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Potent Bioactivity of Bacteriocin-producing LAB Strains against Multi-drug-resistant Pathogens Isolated from Dairy Cattle with Clinical Mastitis

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The high prevalence of antimicrobial resistance (AMR) among mastitis-causing pathogens underscores the urgent need for new types of antimicrobial agents. However, the slow discovery and development of antibiotics are failing to keep pace with the rapidly increasing AMR. Consequently, bacteriocins have emerged as a promising alternative antimicrobial agent, drawing heightened attention due to their natural and safe properties. Thus, this study evaluated the potential of bacteriocin-based formulation against multi-drug-resistant (MDR) Gram-positive and negative pathogens causing clinical mastitis among dairy cattle. The bioactivity of bacteriocin-producing LABs was tested against Gram-positive strains using a colony overlay assay, and the effectiveness of the bacteriocin nisin was assessed against Gram-negative pathogens in combination with membrane-permeabilizing agents chelator (EDTA), organic acids (citric and lactic acid), and surfactants (Triton-X and Tween 80). The combined inhibitory action of nisin and membrane permeabilizing agents was estimated by calculating the growth reduction after exposure of the test organisms to the membrane permeabilizing agents and bacteriocin. All bacteriocin-producing LAB strains demonstrated robust bioactivity when tested against 24 locally isolated Gram-positive mastitis-causing bacterial species. While combining 100 µM of the bacteriocin nisin with 40 mM EDTA significantly inhibited the growth of representative Gram-negative mastitis-causing strains, with complete inhibition observed at 500 µM nisin concentration. Pre-exposure to citric acid or lactic acid and at least 100 mM EDTA resulted in over double the growth reduction of *K. pneumoniae* AG-ES-14. Similar results were observed with the surfactants Triton-X and Tween 80 were sequentially used with bacteriocin treatment for this pathogen. Almost 100% growth reduction was achieved when nisin treatment was preceded by treatment at higher concentrations of EDTA (800 mM) and, combined with either surfactant or organic acid, led to almost complete growth reduction. The results of this study present promising opportunities for developing a powerful biocontrol agent to combat mastitis infections caused by MDR pathogens.

Keywords: antimicrobial resistance, bacteriocin, bacteriocin-producing LAB, mastitis, multi-drug resistance

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INTRODUCTION

Mastitis is a condition wherein there is an inflammation of the udder tissue in the mammary gland of dairy animals. It is the most prevalent disease in the dairy sector posing significant issues with animal welfare, food safety, and milk production. Mastitis can emanate from physical injury or microbial infections. Microorganisms causing mastitis are classified as either contagious or environmental. Commonly isolated Gram-positive mastitis pathogens *Staphylococcus* and *Streptococcus* are the major sources of contagious mastitis (Sharun *et al.* 2021). Environmental streptococci and coliforms such as Gram-negative *Escherichia* and *Klebsiella*, on the other hand, are the most common etiological agents of environmental mastitis. *Serratia*, *Pseudomonas*, and *Proteus* species are among the Gram-negative bacteria frequently detected in cases of intra-mammary infections. (Hogan and Smith 2012).

Bovine mastitis has traditionally been prevented and treated using antimicrobial agents. Thus, dependence on antimicrobials has become a widespread phenomenon in dairy farms (Cheng *et al.* 2019). However, the indiscriminate overuse of antibiotics in dairy animals leads to an increase in the antimicrobial resistance (AMR) of mastitis pathogens. In fact, the prevalence of multidrug resistance (MDR) or resistance to more than three antimicrobial classes in Gram-positive and negative mastitis pathogens has been globally investigated numerous times recently (Shrestha *et al.* 2021; Bag *et al.* 2021; Ahmed and Shimamoto 2011; Yang *et al.* 2020; Tartor *et al.* 2021). Moreover, the incidence of AMR in mastitis presents a difficulty not only for clinical management of the disease but also a public health problem, given the potential of AMR bacteria and its genetic factors to be transferred from animals to people *via* the food chain (Majumder *et al.* 2021).

As a result of rising AMR in mastitis pathogens and the slow discovery and development of new antibiotics that are unable to keep up with the rapidly increasing AMR, bacteriocin has drawn more attention as a natural and safe antimicrobial agent. Bacteriocins are proteinaceous compounds that are ribosomally synthesized by bacteria that have antimicrobial properties against bacteria that are related to the producing strain (Azhar *et al.* 2017). Typically, these peptides display robust thermal and bioactivity stability over a broad pH spectrum, commonly found in various food systems. Moreover, they lack sensory characteristics that could affect food quality (Perez *et al.* 2022). Therefore, bacteriocins, which are mostly produced from "generally recognized as safe" lactic acid bacteria (LABs), are considered suitable for use in food (Xu *et al.* 2021). Thus, the promising attributes of bacteriocin – including its impact on food safety and quality, as well as its effectiveness as an antimicrobial – position it as a potential biological agent against mastitis.

In recent studies, several Gram-positive bacteria and a significant portion of Gram-negative bacteria isolated from dairy cattle with mastitis were found to be virulent and MDR (Manzanilla and Pilapil-Amante 2021; Talebi *et al*. 2021; Perez and Ancuelo 2022; Ancuelo and Perez 2023a, b). Considering various antibiotics have been discovered to be ineffective in combating these virulent microorganisms, an alternate treatment must be employed to decrease and prevent mastitis occurrence. Therefore, this study aims to evaluate the potent bioactivity of bacteriocins from LABs – along with chelating agents, organic acids, and surfactants – against each representative previously isolated Gram-positive and Gram-negative pathogenic MDR mastitis pathogens, which can be used as a benchmark for future development of agents for the prevention and cure of this costly infection in dairy cattle.

MATERIALS AND METHODS

Bacterial Strains and Culture Condition

Mastitis-causing MDR isolates used in this study were cultivated in trypticase soy broth (Titan Biotech Ltd., India) with 0.6% yeast extract (Titan Biotech Ltd.) or TSBYE and kept at 37 °C for 24 h (Table 1). Bacteriocin-producing LABs were cultivated in deMan, Rogosa, and Sharpe (MRS) broth (Oxoid, UK) and kept at 30 °C for 24 h (Table 2). All bacterial cultures were preserved in 30% glycerol and stored at –80 °C. Prior to each use, they were thawed and revived through two rounds of cultivation.

Chemicals and Reagents

Ethylenediaminetetraacetate (EDTA) (Scharlab, Spain) (a strong chelator), surfactants Tween 80 (Scharlab) and Triton-X (Vivantis Technologies, Malaysia), and organic acids – namely citric acid (Wako, Japan) and lactic acid (Wako) – were used as membrane permeabilizing agents. The bacteriocin nisin A from *Lactococcus lactis* in 2.5% purity powder form (Sigma-Aldrich, Germany) was used. The antibiotic tetracycline (Sigma-Aldrich) was dissolved in Tris-HCl buffer (pH 7.4).

Bacteriocin Bioassay

Against Gram-positive bacterial strains. The bioactivity of bacteriocins toward Gram-positive indicator strains was evaluated using colony overlay assay, as described previously with some modifications (Kemperman *et al.* 2003). Briefly, active cultures of the bacteriocin-producing LABs were stabbed in an MRS agar plate

Table 1. Antimicrobial resistance and virulence profiles of selected mastitis-causing bacterial strains.

Mastitis-causing bacterial strain[1]	Antibiotic resistance[2]	MDR	Virulence profile		
			Phenotype		Genotype[3]
			Beta hemolytic	Biofilm	
Gram-positive					
Streptococcus agalactiae AG-JM-35	ERY, STR, PEN, CLI, MY, AML	Yes	+	+	*rib*, *lmb*, *cylE*
Str. equinus AG-AM-12	STR, CLI		+	+	
Staphylococcus aureus AU-OS-85	PEN		+	+	*nuc*
Staph. chromogenes AU-MS-79	ERY, TET, STR, PEN, CLI, MY, AML	Yes	+	+	
Staph. haemolyticus AG-FS-89	PEN		+	+	
Bacillus subtilis AG-MM-109	STR, PEN, MY	Yes	+	+	
B. macroides AU-KS-66	MY		+	+	
Lysinibacillus fusiformis AU-KS-65	ERY, STR, CLI, MY	Yes	+	+	
Gram-negative					
Proteus mirabilis AG-AS-4	ERY, TET, STR, PEN, CLI, MY	Yes		+	*ucaA*, *pmfA*, *atfA*, *ptA*, *zapA*, *hpmA*, *ureG*, *fliC*
Klebsiella pneumoniae AG-ES-14	ERY, TET, STR, PEN, CLI, MY, AML	Yes		+	*mrkD*, *entB*
Escherichia coli AG-EM-20	ERY, TET, STR, PEN, CLI, MY, AML	Yes	+	+	
Providencia stuartii AU-DS-49	ERY, TET, STR, PEN, CLI, MY, AML	Yes	+	+	*ireA*, *ucaA*, *atfA*, *zapA*, *ureG*
Wohlfahrtiimonas chitiniclastica AU-BS-39	ERY, STR, PEN, CLI, MY	Yes	+	+	

[1]Isolates from the laboratory collection as previously reported (Perez and Ancuelo 2022; Ancuelo and Perez 2023a, b, c)
[2][ERY] erythromycin; [TET] tetracycline: [STR] streptomycin; [PEN] penicillin; [CLI] clindamycin; [MY] lincomycin; [AML] amoxicillin
[3]*rib*, *lmb*, and *cylE* are known *Streptococci* virulence-associated genes; *nuc* is a known *Staphylococci* virulence gene; *ucaA*, *pmfA*, *atfA*, *ptA*, *zapA*, *hpmA*, *ureG*, *fliC*, *mrkD*, *entB*, and *ireA* are known Enterobacteriaceae virulence-associated genes

Table 2. Bacteriocin-producing lactic acid bacterial strains used in this study.

LAB strain	Bacteriocin[1]	Source
Lactococcus lactis BIOTECH 10612	Nisin A	PNCM[2]
L. lactis BIOTECH 10613	Nisin Z	PNCM
Lactiplantibacillus plantarum BS	Plantaricin	Lab collection
Enterococcus faecalis MGL-3	Two-peptide lantibiotic	Lab collection

[1]Bacteriocin identity was confirmed *via* DNA sequencing of the bacteriocin structural gene or whole genome of the producer strain
[2]Philippine National Collection of Microorganisms

using a sterile toothpick and incubated overnight at 30 °C. The stab cultures were then overlaid with molten TSBYE agar previously inoculated with 1% active culture of the indicator strains. The degree of sensitivity of the Gram-positive mastitis causing MDR strains toward the bacteriocin is manifested as the size of the inhibition zone around the stab culture after overnight incubation at 37 °C.

Against Gram-negative bacterial strains. Bacteriocins from LABs generally do not exert bioactivity against Gram-negative bacterial strains due to the presence of the lipopolysaccharide (LPS) membrane in their cell wall. In this study, the bioactivity of bacteriocins toward Gram-negative indicator strains was initially evaluated by monitoring the growth of the strains cultivated in its culture media containing different concentrations of the bacteriocin nisin A (100, 250, and 500 µM) and 40 mM EDTA, a strong chelator to disrupt the integrity of the LPS membrane of the Gram-negative bacterial cell wall, thereby making it vulnerable to the antimicrobial action of bacteriocin. Bacterial growth was monitored through the absorbance (OD_{600nm}) of the culture broth taken every 4 h using a UV-1800 UV/ Visible Scanning Spectrophotometer (Shimadzu, Japan). Tetracycline was used as positive control.

In order to further assess the potency of bacteriocin against Gram-negative pathogens, a quick exposure assay

was devised. Bacteriocin potency was expressed as log reduction and percent growth reduction of the viable cells after quick exposure to different concentrations of bacteriocins combined with membrane permeabilizing agents such as EDTA, organic acid, and surfactant. Briefly, the actively growing culture of the Gram-negative indicator strain was normalized to an optical density (OD_{600}) of 0.2 using sterile distilled water. The bacterial cell pellet was then collected by centrifugation at 13,500 rpm for 15 min and resuspended in different concentrations of the chelator EDTA (100, 400, and 800 mM). The combinational effect of bacteriocins with other membrane-disrupting agents such as surfactants (Triton-X and Tween 80) and organic acids (citric acid and lactic acid) was also evaluated. After disrupting the membrane, an aliquot of bacteriocin solution (500-µM final concentration) was then added to the bacterial suspension after it was left to stand for 30 min. After allowing it to react for another 30 min, the resuspension was plated in TSBYE agar and incubated overnight at 37 °C. A bacterial cell pellet resuspended in sterile phosphate buffer was used as the negative control. After CFU/mL was determined, log reduction, percent growth reduction, and percent survival were calculated accordingly using the formula:

$$Growth\ reduction = \frac{A - B}{A} \quad (1)$$

$$log\ reduction = log10(A) - log10(B) \quad (2)$$

where A represents the count of viable microorganisms before treatment, and B represents the count of viable microorganisms after treatment.

Statistical Analysis

All data are expressed as means from three replicates. The associations between the types of surfactants and different concentrations of chelator used were evaluated using two-way ANOVA at a 5% level of significance performed using the Microsoft Excel 2013 program (Microsoft Corp., Redmond, WA, USA).

RESULTS AND DISCUSSION

Sensitivity of Gram-positive Mastitis-causing Pathogens to Bacteriocins

All four bacteriocin-producing LAB strains when tested against 24 Gram-positive locally isolated mastitis-causing bacterial species exhibited potent bioactivity. *L. lactis* BIOTECH 10612, *L. lactis* BIOTECH 10613, *Lb. plantarum* BS, and *E. faecalis* MGL-3 showed very potent inhibitory activity against different pathogenic species of the genera *Streptococcus*, *Staphylococcus*, *Enterococcus*, *Bacillus*, *Lysinibacillus*, and *Clostridium* (Table 3). Noticeably, the nisin-producing strains BIOTECH 10612 and BIOTECH 10613 exhibited very potent inhibitory activities toward the indicator strains, as shown by the very large inhibition zones of up to 33 mm in diameter (Figure 1).

Bacteriocins from Gram-positive bacteria, including LAB, are generally known to exhibit potent inhibitory activity toward closely related organisms. The bacteriocin produced by both *L. lactis* strains BIOTECH 10612 and BIOTECH 10613 is a lantibiotic bacteriocin – namely nisin, which typically has a broader spectrum of activity across Gram-positive strains due to its dual mode of action inhibitory mechanism (Wiedemann *et al*. 2001; Hsu *et al*. 2004). Thus the potency of the bacteriocins from strains BIOTECH 10612 and BIOTECH 10613 toward all tested Gram-positive indicator strains is not surprising. Bacteriocins from other LAB strains exhibiting potent bioactivities toward distantly related genera are also not uncommon. In a study by Kondrotienė *et al.* (2018), all isolated strains of *L. lactis* isolated from goat and cow milk, fermented wheat, and buckwheat samples producing bacteriocin nisin A, Z, or novel variant GLc03 showed clear antagonistic activity against tested food spoilage and pathogenic bacteria, including *Listeria monocytogenes*. While the bacteriocin-producing *Lb. plantarum* SHY 21–2, isolated from yak yogurt, demonstrated antimicrobial activity against various microorganisms, including *S. aureus* (Peng *et al.* 2021). A bacteriocin synthesized by *E. faecalis* KT11, obtained from traditional Kargı Tulum cheese, also exhibited antagonistic efficacy against a range of Gram-positive bacteria such as *L. monocytogenes*, *S. aureus*, and *B. subtilis* (Abanoz and Kunduhoğlu 2018).

Sensitization of Gram-negative Mastitis Causing Pathogens toward Bacteriocins

As stated earlier, the presence of LPS in the outer membrane of Gram-negative bacteria cells prevents the bacteriocin molecule from electrostatically interacting with the bacterial cell wall, thereby effectively blocking the bacteriocin from exerting its inhibitory activity. The growth of the five selected Gram-negative mastitis-causing bacterial strains isolates *E. coli* AG-EM-20, *K. pneumoniae* AG-ES-14, *P. mirabilis* AG-AS-4, *P. stuartii* AU-DS-49, and *W. chitiniclastica* AU-BS-39 were significantly inhibited in the presence of at least 100-µM nisin when combined with 40 mM EDTA in the culture medium. At a higher bacteriocin concentration of 500 µM, the growth of all five pathogenic strains was completely inhibited (Figure 2). The growth of these strains in the culture medium containing EDTA or bacteriocin alone was not significantly inhibited with the exception of *P. stuartii* AU-DS-49, which showed lower when grown with 40-mM EDTA. Furthermore, these mastitis

Table 3. Sensitivity of local mastitis pathogens against the bacteriocin of different LABs using colony overlay assay method.

Local mastitis pathogens	Zone of inhibition (mm)			
	Bacteriocinogenic LAB[1]			
	A	B	C	D
Streptococcus agalactiae AG-JM-35	27	18	28	12
S. bovis AG-BM-41	10	11	6	6
S. equinus AG-AM-12	26	11	33	7
S. gallolyticus AG-BM-46	18	11	19	10
S. uberis AG-HS-29	15	15	16	15
Enterococcus casseliflavus AG-HS-28	16	14	16	10
E. durans AG-CM-53	5	10	5	6
E. faecalis AG-FS-90	9	10	8	7
E. faecium AG-IS-34	7	11	8	10
E. hirae AG-CM-58	10	11	6	6
Staphylococcus haemolyticus AG-FS-89	18	15	22	15
S. saprophyticus AU-OM-83	10	20	9	16
S. aureus AU-OS-85	10	19	12	13
S. agnetis AU-LM-69	15	19	15	20
S. arlettae AU-NS-80	20	18	21	17
S. chromogenes AU-MS-79	23	32	28	25
S. epidermidis AU-AF-9	13	22	14	12
S. simulans AU-AS-6	25	10	27	17
Bacillus subtilis AG-MM-109	11	23	12	16
B. macrolides AU-KS-66	10	15	11	15
B. megaterium AU-OS-84	6	11	8	15
Lysinibacillus fusiformis AU-KS-65	10	13	10	13
L. macrolides AU-AS-5	11	14	10	15
L. xylanilyticus AU-AS-2	8	13	9	14
Clostridium bifermentas AU-ES-14	10	8	10	10

[1][A] *Lactococcus lactis* BIOTECH 10612; [B] *Lactobacillus plantarum* BS; [C] *L. lactis* BIOTECH 10613; *Enterococcus faecalis* MGL-3

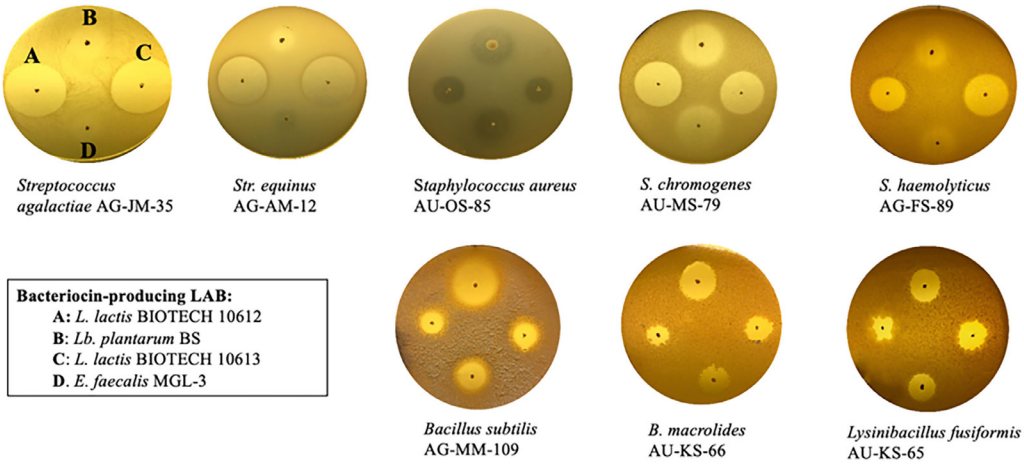

Figure 1. Bacteriocin-producing LABs showed potent bioactivity against different Gram-positive mastitis-causing multi-drug-resistant bacterial isolates.

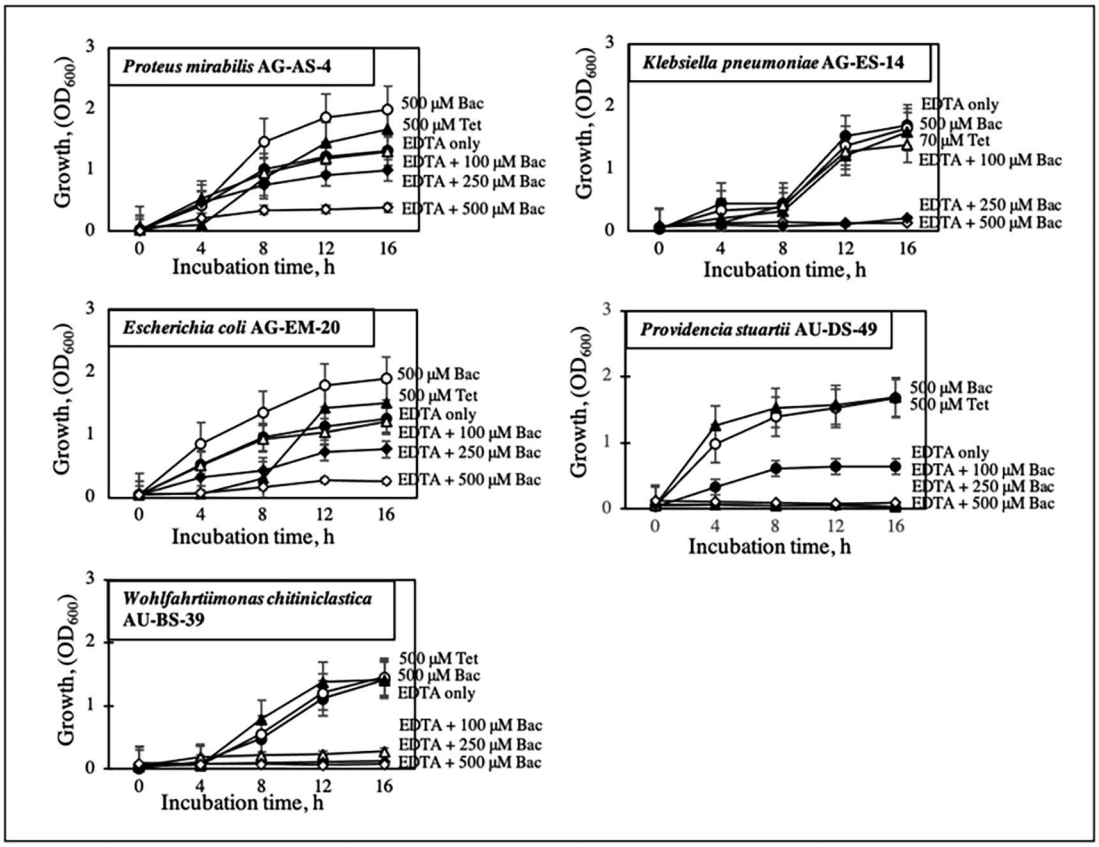

Figure 2. Bacterial growth curve of selected Gram-negative mastitis-causing multidrug-resistant (MDR) mastitis pathogens treated with different concentrations of the bacteriocin nisin A (indicated in the figure as Bac) combined with 40 mM of EDTA. The antibiotic tetracycline (Tet) was used as positive control.

pathogens exhibited strong resistance to tetracycline, as these isolates were not inhibited in the presence of the antibiotic up to 500 µM, highlighting its AMR nature. Only *K. pneumoniae* AG-ES-14 showed lower resistance to tetracycline but nonetheless still able to resist up to 70 µM of the antibiotic (Figure 2).

Although the growth of the Gram-negative strains was shown to be inhibited when the bacteriocin was combined with EDTA, the set-up of constant exposure of the pathogen with the growth inhibitory agents, *i.e.* the combination of bacteriocin and EDTA, is not doable in actual farm setting; hence, the short exposure assay was designed. In this experiment, the strain *K. pneumoniae* AG-ES-14 was used as the representative model strain. Results showed that even at very high concentrations of EDTA up to 800 and 500 µM of bacteriocin, the growth reduction of the Gram-negative strain *K. pneumoniae* AG-ES-14 was only at 78% (Table 4). This finding suggests that the membrane permeation caused by the chelator EDTA is not enough to enable the bacteriocin to interact with the bacterial cell wall for it to exert its bioactivity. However, it is also important to note that there is a significant relationship between different concentrations of the chelator used. In order to address this issue, other membrane permeabilizing agents such as organic acids and surfactants were combined with EDTA to improve the disruption of the LPS membrane of the Gram-negative indicator strain. As expected, the combined effect of EDTA and organic acid or surfactant significantly improved the potency of the bacteriocin. The exposure of the bacterial cells with the combined solution containing 2.5% of hydrous citric acid or lactic acid and at least 100 mM of EDTA prior to bacteriocin exposure (500-µM nisin) resulted in more than double growth reduction of the Gram-negative indicator strain *K. pneumoniae* AG-ES-14 (Table 4). A similar observation was also noted when EDTA was combined with a 2% hydrous solution of Triton-X and Tween 80 surfactants. When a higher concentration of EDTA (800mM) was combined with either surfactant or organic acid, almost 100% growth reduction was observed. The results also indicated a significant relationship between the types of surfactants used.

While the antimicrobial efficacy of many bacteriocins is primarily directed toward Gram-positive bacteria, it is widely recognized that nisin and other bacteriocins

Table 4. Growth inhibition of MDR *K. pneumoniae* after treatment with bacteriocin (500 µM nisin A) combined with membrane permeabilizing agents.

Membrane permeabilizing agent		Log_{10} reduction	% growth reduction
	EDTA (mM)		
Negative control	100	0.20	36.92
	400	0.37	57.69
	800	0.67	78.46
Organic acid[1]			
Citric acid	100	0.63	76.67
	400	1.00	90.00
	800	1.602	97.50
Lactic acid	100	1.398	96.00
	400	1.1447	92.833
	800	2.426	99.625
Surfactant[2]			
Triton-X	100	0.88	86.92
	400	1.35	95.54
	800	2.64	99.77
Tween 80	100	0.70	80.00
	400	1.00	90.00
	800	1.81	98.46

[1]Hydrous solution of organic acid 2.5% concentration
[2]Hydrous solution of surfactant at 2% concentration

can exhibit heightened potency against Gram-negative pathogens under conditions where agents potentially compromising the integrity of their outer membranes are present (Perez *et al.* 2014). It has been proven that EDTA and nisin increase the antimicrobial action against Gram-negative bacteria from different sources according to several studies. The antibacterial activity of nisin against common causative agents of foodborne diseases such as Gram-negative *E. coli* and *Salmonella* Typhimurium was improved by Na-EDTA (Khan *et al.* 2015). The lag phase of growth of *E. coli* strains of swine origin was extended with the use of nisin, essential oils such as cinnamaldehyde, and EDTA (Field *et al.* 2017). The combination of these substances may have facilitated simultaneous action on many sites in the bacterial cell membrane (Khan *et al.* 2015).

Other membrane permeabilizing agents such as organic acids and surfactants have also been demonstrated to enhance the bioactivity of inhibitory compounds such as bacteriocins toward Gram-negative strains. In a study by Burel *et al.* (2020), tribasic citric acid effectively reduced a high concentration of Gram-negative bacteria such as *K. aerogenes* and *E. coli*, resulting in membrane damage and loss in viability. The impact of lactic acid on the permeability of the outer membrane was also investigated in *E. coli*, *P. aeruginosa*, and *S. enterica* serovar Typhimurium, where significant permeabilization was observed in each species upon exposure to specific doses of lactic acid (Alakomi *et al.* 2000). Moreover, the incorporation of surfactants as additives has shown a significant effect on increasing the sensitivity of the outer membrane of Gram-negative bacteria. Brown and Winsley (1969) observed the direct impact of Tween 80 on the permeability barrier of *P. aeruginosa* cells. Triton-X was reported effective in lysing Gram-negative bacteria such as *E. coli* and *P. aeruginosa*, even at low concentrations (Miozzari *et al.* 1978). It was shown to compromise the outer membrane integrity of *Salmonella* Typhimurium as well (Miki and Hardt 2013). Thus, the above-mentioned observation on the improved inhibitory activity of bacteriocins when combined with organic acids and surfactants toward MDR Gram-negative strains highlights its strong practical implication toward its utility in controlling these MDR pathogens.

Potential of Bacteriocins in Controlling MDR Mastitis-causing Pathogens

The utility of bacteriocins as food-grade antimicrobial agents is well established. The expansion of its applications in different industries such as medical and veterinary has also been studied (Cotter *et al*. 2013). In this present study, the utility of a bacteriocin-based formulation as a biocontrol agent against MDR mastitis pathogens has been demonstrated. Most studies that have investigated the capabilities of bacteriocin-producing strains in addressing mastitis infections are focused on their bioactivity against Gram-positive pathogens. This is primarily due to the above-mentioned limitation of the bioactivity of bacteriocins that target only the Gram-positive strains. Bacteriocins synthesized by *B. subtilis* exhibited a lethal effect on biofilm-forming and mastitis-causing strains of *Staphylococcus* and *Streptococcus* species (Raheel *et al.* 2023). Bovicin HC5, a ruminal bacteriocin produced by *S. equinus* HC5, effectively suppressed the growth of two major mastitis-causing pathogens – namely *S. aureus* and *S. agalactiae* (Godoy-Santos *et al.* 2019). However, this present study clearly demonstrated that the MDR Gram-negative mastitis-causing pathogens can also be effectively inhibited by the bacteriocin-based formulation, *i.e.* bacteriocin combined with membrane permeabilizing agents such as chelator, surfactant, and organic acids.

It is worth noting that recent studies have shown that Gram-negative pathogens were predominant among the bacterial pathogens obtained from mastitis in dairy cattle in the Philippines (Ylagan *et al*. 2022; Perez and Ancuelo 2022; Ancuelo and Perez 2023a, b, c). Moreover, 100% of these Gram-negative pathogens belonging to Enterobacteriaceae were MDR pathogens (Table 1). Thus,

findings from this present study offer exciting prospects for formulating a potent biocontrol agent against mastitis infections caused by these MDR Gram-positive and Gram-negative pathogens.

Nonetheless, while bacteriocins show promise in controlling mastitis infections, the expensive nature of their high production has remained a major challenge in their actual application. Current production methods involve complex cultivation and purification processes are still resource-intensive, making it impractical in resource-limited settings and small-scale dairy operations. Thus, developing a low-cost production system for bacteriocins is imperative in order to materialize its utility as an anti-mastitis biocontrol agent.

CONCLUSION

This study highlights the potential of LAB-produced bacteriocins as effective agents against mastitis-causing pathogens. Bacteriocins from *L. lactis* BIOTECH 10612 and 10613, *Lb. plantarum* BS, and *E. faecalis* MGL-3 showed strong inhibitory activity against various Gram-positive bacteria. The combination of bacteriocins with EDTA, organic acids, and surfactants significantly improved their efficacy against MDR Gram-negative pathogens. The bacteriocin-based biocontrol formulation offers an alternative to conventional antibiotics, especially for cases with MDR pathogens, mitigating the spread of AMR, a growing concern in human and veterinary medicine. Its adoption could enhance animal welfare and productivity by reducing mastitis infections – leading to increased milk production, improved profitability for dairy farmers, and enhanced food security in the Philippines.

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