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Original Article

Recombinant expression and antimicrobial activity of Aureocin A53 against *Staphylococcus aureus* isolated from bovine intramammary infections

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Abstract

Background: Bovine mastitis causes major economic losses in dairy production. *Staphylococcus aureus*, the most prevalent causative pathogen of mastitis, is often resistant to antibiotics. Bacteriocins, like Aureocin A53 (A53), are antimicrobial peptides that can provide an alternative treatment. **Aims:** The present study aimed to express a recombinant peptide (A53) in *Escherichia coli* and evaluate its antimicrobial activity against *S. aureus* isolated from bovine mastitis. **Methods:** The *A53* gene was cloned into pET24a and expressed in *E. coli* strains BL21(DE3), Tuner (DE3), and Rosetta (DE3), in different induction conditions. **Results:** Among all the evaluated conditions, A53-pET24a in *E. coli* Rosetta (DE3) induced with IPTG 0.2 mM overnight appeared to yield slightly higher expression levels than the other conditions tested. Cell lysates supernatants containing recombinant A53 showed inhibitory activity against two *S. aureus* indicator strains in agar diffusion assays. Microtiter plate assays conducted with *S. aureus* isolated from bovine mastitis showed that purified recombinant A53 significantly reduced bacterial growth after 24 h of incubation, in comparison with non-treated cultures. **Conclusion:** Our results underscore the feasibility of producing active recombinant A53. Further studies are needed to evaluate its effectiveness against additional *S. aureus* isolates and to optimize expression and recovery processes. Improving production scalability will be essential to evaluate the stability and efficacy of peptide in *in vivo* models, such as the bovine mammary gland, thereby supporting its potential therapeutic application.

Key words: Aureocin A53, Bacteriocins, Bovine mastitis, *Escherichia coli*, *Staphylococcus aureus*

Introduction

Bovine mastitis is a disease of dairy cattle that causes enormous economic losses to both the producer and the industry. Among the infecting pathogens, *Staphylococcus aureus* is the most prevalent in cases of bovine mastitis in Argentina and worldwide (Krishnamoorthy *et al*., 2021). Current mastitis control programs are mainly based on hygiene during milking and antibiotic therapy. However, increasing rates of antimicrobial resistance (Molineri *et al*., 2021) have created the need to explore innovative alternatives for the control and prevention of intramammary infections. One of these alternatives is the use of natural antimicrobial molecules, such as bacteriocins.

Bacteriocins are antimicrobial peptides produced by bacteria, which are active against related or unrelated bacteria. They are characterized by low toxicity, a

specific mechanism of action, and high stability (Guryanova, 2023). The most recent update regarding the classification of bacteriocins divides them into two main classes: Class I assembles small peptides (<5 kDa) that contain post-translational modifications, while Class II bacteriocins are leaderless peptides of 6-10 kDa, with no post-translational modifications, and may or may not include stabilizing disulfide bridges (Soltani *et al*., 2021).

The isolation of bacteriocins from the producing strains can be laborious and inefficient, while chemical synthesis yields only small amounts of peptide at high costs (Parachin *et al*., 2012). In contrast, heterologous expression of bacteriocins in recombinant systems, such as *E. coli*, has considerable advantages such as higher production yields, lower cost, and easy genetic manipulation to modify properties or facilitate purification. Among the bacteriocins described, the structural characteristics of those belonging to Class II place them in an advantageous position for their correct expression in heterologous organisms, guaranteeing their functionality.

Aureocin A53 (A53) is a class II bacteriocin of 51 amino acid residues produced by *S. aureus* strain A53, isolated from commercial milk (Netz *et al*., 2002a). It is distinguished by its high stability in acidic environments and at high temperatures, as well as its low susceptibility to proteases (Netz *et al*., 2002a). A53 is produced, expressed and exported without a leader sequence or signal peptide. Although the mechanism of action of A53 has not yet been fully elucidated, recent studies indicate that the bactericidal activity of A53 would be mediated by a multimodal membrane disruption mechanism, including the formation of transmembrane lesions, membrane thinning patches and transmembrane pores (Hammond *et al*., 2020; Lander *et al*., 2023).

Aureocin A53 has shown a broad inhibitory spectrum against lactic acid bacteria, *Listeria monocytogenes*, *S. aureus* involved in bovine mastitis, *Micrococcus* and *Enterococcus* (Netz *et al*., 2002b; Varella Coelho *et al*., 2007; Fagundes *et al*., 2016). In a recent study, native A53 exhibited bactericidal activity against 100% of *S. aureus* and *Staphylococcus agalactiae* isolated from bovine mastitis cases. Furthermore, it exerted no adverse side effects on bovine mammary gland epithelial cells in *in vitro* assays and significantly reduced the number of viable cells of both pathogens in an excised teat model (Marques-Bastos *et al*., 2022). Regarding recombinant expression of A53 in *E. coli*, there are few reports, and only one of them evaluated the antimicrobial activity of the obtained molecule, showing antimicrobial activity against Gram-positive bacteria (Acedo *et al*., 2016). However, there is no data regarding the evaluation of the antimicrobial activity of recombinant A53 against *S. aureus* isolated from bovine mastitis cases.

Based on this background, the present work aimed to obtain a recombinant molecule of A53 by heterologous expression in *E. coli*, and to evaluate its antimicrobial activity against *S. aureus* strains isolated from cases of bovine mastitis in Argentina.

Materials and Methods

Construction of expression plasmid

Bacterial strains used in this study for cloning and expression of A53 are listed in Table 1. The coding sequence of A53 (GenBank Accession No. AF447813.1) was obtained by commercial synthesis (gBlocks Gene Fragments, Integrated DNA Technologies, USA). This gene was used as template in polymerase chain reactions (PCR) containing 1× PCR buffer, 2 mM $MgCl_2$, 1 U/µL *Thermus aquaticus* DNA polymerase (Thermo Scientific, USA), 0.2 mM dNTPs (Genbiotech, Argentina), 0.3 µM primers, and 20 ng of synthetic DNA, in a total volume of 50 µL. Specific primers were A53p24Fw (5´-GCC CAT ATG AGT TGG TTA AAT TTT TT-3´) and A53p24Rv (5´-CCG CTC GAG TAA TCC AGC AAT TTT CTT TAA-3´) (Genbiotech, Argentina). Underlined sequences correspond to restriction enzyme sites used for cloning. Amplification was performed in an Ivema T21 termocycler (Ivema, Argentina) using the following program: an initial 3 min denaturation step at 95°C, followed by 10 cycles of 30 s of denaturation at 95°C, 30 s of annealing at 47°C, and 30 s of extension at 72°C, and 20 cycles of 30 s of denaturation at 95°C, 30 s of annealing at 58°C, and 30 s of extension at 72°C; with a final extension step at 72°C for 5 min. PCR products were analyzed by electrophoresis on GelRed (Biotium, USA)-stained 1% agarose gels (Biodynamics, Argentina), and sequenced using an ABI 3500 sequencer (Applied Biosystems, USA). *A53* gene was cloned in frame to a C-terminal 6xHis Tag into the pET24a expression vector (Merck Millipore, Germany). This construction was named A53-pET24a and used to transform competent *Escherichia coli* BL21(DE3), BL21 (DE3) pLysS, Rosetta (DE3) and Tuner (DE3) cells.

Table 1: *Escherichia coli* strains used for cloning and expression of A53 in this study

Strains	Relevant features	Source
BL21 (DE3)	T7 expression system	Novagen (2011), USA
BL21 (DE3) pLysS	T7 expression system; basal expression reduced by T7 lysozyme	Novagen (2011), USA
Tuner (DE3)	T7 expression system; $LacY^-$ cells produce a concentration dependent, homogeneous level of induction	Novagen (2011), USA
Rosetta (DE3)	T7 expression system; supply of rare tRNA codons	Novagen (2011), USA

Abbreviation: LacY, lactose permease

Protein expression

Two methods for protein expression were evaluated:
i) A high cell density method using Isopropyl β-d-1-thiogalactopyranoside (IPTG) (Promega, USA), for A53-pET24a in *E. coli* BL21(DE3), Rosetta(DE3), Tuner(DE3) and BL21(DE3)pLysS
ii) An autoinduction method, for A53-pET24a in *E. coli* BL21(DE3) and BL21(DE3)pLysS

High cell density method using IPTG was conducted according to Sivashanmugam *et al*. (2009). Cells were cultured in 15 ml of Luria-Bertani (LB) supplemented with 1% glucose, 0.025 mg/ml of kanamycin, and 0.017 mg/ml of chloramphenicol for *E. coli* Rosetta (DE3) and BL21(DE3) pLysS cultures, for 7h at 37°C. Cells were pelleted through centrifugation at 2800 g and resuspended in M9 minimal medium (MM) supplemented with 0.025 mg/ml of kanamycin, and 0.017 mg/ml of chloramphenicol for *E. coli* Rosetta (DE3) and BL21(DE3) pLysS cultures, in the necessary volume to obtain a final DO_{600} = 3.5. Cells were incubated for 1 h at 37°C. Then, IPTG at 0.2 mM or 0.5 mM final concentration was added and incubated at 37°C with shaking for 4 h or 16 h. After incubation, cells were pelleted through centrifugation at 2800 g and washed 3 times with sterile phosphate-buffered saline (PBS) to eliminate the rest of antibiotics. Finally, cells were resuspended in resuspension buffer (RB) (50 mM Tris pH 8.0, 0.3 M NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF).

Autoinduction method was conducted according to Studier (2005). Cells were cultured in 2 ml MM supplemented with 0.025 mg/ml of kanamycin, and 0.017 mg/ml of chloramphenicol for BL21(DE3) pLysS cultures, for 6 h at 37°C with shaking. Then, cells were harvested through centrifugation at 2800 g and resuspended in necessary volume of ZYP-5052 medium supplemented with 0.025 mg/ml of kanamycin and 0.017 mg/ml of chloramphenicol for BL21(DE3) pLysS cultures, to obtain a final DO_{600}= 0.3, and incubated overnight at 37°C with shaking. Cells were pelleted through centrifugation at 2800 g and washed 3 times with sterile PBS to eliminate the rest of antibiotics. Finally, cells were resuspended in RB.

For both methods, cells resuspended in RB were then lysed by two passes through a cell disruptor (Avestin, Ottawa, Ontario, Canada) at 20,000 psi. The supernatants were separated from the cell pellets by centrifugation at 13400 g and analyzed by 16% Tricine-SDS PAGE using an Unstained Protein Molecular Weight Marker (Thermo Scientific, USA) and Western blot. The supernatants containing A53 were then stored at 4°C until use for the agar diffusion test (see below).

Western blot

Supernatants were separated by SDS-PAGE and transferred to 0.45 µm nitrocellulose membranes (Mini Trans-BlotCell, BioRad, USA). Membranes were first incubated with PBS with low fat goat milk 5%, and then with monoclonal anti-His tag antibody (Thermo Scientific, USA), diluted in PBS with low fat goat milk 1%. Finally, membranes were incubated with peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich, USA) diluted in PBS with low fat goat milk 1%. Between each step, membranes were washed three times with 0.05% Tween 20 in PBS. All incubations were 1 h at 37°C. Lastly, enzyme substrate (H_2O_2/ diaminobenzidine, Sigma-Aldrich, USA) was added. The reaction was stopped by submerging the membranes in distilled water. A positive control consisting in Thioredoxin fused to HisTag (TRX-His), and a negative control consisting in supernatant from A53-pET24a in *E. coli* BL21(DE3) uninduced, were included. A PageRuler Prestained Protein Ladder (Thermo Scientific, USA) was used as molecular weight marker.

Protein purification

A53 expression was induced in 100 ml culture following the conditions that yielded the best results in the protein expression assays. Cell lysate supernatant containing A53 was filtered through a 0.45 µm filter, loaded by gravity onto NTA-Ni-Sepharosa column (Thermo Scientific, USA) and eluted into different fractions, using RB plus 50, 100, and 250 mM imidazole, consecutively. Eluted fractions containing A53 were dialyzed against PBS and concentrated 10 times with Ultra Centrifugal Filter 3 kDa (Amicon, Merck Millipore, Germany), aliquoted, and stored at 4°C for no more than 48 h until use. Purity of A53 was analyzed by 16% Tris-tricine gels and identity was confirmed by Western Blot as described above.

Antimicrobial activity of A53

Agar diffusion test

This test was performed according to Jaouani *et al*. (2014) with some modifications. Forty µL of a suspension containing 1×10^8 CFU/ml of *S. aureus* ATCC 29213 or *S. aureus* Newbould 305 (ATCC 29740) were streaked onto 10 ml Brain Heart Infusion (BHI) 0.8% agar plates. Small wells (5 mm in diameter) were made on the agar, which were filled with 25 µL of supernatants containing A53. A commercial bacteriocin (Nisin-A, Sigma-Aldrich, USA; 5 mg/ml) was used as positive control, while the cell lysates supernatants from cultures of *E. coli* strains transformed with pET24a without an insert were used as negative controls. The presence or absence of inhibition zones was registered after 24 h of incubation at 37°C.

Microplate assay

This test was performed according to Ceotto *et al*. (2010) with some modifications. Four *S. aureus* strains were used: ATCC 29213 and three strains isolated from bovine mastitis cases in Argentina; all of them belonged to the strain collection of the Laboratorio de Sanidad Animal at EEA INTA Rafaela. Two ml of Mueller Hinton (MH) medium were inoculated with a single colony of the indicator strain and incubated at 37°C with shaking, 4 h. OD_{600} was then measured, and adjusted with MH to a final concentration of 1×10^7 CFU/ml. One hundred µL of these cultures were plated on U-

shaped bottoms 96-well plates along with 100 µL of purified A53 in PBS, 100 µL of ampicillin (0.1 mg/ml in PBS; positive control), or 100 µL of PBS (negative control). Plates were incubated at 37°C for 24 h in a microplate incubator (Synergy HT, Biotek, USA). Bacterial growth was recorded by measuring OD_{600} every 30 min, with shaking before each OD reading. Tests were performed in duplicate.

Statistical analysis

Differences in OD_{600}, obtained in antimicrobial activity microplate assay, were analyzed by a generalized linear mixed model using an identity link function with OD as the outcome variable, strain as a random factor, and treatment as a fixed factor.

Results

Protein expression

In general, A53 expression levels were low. The induction conditions in which detectable bands of A53 (below 14.4 kDa) were observed were:

i) IPTG 0.2 mM 16 h in *E. coli* BL21(DE3) cells (Fig. 1a), *E. coli* BL21(DE3) pLysS (Fig. 1b) and *E. coli* Rosetta (DE3) (Fig. 1c)
ii) ii) IPTG 0.5 mM 16 h in *E. coli* Rosetta (DE3) (Fig. 1c)
iii) iii) Autoinduction 16 h in *E. coli* Bl21(DE3) pLysS (Fig. 1b). No detectable levels of A53 were observed in *E. coli* Tuner (DE3), for any evaluated condition (not shown)

Cell lysate supernatants from A53-pET24a in *E. coli* Rosetta (DE3) and BL21(DE3), both with IPTG overnight 0.2 mM, and A53-pET24a in *E. coli* BL21(DE3) pLysS with autoinduction ON, were also evaluated by Western Blot. In all the conditions evaluated, a band of approximately 10 kDa was observed, consistent with the expected size of A53 (theorical size ~7 kDa) (Fig. 1d). Visual estimation of Western blot band intensity suggested that *E. coli* Rosetta (DE3) induced with 0.2 mM IPTG overnight yielded slightly higher expression levels than the other conditions tested. Therefore, this condition was selected to continue working with.

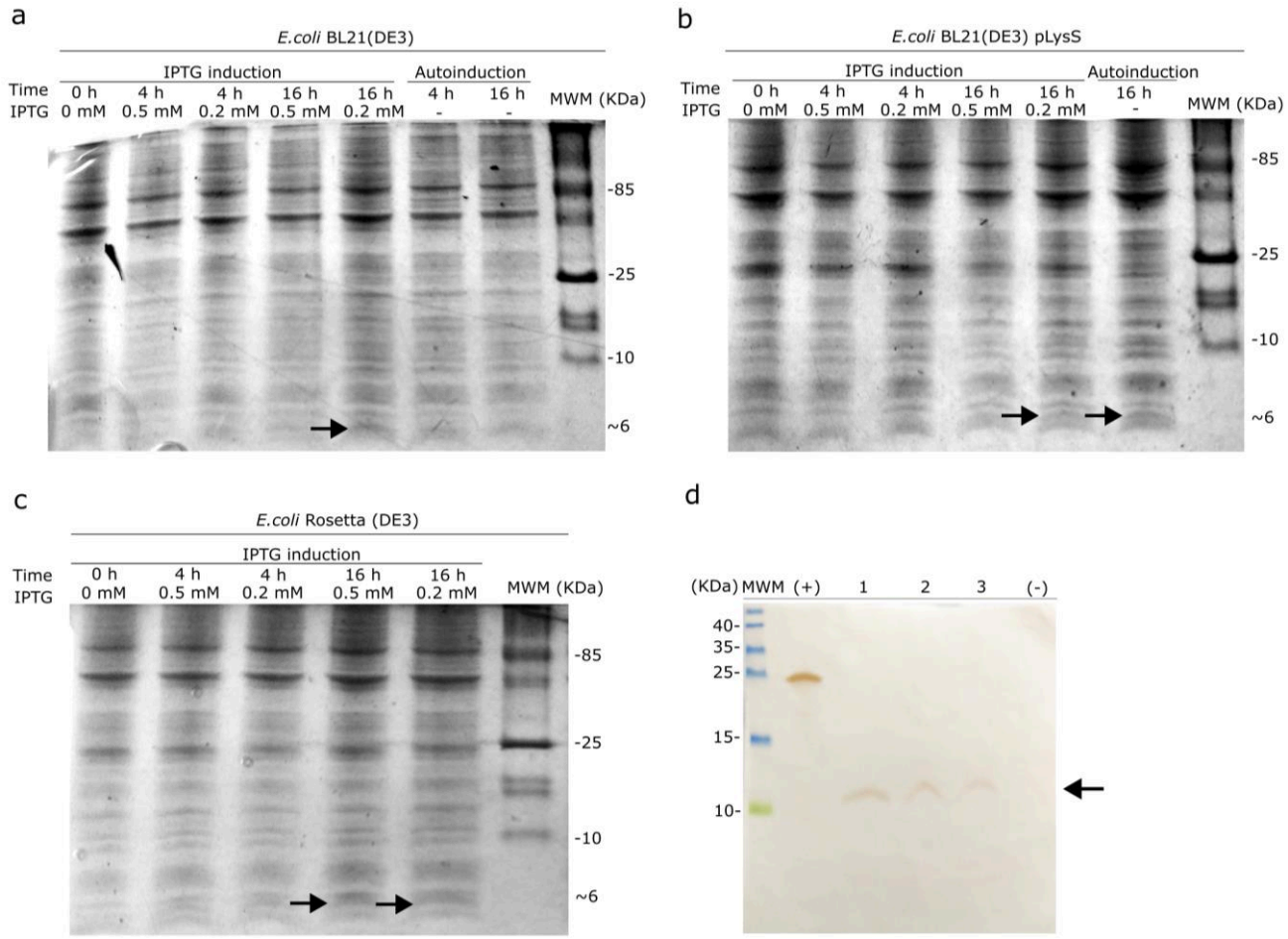

Fig. 1: Assessment of A53 expression in different *E. coli* strains. The overexpression band is indicated with a black arrow. (**a-c**) Tricine-SDS-PAGE analysis of A53 protein expression following IPTG induction and autoinduction, and (**d**) Confirmation of A53 identity by Western blot using monoclonal anti-HisTag antibody; (+) Positive control (TRX-His), (1) IPTG induction in Rosetta (DE3) for 16 h with 0.2 mM IPTG, (2) BL21 (DE3) pLysS autoinduction for 16 h, (3) IPTG induction in BL21 (DE3) for 16 h with 0.2 mM IPTG, (-) Negative control (A53-pET24a in *E. coli* BL21 (DE3) uninduced), and MWM: Molecular weight marker

Preliminary antimicrobial activity of A53 (agar diffusion test)

Antimicrobial activity of cell lysate supernatant containing A53 from induction with IPTG overnight 0.2 mM in *E. coli* Rosetta (DE3) was preliminarily evaluated in agar diffusion tests to validate the production of a biologically active recombinant peptide. *S. aureus* Newbould 305 (ATCC 29740) (Fig. 2a) and *S. aureus* ATCC 29213 (Fig. 2b) were used as indicator strains. After 24 h of incubation at 37°C, inhibition zones of approximately 15 mm were observed in the wells filled with the A53-containing supernatant, for both indicator strains. No growth inhibition was observed in the wells filled with the negative control, while Nisin-A generated inhibition zones of approximately 15 mm and 9 mm in diameter for *S. aureus* Newbould 305 and *S. aureus* ATCC 29213, respectively.

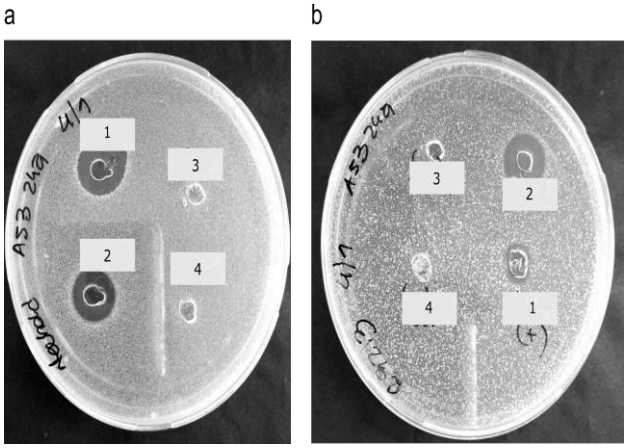

Fig. 2: Antimicrobial activity of supernatant containing A53 by agar diffusion test. (**a**) *S. aureus* Newbould 305 (ATCC 29740), and (**b**) *S. aureus* ATCC 29213. (1) Positive control (Nisin-A), (2) Cell lysate supernatant containing A53, (3) Negative control (supernatant of BL21(DE3)-pET24a), and (4) Negative control (supernatant of Rosetta (DE3)-pET24a)

Purification of A53

An induction of A53-pET24a was performed in *E. coli* Rosetta (DE3) under the previously established condition of 0.2 mM IPTG 16 h. The purification of A53 was carried out through Ni^{2+} affinity chromatography, and the obtained fractions were analyzed by 16% Tris-Tricine gels followed by Western Blot. Most of the A53 remained in the flow-through fraction, and no detectable levels of A53 were observed by SDS-PAGE in the eluted fractions. However, Western blot confirmed the presence of A53 in eluted fraction 1, suggesting a protein concentration below the Coomassie Brilliant Blue R-250 detection limit (Supplementary Figures 1a and b (SF1a and b)). This fraction was dialyzed against PBS and stored at 4°C for antimicrobial activity assessment.

Antimicrobial activity of A53 (microplate assay)

Bacterial growth in the presence of A53, ampicillin (positive control) or PBS (negative control) was recorded as OD_{600} every 30 min during a 24 h incubation period (Fig. 3a). At the end of the incubation period, bacterial growth differed significantly among the three evaluated treatments (P<0.001) (Fig. 3b). Incubation with purified A53 significantly reduced bacterial growth compared with the untreated control [A53 OD_{600}=0.73 (95% CI: 0.544-0.923) vs PBS OD_{600}=1.27 (95% CI: 1.082-1.461); P<0.001], representing a growth inhibition of 42.5%. However, A53 treatment was less effective in growth reduction than the antibiotic treatment [A53 OD_{600}=0.73 (95% CI: 0.544-0.923) vs ampicillin OD_{600}=0.10 (95% CI: 0.088-0.292); P<0.001] (Fig. 3b).

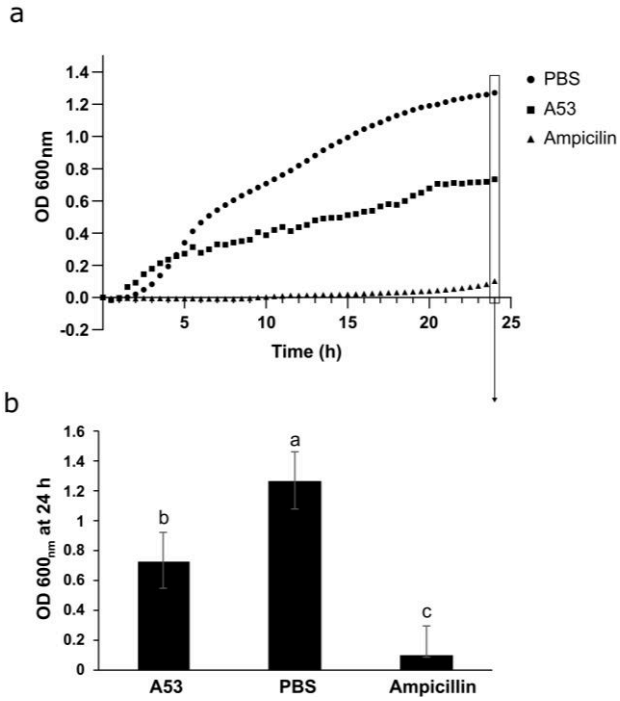

Fig. 3: Antimicrobial activity of A53 against *S. aureus* isolated from bovine intramammary infections. (**a**) Mean growth kinetics, expressed as OD_{600nm} of *Staphylococcus aureus* in presence of purified A53 (●), PBS (negative control, ▲) and ampicillin (positive control, ■), and (**b**) Comparison of bacterial growth means after 24 h incubation period under the three conditions analyzed. The bars represent the 95% CI and Different letters indicate significant differences (P<0.001)

Discussion

In the present study, we aimed to produce recombinant A53 in *E. coli* and evaluate its antimicrobial activity against *S. aureus* isolated from bovine mastitis cases. A53 was selected for this study primarily because it does not undergo any post-translational processing, so it can be easily introduced into heterologous expression systems simply by cloning its structural gene (Perez *et al.*, 2018). For production purposes, the *E. coli* expression system was chosen given its wide use, easy handling, high yields, and low costs (Rosano and Ceccarelli, 2014). Our experimental results confirm that A53 can be successfully expressed in *E. coli* while maintaining its antimicrobial activity, validating our expression strategy.

Our expression optimization experiments suggest that the Rosetta (DE3) strain could be the most suitable host

for producing recombinant A53. The potential of this system, further supported by the antimicrobial activity observed in the agar diffusion assays, can be explained because Rosetta (DE3) contains extra copies of tRNA genes that most frequently limit protein translation in *E. coli*. In one of the few existing reports describing recombinant A53 production, Acedo *et al*. (2016) cloned the *A53* gene with codon optimization for *E. coli* fused to SUMO protein, obtaining high yields of protein. The absence of codon optimization in our study may have significantly impacted expression levels, as suggested by the improved yields when using Rosetta (DE3). Furthermore, the expression of antimicrobial peptides fused to a carrier protein is proposed to protect both cells from toxicity and peptides from proteolysis (Parachin *et al*., 2012). In the present work, A53 was expressed without a fusion protein, which suggests that proteolytic degradation could be one of the causes of the low production levels observed. Future work should incorporate codon optimization and fusion partners to enhance both expression and solubility, thereby improving yields while maintaining antimicrobial activity.

The highest yields of A53 were obtained using the high cell density method (IPTG) (Sivashanmugam *et al*., 2009). In this method, bacteria are allowed to grow in a rich medium up to about half of the saturation OD_{600} and then transferred to a minimal medium in the presence of an inducer. This way, protein production is prioritized over cell growth. In this work, the *A53* gene was cloned without fusion protein and, therefore, the bacteriocin would already be active immediately after being produced and secreted, becoming potentially toxic to the host cell and stopping cell growth. In this context, the use of a method in which bacteriocin production starts at high cell density would be beneficial to obtain higher expression levels. An agar diffusion assay was performed, as an initial evaluation of the antimicrobial activity of recombinant A53, to validate the expression of an active molecule before proceeding to protein purification, using two *S. aureus* ATCC as indicator strains. In a study by Varella Coelho *et al*. (2007), 74.4% of 165 *S. aureus* isolated from bovine mastitis cases from Argentina and Brazil were sensitive to native A53 (inhibition halos >14 mm). In subsequent work, recombinant A53 showed activity against *S. aureus* ATCC 29213 in spot agar assays (Acedo *et al*., 2016), although the halo diameter cut-off was not reported. In the present work, the cell lysate supernatant containing recombinant A53 exhibited inhibition zones of approximately 15 mm for both *S. aureus* indicator strains, ATCC 29213 and ATCC 29740, which were similar to or greater than those observed for Nisin-A. This preliminary observation led us to continue evaluating the activity of A53.

Recombinant A53 was purified by Ni+2 affinity chromatography. However, purification yields were low, and most of the protein remained in the flow through fraction, possibly because low expression levels resulted in concentrations below the binding capacity of the affinity resin, allowing bacterial lysate components to compete for binding sites. Additional optimization strategies will be required in the future to improve recovery yields. Nevertheless, the presence of A53 in the purification eluate was confirmed by Western Blot.

In the antimicrobial microplate assays, 4 strains of *S. aureus* were used: ATCC 29213 and 3 isolates from bovine mastitis cases. After 24 h of incubation with purified A53, a significant reduction in bacterial growth was observed, in comparison with non-treated cultures. In a previous work by Netz *et al*. (2002b), native A53 showed minimum inhibitory concentrations (MICs) of approximately 1 µg/ml for two clinical isolates of *S. aureus* in microdilution plate assays. Later, Marques-Bastos *et al*. (2022) reported that after 24 h no viable cells were recovered from *S. aureus* bovine mastitis cultures treated with A53, although inhibitory titters were expressed in arbitrary units, precluding direct comparison of concentrations. Recently, Lander *et al*. (2023) found that native A53 and 9 of 12 synthetic variants presented MICs lower than 64 µg/ml against 4 *S. aureus* indicator strains. To the best of our knowledge, there is only one report evaluating antimicrobial activity of recombinant A53 against *S. aureus* (Acedo *et al*., 2016). In that work, recombinant A53 showed MICs of 128 µM (600 µg/ml approx.) against *S. aureus* ATCC 29213, but no inhibition against *S. aureus* ATCC 6538, in spot-on-lawn assays. In the present work, an inhibitory concentration was not reached with A53. However, considering that our protein could not be visualized by Coomassie-stained SDS-PAGE (detection limit ~100 ng; Brunelle and Green, 2014), we estimate that A53 was evaluated at less than 20 µg/ml. Despite this low concentration, a marked inhibition of *S. aureus* growth was observed in the presence of recombinant A53. The antimicrobial activity observed for the recombinant A53 protein against *S. aureus* isolates merits further study to improve its expression and purification, to obtain it at higher concentrations and with greater purity. This will allow the determination of MICs and facilitate a comprehensive evaluation of its efficacy against a larger number of bovine mastitis *S. aureus* isolates, with different clinical characteristics and from different dairy regions. Furthermore, scaling up production will be essential to evaluate its stability and therapeutic potential in *in vivo* models, such as the infected bovine mammary gland, thereby supporting its potential therapeutic application.

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Conflict of interest

The authors have no conflicts of interest associated

with this research work. Generative AI tools were used solely for language editing. No AI was used for data generation, analysis, or scientific content.

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Use of generative AI and AI-assisted technologies statement

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