparation could inhibit biofilm formation, degrade mature biofilms, and have well-defined microbicidal effects on planktonic and biofilm bacteria. The respective clinical and bacteriological cure rates were 100% and 80% for enro-C/DMSO/chitosan and 41.7% and 25% for enro-C alone.
Conclusions: Enro-C/DMSO/chitosan eliminates bp-Sa and bp-Scn from cases of chronic bovine mastitis.

Keywords: Dairy cow; biofilm; chronic mastitis; *Staphylococcus aureus*; enrofloxacin

INTRODUCTION

Bovine mastitis is a costly disease that results in decreased milk production [1,2]. Despite treatment, a mastitis episode may often become chronic [3], accompanied by sustained high

ORCID iDs

Diana Córdova-González
https://orcid.org/0009-0008-4451-0519
Edgar Alfonseca-Silva
https://orcid.org/0000-0003-3274-9596
Lilia Gutiérrez
https://orcid.org/0000-0002-4823-0388
Graciela Tapia-Pérez
https://orcid.org/0000-0002-3181-8441
Héctor Sumano
https://orcid.org/0000-0002-8802-5274

Author Contributions

Conceptualization: Sumano H, Gutiérrez L; Data curation: Córdova-González D, Sumano H; Formal analysis: Tapia-Pérez G; Funding acquisition: Sumano H; Investigation: Córdova-González D, Alfonseca-Silva E, Sumano H; Methodology: Córdova-González D, Alfonseca-Silva E, Sumano H; Project administration: Sumano H, Gutiérrez L; Resources: Sumano H; Software: Tapia-Pérez G; Supervision: Sumano H, Alfonseca-Silva E, Gutierrez L; Validation: Sumano H, Alfonseca-Silva E, Tapia-Pérez G; Writing - original draft: Córdova-González D, Alfonseca-Silva E, Sumano H; Writing - review & editing: Córdova-González D, Alfonseca-Silva E, Sumano H, Tapia-Pérez G.

Conflict of Interest

The authors declare no conflicts of interest. The National Autonomous University of Mexico (UNAM), owner of the patent for enro-C, is open to licensing it.

Funding

This study was supported by the research program PAPIIT at UNAM No. ITT200322. Diana Cordova was awarded a CONAHCYT, Mexico graduate scholarship. A sabbatical program from the PASPA-DGAPA-Universidad Nacional Autónoma de México supported Edgar Alfonseca-Silva.

somatic cell counts (SCCs) and various outbreaks of clinical mastitis in the same mammary quarter during the ongoing lactation cycle. These cases are often associated with biofilm-forming bacteria, particularly *Staphylococcus* spp. [4-6].

A biofilm is a bacterial virulence factor that contains bacteria in an extracellular matrix of exopolysaccharides. The biofilm adheres to the epithelium of the mammary gland where bacteria agglomerate can evade the host's immune system, resist the effect of antimicrobials, and colonize the host's nearby tissue through a cycle of adhesion-multiplication-exodus-maturation and dispersion [7,8]. Treating chronic mastitis linked to biofilm-producing pathogens is not recommended because of the low efficacy and the risk of horizontal spread [9]. In many developing nations, however, this problem is rarely diagnosed, and cows are not culled. Hence, a viable treatment is needed. The modified antimicrobial enrofloxacin hydrochloride-dihydrate (enro-C) (UNAM Patent 472715-INPI, Mexico) is a more water-soluble solvate than enrofloxacin that does not irritate the mammary gland. It has a pH of 6.5 in suspension at 30 mg/mL and achieves high concentrations in mammary tissue. Enro-C has shown high success in treating uncomplicated cases of mastitis [10-12] and recurrent chronic cases caused by bacteria resistant to fluoroquinolones [13].

This paper proposes that in conjunction with pharmaceutical vehicles that destroy or weaken the biofilm without affecting the glandular tissue, an experimental preparation containing enro-C, low molecular weight chitosan, and dimethyl sulfoxide (DMSO) could effectively treat chronic mastitis. Chitosan is a biocompatible, biodegradable, and non-toxic substrate obtained from the exoskeleton of marine crustaceans and has been recognized to reduce preformed biofilms and limit the adhesion of pathogens to epithelia [14]. DMSO is a pharmaceutical vehicle that increases the diffusion of drugs to the target site, possesses antimicrobial activity, and is safe [15-17].

MATERIALS AND METHODS

This study was ethically approved at the National Autonomous University of Mexico (UNAM) (ID No: SICUAE.MC-2021/4-2). The elaboration of the intramammary preparations was carried out in the Department of Physiology and Pharmacology, UNAM, under good manufacturing practices. The bacteriological diagnosis and the *in vitro* evaluation of the preparation used were carried out in the Department of Microbiology and Immunology at UNAM, and the clinical evaluation was conducted on a farm in Chipilo, Puebla, Mexico.

Intramammary preparations

A control group was treated with a suspension based on enro-C (300 mg in 10 mL), commercially available as Enromastic (Laboratorios Aranda S.A. de C.V., México). The experimental group was treated with a suspension based on enro-C (300 mg in 10 mL), DMSO (Chromasolv, USA) (20 mg/10 mL), and low molecular chitosan (Pharmachem S.A. de C.V., Mexico) (50 mg/mL) (enro-C/DMSO/chitosan). These components were weighed and re-suspended in distilled water, and the pH was maintained at 6 using a buffer solution. The mixture was stirred for 24 h. When the suspension turned visibly homogeneous, 10 mL intramammary syringes were filled and stored at 4°C, together with the Enromastic syringes. Before administering either preparation, the syringes were gently shaken to homogenize the suspension, and their content was inserted into the distal third teat using an aseptic technique. The treatments were given twice a day for eight days based on similar trials

[18,19], and the dose was maintained at 300 mg per quarter, as previous work revealed high efficacy and a lack of apparent tissue toxicity [12].

Study population

The initial selection of second and third-pregnancy Holstein/Friesian cases of mastitis was carried out using a direct visual evaluation of stripped milk aided by a black bowl, a California mastitis test on each quarter of the affected mammary gland, and by checking the cow's records as having had at least three or more previous treatments in the current milking cycle in a given mammary quarter. A SCC greater than 200,000 cells/mL in the last two months was set as a prerequisite to enter this trial, and no treatment had been administered in the last two weeks [4,6]. Thirty cows, with 98 mammary quarters with mastitis, were initially regarded as potentially infected with biofilm-producing *Staphylococcus* spp., and suffering from chronic mastitis. Their milk was studied to isolate the pathogen and identify the possible biofilm-forming bacteria.

Bacteriological identification

Twenty mL of milk from each mammary quarter of initially chosen cows were collected in 50 mL sterile conical centrifuge tubes, kept at 4°C, and processed within the next 4 h for bacteriological diagnosis. Bacterial isolation was first carried out on blood agar (Bioxon; Becton Dickinson, Mexico). Gram staining, catalase tests, coagulase tests, and isolation in salt and mannitol agar (Bioxon; Becton Dickinson), anaerobic mannitol (Hycel; REGUSA, Mexico), and P agar (Bioxon; Becton Dickinson) were performed to differentiate *Staphylococcus* species. A pure culture on CAMP-esculin agar (Bioxon; Becton Dickinson) for the identification and differentiation of *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, or *Streptococcus uberis*, was performed when the isolates did not agree with the genus *Staphylococcus* and were negative to catalase [20].

Biofilm formation ability

Having identified *Staphylococcus* spp., the modified Congo red agar qualitative phenotypic test was performed as proposed by Mariana et al. [21]. Briefly, the strains that formed black colonies were considered biofilm producers and those that formed bright red colonies were not. The quantitative phenotypic determination was made with the biofilm microtiter technique in 96-well flat-bottom tissue culture plates (Greiner Bio-One, Germany) [22] at a wavelength of 620 nm. The utilized technique allows a quantitative classification of biofilm production in the strains of interest: non-biofilm producers, low biofilm producers, medium biofilm producers, and high biofilm producers.

DNA was extracted and purified from the isolated strains using a CTAB-chloroform:isoamyl alcohol methodology. The concentration and integrity of the DNA were measured by (NanoDrop ND-1000, Wilmington, USA), and the samples were standardized to a concentration of 40 ng/μL. The selected biofilm production genes were those associated with the *ica* operon (*icaA* and *icaD*) for the production of the Intercellular Adhesion Polysaccharide (IAP), the most relevant in biofilm production by the *Staphylococcus* genus and were detected by a polymerase chain reaction (PCR) using a MyTaq DNA polymerase kit (Bioline; Meridian Life Science, USA). The PRIMER-BLAST tool was selected for primer design (https://www.ncbi.nlm.nih.gov/tools/primer-blast/): primers for the *ica*A gene (321 bp), F – 5'TTA GCA CAA TGA AAC GA AAA GGT3' y R – 5'CGA CAA GAA CTA CTG CTG CGT3' and primers for the *ica*D (78 pb) F – 5'AGC CCA GAC AGA GGG AAT AC3' y R – 5'ACG ATA TAG CGA TAA GTG CTG TTT3'. The PCR conditions were as follows: activation at 95°C for 2 min,

denaturation at 95°C for 45 sec, alignment at 62°C for 30 sec, amplification at 72°C for 45 sec (25 cycles), and a final extension at 72°C for 2 min. The size of the amplicons was checked by electrophoresis in a 2% agarose gel stained with Diamond (Promega, USA), which was run at 80 V for 45 min to examine the gel in a transilluminator and determine the amplicons obtained from PCR. The positive and negative controls for biofilm production were *Staphylococcus aureus* Cowan strain and *Escherichia coli*, respectively.

Antibacterial activity and biofilm synthesis and degradation effects

The results were compared with a reference *S. aureus* strain from case 10 because it was classified as a phenotypically and genotypically high biofilm producer. The effects of the enro-C alone and enro-C/DMSO/chitosan preparation on biofilm synthesis and their antibacterial activity were evaluated in 96-well flat-bottom microplates (Greiner Bio-One). The standardized inoculum was exposed 1:1 to two different concentrations of enro-C or the experimental preparation (low concentration: 10 μg enro-C and medium concentration: 50 μg enro-C per well). The control for bacterial growth and biofilm formation without treatment was also set. The plates were incubated at 37°C for 24 h. The antibacterial activity of the preparation was evaluated on planktonic bacteria (BPl) in the supernatant and on bacteria contained within the biofilm (BBi). A micro-brush was used to break the matrix and release the bacteria inside the biolayer. The effects of the treatments were determined by preparing serial tenfold dilutions and comparing the colony-forming units (CFU).

The effects of the enro-C/DMSO/chitosan preparation on biofilm degradation and antibacterial activity on microorganisms present within the biofilm were evaluated by copying the previous experimental settings and exposing the wells exposed in a 1:1 ratio to three different concentrations of enro-C alone or with the experimental preparation at three concentrations (enro-C: low [10 μg], medium [50 μg], and high [100 μg]). An untreated set of wells served as a control for bacterial growth and biofilm formation. The plates were incubated as described elsewhere [13], and evaluations were carried out 24 h and 72 h later. The supernatant was removed daily, and glucose–trypticasein soy broth (Bioxon; Becton Dickinson) plus enro-C or the preparation was added to each well. At the end of the procedures, degradation of the previously formed biofilm and the antibacterial activity on BPl and BBi were determined from the CFU count compared with the controls, as described elsewhere [13]. The results are expressed as the mean of three independent experiments with two internal replicates.

Clinical and bacteriological efficacies

The treatments of the mammary quarters with either Enromastic or the enro-C/DMSO/chitosan preparation were assigned randomly (by blocks due to bacteria). Daily clinical evaluations of mammary-quarter conformation, milk organoleptic characteristics, and California mastitis tests were performed to determine the case evolution. The end-point clinical efficacy was set when milk was shown to be free of organoleptic alterations, glands free of pain and swelling, and the California score test was zero (no evidence of inflammation) [23]. The end-point for bacteriological efficacy was determined ten days after the clinical results were recorded and was based on the negative growth/isolation of *S. aureus.*

Statistical analysis

The statistical package IBM SPSS Version 25 64-bit Edition, 2017 (IBM, USA) was used. The Kruskal-Wallis test for independent samples was used to determine the statistical significance ($p < 0.05$) of the antibacterial activity of the experimental preparation under the

inhibition and degradation of biofilms. In addition, the inhibition effect of the preparations on the formation of biofilms and degradation of the mature biofilm at one and three days were examined. The statistical significance ($p < 0.05$) of the clinical and bacteriological efficacy was determined between groups with chronic bovine mastitis due to *Staphylococcus* spp. biofilm producer treated with the proposed preparations. The statistical significance ($p < 0.05$) of the effect of the drug on the etiological agent causing mastitis based on its biofilm production capacity was assessed using a Pearson's χ^2 test. Bootstrap [24] was also used (n = 1,000, confidence interval = 95). Post hoc analysis was performed using G*Power [25] to corroborate the adequacy of the sample size with the actual P and η values.

RESULTS

Bacteriological identification

Samples of 98 mammary glands from 30 cows with chronic inflammatory processes were studied. In 57% (56/98) of cases, *Staphylococcus* spp. was isolated; 25% (24/98) were linked to *Streptococcus* spp.; 8% (8/98) presented a mixed infection (*Staphylococcus* coagulase-negative with *S. agalactiae*); 10% (10/98) did not show bacterial growth. From the *Staphylococcus* genus, 50% were *S. aureus* (28/56); 46% (26/56) were *Staphylococcus* coagulase-negative; 4% (2/56) were *S. pseudintermedius*. Of the 24 samples positive to *Streptococcus* spp., 67% (16/24) and 33% (8/24) were *S. uberis* and *S. agalactiae*, respectively. The *Staphylococcus* genus was the leading etiological agent of chronic bovine mastitis, identified in 65% of the samples.

Phenotypic and genotypic characterization of the isolates

Table 1 lists the ability to produce biofilm. Based on their phenotype, the isolated species of the genus *Staphylococcus* were biofilm producers: 100% for *S. aureus* and 77% for *S.* coagulase-negatives. **Fig. 1** shows a representative image of the amplification of the *icaA* and *icaD* genes associated with biofilm production in the *Staphylococcus* genus.

Antibacterial activity on biofilm production and degradation

The experimental preparation showed a well-defined inhibitory effect on biofilm formation compared to the biofilm without the drug ($p < 0.01$ in all cases) (**Fig. 2A**). In addition, a significant decrease in bacterial growth of *S. aureus* was observed, both in planktonic bacteria, from 1.8×10^8 CFU/mL to 4.1×10^5 CFU/mL with the low concentration preparation ($p = 0.002$) and up to 4×10^4 CFU/mL with the medium concentration preparation ($p < 0.001$). A similar scenario was also observed in bacteria within the biofilms, i.e., from 2.5×10^{11} CFU/mL to 6.4×10^7 CFU/mL, and 2.5×10^6 CFU/mL with the low and medium concentrations of the preparation ($p = 0.02$ and $p < 0.001$, respectively), respectively, as summarized in **Table 2**. As expected, the preparations on bacterial growth proportionally affect biofilm formation.

The enro-C/DMSO/chitosan preparation in the biofilm degradation assay had an antibacterial effect against *S. aureus*, reducing the number of planktonic bacteria from 1.3×10^{11} CFU/mL to

Table 1. Bacteriological identification and the phenotypic and genotypic characterization of bacteria isolated from bovine milk with chronic mastitis

Bacteria	Biofilm phenotypic production (%)						Identification of *ica* operon genes	
	Modified Congo red agar		Plate microtiter (biofilm producer)					
	+	–	Negative	Low	Moderate	High	+	–
Staphylococcus aureus	100.0	-	7.1	50.0	21.4	21.4	85.7	14.3
Staphylococcus coagulase-negative	76.9	23.1	53.8	15.4	15.4	15.4	23.1	76.9
Staphylococcus pseudintermedius	-	100.0	100.0	-	-	-	-	100.0

https://doi.org/10.4142/jvs.23245

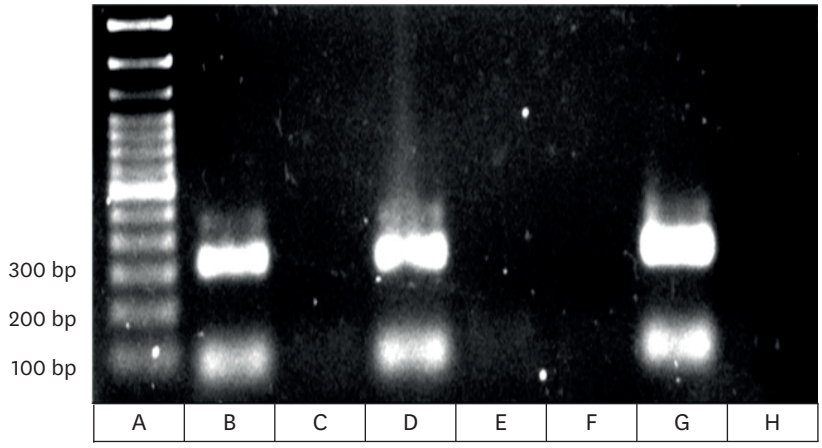

Fig. 1. Detection by multiplex PCR in 2% agarose gel of the *icaA* (321 bp) and *icaD* (78 bp) genes associated with biofilm production of *Staphylococcus aureus* isolated from dairy cows suffering chronic mastitis. A: 100 bp Molecular Weight Marker; B: *S. aureus* DNA: *icaA* and *icaD* genes; C: *S. aureus* DNA: negative; D: DNA from *Staphylococcus* coagulase-negative: *icaA* and *icaD* genes; E: DNA from *Staphylococcus* coagulase-negative: negative; F: *Escherichia coli* DNA: negative control; G: *S. aureus* Cowan DNA: positive control *icaA* and *icaD* genes; H: no DNA: negative control.

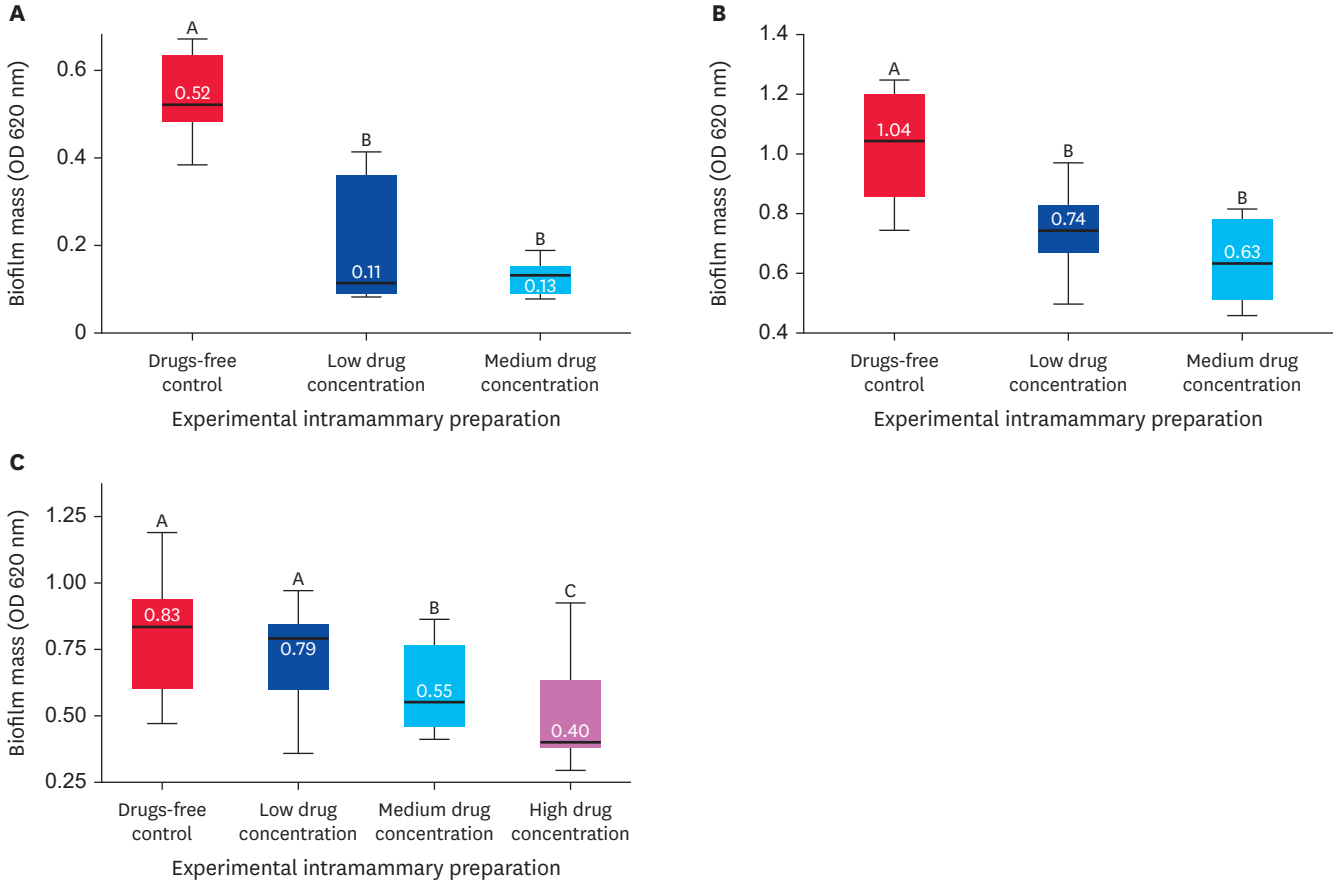

Fig. 2. Effect of the intramammary preparation of enrofloxacin hydrochloride-dihydrate/dimethyl sulfoxide/chitosan on the biofilm production of *Staphylococcus aureus* isolated from chronic bovine mastitis in 96-well polystyrene microplates. (A) Inhibition effect on the formation of *S. aureus* biofilm. (B) Effect of degradation on the mature biofilm of *S. aureus* (1 day). (C) Effect of degradation on the mature biofilm of *S. aureus* (three days). Box plots represent the non-outlier extreme values, 25th and 75th percentiles and the median of three independent experiments.
OD, optical density.
[A,B,C]The different letters indicate significant differences between the groups ($p < 0.05$) according to a two independent groups Wilcoxon test.

https://doi.org/10.4142/jvs.23245

Table 2. Median values of the CFU/mL obtained with each treatment using the intramammary preparation prototype in the test of enro-C/DMSO/chitosan against *Staphylococcus aureus*

Treatment	Biofilm formation inhibition assay (CFU/mL)		Mature biofilm degradation assay (CFU/mL)	
	Planktonic bacteria	Bacteria in biofilm	Planktonic bacteria	Bacteria in biofilm
Control bacteria without treatment	1.8×10^{8a}	2.5×10^{11A}	1.3×10^{11a}	1.8×10^{12A}
Low concentration of enro-C (10 μg), DMSO (0.00017 μL), and chitosan (1.67 μg) per well	4.1×10^{5b}	6.4×10^{7B}	6.7×10^{8b}	1.8×10^{9B}
Medium concentration of enro-C (50 μg), DMSO (0.00083 μL), and chitosan (8.33 μg) per well	4×10^{4c}	2.5×10^{6C}	5.4×10^{6c}	1.7×10^{9B}

CFU, colony-forming units; enro-C, enrofloxacin hydrochloride-dihydrate; DMSO, dimethyl sulfoxide.
[a,b,c]Different lowercase letters indicate the significant differences between the groups of planktonic bacteria in each assessment ($p < 0.05$). Independent two-group Wilcoxon median comparison tests after the Kruskal-Wallis tests.
[A,B,C]Different capital letters indicate significant differences between the groups of bacteria contained in the biofilm in each assessment ($p < 0.05$). Independent two-group Wilcoxon median comparison tests after the Kruskal-Wallis tests.

6.7×10^8 CFU/mL with the low concentration preparation ($p = 0.02$), and up to 5.4×10^6 CFU/mL with the medium concentration ($p < 0.001$). A similar pattern was observed with the number of bacteria in the biofilm, i.e., from 1.8×10^{12} CFU/mL to 1.8×10^9 CFU/mL and 1.7×10^9 CFU/mL, with the low or medium concentration preparations, respectively ($p < 0.001$ for both cases) (**Table 2**). The experimental preparation at both low and medium concentrations degraded the one-day-old biofilm formed by *S. aureus* by 30% ($p = 0.008$) and 40% ($p < 0.001$), respectively (**Fig. 2B**). **Fig. 2C** shows how the medium and high concentrations administered for three consecutive days can have a more significant degradation effect on the mature biofilm formed by *S. aureus* compared to the low concentration (44% and 52%, respectively; $p = 0.05$ and $p = 0.003$, respectively).

Clinical efficacy

Table 3 lists the results obtained from the clinical cure and bacteriological cure evaluations in treating dairy cattle suffering from chronic mastitis. The enro-C/DMSO/chitosan preparation had a 100% clinical-cure efficacy and an 80% bacteriological cure. In contrast, the preparation containing only enro-C from Enromastic had a clinical and bacteriological cure of 41.7% and 25%, respectively ($p = 0.04$). **Table 4** lists the bacteriological efficacy of

Table 3. Clinical and bacteriological efficacy of the experimental preparation of enro-C/DMSO/chitosan compared with enro-C from Enromastic (Laboratorios Aranda S.A. de C.V., Mexico) in cases of chronic bovine mastitis

Treatment	Clinical cure (%)	Bacteriological cure rate (%)	Bacteriological cure failure (%)	Mammary quarters (No.)	Pearson's χ^2 test (p)
Enro-C/DMSO/chitosan	100.0	80.0	20.0	40.0	-
Enro-C	41.7	25.0	75.0	48.0	0.042

Enro-C, enrofloxacin hydrochloride-dihydrate; DMSO, dimethyl sulfoxide.

Table 4. Efficacy of the enro-C/DMSO/chitosan preparation in comparison with enro-C from Enromastic (Laboratorios Aranda S.A. de C.V., Mexico) on biofilm-producing and non-producing bacteria obtained from chronic bovine mastitis cases

Isolated bacteria	Bacteriological efficacy	Treatment (%)		Total MQ (%)	Pearson's χ^2 test (p)
		Enro-C/DMSO/chitosan	Enro-C		
Streptococcus spp.	Cured	41.7	58.3	100 (24 MQ)	-
Staphylococcus non-biofilm producer	Cured	33.3	11.1	100 (18 MQ)	0.068
	Failed	11.1	44.4		
Staphylococcus low biofilm producer	Cured	22.2	33.3	100 (18 MQ)	0.070
	Failed	0.0	44.4		
Staphylococcus moderated biofilm producer	Cured	60.0	40.0	100 (10 MQ)	-
Staphylococcus high biofilm producer	Cured	40.0	20.0	100 (10 MQ)	0.532
	Failed	20.0	20.0		
Mixed infection	Cured	25.0	25.0	100 (8 MQ)	0.147
	Failed	50.0	0.0		
Total	Cured	36.4	34.1	100 (88 MQ)	0.144
	Failed	9.1	20.5		

Enro-C, enrofloxacin hydrochloride-dihydrate; DMSO, dimethyl sulfoxide; MQ, mammary quarters.

both preparations based on the biofilm production capacity of the isolated bacteria. *Post hoc* analysis revealed a 0.85 power of this clinical test.

DISCUSSION

The enro-C/DMSO/chitosan components were selected based on the antimicrobial potency, enhanced tissue diffusion, and antibiofilm-reported effects, respectively. Enro-C, being a zwitterion, can achieve high concentrations beyond the mammary gland cistern and diffuse into the interstitial space where the biofilm is present. In addition, it can diffuse into the intracellular level where *S. aureus* can access, survive, and grow [8,10,26]. Low molecular weight chitosan was added because of its anti-biofilm effect owing to the electrostatic reaction between the positive charges of its NH_2^+ groups with the negative charges of the exopolymeric proteins, extracellular DNA, and other proteins comprising the biolayer [27]. In addition, low molecular weight chitosan has better water solubility and can inhibit bacterial enzymatic activity through cation chelation mechanisms. This will block mRNA transcription and various metabolic pathways [28]. DMSO allows better tissue diffusion of components and has anti-biofilm properties [16,29].

Biofilm production by *Staphylococcus* spp. is initiated by synthesizing IAP. In this study, three different methodologies were used to identify IAP in the bacteria associated with cases of chronic bovine mastitis. (1) Congo red agar forms hydrogen bonds between the dye and the IAP, and with an improved formula proposed by Mariana and Salman [21] that produces better pigmentation in the biofilm-producing colonies, it is evidenced by the growth of black colonies in the medium. This stain is an easy, fast, and economical alternative, but it depends on the experience and interpretation of the personnel, which increases the error. (2) A plate microtiter is considered the gold standard test to quantify biofilm production [30], particularly with the modified steps proposed by Peña and Uffo [22], i.e., transferring bacteria to the microplate and heating them to avoid losing bacterial cells and polysaccharides attached to the walls of the well. Unlike Congo red agar, which only determines if the strain can produce the biofilm, biofilm microtitration allows the bacteria to be classified according to their level of IAP production into high, moderate, low, and non-biofilm producers. In this context, *S. aureus* isolates exhibited more biofilm-producing bacteria than *Staphylococcus* coagulase-negative. (3) The production capacity of IAP is encoded in the *ica* operon, composed of the *icaA*, *icaB*, *icaC*, and *icaD* genes. In this study, the *icaA* and *icaD* genes were selected for amplification to verify that the phenotypical expression of biofilm-forming ability was linked to the referred genes [31]. A comparison of the phenotypic methodologies used for biofilm detection showed no concordance when classifying the strains. On the other hand, similar percentages of biofilm-producing strains are observed when comparing the phenotypic methodologies with the PCR results in the *S. aureus* isolates. This was not repeated for *Staphylococcus* coagulase-negative bacteria, as the phenotypic tests identified more biofilm producers than PCR. Considering the above, the most reliable method to assess biofilm production is the microtiter biofilm test, accompanied by identifying the genes related to this virulence factor.

A plate microtiter was also used to assess the anti-biofilm capacity of the enro-C/DMSO/chitosan preparation. The determination to carry out studies at one and three days was based on the fact that after one day of *in vitro* culture, a biofilm layer within the well was obtained, reaching its maximum point on day three of culture [32]. The enro-C/DMSO/chitosan preparation had an excellent antimicrobial effect *in vitro* and reduced biofilm

production by *S. aureus* isolated from clinical cases of chronic bovine mastitis in planktonic and biofilm bacteria. Similarly, Felipe et al. [33] reported that chitosan at 100 and 1,600 μg/mL inhibited and degraded the formation of *S. aureus* biofilms, respectively. Breser et al. [34] found that a combination of chitosan and cloxacillin-Na against *Staphylococcus* coagulase-negative, causative of chronic bovine mastitis, inhibited biofilm formation more efficiently than each independently. The effects of enro-C/DMSO/chitosan on biofilm formation and the degradation of mature biofilm were achieved with the lowest chitosan concentration. i.e., 10 to 100 times lower than the reported concentrations [34]. Hence, the components of the enro-C/DMSO/chitosan may act synergistically against the biofilms tested.

The clinical evaluation of enro-C/DMSO/chitosan showed that the bacteriological cure rate was better than those obtained with the Enromastic enro-C alone preparation. On the other hand, more studies with a larger number of cows are needed to assess the scope of this therapy. The American Academy of Bovine Practitioners recommends that cows suffering from chronic mastitis be culled [9]. In contrast, most dairies in Latin America lack the diagnostic resources nor the will to follow this recommendation. Hence, conducting clinical studies to find and propose new treatment options with better efficacies is relevant. With the enro-C/DMSO/chitosan prototype tested, a 100% clinical cure and 80% bacteriological cure were achieved, and the inclusion criteria of the clinical cases allowed a well-defined set of chronic cases of bovine mastitis caused by biofilm-forming bacteria. Nevertheless, it is essential to scale up the results to define how cattle from different ecological niches respond to this therapy. Moreover, hygienic and biosecurity measures must be improved on each farm. Enro-C, as an intramammary preparation, was reported to treat mastitis successfully [11-13]. On the other hand, their case-inclusion criteria and treatment protocols make comparisons difficult. For example, the absence of recidivism of the mammary glands treated tested 21 days after cure, as carried out by Martínez-Cortés et al. [11], was a requisite that could not be implemented in this study due to management issues imposed in the farm. There are areas of opportunity to improve this preparation, i.e., formulating nano particles of the active principles [35] and establishing sequential treatment schemes, if necessary, as proposed by Alfonseca-Silva et al. [13] using ceftiofur. In addition, adding some non-antibiotic elements may improve efficacy, i.e., vitamin D3 [36-38].

In conclusion, biofilm-producing *Staphylococcus* species are associated with chronic mastitis in cattle. The enro-C/DMSO/chitosan intramammary preparation inhibits and degrades the biofilm and eliminates both *S. aureus* contained within the exopolysaccharide matrix and planktonic bacteria. Therefore, the preparation presented in this paper is an acceptable option for treating this type of mastitis.

REFERENCES

1. Kalińska A, Wójcik A, Slósarz J, Kruzińska B, Michalczuk M, Jaworski S, et al. Occurrence and aetiology of staphylococcal mastitis – a review. Anim Sci Pap Rep. 2018;36(3):263-273.
2. Saeed SI, Vivian L, Zalati CW, Sani NI, Aklilu E, Mohamad M, et al. Antimicrobial activities of graphene oxide against biofilm and intracellular *Staphylococcus aureus* isolated from bovine mastitis. BMC Vet Res. 2023;19(1):10. PUBMED | CROSSREF
3. Nagahata H, Kawai H, Higuchi H, Kawai K, Yayou K, Chang CJ. Altered leukocyte responsiveness in dairy cows with naturally occurring chronic *Staphylococcus aureus* mastitis. J Vet Med Sci. 2011;73(7):885-894. PUBMED | CROSSREF

4. Kampa J, Sukolapong V, Chaiyotwittakun A, Rerk-u-suke S, Polpakdee A. Chronic mastitis in small dairy cattle herds in Muang Khon Kaen. Thai J Vet Med. 2010;40(3):265-272.
5. Piessens V, De Vliegher S, Verbist B, Braem G, Van Nuffel A, De Vuyst L, et al. Characterization of coagulase-negative staphylococcus species from cows' milk and environment based on *bap*, *icaA*, and *mecA* genes and phenotypic susceptibility to antimicrobials and teat dips. J Dairy Sci. 2012;95(12):7027-7038. PUBMED | CROSSREF
6. Cardozo LL, Thaler Neto A, Souza GN, Picinin LC, Felipus NC, Reche NL, et al. Risk factors for the occurrence of new and chronic cases of subclinical mastitis in dairy herds in southern Brazil. J Dairy Sci. 2015;98(11):7675-7685. PUBMED | CROSSREF
7. Pasachova GJ, Ramírez MS, Muñoz LM. *Staphylococcus aureus*: generalidades, mecanismos de patogenicidad y colonización celular. NOVA. 2019;17(32):25-38. CROSSREF
8. Bohl LP, Isaac P, Breser ML, Orellano MS, Correa SG, Tolosa de Talamoni NG, et al. Interaction between bovine mammary epithelial cells and planktonic or biofilm *Staphylococcus aureus*: the bacterial lifestyle determines its internalization ability and the pathogen recognition. Microb Pathog. 2021;152:104604. PUBMED | CROSSREF
9. Royster E, Wagner S. Treatment of mastitis in cattle. Vet Clin North Am Food Anim Pract. 2015;31(1):17-46. PUBMED | CROSSREF
10. Miranda-Calderón JE, Gutiérrez L, Flores-Alamo M, García-Gutiérrez P, Sumano H. Enrofloxacin hydrochloride dihydrate. Acta Crystallogr Sect E Struct Rep Online. 2014;70(Pt 4):o468-o469. PUBMED | CROSSREF
11. Martínez-Cortés I, Gutierrez L, Tapia G, Ocampo L, Sumano H. Serum and milk concentrations of enrofloxacin in cows intramammarily treated with a new enrofloxacin-polymorph. Med Weter. 2016;72(11):686-692. CROSSREF
12. Viveros M, Lopez-Ordaz R, Gutiérrez L, Miranda-Calderón JE, Sumano H. Efficacy assessment of an intramammary treatment with a new recrystallized enrofloxacin vs ceftiofur and parenteral enrofloxacin in dairy cows with nonsevere clinical mastitis. J Vet Pharmacol Ther. 2018;41(1):e1-e9. PUBMED | CROSSREF
13. Alfonseca-Silva E, Cruz-Villa JC, Gutiérrez L, Sumano H. Successful treatment of recurrent subclinical mastitis in cows caused by enrofloxacin resistant bacteria by means of the sequential intramammary infusion of enrofloxacin HCl-2H_2O and ceftiofur HCl: a clinical trial. J Vet Sci. 2021;22(6):e78. PUBMED | CROSSREF
14. Silva-Dias A, Palmeira-de-Oliveira A, Miranda IM, Branco J, Cobrado L, Monteiro-Soares M, et al. Anti-biofilm activity of low-molecular weight chitosan hydrogel against *Candida* species. Med Microbiol Immunol. 2014;203(1):25-33. PUBMED | CROSSREF
15. Hebling J, Bianchi L, Basso FG, Scheffel DL, Soares DG, Carrilho MR, et al. Cytotoxicity of dimethyl sulfoxide (DMSO) in direct contact with odontoblast-like cells. Dent Mater. 2015;31(4):399-405. PUBMED | CROSSREF
16. Yahya MF, Alias Z, Karsani SA. Antibiofilm activity and mode of action of DMSO alone and its combination with afatinib against Gram-negative pathogens. Folia Microbiol (Praha). 2018;63(1):23-30. PUBMED | CROSSREF
17. Deutch CE. Inhibition of urease activity in the urinary tract pathogens *Staphylococcus saprophyticus* and *Proteus mirabilis* by dimethylsulfoxide (DMSO). J Appl Microbiol. 2020;128(5):1514-1523. PUBMED | CROSSREF
18. Swinkels JM, Krömker V, Lam TJ. Efficacy of standard vs. extended intramammary cefquinome treatment of clinical mastitis in cows with persistent high somatic cell counts. J Dairy Res. 2014;81(4):424-433. PUBMED | CROSSREF
19. McDougall S, Clausen L, Hintukainen J, Hunnam J. Randomized, controlled, superiority study of extended duration of therapy with an intramammary antibiotic for treatment of clinical mastitis. J Dairy Sci. 2019;102(5):4376-4386. PUBMED | CROSSREF
20. Aguilar RF, Anguiano BR, Atilano LD, Bazán ME, Castañeda RA, Castañón OL, et al. *Atlas Fotográfico de Laboratorio de Bacteriología y Micología Veterinaria*. 1st ed. Mexico City: Faculty of Veterinary Medicine, National Autonomous University of Mexico; 2014.
21. Mariana NS, Atshan S, Neela V, Sekawi Z. Evaluation of modified Congo red agar for detection of biofilm produced by clinical isolates of methicillin-resistance *Staphylococcus aureus*. Afr J Microbiol Res. 2009;3(6):330-338.
22. Peña J, Uffo O. Producción de biofilme en genotipos de *Staphylococcus aureus* aislados de mastitis bovina en Cuba. Rev Salud Anim. 2013;35(3):189-196.
23. Pampariene I, Veikutis V, Oberauskas V, Zymantiene J, Zelvyte R, Stankevicius A, et al. Thermography based inflammation monitoring of udder state in dairy cows: sensitivity and diagnostic priorities comparing with routine California mastitis test. J Vibroeng. 2016;18(1):511-521.
24. Lin JJ, Chang CH, Pal N. A revisit to contingency table and tests of independence: bootstrap is preferred to Chi-square approximations as well as Fisher's exact test. J Biopharm Stat. 2015;25(3):438-458. PUBMED | CROSSREF

25. Faul F, Erdfelder E, Lang AG, Buchner A. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods. 2007;39(2):175-191. PUBMED | CROSSREF
26. Sumano LH, Ocampo CL, Gutiérrez OL. *Farmacología Veterinaria*. 4th ed. Mexico City: Aranda; 2015.
27. Khan F, Pham DT, Oloketuyi SF, Manivasagan P, Oh J, Kim YM. Chitosan and their derivatives: antibiofilm drugs against pathogenic bacteria. Colloids Surf B Biointerfaces. 2020;185:110627. PUBMED | CROSSREF
28. Confederat LG, Tuchilus CG, Dragan M, Sha'at M, Dragostin OM. Preparation and antimicrobial activity of chitosan and its derivatives: a concise review. Molecules. 2021;26(12):3694. PUBMED | CROSSREF
29. Hoang C, Nguyen AK, Nguyen TQ, Fang W, Han B, Hoang BX, et al. Application of dimethyl sulfoxide as a therapeutic agent and drug vehicle for eye diseases. J Ocul Pharmacol Ther. 2021;37(8):441-451. PUBMED | CROSSREF
30. Lee JS, Bae YM, Han A, Lee SY. Development of Congo red broth method for the detection of biofilm-forming or slime-producing *Staphylococcus* sp. Lebensm Wiss Technol. 2016;73:707-714. CROSSREF
31. Idrees M, Sawant S, Karodia N, Rahman A. *Staphylococcus aureus* biofilm: morphology, genetics, pathogenesis and treatment strategies. Int J Environ Res Public Health. 2021;18(14):7602. PUBMED | CROSSREF
32. Yang B, Lei Z, Zhao Y, Ahmed S, Wang C, Zhang S, et al. Combination susceptibility testing of common antimicrobials *in vitro* and the effects of sub-MIC of antimicrobials on *Staphylococcus aureus* biofilm formation. Front Microbiol. 2017;8:2125. PUBMED | CROSSREF
33. Felipe V, Breser ML, Bohl LP, Rodrigues da Silva E, Morgante CA, Correa SG, et al. Chitosan disrupts biofilm formation and promotes biofilm eradication in *Staphylococcus* species isolated from bovine mastitis. Int J Biol Macromol. 2019;126:60-67. PUBMED | CROSSREF
34. Breser ML, Felipe V, Bohl LP, Orellano MS, Isaac P, Conesa A, et al. Chitosan and cloxacillin combination improve antibiotic efficacy against different lifestyle of coagulase-negative *Staphylococcus* isolates from chronic bovine mastitis. Sci Rep. 2018;8(1):5081. PUBMED | CROSSREF
35. Verderosa AD, Totsika M, Fairfull-Smith KE. Bacterial biofilm eradication agents: a current review. Front Chem. 2019;7:824. PUBMED | CROSSREF
36. Téllez-Pérez AD, Alva-Murillo N, Ochoa-Zarzosa A, López-Meza JE. Cholecalciferol (vitamin D) differentially regulates antimicrobial peptide expression in bovine mammary epithelial cells: implications during *Staphylococcus aureus* internalization. Vet Microbiol. 2012;160(1-2):91-98. PUBMED | CROSSREF
37. Yue Y, Hymøller L, Jensen SK, Lauridsen C, Purup S. Effects of vitamin D and its metabolites on cell viability and *Staphylococcus aureus* invasion into bovine mammary epithelial cells. Vet Microbiol. 2017;203:245-251. PUBMED | CROSSREF
38. Merriman KE, Powell JL, Santos JE, Nelson CD. Intramammary 25-hydroxyvitamin D_3 treatment modulates innate immune responses to endotoxin-induced mastitis. J Dairy Sci. 2018;101(8):7593-7607. PUBMED | CROSSREF