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In vitro Inhibition of Mastitis Pathogens by Bacteriocin RN 86 Produced by an Indigenous *Lactobacillus casei* Isolate

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Abstract: A broad-spectrum bacteriocin produced by *L. casei* RN 86 was a stable and relatively small molecule (5.5 KDa) being bactericidal against a range of mastitis-causing bacterial pathogens. The antimicrobial activity of the peptide against the sensitive cells was 10 fold increased (AU mL^{-1}) after partial purification by 20% ammonium sulphate precipitation. The partially purified peptide indicated to be bactericidal against *S. aureus* and *S. agalactiae* cells and killed approximately 10^6 to 10^9 of the actively growing sensitive cells within an hour of incubation. It caused rapid cessation of the growth of the sensitive strains within few minutes of contact, while almost 99.9% of the cells were killed within an hour of incubation with 10240 AU mL^{-1} of its concentrations. Owing to the wide spectrum activity, efforts were made to investigate the *in vitro* efficacy of this antibacterial compound in combination with a teat seal formulation, against mastitis pathogens. A new prototype formulation containing the bacteriocin RN 86 with or without minimum concentrations of antibiotics and tween 80, which were the active ingredients of a teat seal used for the treatment of bovine mastitis. The results showed synergistic effect of all three components in combination and were the most effective in controlling the growth of *Staphylococcus aureus* and *Streptococcus agalactiae* during *in vitro* experiments.

Key words: Bovine mastitis, *L. casei*, bacteriocins, bactericidal, antibiotics, teat seals, *in vitro*

INTRODUCTION

Bovine mastitis is one of the most persistent and costly diseases in dairy cows which is usually treated or prevented with intramammary antibiotic formulations (Meaney, 1977; Sears *et al*., 1992; 1995). Although normally the use of these antibiotics to control mastitis has been very effective but it has some disadvantages, including the appearance of residues in the milk of treated cows (Craven, 1987). As a result, milk is normally withheld for a period of time following antibiotic administration, with concomitant economic losses. The continued use of antibiotics even during the dry period in these cows, for either therapeutic or prophylactic purposes has some disadvantages, including the perceived connection to the emergence of antibiotic-resistant human pathogens (Van Veen *et al*., 1999). Thus it is very likely that the widespread use of antibiotics, particularly for prophylactic application would be restricted in the future. Such concerns have prompted the World Health Organization to issue recommendations on global programmes to reduce the use of antibiotic therapies for both human and animal applications in the future. As a consequence, there is now a growing requirement for effective alternatives to prevent and cure diseases including mastitis. To extend the range of therapeutic options, non-peptide antibiotics could be used in combination with cationic peptides such as bacteriocins. There are numerous reports which have indicated that broad-spectrum bacteriocins produced by lactic acid bacteria may provide valuable alternatives to antibiotics for the treatment of this disease. One such bacteriocin, nisin, which is produced by *Lactococcus lactis*, is effective against a wide range of gram-positive bacteria, including mastitis pathogens (Morgan *et al*., 2006; Broadbent *et al*., 1989; Ingham *et al*., 2003). Nisin in combination with lysostaphin (Oldham and Daley, 1991) has been shown to be an effective treatment for mastitis and is now the active ingredient of a teat wipe used for the prevention of mastitis.

In recent years a number of other bacteriocins besides nisin, has been isolated and characterized for their vast antibacterial potentials. One such bacteriocin is lacticin 3147, produced by *Lactococcus lactis* ssp. lactis DPC3147, which has been shown to be effective against all Gram-positive bacteria tested to date including mastitis-causing pathogens (Ryan *et al*., 1999). Owing to

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these facts, Meany *et al.* (2001) formulated a new prototype formulation containing lacticin 3147 and teat seal, which was shown to be effective in controlling *Streptococcus dysgalactiae* and *Staphylococcus aureus* using experimental infection models both in non-lactating and lactating dairy cows. In this study we describe the development and formulation of a teat seal and bacteriocin RN 86 used in combination for the effective prevention of mastitis pathogens in *in vitro* conditions only.

MATERIALS AND METHODS

Bacterial strains and growth conditions: A bacteriocin producing strain of *L. casei* RN 86 isolated from a chicken intestinal specimens (unpublished data) was grown in MRS (deMan Rogosa and Sharpe, Himedia India) broth media at 37°C. The mastitis causing indicator strains (standard ATCC; American type culture collection, RTCC; Razi type culture collection; or local isolates R) listed in Table 1 were grown in Brain Heart Infusion broth (Merck, Germany) at 37°C for 24 h. The cultures were maintained at -70°C with glass beads in small vials containing BHI broth and 5% glycerol and were sub-cultured twice on specific media before use.

Antimicrobial assay: The test for detection of antagonistic activity was performed by agar well diffusion method described by Toba *et al.* (1991). The solidified agar plates were overlaid with 5 mL semisolid media seeded with 100 μL indicator organism (approximately 10^7 cells). Wells of 5 mm diameter were punched into these plates by using a sterile cork borer and 100 μL of the neutralized (3M NaOH) culture supernatant fluid of the producer strain collected by centrifugation (10,000 rpm,15 min at 4°C) was added to each well. All the plates were incubated at 4°C for 2-4 h prior to overnight incubation at 37°C and observed for the presence of zone of inhibition around the wells. The extent of inhibition was estimated by measuring the zone diameter in millimeters.

Table 1: Inhibitory spectrum of bacteriocins RN86 against mastitis pathogens

No.	Indicator orgs	Gram reaction	Inhibitory activity	AUmL^{-1}
1	*B. cereus ATCC 6464*	G+ve	-ve	-
2	*L. ivaovii RTCC 1331*	G+ve	-ve	-
3	*L. monocytogenes ATCC1315*	G+ve	+ve	640
4	*S. aureus RTCC 1263*	G+ve	+ve	320
5	*S. aureus ATCC 12601*	G+ve	+ve	1280
6	*St. agalactieae RTCC 1291*	G+ve	+ve	320
7	*S. agalactieae R 011*	G+ve	+ve	320
8	*S. pyogenes R 210*	G+ve	+ve	2560
9	*S. epidermidis ATCC 29887*	G+ve	+ve	1280
10	*S. dysagalactieae R41*	G+ve	+ve	640
11	*S. fecalis R 312*	G+ve	-ve	-

+ ve = Zone of inhibition, -ve = No zone of inhibition

Critical dilution assay: The method described by Mayr-Hartings *et al.* (1992) was followed. A two-fold dilution of the supernatant fluid from the producer organism was made in MRS broth and the remaining activity in each dilution was determined by agar well diffusion assay. The highest dilution showing antibacterial activity was recorded and the reciprocal of this dilution was expressed as arbitrary units per milliliter (AU mL^{-1}).

Survival assay: A two fold dilution of neutralized culture supernatant of RN 86 was prepared in MRS broth and 50 μL of overnight culture of *S. aureus* R1 and *S. agalactiae* R1 as an indicator were added to each dilution, respectively and incubated at 37°C for 2 h. At every 30 min of time intervals the absorbance (OD) at 660 nm, the viable count cfu mL^{-1} and the remaining antibacterial activity in each dilution was assessed.

Partial purification of bacteriocin RN 86

Ammonium sulphate precipitation: The antimicrobial compound produced by the strain (RN 86) was partially purified by four rounds of 20% ammonium sulphate precipitation. After subjecting the neutralized cell free supernatant fluids of RN 86 to increasing concentrations of ammonium sulphate salts at 4°C, the precipitates were collected by centrifugation at 10,000 rpm (4°C, 15 min). Remaining salts was removed from the precipitates by dialysis against distilled water. The titer (AU mL^{-1}) in the precipitate at each step was determined by critical dilution assay described earlier.

SDS-PAGE analysis: The partially purified antimicrobial peptide was analyzed by subjection to SDS-PAGE (Schagger and von Jagow, 1987). The gel was run for two hours at 60 V and later assayed for molecular weight estimation. A molecular weight marker kit (Invitrogen) was used for standard protein.

Antibacterial activity of partially purified bacteriocin RN 86: The antagonistic effect of the partially purified bacteriocins against bovine mastitis pathogens was evaluated by agar well diffusion assay. The mastitis causing bacterial pathogens were used as indicator organism and the AU mL^{-1} of the bacteriocins active against these pathogens were determined by critical dilution assay. Sensitivity of the results was determined from triplicate experiments and by measuring the zone diameters and the titer.

Synergistic effect of bacteriocin RN 86 and teat seal preparations: A liquid preparation of bacteriocin RN 86 was prepared and blended with teat seal ingredients. The

formulation containing bacteriocin RN 86 with or without minimum concentrations of streptomycin (35 mg mL^{-1}) and or Tween 80 (2% w/v) was mixed to form an emulsion before evaluation. The teat seal preparations were carefully dispensed into the wells so that the seals were in contact with the walls of the wells. The efficacy of the teat seal containing the bacteriocin was assessed *in vitro* by challenge against mastitis pathogens using agar well diffusion method. The impact of the bacterial challenge was manipulated by increasing the number of colony forming units of the sensitive strains inoculated in the semisolid agar or by increasing the concentrations of the bacteriocins AU mL^{-1} introduced into the teat seal preparations. A commercially available teat seal, Penstreson MC (containing Penicillin G procain 300,000 IU, Dihydro streptomycin 300 mg and Dexamethasome 1.5 mg (Kimia Biotechnology Co. Iran) was also used along the experiments for comparisons. Different concentrations of streptomycin, Tween 80 and bacteriocin RN 86 were assessed individually and in combination for evaluating their synergistic antimicrobial potential.

RESULTS

The bacteriocin RN 86 possessed wide inhibitory spectrum against a number of gram positive bacteria against which tested in this study (Fig. 1) of these pathogens Streptococci and Staphylococci were the most dominant bacteria evaluated for their sensitivity to bacteriocin RN 86 (Table 1). To determine the exact concentration of the bacteriocins effective against the pathogens the critical dilution assay was performed and the inhibitory agents were quantified in AU mL^{-1}. The highest dilutions giving noticeable zone of inhibition were defined as its titer and the reciprocal of the dilutions as one arbitrary unit per milliliter AU mL^{-1}. Figure 2 indicates the titer of RN 86 bacteriocin against the indicator cells. The results showed *S. aureus* (RTCC 1263) and a locally isolated *S. agalactiae* (R 011), to be the most sensitive of all indicator strains used in study. Minimum concentrations of only 320 AU mL^{-1} of the bacteriocin inhibited the growth of these pathogens within an hour of incubation. These two mastitis pathogens were used throughout studies as indicator cells.

Efforts to partially purify the bacteriocin by ammonium sulphate precipitation, by subjecting the supernatant fluids to four rounds of 20% ammonium sulphate salt (4°C) resulted in a gradual increase in the antimicrobial activity after each round of precipitation. All the activity was restored in the pellet with no activity in the supernatant fluid at 80% of saturation. The data presented in Table 2 indicates a 50 fold increase in purification and a recovery of about 28% at the end of fourth round of precipitation. The inhibitory activity of bacteriocin RN 86 against the sensitive cells was at its maximum at 102400 AU mL^{-1}.

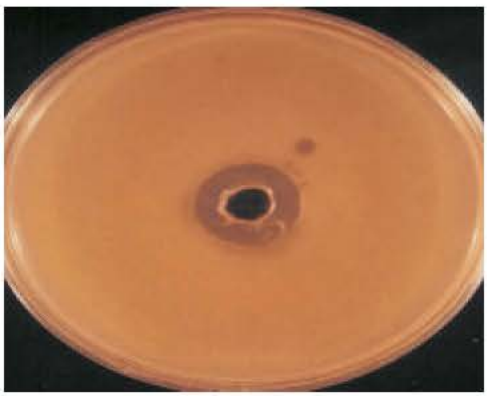

Fig. 1: Antibacterial effect of bacteriocin RN 86 against *S. aureus* by agar well diffusion assay

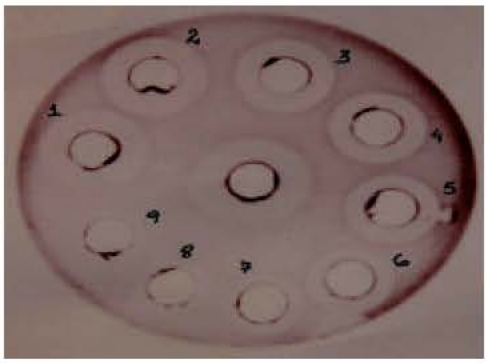

Fig. 2: The antibacterial titer of bacteriocin RN 86 against *S. aureus*, in AU mL^{-1} determined by critical dilution assay

Table 2: Recovery of bacteriocin RN 86 during ammonium sulphate precipitation

Purification steps	Volume (mL)	Total protein (mg)	Total activity (AU)	Specific activity (sp) (AU mg^{-1})	Increase in sp activity	Yield (%)
Conc. supernatant	100	2160	1024000	474.0	1.0	100
NH_4-SO_4	9	27	92160	3413.3	7.2	9
Dialyzed precipitate	7	12	286720	23893.0	50.4	28

The SDS-PAGE analysis of the partially purified sample confirmed the presence of unique band which approximated 5.5 KDa by Coomassie blue tinction of the gel.

Figure 3 shows the survival of *S. aureus* cells in the presence of different concentrations (AU mL^{-1})

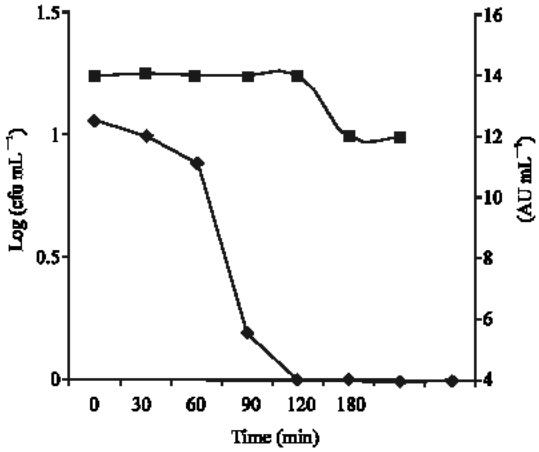

Fig. 3: Influence of bacteriocin RN 86 on viability cfu mL^{-1} of sensitive *S. aureus* cells. (A) indicates the decrease in activity after addition of sensitive cells to the culture supernatant of RN 86. (B) indicates the concentration of bacteriocin in the fluid

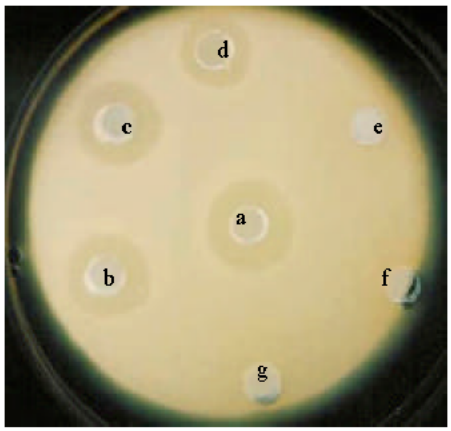

Fig. 4: Synergistic effect of bacteriocin RN 86 in a teat seal formulation against *S. agalacteae*. (a) Bacteriocin RN 86 + Streptomucin + Tween 80 (b) Bacteriocin RN 86 + Tween 80 (c) Bacteriocin RN 86 + Streptomycin (35 mg) (d) Bacteriocin RN 86 (e) Streptomycin (f) Tween 80 (g) Negative control

of the bacteriocin RN 86. A decline in the viable count (cfu mL^{-1}) of *S. aureus* was observed with the increasing concentrations of the bacteriocin, while inversely, when the concentration of the bacteriocin was decreased the number of viable colonies of indicator cells increased significantly. Bacteriocin RN 86 was shown to cause rapid cell death when added to actively growing cells of the sensitive organisms. This bactericidal mode of action of RN 86 was examined using *Streptococcus agalactiae* and *S. aureus* and addition of 640 AU mL^{-1} of the bacteriocin to approximately 10^6 to 10^7 of the actively growing sensitive cells caused a rapid loss of viability in comparison to control cultures to which no bacteriocin was added. Moreover, by adding increasing concentrations of bacteriocin RN 86, an increased rate of this killing effect was observed and only 3 of 10^6 *S. aureus* cells survived exposure to 10,240 AU mL^{-1} after 1 h of exposure.

The partially purified bacteriocin RN 86 was active against mastitis pathogens at its minimum concentration of 320-640AU mL^{-1} of activity. To evaluate the efficacy and synergism of bacteriocin in teat seal preparations, the bacteriocin RN 86 was mixed with the minimum doses of antibiotic (streptomycin 35 mg mL^{-1}) and tween 80; the active ingredients of the teat seal. After addition of this emulsion to the growth of sensitive cells the growth was inhibited and a clear zone of inhibition around the wells in the agar-well diffusion assay was observed (Fig. 4). Results indicated the synergistic effect of bacteriocin RN 86 and streptomycin as in combination both could inhibit most of the mastitis pathogens against which tested. When used individually, high concentrations of streptomycin (250 mg) was able to kill the growth of pathogens and lower doses were ineffective. The synergistic effect was significantly enhanced in the presence of 2% w/v Tween 80 and the formulation proved to be effective in controlling the growth of bovine mastitis causing Staphylococci and Streptococci. The same concentrations of the antibiotic and tween 80 were almost insignificant in their activity when examined individually against the pathogens.

DISCUSSION

Antimicrobial peptides provide a new structural class of highly active antimicrobial agents and offer a new resource for the development of novel antimicrobial agents (Van Veen *et al*., 1999). These peptides, including both cationic and neutral peptides, are secreted from a number of gram positive and gram-negative bacteria. They have been classified within the bacteriocins and are reported to be inhibitory peptides having wide spectrum of activity and thus possibly have a broader application (Hancock and Chapple, 1999; Cleveland *et al*., 2001; Minahk *et al*., 2004).

The development of non-antibiotic formulations for the prevention of mastitis in cows has the potential to reduce the dependence on antibiotics for prophylactic therapies in the future (Ryan *et al*., 1999). It has been well documented that some mixtures have synergic interactions; nevertheless the mechanisms of these

positive interactions appear to be complex and are not fully understood (Giacometti *et al*., 2000). The trials we report here form the part of a more comprehensive data set on a non-antibiotic approach to mastitis prevention in milking cows. Given that *L. casei* RN 86 bacteriocin was effective in inhibiting various mastitis pathogens, the potential for incorporating this bacteriocin into a teat seal and their synergistic effect in the formulation was investigated. When the partially purified bacteriocin was mixed with a teat seal preparations (minimum concentrations of streptomycin and tween 20), it proved to be effective in controlling mastitis pathogens under *in vitro* conditions.

A number of researches reported that broad-spectrum bacteriocins produced by lactic acid bacteria may provide valuable alternatives to antibiotics for the treatment of diseases (Delves-Broughten, 1990; Meaney *et al*., 2001). Up to date one such bacteriocin, nisin, which is produced by *Lactococcus lactis*, effective against a wide range of gram-positive bacteria, includes mastitis pathogens (Wirawan *et al*., 2006; Broadbent *et al*., 1989). Using nisin as an alternative to antibiotics does not compromise any human applications and in addition is readily degraded by digestive enzymes, therefore, nontoxic (Oldham and Daley, 1991). Sears *et al*. (1992, 1995) also showed that nisin is an effective treatment for mastitis, with cure rates of 66, 95, and 100% reported for animals infected with *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus uberis*, respectively.

Similar to the conclusions made by the researchers regarding nisin and lacticin 3147 (McAuliffe *et al*., 1998; Guerra and Castro, 2002), bacteriocin RN 86 exhibited a broad spectrum of inhibition against gram-positive bacteria especially mastitis pathogens. The sensitivities of 15 mastitis causing pathogens to the bacteriocins RN 86 showed that it inhibited maximum strains against which tested, although their sensitivities varied considerably as seen by their zone diameters and activity in AU mL^{-1}. RN 86 bacteriocin was shown to cause rapid cell death when added to actively growing cells of sensitive organisms. Moreover, by adding increasing concentrations of bacteriocin, an increased rate of this killing effect was observed indicating that the residual populations are not resistant and can be killed by merely increasing the bacteriocin concentration. Thus it could be suggested that the killing rate of sensitive cells is directly proportional to the bacteriocin RN 86 concentrations. Similar observations were made by Coakley *et al*. (1997) who also reported the bactericidal mode of action of lacticin on *S. aureus*.

The main advantages of bacteriocin RN 86 containing teat seal appeared to be twofold; first, the seal itself provides an effective physical barrier against infection at the teat orifice and second, the bacteriocin can inhibit any infectious gram-positive microorganisms which have the potential to evade the teat seal.

To conclude we might suggest that although bacteriocin RN 86 might not be considered a complete alternative or replacement of the non-peptide antibiotics, but could provide an effective protection against mastitis pathogens by significantly decreasing the concentrations or the dose of antibiotics used for the treatment of this disease in milking cows. However to prove our findings it is obligatory to test the performance of the developed teat seal formulation against the mastitis pathogens in *in vivo* conditions. The results could form the basis of an improved treatment for the prevention of mastitis in cows in near future.

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