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Article

Nisin and Chitosan Enhance the Antimicrobial Activity of Ceftiofur Against Antibiotic-Resistant *Staphylococcus aureus* and Have Anti-Biofilm Effect

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Abstract

Mastitis is one of the major diseases affecting dairy cattle worldwide. Antibiotic therapy remains the most widely used treatment. However, its effectiveness has been compromised due to the selection of antibiotic-resistant and biofilm-producing pathogenic bacteria. This promotes the search for alternatives that increase the antibacterial and anti-biofilm efficacy of antibiotics such ceftiofur (CFT). Biocompounds that, combined with antibiotics, exhibit these characteristics are gaining attention. Nisin (N) and chitosan (CH) may possess these properties. The aim of this study was to evaluate whether N+CFT and CH+CFT enhance the antibacterial activity of the antibiotic on *Staphylococcus aureus* associated with bovine mastitis, as well as its anti-biofilm effect. Two clinical isolates of *S. aureus* (AMC-43 and AMC-48) and the reference strain ATCC 27543 resistant to CFT were used. The combination of sub-inhibitory concentrations of N (320 µg/mL) and CH (400 µg/mL) with CFT (1, 2, 4, and 8 µg/mL) significantly reduced bacterial growth (expressed in logarithmic units); however, the CH+CFT mixtures were the most efficient. Anti-biofilm activity was also detected, both in biofilm synthesis and in the reduction of living bacterial cells located inside this preformed structure. These results highlight N and CH as potential agents for the prevention or control of bovine mastitis.

Keywords: *Staphylococcus aureus*; antibiotic resistance; ceftiofur; nisin; chitosan; antibiotic enhancers

1. Introduction

Mastitis or the inflammation of the mammary gland is the most costly and prevalent disease in dairy herds. Globally, millions of dollars are lost annually by the disease and markedly impact dairy farming. The multifactorial etiology of the disease complicates its treatment. However, Gram-positive and Gram-negative microorganisms are prominent as causal agents [1]. Among these, the most important species capable of causing inflammation to belong to a few groups, which are categorized as contagious and environmental pathogens according to their reservoir and transmission mechanisms [2].

Staphylococcus aureus is the most frequently isolated contagious pathogen from milk samples of cows with clinical and subclinical mastitis [3]. This pathogen causes the most virulent forms of the disease and poses one of the greatest challenges to milk production worldwide [4]. Antibiotic therapy is the primary strategy that is used to control bacterial infections; especially β-lactam antibiotics such as ceftiofur and oxacillin [5]. However, this therapy fails due to extended and inappropriate antibiotic use. This increases bacterial selection pressure, which led to the emergence of resistant strains, along

with the rapid dissemination of resistance determinants among these and other pathogenic microorganisms. Added to this is its natural resistance to antibiotics [6].

Resistance of *S. aureus* strains to penicillin and its variants implicated in mastitis (β -lactams) is perhaps the most recognized form of resistance, ranging 30-90% with variations from one region to another where dairy herds exist [7]. *S. aureus* strains such as methicillin-resistant *S. aureus* (MRSA) that are resistant to multiple antibiotics, are common [6]. During bovine mastitis development, some *S. aureus* strains utilize virulence factors, such as biofilm synthesis, to establish themselves in the host [8]. Furthermore, biofilms might provide protection against antimicrobial strategies owing to the low diffusion capacity of antibiotics through biofilm components [9]. Both bacterial strategies, resistance to antibiotics and biofilm synthesis, allow the development of recurrent intramammary infections. Lack of new antibiotics from the pharmaceutical industry constitutes a major challenge for the veterinary sector and global public health [5].

These findings underscore the need to integrate innovative strategies to enhance the efficacy of traditional antibiotics, inhibit the biofilm synthesis, contribute to the sustainable management of antibiotics, and potentially assist in controlling intramammary infections. Biocompounds that, combined with antibiotics at sub-inhibitory concentrations, improve the antibiotic's efficacy against resistant bacteria, possess antibiofilm properties, and are safe for human and animal health are receiving increasing attention [10,11].

Nisin is a cationic peptide of 34 amino acids produced by *Lactococcus lactis* subsp. *lactis*, with antibacterial activity, particularly against Gram-positive bacteria. Recognized as a safe additive, this peptide has been proposed as a potential therapeutic agent for control of bacterial infections of the mammary gland [12]. Chitosan is a cationic polymer of natural origin with biological activity against Gram-positive and Gram-negative pathogens [13]. This biodegradable, and nontoxic biocompound has been proposed for various biomedical therapies [14]. Both biocompounds have exhibited antibacterial activity against bovine mastitis-associated *S. aureus* isolates [7].

However, their ability to enhance the antibacterial effect of conventional antibiotics, like ceftiofur, and their effects on biofilm synthesis and the bacterial viability inside the biofilm remain unknown. Therefore, the aim of this study was to assess the enhancer effect of the combination of nisin and chitosan with ceftiofur on its antibiotic activity on antibiotic-resistant *Staphylococcus aureus*, as well as the effect of the combinations on biofilm synthesis and bacterial viability within this preformed structure.

2. Materials and Methods

2.1. Bacterial Isolates and Growth Conditions

Two isolates of *S. aureus* bovine-mastitis associated, including AMC-43 and AMC-48 were used. These clinical isolates were obtained from milk samples from cows diagnosed with clinical and subclinical mastitis (California mastitis test) from 34 dairy herds in the Ciénega de Chapala region, Michoacán, Mexico. The isolates were characterized microscopically (morphology), biochemically (Gram staining, catalase, coagulase, tellurite reduction, and lipolytic activity tests in Petri dishes with Baird Parker agar and growth on salt and mannitol agar), and molecularly (amplification of a fragment of the *nuc* gene, which encodes the thermostable nuclease of *S. aureus*) [7]. The certified *S. aureus* subsp. *aureus* ATCC (27543) strain, which was isolated from a clinical case of bovine mastitis, was used as positive control. Bacteria were cultured in nutrient agar (BD Bioxon®, Mexico) at 37 °C for 24 h and maintained at 4 °C until further use. Due to the nature of the research and the way the milk samples were obtained, approval from an ethics committee was not required.

2.2. Nisin and Chitosan Antibacterial Activity and Ceftiofur Resistance Assays

The antimicrobial peptide nisin (N) (Nisina Raff®, Mexico), polymer chitosan (CH) (Chitosan $\geq$ 90% deacetylation grade, Future Foods®, Mexico), and β -lactam antibiotic ceftiofur (CFT) (Sigma Aldrich, Vetranal®, USA), were prepared following the manufacturer's instructions. Minimum

inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of N, CH, and CFT were determined using the microdilution method in Mueller–Hinton broth (MHB) (BD Bioxon®, Mexico) in 96-well microplates (Celltreat®, China), following the Clinical Laboratory Standards Institute (CLSI) [15] guidelines and methodology of Ster et al. [16] with some modifications. Overnight bacterial cultures in MHB at 37 °C were adjusted to ~0.2 optical density (OD) at 600 nm (Thermo Fisher Scientific® spectrophotometer, USA), equivalent to approximately 4.8×10^6 CFU/mL (6.68 log). These cultures were incubated for 24 h at 37 °C in 96-well microplates in different concentrations of N, CH, and CFT with a final volume of 150 µL per well. Initial concentrations of 5120; 6400; and 2048 µg/mL were used for N, CH, and CFT, respectively, and then were diluted. Resistance to CFT was confirmed by inoculating bacteria on Mueller–Hinton agar (MHA) plates containing the indicated antibiotic concentrations.

MIC was defined as the concentration of antibiotic or biocompounds that prevented bacterial growth. After, the content of each well equal to or greater than the MIC was streaked on MHA plates and incubated at 37 °C to analyze bacterial growth 24 h after plating. The lowest concentration without visible bacterial colony growth was considered MBC. *S. aureus* was considered resistant to CFT when it grew at a concentration $\geq$ of 8 µg/mL after 24 h of incubation [5,16,17]. MHB–*S. aureus* was used as the negative control growth, while MHB+*S. aureus* as the positive control growth. For CH, a second growth control was used: MHB+*S. aureus*+1% acetic acid (pH 5.6). The treatments evaluated were MHB+*S. aureus*+N; MHB+*S. aureus*+CH; and MHB+*S. aureus*+CFT.

2.3. Inhibition Assays of *S. aureus* by N, CH, CFT, and N+CFT and CH+CFT Combinations

The antibacterial effect of N+CFT and CH+CFT combinations on antibiotic-resistant *S. aureus* was investigated using the microdilution method. Overnight bacterial cultures in MHB at 37 °C were adjusted to an OD of ~0.2 and incubated for 24 h at 37 °C in the presence of sub-inhibitory concentrations (sub-MICs) of N (320 µg/mL) and CH (400 µg/mL), combined with different concentrations of CFT (1, 2, 4, and 8 µg/mL). For example: (320+1) means that the combination contained 320 µg/mL of N and 1 µg/mL of CFT, and so on. It also applied to the mixture of CH with CFT (400+1). The positive and negative controls for bacterial growth were MHB+*S. aureus* and MHB–*S. aureus*, respectively. The following treatments were evaluated: MHB+*S. aureus*+N; MHB+*S. aureus*+CH; MHB+*S. aureus*+N+CFT; MHB+*S. aureus*+CH+CFT. The reduction in bacterial growth was verified by measuring the OD of each treatment and quantifying the CFU/mL, which were expressed in logarithmic units, after 24 h of incubation in MHA plates. In addition, the logarithmic scale reduction factor (Log_{10}) was calculated using the formula $\text{RF} = \text{Log}_{10}(\text{A}) - \text{Log}_{10}(\text{B})$, where A is the number of colonies recovered from the unexposed (control) and B is the number of colonies recovered from the exposed (test) to treatments. Using the average values of these counts, the microbial reduction percentages were calculated according to the following formula: Microbial reduction percentage (%) = $(\text{control CFU} - \text{test CFU}) / \text{control CFU} \times 100$ [18].

2.4. Biofilm Production Assays of *S. aureus* and Inhibition by N, CH, CFT, and N+CFT and CH+CFT Combinations

To assess the inhibitory capacity of N, CH, CFT, and their corresponding combinations on the synthesis of *S. aureus* biofilms, the ability of the bacteria to produce this virulence factor was determined, followed by inhibition analysis. Biofilm synthesis was determined using the crystal violet staining methodology reported by Guzmán-Rodríguez et al. [19], with some modifications. Assays were performed in 96-well plates, where 150 µL of each bacterial culture grown in MHB was adjusted to an OD of ~0.2 (600 nm) was placed. The plates were incubated for 24 h at 37 °C. Next, the wells were washed with phosphate-buffered saline (PBS), 150 µL of 0.5% crystal violet (Golden Bell®, Mexico) was added, and plates were maintained for 15 min at room temperature (25 ± 2.0 °C). Subsequently, the dye was discarded, and the wells were washed twice with PBS. Finally, 150 µL of 33% acetic acid (Merk®, Mexico) was added, the plate was thoroughly mixed for homogenization, and the absorbance was read at 595 nm using a microplate spectrophotometer. The classification by

Stepanović et al. [20] was used to categorize bacteria as non-biofilm producers (0), weak biofilm producers (+ or 1), moderate biofilm producers (++ or 2), and strong biofilm producers (+++ or 3) based on the OD values of the samples analyzed according to the following calculations: $OD \leq OD_{control}$ = non-biofilm producer; $OD_{control} < OD \leq 2 \times OD_{control}$ = weak biofilm producer; $2 \times OD_{control} < OD \leq 4 \times OD_{control}$ = moderate biofilm producer; $4 \times OD_{control} < OD$ = strong biofilm producer; where $OD_{control}$ is the OD of the broth without bacteria.

Then, the antibiofilm effects of the biocompounds, antibiotic, and their combinations were determined using the same methodology of Guzmán-Rodríguez et al. [19], except that, in this case, bacterial cultures grown in MHB and adjusted to an OD of ~0.2 were added to the 96-well plates, along with sub-MIC concentrations of N (320 µg/mL), CH (400 µg/mL), and the breakpoint concentration of CFT (8 µg/mL), as well as the corresponding N+CFT, and CH+CFT combinations. The plates were incubated for 24 h at 37 °C. The positive and negative controls for biofilm formation were MHB+*S. aureus* and MHB-*S. aureus*, respectively. The treatments included MHB+*S. aureus*+N; MHB+*S. aureus*+CH; MHB+*S. aureus*+N+CFT, and MHB+*S. aureus*+CH+CFT. The antibiofilm effects of the controls and treatments were expressed as inhibition percentages, with 100% representing the biofilm production of each isolate. Likewise, the effect of the combinations on this virulence factor allowed the bacteria to be recategorized according to Stepanović et al. [20].

2.5. Antibacterial Activity on *S. aureus* Cells Living Within Preformed Biofilms

These experiments were only performed on the clinical isolate of *S. aureus* that was classified as the strongest biofilm producer (AMC-48). Bacterial cultures grown in MHB and adjusted to an OD of ~0.2 were added to the 96-well plates. Plates were statically incubated for 24 h at 37 °C to allow cell adhesion and biofilm synthesis. After the incubation period, supernatants were removed and 150 µL of sterile PBS were added (twice) to remove non-adherent bacteria. The preformed biofilms were treated with N, CH, CFT, and their corresponding combinations for 24 h at 37 °C. Once again, the supernatant was removed, and the biofilm was washed with 150 µL of sterile PBS (twice). After these washes, 150 µL of 0.1% Triton X-100 (Hycel, Mexico) was added to break down the extracellular matrix and disperse the biofilm produced. The microplate was sealed with parafilm and placed in a sonicator (Branson Ultrasonics CPX-952-238R®, USA) to 42 kHz by 5 min. Dilutions were then made and plated on MHA, incubated for 24 h at 37 °C, and the bacterial count was performed, which was expressed as logarithmic units [21].

2.6. Statistical Analysis

A completely randomized experimental design was used, comprising three independent experiments (biological replicas) with three repeats per treatment. Data were analyzed using analysis of variance, with a significance level of ($p \leq 0.05$), followed by Tukey's test ($p \leq 0.05$). The percentages of biofilm synthesis inhibition were transformed using square root transformation ($\sqrt{x + 0.5}$). Statistical analyses were performed using IBM SPSS Statistics version 25.

3. Results

3.1. Resistance and Susceptibility of *S. aureus* to CFT, N and CH

The three bacterial members of *S. aureus* exhibited resistance to CFT, since ATCC 27543, AMC-43, and AMC-48 grew in the presence of ≥ 8 µg/mL of the antibiotic after 24 h of incubation (Table 1). CFT MICs ranged between 256 at 2048 µg/mL, while MBCs ranged from 512 at 2048 µg/mL. On the other hand, N exhibited antibacterial effect only against the ATCC 27543 strain, with a MIC of 640 µg/mL; however, the MBC at the highest peptide concentration used (5120 µg/mL) could not be determined. MICs and MBCs could not be determined for AMC-43 and AMC-48 either. In contrast, CH showed antibacterial effects against all evaluated bacteria, whose MICs and MBCs were 6400 µg/mL (Table 1).

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal (MBC) of ceftiofur (CFT), nisin (N), and chitosan (CH) against bovine mastitis-associated *S. aureus*.

	CFT (µg/mL)		N (µg/mL)		CH (µg/mL)	
	MIC	MBC	MIC	MBC	MIC	MBC
ATCC 27543	256	512	640	ND ^a	6400	6400
AMC-43	2048	2048	ND ^a	ND ^a	6400	6400
AMC-48	512	2048	ND ^a	ND ^a	6400	6400

^a ND: Not determined at the highest concentration evaluated in this study (5120 µg/mL). Values represent the means of three replicates and three independent experiments.

3.2. Increasing the Efficacy of CFT Against *S. aureus* by the Addition of N and CH

The use of biocompounds as enhancers of the antibacterial effect of antibiotics is based on the use of sub-MICs of the former. To test the hypothesis that sub-MICs concentrations of N and CH increase the efficacy of CFT, the next concentrations were selected because of the antibacterial effect on *S. aureus* was minimal or not significant: N (320 µg/mL) and CH (400 µg/mL), which were combined with CFT (1, 2, 4, and 8 µg/mL), and their effects on bacterial growth, biofilm synthesis and bacterial viability within biofilms were evaluated. Regarding N and CFT mix, the results showed that all compounds' combinations reduced bacterial growth (expressed as logarithmic reduction scale, LRS) of ATCC 27543, AMC-43, and AMC-48 compared to controls and individual treatments with N and CFT added individually ($p \leq 0.05$) (Table 2). As can be seen, in strain ATCC 27543, the combinations of N+CFT showed logarithmic reductions in a range from 2.24 to 2.76 units. In the case of the AMC-43 clinical isolate, the reduction in bacterial growth was more pronounced, with decreases within the range of 4.35 to 4.86 units. A similar result was observed with the isolate AMC-48, whose reductions in bacterial growth were also substantial, with a range of 3.66 at 4.22 units (Table 2). These results indicate that N addition improved the efficacy of CFT by reducing the bacterial growth of the three pathogenic microorganisms in an inhibitory range of 30.9-66.7%, with a clear tendency towards bacterial reduction in all combinations.

Table 2. Logarithmic growth reduction of *S. aureus* by nisin (N), ceftiofur (CFT), and N+CFT combination after 24 h of incubation.

ATCC 27543							
Time (h) /tmts ¹	Control ²	N 320	CFT 8	N+CFT 320+1	N+CFT 320+2	N+CFT 320+4	N+CFT 320+8
0	6.63±0.08	6.63±0.08	6.63±0.08	6.63±0.08	6.63±0.08	6.63±0.08	6.63±0.08
24	7.26±0.05 ^a	7.34±0.02 ^a	6.31±0.25 ^b	5.02±0.82 ^c	4.50±0.45 ^c	4.55±0.36 ^c	4.65±0.53 ^c
LSR ⁵	--	--	0.95	2.24	2.76	2.71	2.61
AMC-43							
Time (h) /tmts ¹	Control ³	N 320	CFT 8	N+CFT 320+1	N+CFT 320+2	N+CFT 320+4	N+CFT 320+8
0	6.56±0.27	6.56±0.27	6.56±0.27	6.56±0.27	6.56±0.27	6.56±0.27	6.56±0.27
24	7.29±0.26 ^a	6.53±0.09 ^{ab}	5.08±0.29 ^b	2.94±0.55 ^c	2.43±0.29 ^c	2.72±0.57 ^c	2.54±0.38 ^c
LSR ⁵	--	0.76	2.21	4.35	4.86	4.57	4.75
AMC-48							
Time (h) /tmts ¹	Control ⁴	N 320	CFT 8	N+CFT 320+1	N+CFT 320+2	N+CFT 320+4	N+CFT 320+8
0	6.81±0.02	6.81±0.02	6.81±0.02	6.81±0.02	6.81±0.02	6.81±0.02	6.81±0.02
24	7.25±0.09 ^a	7.19±0.05 ^a	6.13±0.23 ^b	3.03±0.76 ^c	3.59±1.05 ^c	3.19±0.86 ^c	3.38±0.97 ^c
LSR ⁵	--	0.06	1.12	4.22	3.66	4.06	3.87

Different letters indicate significant differences according to Tukey's test ($p \geq 0.05$). The data are presented as Log₁₀ CFU/mL ± SD. ¹ Treatments: µg/mL. ² Control: *S. aureus* ATCC 27543 without treatment. ³ Control: *S. aureus* AMC-43 without treatment. ⁴ Control: *S. aureus* AMC-48 without treatment. ⁵ LRS: logarithmic reduction scale.

In addition, mixtures of CH and CFT showed that all combinations also reduced bacterial growth of ATCC, AMC-43 and AMC-48 when compared to controls and individual CH and CFT treatments ($p \leq 0.05$) (Table 3). As can be seen, in strain ATCC 27543, the N+CFT combinations showed logarithmic reductions in bacterial growth of 7.21 units (0 CFUs recovered) in all combinations, showing substantial differences with control, CH and CFT. An identical result was observed with the clinical isolate AMC-43, since bacterial growth was also reduced to 7.39 units (0 CFUs recovered) in all combinations evaluated. Finally, although the inhibition of the AMC-48 isolate was less pronounced, this decrease was considerable, with a range of 4.96 to 5.67 units (Table 3). These results indicate that CH addition improved the efficacy of CFT by reducing the bacterial growth of the three bacterial pathogens in an inhibitory range of 66.7 to 100%, again, showing a clear trend towards bacterial reduction in all combinations. Importantly, the vehicle did not inhibit bacterial growth of any of the three pathogens, as shown in Table 3.

Table 3. Logarithmic growth reduction of *S. aureus* by chitosan (CH), ceftiofur (CFT), and CH+CFT combination after 24 h of incubation.

ATCC 27543								
Time (h) /tmts ¹	Control ²	Vehicle ⁵	CH 400	CFT 8	CH+CFT 400+1	CH+CFT 400+2	CH+CFT 400+4	CH+CFT 400+8
0	6.68±0.30	6.68±0.30	6.68±0.30	6.68±0.30	6.68±0.30	6.68±0.30	6.68±0.30	6.68±0.30
24	7.21±0.06 ^a	7.24±0.07 ^a	6.56±0.02 ^b	6.32±0.26 ^c	0±0 ^d	0±0 ^d	0±0 ^d	0±0 ^d
LSR ⁶	--	--	0.65	0.89	7.21	7.21	7.21	7.21
AMC-43								
Time (h) /tmts ¹	Control ³	Vehicle ⁵	CH 400	CFT 8	CH+CFT 400+1	CH+CFT 400+2	CH+CFT 400+4	CH+CFT 400+8
0	6.88±0.07	6.88±0.07	6.88±0.07	6.88±0.07	6.88±0.07	6.88±0.07	6.88±0.07	6.88±0.07
24	7.39±0.16 ^a	7.41±0.16 ^a	6.52±0.34 ^b	5.08±0.29 ^c	0±0 ^d	0±0 ^d	0±0 ^d	0±0 ^d
LSR ⁶	--	--	0.87	2.31	7.39	7.39	7.39	7.39
AMC-48								
Time (h) /tmts ¹	Control ⁴	Vehicle ⁵	CH 400	CFT 8	CH+CFT 400+1	CH+CFT 400+2	CH+CFT 400+4	CH+CFT 400+8
0	6.80±0.17	6.80±0.17	6.80±0.17	6.80±0.17	6.80±0.17	6.80±0.17	6.80±0.17	6.80±0.17
24	7.44±0.15 ^a	7.45±0.15 ^a	6.63±0.32 ^a	6.14±0.18 ^a	2.17±0.81 ^b	2.48±0.78 ^b	1.77±0.63 ^b	1.96±0.59 ^b
LSR ⁶	--	--	0.81	1.3	5.27	4.96	5.67	5.48

Different letters indicate significant differences according to Tukey’s test ($p \geq 0.05$). ¹Treatments: µg/mL.
²Control: *S. aureus* ATCC 27543 without treatment. ³Control: *S. aureus* AMC-43 without treatment. ⁴Control: *S. aureus* AMC-48 without treatment. ⁵Vehicle: *S. aureus* ATCC, AMC-43, and AMC-48 containing acetic acid 1%, pH 5.6. ⁶LRS: Logarithmic reduction scale.

3.3. Inhibition of *S. aureus* Biofilm Synthesis After Adding N, CH, N+CFT, and CH+CFT

According to the classification of Stepanović et al. [20], the strain ATCC 27543 and isolate AMC-43 were classified as moderate biofilm producers (++), whereas isolate AMC-48 was considered a strong biofilm producer (+++) (Table 4). Regarding the effect of N, CFT, and N+CFT combinations on biofilm synthesis, all treatments inhibited the synthesis of this virulence factor in the three pathogenic bacteria evaluated, compared to the untreated controls ($p \leq 0.05$) (Figure 1A–C). The inhibition percentage ranged from 67.8 to 95.9% among the three bacteria. However, no differences were observed in the inhibition of most combinations of N+CFT and individual treatments of N and CFT. These results show that it was not possible to detect an enhancing effect of N on CFT inhibiting biofilm synthesis. Nevertheless, one noteworthy aspect is that all treatments inhibited the synthesis of biofilm, which was additionally reflected in the recategorization of the bacteria as follows: *S. aureus* ATCC 27543 was reclassified from moderate to weak producer; AMC-43 from moderate to non-producer; and AMC-48 from strong to weak producer (Table 4).

Table 4. Biofilm production by bovine mastitis-associated *S. aureus* and change in bacterial classification due to the anti-biofilm effect of N+CFT and CH+CFT combinations.

<i>S. aureus</i>	Biofilm production ^a	Classification ^b	Classification after N+CFT exposition	Classification after CH+CFT exposition
ATCC 27543	0.29 ± 0.05	++	+	+
AMC-43	0.25 ± 0.04	++	(0)	+
AMC-48	1.28 ± 0.09	+++	+	++

^aBiofilm quantification was determined by OD (595 nm): the average of three replicates obtained from three independent experiments ± standard deviation. ^b(0): no biofilm producer; (+): weak biofilm producer; (++) moderate biofilm producer; (+++): strong biofilm producer Stepanović et al. [20].

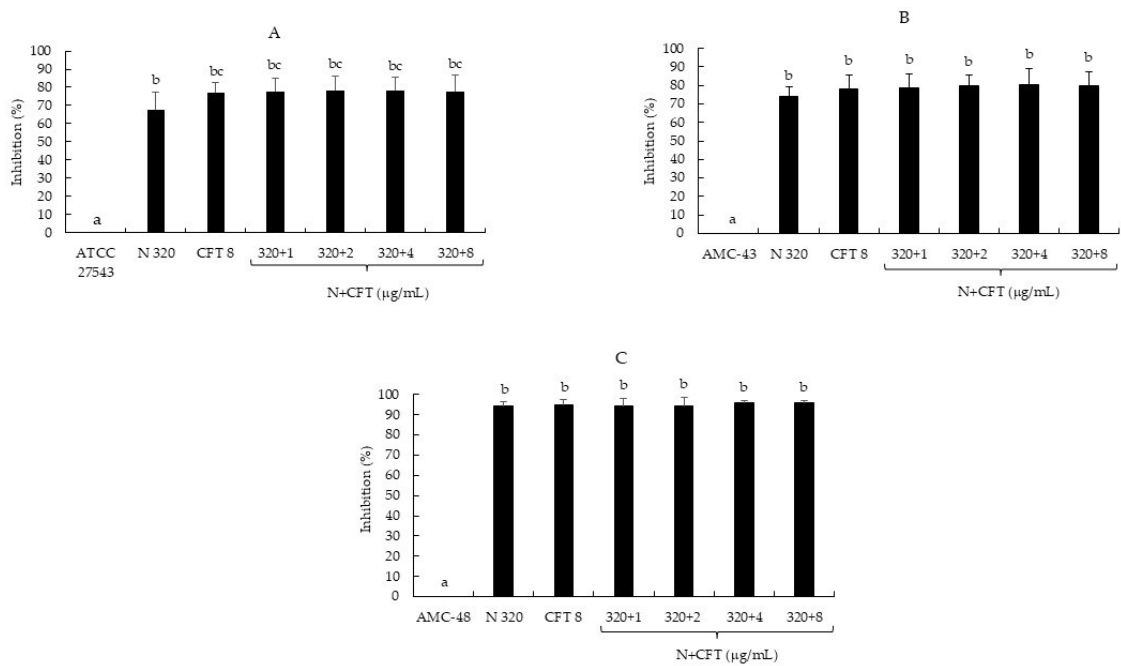


Figure 1. Inhibition percentage of *S. aureus* biofilm production by nisin (N), ceftiofur (CFT), and N+CFT after 48 h of incubation. (A) *S. aureus* ATCC 27543. (B) *S. aureus* AMC-43. (C) *S. aureus* AMC-48. The negative inhibition control was plotted as the optical density (OD) of each bacterium. Evaluation of different combinations of N+CFT are shown. Graph represents the mean ± standard deviation. Different letters within each column indicate significant differences according to Tukey’s test ($p \leq 0.05$).

On the other hand, most of CH+CFT combinations (91.6%) reduced biofilm synthesis compared to the untreated controls ($p \leq 0.05$) (Figure 2A–C). The inhibition range on the three bacteria was 32.4 to 74.4%. When comparing the effect of the mixtures with the individual compounds, the trend showed differences between CH+CFT and CH alone, as inhibition was more pronounced in the combinations than in CH. However, this was not observed between CH+CFT and CFT alone, as the antibiotic inhibited biofilm synthesis (Figure 2A–C). Once again, these results show that it was not possible to detect an enhancing effect of CH on CFT in inhibiting the synthesis of *S. aureus* biofilm. Nevertheless, it is important highlight that the treatments that inhibited the synthesis of biofilm, caused a recategorization of the bacteria as follows: *S. aureus* ATCC 27543 and AMC-43 were reclassified from moderate to weak producers; and AMC-48 from strong to moderate producer (Table 4). Finally, it was also noted that the control (vehicle: 1% acetic acid, pH 5.6) showed no significant inhibition ($p \leq 0.05$) of biofilm production in any of the microorganisms evaluated (Figure 2A–C).

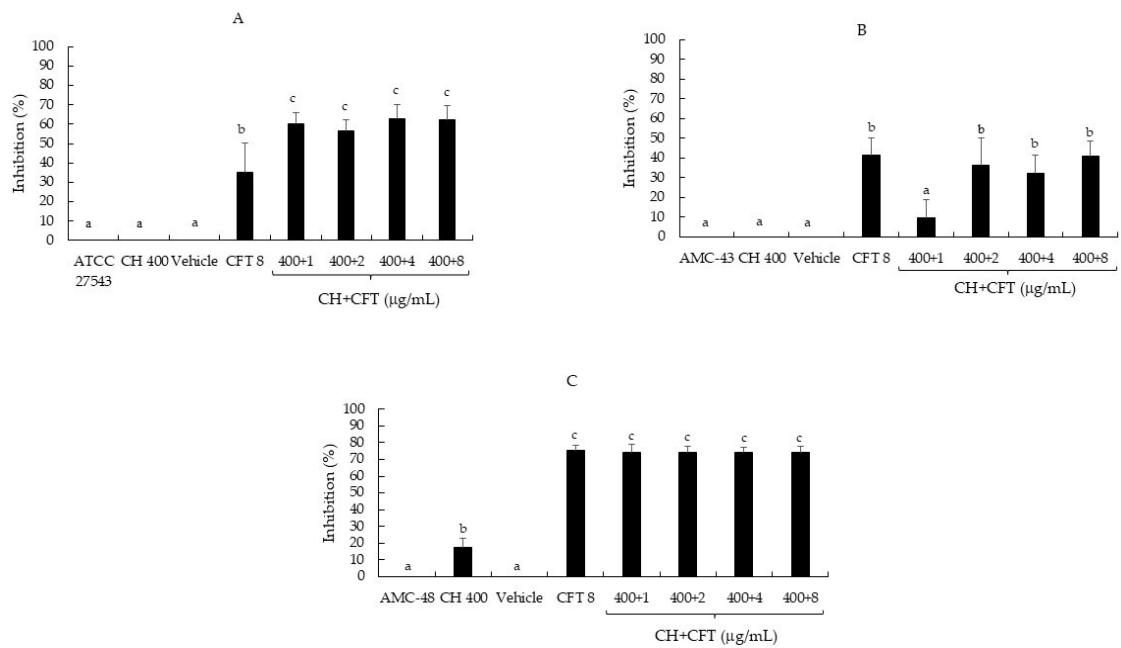


Figure 2. Inhibition percentage of *S. aureus* biofilm production by chitosan (CH), ceftiofur (CFT), and CH+CFT after 24 h of incubation. (A) *S. aureus* ATCC 27543. (B) *S. aureus* AMC-43. (C) *S. aureus* AMC-48. The negative inhibition control was plotted as the optical density (OD) of each bacterium, together with the OD of the vehicle used: 1% acetic acid, pH 5.6. Evaluation of different combinations of CH+CFT are shown. Graph represents the mean ± standard deviation. Different letters within each column indicate significant differences according to Tukey's test ($p \leq 0.05$).

In addition to analyzing the effect of combinations of biocompounds and CFT on the synthesis of biofilm produced by *S. aureus*, we were interested in determining whether these treatments could penetrate the preformed biofilm and reduce the CFUs inside the biofilm. The results showed that all N+CFT combinations reduced bacterial growth by up to 2.5 logarithmic units, showing significant differences compared to the control ($p \leq 0.05$) (Figure S1-A). However, as shown in Figure S1-B, the antibacterial effect of the CH+CFT combinations was more effective, as the combinations of 400+4 and 400+8, respectively, reduced the recovered CFUs to 0.

4. Discussion

Bovine mastitis caused by *S. aureus* affects dairy cattle worldwide and causes major economic losses [22]. As the disease is difficult to eradicate due, in part, to the high rate of bacterial resistance to antibiotic therapy and biofilm formation, studies should focus on development of new antibiotics and sustainable management models. This can enhance antibiotic efficacy, reduce the evolution of natural resistance, and inhibit virulence factors, such as biofilm synthesis [23]. In this regard, molecules of natural origin, such as essential oils, antimicrobial peptides, carbohydrate polymers, and synthetic chemical compounds, are garnering attention as enhancers of antibiotic efficacy, inhibitors of antibiotic resistance and virulence factor [5,12,24,25].

The present study contributes to this alternative by selecting two clinical isolates of *S. aureus* (AMC-43 and AMC-48) including the reference strain *S. aureus* ATCC 27543, because of their resistance to the β -lactam antibiotic CFT, as shown in Table 1. The MICs of CFT ranged from 256 to 2,048 $\mu\text{g/mL}$, whereas the MBCs fluctuated between 512 and 2,048 $\mu\text{g/mL}$. These results confirmed that the bacteria analyzed in this study were resistant to CFT, as the breakpoint for this antibiotic was 8 $\mu\text{g/mL}$ [15,16]. This antibiotic is a third-generation cephalosporin that has been widely used for over two decades for mastitis treatment [26]. However, Molineri et al. [27] and Morar et al. [28] reported that increased bacterial resistance to conventional antibiotics over time has become a

common characteristic, including resistance to CFT, which is consistent with the findings of this study.

Employing enhancers of the antibacterial effect of antibiotics relies on MIC or sub-MIC utilization. To select the sub-MIC of N and CH, the MICs of both biocompounds were also determined. The results showed an antibacterial effect of N only on the certified strain of *S. aureus* ATCC 27543, with an MIC of 640 µg/mL. Previous studies have reported the antibacterial effect of this peptide on bovine mastitis-associated *S. aureus* [29] (Bennett et al. 2021). The antimicrobial effect of N is based on its net positive charge, which allows it to bind to the peptidoglycan of the wall through electrostatic attractions and destabilizes it. This allows peptide to insert into the bacterial cell membrane to form pores that cause the microorganism death [30]. Notably, the low susceptibility of clinical isolates to N is striking, since this peptide is not used in herds of the region as a therapeutic alternative against bovine mastitis. However, *S. aureus* has a high potential to develop natural resistance or low susceptibility to N in vitro [31]. This could explain its null antibacterial effect on the AMC-43 and AMC-48 isolates. However, further experimental work is needed to understand the mechanism of low susceptibility of the clinical isolates to N used in this study.

In contrast, CH showed antibacterial effects against all evaluated bacteria, whose MICs and MBCs were 6400 µg/mL (Table 1). CH is an efficient antibacterial agent that inhibits the growth of Gram-positive and Gram-negative bacteria [32]. These precedents include the antibacterial effect on bovine mastitis-associated *S. aureus* [22], which corroborates the results of this study. The antibacterial activity of CH is based on its net positive charge, which allows it to efficiently bind to negatively charged cell wall components such as teichoic and lipoteichoic acids. This allows the formation of membrane pores and bacterial death [32].

Regarding the use of biocompounds as enhancers of the antibacterial effect of antibiotics, the results showed that all combinations of N+CFT reduced bacterial growth (expressed as logarithmic reduction) of ATCC 27543, AMC-43, and AMC-48 compared to controls and individual treatments with N and CFT added individually ($p \leq 0.05$) (Table 2). The increased efficacy of β -lactam antibiotics such as penicillin and CFT, in combination with N, has been evaluated against *S. aureus* (SA113), *S. pseudintermedius* (DSM21284), and *Streptococcus suis*, with positive results showing a greater reduction in bacterial growth [33,34]. However, this is the first study that evaluates the restoration of CFT's antibiotic efficacy when combined with N on *S. aureus* associated with bovine mastitis. Notably, the combined use of antimicrobial agents with conventional antibiotics, which have different cellular targets, could reduce the selection of resistant bacteria, thereby potentially allowing for the reduction of antibiotic therapeutic doses [33]. However, to know the type of interaction that allows this biological effect between the peptide and the antibiotic, further experiments are needed.

In addition, the CH and CFT mixtures showed that all combinations also reduced the bacterial growth of ATCC, AMC-43, and AMC-48 more efficiently compared to the controls and individual treatments with CH and CFT (Table 3). According to these results, other authors have reported an increase in the efficacy of antibiotics such as cloxacillin, oxacillin, ampicillin, penicillin, and cefotaxime when combined with CH or CH nanoparticles against coagulase-negative *Staphylococcus*, *Staphylococcus* spp., and enterobacteria [5,35,36]. However, this is the first study to evaluate the restoration of susceptibility of *S. aureus* associated with bovine mastitis to CFT, through the addition of subMIC CH. Similarly, these data suggest that combination therapy with CH and CFT could increase bacterial susceptibility, as well as the therapeutic dose of the antibiotic. Additionally, to provide further evidence to strengthen these results, it is necessary to know the type of interaction between CH and CFT, so further studies are needed.

Regarding bacterial reduction expressed in logarithmic units, authors such as Boddie et al. [37], Fitzpatrick et al. [38], and the National Mastitis Council [39], indicate that a reduction of 3 units is acceptable, but a reduction of 4-5 logarithmic units is preferable. In this study, the reduction of N+CFT combination ranged from 2.24 to 4.86 on the three bacteria. In this case, the combination would only be acceptable in the AMC-43 (4.35-4.86) and AMC-48 (3.66-4.22) clinical isolates, but not in the ATCC 27545 strain (2.24-2.76), so this combination should be evaluated in a larger number of clinical isolates

of *S. aureus* to obtain more consistent data. On the other hand, the reduction of CH+CFT combination ranged from 4.96 to 7.21 on all pathogenic bacteria. As can be seen, the combination is acceptable for ATCC 27543 (7.21), AMC-43 (7.39), and AMC-48 (4.96-5.67). Nevertheless, this combination should also be evaluated in a larger number of clinical isolates of *S. aureus* to strengthen the results of the CH+CFT combination.

Regarding the effect of N, CFT, and N+CFT combinations on biofilm synthesis, all treatments inhibited the synthesis of this virulence factor in the three pathogenic bacteria evaluated, compared to the untreated controls ($p \leq 0.05$) (Fig. 1A–C). The bacteria were even reclassified according to Stepanović et al. [20] categorization (Table 4). Similar of these results, other author has reported the effect of N sub-MICs in inhibiting biofilm synthesis by *S. aureus*, suggesting that this allows the peptide to inhibit biofilm synthesis by this pathogen without the need for a combination [40]. Additionally, Ster et al. [16] reported that concentrations below the CFT breakpoint (0.25 and 1.0 µg/mL) inhibit biofilm synthesis by *S. aureus*, which aligns with the findings in this study. On the other hand, most of CH+CFT combinations (91.6%) reduced biofilm synthesis compared to the untreated controls ($p \leq 0.05$) (Fig. 2A–C). Once again, the bacteria were also reclassified (Table 4). The effect of CH on biofilm synthesis by *S. aureus* has been documented [41]. However, this is in contrast with our results, at least for ATCC 27543 and AMC-43, where no inhibition was observed. Nevertheless, CH concentrations that inhibit biofilm synthesis can reach up to 1600 µg/mL, which could explain the differences observed in this study, where only just 400 µg/mL were used for CH [22].

Interestingly, when analyzing the effect of the combinations on the cells inside the biofilm, all N+CFT combinations reduced bacterial growth by up to 2.5 logarithmic units (Figure S1-A). However, the antibacterial effect of the CH+CFT combinations was more effective, as the combinations of 400+4 and 400+8, respectively, reduced the recovered CFUs to 0 (Figure S1-B). The above shows an enhancing effect of biocompounds on the antibacterial effect of CFT and suggests that these combinations appear to be penetrating the biofilm, as significant reductions in recovered CFUs were recorded. However, further studies are needed to strengthen this assumption.

5. Conclusions

This study demonstrated a significant increase in the antibacterial activity of the antibiotic CFT when combined with most of sub-MICs of N and CH on the ATCC 27543 strain of *S. aureus* and the clinical isolates AMC-43 and AMC-48, all resistant to CFT and associated with bovine mastitis, which was reflected in the reduction in bacterial logarithmic units as recommended by the NMC. Although the results varied with the evaluated bacteria and the individual biocompounds, a reduction in biofilm synthesis by the three bacteria was observed with N+CFT and CH+CFT combinations, as well as with individual treatments of N, CFT and to a lesser extent CH. However, it was not possible to detect a clear enhancer effect of the N+CFT and CH+CFT mixtures in inhibiting the synthesis of the *S. aureus* biofilm, in relation to the individual biocompounds; effect that was well established on the cells living inside the biofilms. Finally, these findings indicate that N and CH could have potential applications in the reuse of CFT in dairy herds and could have possible uses on prevention and control of bovine mastitis caused by *S. aureus*.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/doi/s1, Figure S1>.

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