Short communication: Activity of nisin, lipid bilayer fragments and cationic nisin-lipid nanoparticles against multidrug-resistant *Staphylococcus* spp. isolated from bovine mastitis

L. Castelani,^{1,2*} J. R. P. Arcaro,¹ J. E. P. Braga,¹ A. S. Bosso,¹ Q. Moura,² F. Esposito,³ I. P. Sauter,⁴ M. Cortez,⁴ and N. Lincopan^{2,3*}

¹Institute of Animal Science, Dairy Cattle Center, Nova Odessa, São Paulo, Brazil 13380-011

²Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil 05508-900

³Department of Clinical Analysis, School of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil 05508-900

⁴Department of Parasitology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil 05508-900

ABSTRACT

Staphylococci are the main etiological agents of bovine mastitis. Bacteriocins and nanoparticles have emerged as promising alternatives for the future development of antimicrobial agents. This study evaluated the activity of the bacteriocin nisin and bicelles of the synthetic cationic lipid dioctadecyldimethylammonium bromide, alone and in combination, against multidrug-resistant *Staphylococcus* spp. strains isolated from bovine mastitis. In summary, cationic nisin/dioctadecyldimethylammonium bromide nanoparticles are shown to be a promising alternative for the control of mastitis caused by multidrug-resistant *Staphylococcus* spp.

Key words: bacteriocin, cationic lipid, bicelle, antibacterial activity

Short Communication

Mastitis is the most prevalent and costly disease in dairy herds worldwide. Several *Staphylococcus* species have been implicated in mastitis, and resistance to drugs has contributed to treatment failure, particularly in infections caused by *Staphylococcus aureus*. However, although antimicrobial resistance can be a feature of treatment failure, overall it does not appear to be a frequent or main reason for negative treatment outcomes (du Preez, 2000).

Nisin (NS), a bacteriocin produced by *Lactococcus lactis* ssp. *lactis*, exhibits high antimicrobial activity against many gram-positive bacteria and foodborne pathogens (McAuliffe et al., 2001). The therapeutic potential of NS in veterinary medicine has been

demonstrated for the treatment of infectious diseases, including methicillin-resistant *Staphylococcus aureus* (MRSA) as an alternative to traditional antibiotics (Field et al., 2012).

Synthetic cationic lipids, such as dioctadecyldimethylammonium bromide (DDA), are versatile compounds that exhibit microbicidal activity against fungi and bacteria (Carmona-Ribeiro et al., 2006; Lincopan and Carmona-Ribeiro, 2006). Dioctadecyldimethylammonium bromide contains a quaternary ammonium moiety as cationic polar head, which imparts outstanding antimicrobial properties to this cationic lipid. The DDA self-assemble in aqueous solution yielding closed bilayers (vesicles) or disrupted vesicles, depending on the procedure used for dispersing the lipid. Interestingly, previous studies have showed that antimicrobial drugs self-assemble and solubilize at hydrophobic sites of DDA bilayer fragments (DDA BF), in water solution, exhibiting in vitro and in vivo activity (Lincopan et al., 2005; Vieira and Carmona-Ribeiro, 2008). Moreover, DDA BF have displayed broad-spectrum activity against clinically significant bacteria (Carrasco et al., 2015, 2016).

In this study, we evaluated the bactericidal activity of the bacteriocin NS and the lipid DDA, alone and in combination, against antibiotic-resistant *Staphylococcus* spp. isolated from bovine mastitis and from a collection of reference strains.

Thirty mastitis isolates of *S. aureus* (n = 14), *S. capitis* (n = 2), *S. chromogenes* (n = 9), *S. epidermidis* (n = 1), *S. haemolyticus* (n = 2), *S. sciuri* (n = 1), and *S. warneri* (n = 1) were used in this study (Table 1). The different species were chosen on the basis of susceptibility profile (i.e., resistance to at least one antimicrobial class). They belong to a collection of clinical mastitis isolates from dairy cows of 7 dairy farms located in the region of Campinas, Southeastern Brazil (Pilon et al., 2016). After isolation from milk samples, bacteria were

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*Corresponding authors: livia_cast@yahoo.com.br and lincopan@usp.br

Table 1. Antimicrobial resistance profile and minimum bactericidal concentrations (MBC) for nisin (NS), dioctadecyldimethylammonium bromide (DDA) bilayer fragments (BF), and NS/DDA complexes against *Staphylococcus* spp. isolated from bovine mastitis and *Staphylococcus aureus* reference strains

Strain	Species	Resistance profile ¹	MBC (µg/mL)			FBC ²			Interpretation ³
			NS	DDA BF	NS/DDA	NS	DDA BF	ΣFBC	
45T	<i>S. aureus</i>	Ery, Ffc, Kan, Lzd	50	4	3/2	0.06	0.50	0.56	PS
558T	<i>S. aureus</i>	Ffc, Lzd	50	8	3/2	0.06	0.25	0.31	S
731T	<i>S. aureus</i>	Amp, Pen	50	4	3/4	0.06	1.00	1.06	I
906T	<i>S. aureus</i>	Amp, Pen	50	4	3/4	0.06	1.00	1.06	I
969T	<i>S. aureus</i>	Amp, Cli, Ery, Pen, Tet	50	4	3/4	0.06	1.00	1.06	I
1025T	<i>S. aureus</i>	Amp, Pen, Tet	50	4	3/4	0.06	1.00	1.06	I
1027T	<i>S. aureus</i>	Amp, Pen, Tet, Str	100	4	3/2	0.03	0.50	0.53	PS
1037T	<i>S. aureus</i>	Amp, Pen, Tet, Str	50	2	3/2	0.06	1.00	1.06	I
1079T	<i>S. aureus</i>	Amp, Pen, Tet, Str	50	4	3/4	0.06	0.50	0.56	PS
1705T	<i>S. aureus</i>	Cip, Cli, Kan, Lzd, Sxt,	50	8	3/4	0.06	0.50	0.56	PS
1808T	<i>S. aureus</i>	Amp, Pen, Str	50	2	3/2	0.06	1.00	1.06	I
1849T	<i>S. aureus</i>	Amp, Cip, Cli, Ery, Eno, Pen	50	2	3/2	0.06	1.00	1.06	I
1960T	<i>S. aureus</i>	Amp, Eno, Pen	50	2	12/1	0.24	0.50	0.74	PS
111N	<i>S. aureus</i>	Amp, Pen	50	4	6/2	0.12	0.50	0.62	PS
180N	<i>S. capitis</i>	Amp, Pen	50	4	3/2	0.06	0.50	0.56	PS
565T	<i>S. capitis</i>	Amp, Pen, Tet	50	4	3/2	0.06	0.50	0.56	PS
89N	<i>S. chromogenes</i>	Amp, Pen, Str	50	2	3/2	0.06	1.00	1.06	I
90N	<i>S. chromogenes</i>	Amp, Gen, Pen, Str	50	4	3/4	0.06	1.00	1.06	I
118N	<i>S. chromogenes</i>	Amp, Ffc, Pen, Tet	50	4	3/2	0.06	0.50	0.56	PS
120T	<i>S. chromogenes</i>	Cli, Ffc, Lzd, Str, Tet	50	2	3/2	0.06	1.00	1.06	I
121N	<i>S. chromogenes</i>	Amp, Ffc, Pen, Str, Tet	50	4	3/4	0.06	1.00	1.06	I
148N	<i>S. chromogenes</i>	Amp, Ffc, Pen, Tet	50	2	3/2	0.06	1.00	1.06	I
158N	<i>S. chromogenes</i>	Amp, Ffc, Pen, Tet	50	4	3/4	0.06	0.50	0.56	PS
498T	<i>S. chromogenes</i>	Amp, Ffc, Pen	50	4	3/4	0.06	1.00	1.06	I
1501T	<i>S. chromogenes</i>	Oxa, Sxt	50	4	3/2	0.06	0.50	0.56	PS
1043TE	<i>S. epidermidis</i>	Amp, Kan, Pen, Tet	50	4	3/2	0.06	0.50	0.56	PS
75N	<i>S. haemolyticus</i>	Amp, Pen	25	4	3/2	0.12	0.50	0.62	PS
133N	<i>S. haemolyticus</i>	Amp, Ffc, Pen	50	8	3/4	0.06	0.50	0.56	PS
7N	<i>S. sciuri</i>	Oxa	50	32	6/16	0.12	0.50	0.62	PS
107N	<i>S. warneri</i>	Amp, Pen	50	4	3/2	0.06	0.50	0.56	PS
ATCC 25923	<i>S. aureus</i>	—	100	4	3/2	0.03	0.5	0.53	PS
ATCC BAA-976	<i>S. aureus</i>	Amp, Ery, Pen	100	4	3/2	0.03	1.0	1.03	I
ATCC BAA-977	<i>S. aureus</i>	Amp, Ery, Pen	100	32	3/16	0.03	0.5	0.53	PS
ATCC BAA-1026 (MRSA)	<i>S. aureus</i>	Amp, Cf, Cli, Eno, Ery, Gen, Oxa, Pen, Sxt	100	4	3/4	0.03	1.0	1.03	I
N315 (MRSA)	<i>S. aureus</i>	Amp, Oxa, Pen, Tet	50	4	3/2	0.06	0.5	0.56	PS
MU-50 (VISA)	<i>S. aureus</i>	Amp, Cf, Cli, Eno, Ery, Fur, Gen, Oxa, Pen, Tet, Van	50	4	3/4	0.06	1.0	1.06	I

¹Determined by disk diffusion method. Amp = ampicillin (10 µg); Cf = cephalothin (30 µg); Cip = ciprofloxacin (5 µg); Cli = clindamycin (2 µg); Eno = enrofloxacin (5 µg); Ery = erythromycin (15 µg); Ffc = florfenicol (30 µg); Gen = gentamicin (10 µg); Kan = kanamycin (30 µg); Lzd = linezolid (30 µg); Oxa = oxacillin (1 µg); Pen = penicillin (10 UI); Str = streptomycin (10 µg); Sxt = trimethoprim-sulfamethoxazole (1.25/23.75 µg); Tet = tetracycline (30 µg).

²FBC = fractional bactericidal concentration (FBC = MBC combination/MBC alone); ΣFBC = FBC summation.

³The combination is classified as synergistic (S) when ΣFBC is ≤0.5, partially synergistic (PS) when ΣFBC is >0.5 to ≤1.0, indifferent (I) when ΣFBC is >1.0 to 4.0, and antagonistic (A) when ΣFBC is ≥4.0 (Hall et al., 1983).

identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) using the MALDI Biotyper system (Bruker Daltonik), being kept frozen at −80°C until further processing. Additionally, we used 6 *S. aureus* ssp. *aureus* American Type Culture Collection reference strains (ATCC 25923, ATCC BAA-976, ATCC BAA-977, ATCC BAA-1026, MRSA N315, and hetero-VISA Mu-50).

Bacteria were tested for antimicrobial susceptibility by the disk-diffusion method on Mueller-Hinton Agar (Oxoid, Basingstoke, UK; CLSI, 2009, 2015).

Nisin and DDA (99.9% pure) were obtained from Sigma Chemical Co. (St. Louis, MO). The NS stock solution was prepared by dissolving 0.01 g of NS in 10 mL of 0.02 N HCl (pH 2.0) as previously described (Stevens et al., 1991), being sterilized by filtration using a 0.45-µm filter (Millipore, Carrigtohill, Co. Cork, Ireland). The DDA synthetic BF, at 2 mM, were obtained by sonication with a titanium macrotip probe in isotonic glucose-phosphate buffer (IGP, 1 mM potassium phosphate buffer, pH 7.0, supplemented with 287 mM glucose as an osmoprotectant), and decontaminated by

Table 2. Distribution of size (mean diameter, D_z) and zeta potential (ζ) analysis of nisin (NS), dioctadecyldimethylammonium bromide (DDA) bilayer fragments (BF), NS/DDA, methicillin-resistant *Staphylococcus aureus* (MRSA) N315, and different combinations, in isotonic glucose-phosphate buffer

Particles	D_z (nm)	SD	Polydispersity	SD	ζ (mV)	SD
MRSA N315 (1×10^5 cells/mL)	1,073.7	29.5	0.219	0.038	-27.32	1.37
DDA BF 2 mM	69.4	0.7	0.216	0.007	19.46	1.20
NS 300 $\mu\text{g/mL}$	187.9	14.5	0.630	0.055	0.62	0.62
NS/DDA 30/20 $\mu\text{g/mL}$	148.5	22.5	0.461	0.021	8.84	4.83
NS/DDA 3/2 $\mu\text{g/mL}$ + MRSA N315	556.9	14.3	0.380	0.013	-8.12	1.22
NS/DDA 30/20 $\mu\text{g/mL}$ + MRSA N315	462.8	18.8	0.434	0.017	15.28	0.40
NS/DDA 300/200 $\mu\text{g/mL}$ + MRSA N315	270.7	19.5	0.416	0.007	33.56	0.97

filtration (0.45 μm ; Lincopan and Carmona-Ribeiro, 2006).

Minimum bactericidal concentration (**MBC**) values of NS and DDA were determined by the microbroth dilution method (CLSI, 2015), whereas bactericidal activity resulting from interactions between NS and DDA BF was evaluated by the checkerboard method, as previously described (Lincopan and Carmona-Ribeiro, 2006). Briefly, 2-fold serial dilutions of NS and DDA BF were prepared in IGP at a final volume of 100 μL per well, in 96-well flat-bottomed microtiter plates. Final concentrations ranged from 200 to 3.125 $\mu\text{g/mL}$ for NS, and from 64 to 0.5 $\mu\text{g/mL}$ for DDA BF. Each well of the microtiter plate was inoculated with a bacterial cell suspension in IGP at a final concentration of 10^5 cells per mL, and the plates were incubated at $35 \pm 2^\circ\text{C}$ for 24 h. An aliquot (10 μL) of cell suspension was taken from each well, diluted 1:10 and 1:100, and cell viability was determined by plating 0.05 mL on Mueller-Hinton agar plates and incubating for 24 h at $35 \pm 2^\circ\text{C}$. The MBC endpoint was the lowest drug concentration that killed $\geq 99.9\%$ of the inoculum (Lincopan and Carmona-Ribeiro, 2006). The fractional bactericidal concentration (**ΣFBC**) was calculated as follows: $\Sigma\text{FBC} = \text{FBC A} + \text{FBC B}$, where the FBC of each drug (A or B) is defined as the ratio of the MBC of drug used in combination with the MBC of the drug tested alone. The combination is classified as synergistic when ΣFBC is ≤ 0.5 , partially synergistic when ΣFBC is > 0.5 to ≤ 1.0 , indifferent when ΣFBC is > 1.0 to 4.0 , and antagonistic when ΣFBC is ≥ 4.0 (Hall et al., 1983).

For the MRSA strain N315, time kill assays were performed using concentrations of NS and DDA BF (alone and combined) fixed at MBC value. Untreated cells were used as control. In brief, under identical experimental conditions (as above), aliquots of 50 μL of MRSA strain N315 (final concentration of 10^5 cells per mL) were incubated with 950 μL of MBC concentrations of DDA BF (2 and 4 $\mu\text{g/mL}$), NS (3, 25, and 50 $\mu\text{g/mL}$), or their combinations (NS/DDA 3/2 $\mu\text{g/mL}$) in IGP. The effect of NS and DDA BF alone and in

combination on cell viability was evaluated at 0, 1, 2, 4, 8, 12, and 24 h after incubation at $35 \pm 2^\circ\text{C}$, by plating. After incubation, aliquots of 10 μL of each sample were diluted 1:10, 1:100, 1:1,000, 1:10,000, and 1:100,000 and 50 μL of each dilution was plated on Mueller-Hinton agar. The plates were incubated aerobically at $35 \pm 2^\circ\text{C}$ for 24 h. Colony-forming units were counted and plotted ($\log_{10}$ cfu/mL) as a function of time (Balouiri et al., 2016).

Size distribution and surface charge of NS, DDA BF, MRSA N315, and their combinations were determined by the ZetaPals Zeta Potential Analyzer (Brookhaven Instruments Corporation, Holtsville, NY) equipped with a laser of 570 nm, and light scattering dynamic at 90° . Nisin, DDA BF, and the MRSA N315 strain were prepared at different concentrations and combinations (Table 2). Then, size distribution and surface charge were obtained after 1 h interaction at 25°C in IGP.

Staphylococcus species isolated from bovine mastitis displayed resistance to at least one antibiotic class, where 14/30 (47%) isolates (belonging to *S. aureus*, *S. chromogenes*, or *S. haemolyticus*) could be classified as multidrug-resistant because they were nonsusceptible to ≥ 1 agent in ≥ 3 antimicrobial categories (Magiorakos et al., 2012). The minimum bactericidal concentration that killed 50% of the bacteria analyzed (**MBC₅₀**) of NS and DDA BF against *Staphylococcus* spp. was 50 and 4 $\mu\text{g/mL}$, respectively. The checkerboard assay confirmed the in vitro activity of the combination of NS and DDA (NS/DDA complex), which resulted in a reduction of the **MBC₅₀** to 3/2 $\mu\text{g/mL}$. Determination of the ΣFBC revealed synergistic activity of the complex in 2.8% (1/36) of the isolates, partially synergistic in 52.8% (19/36), and indifferent in 44.4% (16/36; Table 1).

The time-kill assay shows that the bactericidal effect of the NS/DDA complex (3/2 $\mu\text{g/mL}$), against MRSA N315 occurred 1 h after interaction, with a reduction of $\geq 3 \log_{10}$ cfu/mL, whereas NS (50 $\mu\text{g/mL}$) and DDA (4 $\mu\text{g/mL}$) achieved bactericidal activity after 2 and 3 h interaction, respectively (Figure 1).

Under the condition studied [i.e., low ionic strength (IGP buffer)], whereas the MRSA N315 strain had a

negative charge, NS and DDA BF displayed a positive charge (Table 2). Moreover, the interaction between these molecules resulted in a cationic complex that promoted adhesion to the negative bacterial surface and favored the bactericidal action of the complex. This was evident when the interaction between NS/DDA complexes (at concentrations of 3/2, 30/20, and 300/200 $\mu\text{g}/\text{mL}$) and bacteria was analyzed after 1 h of incubation, where the observation of a change in the surface charge of bacterial cells from negative to positive, with further reduction of size distributions, suggested destruction of the bacterium. In this regard, previous studies have shown that alteration of zeta potential, in bacteria, induced by cationic agents such as

QAC and polymyxins, is correlated with the enhancement of membrane permeability, membrane damage, and changes in size distribution of bacterial cells, which closely correlate with bactericidal activity produced by these agents (Campanhã et al., 1999; Carmona-Ribeiro et al., 2006; Halder et al., 2015; Carrasco et al., 2016).

In the present study, NS displayed *in vitro* bactericidal activity against drug-resistant *Staphylococcus* spp. isolated from bovine mastitis, as well as against reference strains (Table 1). The US Food and Drug Administration Center for Veterinary Medicine has declared favorably regarding the use of NS for the intramammary treatment of subclinical mastitis (Field et al., 2012). With respect to DDA, in addition to its potent

Figure 1. Time kill curves of methicillin-resistant *Staphylococcus aureus* N315. Bacterial cells were incubated with minimum bactericidal concentrations (MBC) of dioctadecyldimethylammonium bromide (DDA) bilayer fragments (BF; 2 and 4 $\mu\text{g}/\text{mL}$), nisin (NS; 3, 25, and 50 $\mu\text{g}/\text{mL}$), or their combinations (NS/DDA 3/2 $\mu\text{g}/\text{mL}$), in isotonic glucose-phosphate buffer. The effect of NS and DDA BF alone and in combination on cell viability was evaluated at 0, 1, 2, 4, 8, 12, and 24 h after incubation. Colony-forming units were counted and plotted ($\log_{10}$ cfu/mL) as a function of time.

bactericidal activity against many microbial species including MDR bacteria, this versatile compound contains hydrophobic regions able to transport antibiotics, drugs, and antigens that can exert synergistic effects or exhibit bacterial properties themselves (Carmona-Ribeiro et al., 2006; Lincopan and Carmona-Ribeiro, 2006).

In the present study, the NS/DDA complex exerted a partially synergistic and an indifferent effect in 52.8 and 44.4% of the strains, respectively. Nevertheless, the combination of both drugs increased bacterial susceptibility to NS, reducing the MBC from 50 to 3 $\mu\text{g/mL}$. This activity was also observed against MRSA strains (Table 1). In fact, in the time-kill assay, a reduction $\geq 3 \log_{10}$ cfu/mL of *S. aureus* N315 was observed after a 1-h interaction with NS/DDA complex (3/2 $\mu\text{g/mL}$; Figure 1). In this regard, even when drug combinations do not result in synergistic activity, reductions in MIC are important because they suggest the possible administration of these drugs given adjustments in their dosage, thus permitting effective treatment and a reduction in possible side effects (Mitsugui et al., 2011).

Interesting results were also obtained for *S. sciuri* 7N and *S. aureus* ATCC BAA-977 strains (Table 1). Although these strains exhibited a higher MBC for the cationic lipid alone or in combination with NS, compared with the other strains, the NS/DDA combination displayed a partially synergistic effect, with a reduction in the MBC of both drugs.

It is well known that a combination of antimicrobial agents can prevent the development of resistance, reducing side effects (Rahal, 2006). On the other hand, another advantage of combining 2 drugs is the possibility of broadening the spectrum of action. In this respect, it has been demonstrated that, although NS generally acts against gram-positive bacteria, their combined use with conventional antibiotics increased antibacterial activity against gram-negative pathogens (Singh et al., 2013).

Adsorption of NS/DDA nanoparticles on bacterial cells may occur through electrostatic attraction between the cationic NS/DDA complex and the negative surface of bacteria. The negatively charged domains present in the structure of the bacterial cell wall favor this interaction. In this regard, previous studies have demonstrated a clear correlation between the adsorption of cationic vesicles that generate a positive charge on the bacterial cell surface and cell death (Campanhã et al., 1999). The NS/DDA complex probably reverses the cell surface charge and penetrates the bacterial cell wall. Through binding to lipid II, NS then forms pores in the cytoplasmic membrane, followed by membrane disorganization, leakage of intracellular low molecular weight material, and inhibition of cell wall synthesis

(de Kruijff et al., 2008; Field et al., 2012). The NS/DDA combination therefore resulted in a complex whose bactericidal effect was enhanced at much lower concentrations than NS and DDA BF alone.

The physicochemical interactions that occur in the NS/DDA complex were elucidated by light scattering and zeta potential analysis. First, we observed that NS has a flexible structure whose supramolecular arrangement is governed by different physicochemical conditions inherent to the molecule. Nisin contains positively and negatively charged AA, as well as hydrophobic and hydrophilic residues (de Kruijff et al., 2008). Thus, both solubilization and incorporation are pH dependent, a fact that could affect the acid dissociation constant (pK_a) of some residues. In the condition studied [i.e., low ionic strength (IGP buffer, pH = 7.0)], both size distribution and surface charge of the NS/DDA complex demonstrate that NS forms a cationic complex with DDA BF (Table 2). The solubilization of NS, an amphipathic molecule, probably occurs through electrostatic attraction between anionic AA present in the molecule and the cationic polar head of DDA BF. In addition, due to its hydrophobicity, NS can be directly incorporated into the hydrophobic portion of the DDA bilayer.

A limitation of this study was the lack of antibacterial evaluation of the compounds in the presence of milk, which may affect their activity and thus applicability as a treatment for bovine mastitis. In this regard, DDA belongs to the quaternary ammonium compounds (QAC), which constitute a large group of chemicals that are mainly used as antimicrobials and disinfectants (Carmona-Ribeiro et al., 2006). The dairy industry is probably the most important food-related field of QAC use, where they are used to disinfect udder and milking equipment with the goal of preventing mastitis and producing raw milk with low bacterial count, which is preferred both from a hygienic and economic perspective (Xian et al., 2016). On the other hand, NS anti-bacterial peptide has been demonstrated in clinical studies to be effective in the reduction of mastitis-causing organisms in dairy cows, whereas in vitro studies have revealed synergistic activity of NS in combination with antibiotics against *Staphylococcus* spp., even when present in biofilms (Ceotto-Vigoder et al., 2016; Field et al., 2016; Kitazaki et al., 2017; Singh, 2018). Because the application of NS and DDA BF in food systems could be influenced by inadequate physical conditions and chemical composition of food including pH, temperature, nutrients, and so on, a topical application of these compounds may be a more efficient method for supplying antibacterial properties. Moreover, topical preparations of NS/DDA can be used, and more easily accepted, as a form of drug delivery, where

the persistence and stability of NS and DDA BF on the surface of the teat skin is essential.

In summary, cationic NS/DDA nanoparticles are shown to be a promising treatment alternative for the control of mastitis caused by MDR *Staphylococcus* spp., supporting further in vivo investigations.

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