

## EFFECT OF THE BACTERIOCIN PRODUCED BY *LACTOCOCCUS LACTIS* SUBSP. *LACTIS* CCSUB202, ON MODE OF ACTION OF *LACTOCOCCUS LACTIS* SUBSP. *LACTIS* MTCC3038

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**ABSTRACT:** *In the present study we have check mode of action of bacteriocin produced by Lactococcus lactis subsp. lactis CCSUB202 isolated by Indian cheese. The cells of indicator bacterium Lactococcus lactis subsp. lactis MTCC3038 was mixed with four different concentrations of bacteriocin viz. 400, 4000, 40000 and 400000 AU/ml, which were then incubated at 37°C. Growth was monitored at 2-h intervals by determining the OD<sub>600</sub> and by viable cell counts (cfu/ml). The results indicated that addition of higher concentration of bacteriocin brought complete destruction of L. lactis subsp. lactis MTCC3038 in less than 30 min as no survivors could be detected when 0.1 ml undiluted sample was spread on MRS plates. The bacteriocin added at lower concentration viz. 400 and 4000 AU/ml resulted in about 3 and 3.2 log cycle reductions, respectively in the first half-an-hour of addition reaching to 4 and 4.3 log cycle reductions at the corresponding concentrations at the end of 4 h incubation. It may be concluded that bacteriocin of L. lactis subsp. lactis CCSUB202 exhibit a bactericidal non-bacteriolytic mode of action. The indicator cells that survived the bacteriocin treatment were, however, found to be sensitive to the bacteriocin.*

**KEY WORDS:** Bacteriocin, Cheese, *Lactococcus lactis* subsp. *lactis*, Mode of action.

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### INTRODUCTION:

Lactic acid bacteria produce a wide variety of proteinaceous antimicrobial agents named bacteriocins. Bacteriocins are bacterially produced peptide antibiotics with the ability to kill a broad or a wide range of bacteria, usually but not always those that are closely related to the producer bacterium and synthesized ribosomally by

bacteria (Kumari and Garg, 2007; Kumari *et al.*, 2008). The potential application of bacteriocins of lactic acid bacteria as food preservatives requires an in-depth knowledge of how they exert their bactericidal effect. Most bacteriocins whose primary mode of action is known act at the plasma membrane. It has been proposed that these peptides form poration complexes that traverse the phospholipid bilayer. This provokes membrane permeabilization and hence depletion of the proton-motive force of sensitive cells (Ojcius and Young, 1991; Driessen *et al.*, 1995; Abee, 1995). Bacteriocins show a bactericidal mode of action against closely related species (Tagg *et al.*, 1976). These substances are of particular interest as they are proteinaceous and may thus be degraded during digestion in humans and other animals. Many of The lactic acid bacteria (LAB) produce bacteriocins (Klaenhammer, 1988; Piard *et al.*, 1992).

Most bacteriocins from lactic acid bacteria exert their antibacterial effect by permeabilizing the target cell membrane, whereby the cells lose their viability (Abee, 1995; Bruno and Montville 1993; Moll, *et al.*, 1998). Apart from damaging cell membranes, some bacteriocins have also been reported to cause bacteriolysis. Bierbaum and Sahl (1987) were among the first to show the involvement of autolysins in the bacteriolytic effect of a bacteriocin. Autolysins are peptidoglycan hydrolases that are capable of causing bacterial autolysis (Shockman and Hölte, 1994).

### MATERIALS AND METHODS

#### Isolation of bacteriocin producing bacteria

The bacteriocin producing strain used in this study was strain CCSUB202 isolated from Indian cheese by plating method. Briefly, Cheese samples were prepared by transferring 10 g of aseptically weighed sample to 100 ml sterile 2 % sodium citrate solution at 45 to 50°C and homogenized for 3 minutes. Plates of MRS agar were spread with 0.1ml portion of prepared cheese

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sample, and were incubated at 37°C for 24 h. The isolates were identified to genus level by Gram staining (Harrigan and McCance, 1993), observing growth at 10, 15 and 45°C and in saline MRS broth (4 and 6.5% NaCl w/v) by visual turbidity after 72 h incubation. Other tests included measurements of activities of catalase (Harrigan and McCance, 1993), oxidase (Collins and Lyne, 1987) and nitrate reductase and gas production from glucose in sugar basal medium (SBM) broth containing 2% (w/v) glucose dispensed in test tubes containing inverted Durham tubes. The salt tolerance test was done using MRS broth containing 6.5 (w/v) NaCl with incubation time of 4 days at 37°C. Identified to species level by Carbohydrate (glucose, sucrose, raffinose, fructose) fermentation test was done, as above. Arginine deamination was detected in SBM supplemented with 1% (w/v) arginine monochloride, 0.3% (w/v) Bacto-agar and 0.01% (w/v) phenol red, pH 7.2. After inoculation the medium was incubated at 37°C for 3 days. Arginine hydrolysis was observed by the culture turning yellow. *Lactococcus lactis* subsp. *lactis* MTCC3038 collected from the Institute of Microbial Technology (IMTECH), Chandigarh India and were maintained in 30 % sterile skimmed milk in small vials of 2 ml, at - 20°C for further experiments.

### Preparation of culture supernatants

100 ml of Soya nutri nuggets extract broth (SNNEM, Kumari *et al.*, 2008) was inoculated with 1 ml of bacteriocin producing strain and incubated for 24 h at 37°C. Cell free supernatant (CFS) was obtained by centrifuged the culture at 15,000 rpm for 30 min at 4°C in the high-speed refrigerated centrifuge (Sigma 2K15). The resulting CFS was then filtered through a sterile 0.2µm pore size filter (Millipore). CFS thus obtained was stored in vials, at -20°C.

### Determination of antimicrobial spectrum and bacteriocin activity assay

Inhibitory activity titers against the indicator bacteria were determined by agar well diffusion assay as described by Schillinger and Lucke (1989). MRS broth, 30 ml containing 0.75% agar, was inoculated with 10<sup>7</sup> CFU/ml of *L. lactis* subsp. *lactis* MTCC3038, poured into 100 X 15 mm standard Petri dishes and allowed to gel for 30 min at room temperature. Wells of 4 mm in diameter were cut and 80 µl of CFS of the bacteriocin producing strain CCSUB202 were placed into each well. All plates were then incubated at 37°C for 24 h and examined for formation of inhibition zones. Inhibition was scored positive if the width of the clear zone around the well was = 0.5 mm. to quantify the bacteriocin activity; CFS was serially diluted with sterile deionized water and 80 µl of each dilution were added into the wells. The

antimicrobial activity was defined as the reciprocal of the highest dilution, which exerted total inhibition of the indicator lawn and was expressed in activity units (AU) per milliliter (Zamfir *et al.* 1999).

### Determination of mode of action of bacteriocin

The action of the bacteriocin on the resting cells *Lactococcus lactis* subsp. *lactis* MTCC3038 was determined as follows. *Lactococcus lactis* subsp. *lactis* MTCC3038 was grown in MRS broth for 24h at 37°C, centrifuged at 15,000 rpm for 15 min at 4°C, and the cell pellet washed twice with 5mM phosphate buffer (pH 7) to remove any media ingredients. The cell pellet obtained from 1.0 ml MRS broth as described above was dissolved in 1.0 ml of 0.5 M phosphate buffer (pH 7) containing four different concentrations of bacteriocin viz. 400, 4000, 40000 and 400000 AU, which were then incubated at 37°C. Growth was monitored at 2-h intervals by determining the OD<sub>600</sub> and by viable cell counts (cfu/ml). The control sample without bacteriocin and the sample with 4,00,000 units of bacteriocin were also monitored for changes in the optical density values at wavelength viz. 600 and 260 nm after 0, 1, 2 and 4 h.

The surviving cells were grown in MRS broth for 24 h and assessed for bacteriocin sensitivity by well plate method.

## RESULTS AND DISCUSSION

### Isolation and Identification of bacteriocin producing strain

From Indian cheese 27 bacteriocin producing strains were isolate. Strain number CCSUB202 showed highest antibacterial activity against test strain *Lactococcus lactis* subsp. *lactis* MTCC 3038. We selected for detail study. All isolates were identified compared to the Bergey's manual of systematic bacteriology (Holt, *et al.*, 1994). Bacteriocin producing strain CCSUB202 belonged to the species *Lactococcus lactis* subsp. *lactis*. Table 1 and Figure 1 shows antimicrobial spectrum of bacteriocin produced by *Lactococcus lactis* subsp. *lactis* CCSUB202.

TABLE 1. Antimicrobial activity of bacteriocin produced by *Lactococcus lactis* subsp. *lactis* CSUB202 against different test organisms after 24h at 37°C. Antibacterial activity was determined by agar well diffusion method. Formation of a well-defined zone inhibition around the well containing the test sample was measured. R=resistant and S=sensitive.

TEST ORGANISM	ANTIBACTERIAL ACTIVITY			
	Zone of Inhibition (mm)		Mean (mm)	Inference
<i>Lactococcus lactis</i> subsp. <i>lactis</i> MTCC3041	11.0	13.0	12.0	S
<i>Lactococcus lactis</i> subsp. <i>lactis</i> MTCC3038	14.5	15.5	18.0	S
<i>Bacillus subtilis</i> MTCC441	15.0	17.0	16.0	S
<i>Streptococcus pneumoniae</i> MTCC1935	0.0	0.0	0.0	R
<i>Salmonella typhi</i> MTCC734	13.0	12.0	11.0	S
<i>Listeria monocytogenes</i> MTCC657	23.0	21.0	22.0	S
<i>Enterobacter faecalis</i> MTCC439	0.0	0.0	0.0	R
<i>Listeria monocytogenes</i> MTCC1143	18.5	18.5	17.0	S
<i>Staphylococcus aureus</i> MTCC96	12.0	14.0	16.0	S

FIGURE 1. Antimicrobial activity of bacteriocin produced by *L. lactis* subsp. *Lactis* CCSUB202 against pathogens (Fig 1a) and *L. lactis* subsp. *lactis* MTCC3038 (Fig.1b) at 37°C after 24 h (pH-6.5). (Fig. 1a: A- *Bacillus subtilis*, B- *Listeria monocytogenes*, C- *Bacillus mycoides*, D- *Bacillus cereus*).

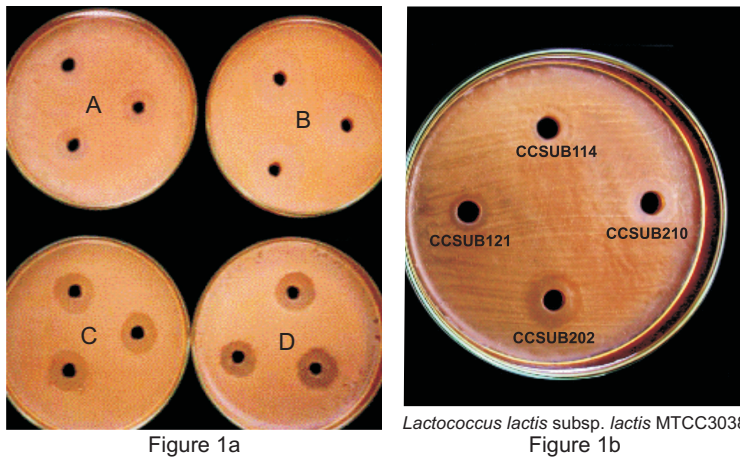


Figure 1a

Lactococcus lactis subsp. lactis MTCC3038  
Figure 1b

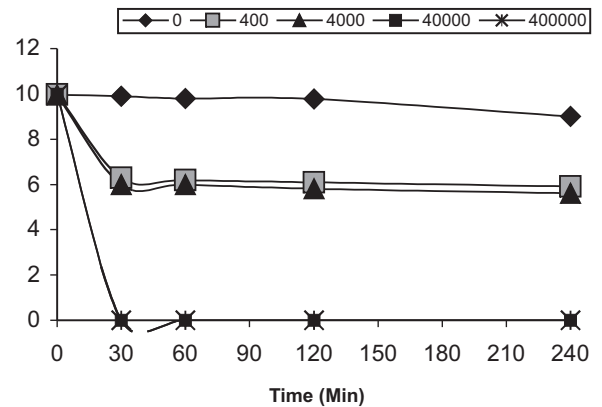
#### Mode of action of bacteriocin

The results regarding the mode of action of bacteriocin on the cells of *L. lactis* subsp. *lactis* MTCC3038 is depicted in Figure 2. It may be discerned that addition of higher concentration of bacteriocin brought about complete destruction of *Lactococcus lactis* subsp. *lactis* MTCC3038 in less than 30 min as no survivors could be detected when 0.1 ml undiluted sample was spread on MRS plates. The bacteriocin added at lower concentration viz. 400 and 4000 AU/ml resulted in about 3 and 3.2 log cycle reductions, respectively in the first half-an-hour of addition reaching to 4 and 4.3 log cycle reductions at the corresponding concentrations at the end of 4 h incubation period at 37°C. The control sample without any bacteriocin showed a 0.7 log cycle reduction in the viable cell counts at the end of the 4 h incubation period. The difference in the viable cell counts between control and experimental (bacteriocin added at a concentration of 400 and 4000 AU/ml) samples were observed to be 3.3 and 3 log cycle after 4 hr of incubation.

TABLE 2. Changes in the optical density values of *Lactococcus lactis* subsp. *lactis* MTCC3038 incubated with bacteriocin in phosphate buffer (pH 7) at 30°C.

SAMPLE	OPTICAL DENSITY AT 600 NM				OPTICAL DENSITY AT 260 NM			
	0 hour	1 hour	2 hour	4 hour	0 hour	1 hour	2 hour	4 hour
Phosphate buffer + bacteriocin	0.062	-	0.06	0.061	1.73	-	1.71	1.73
Phosphate buffer + cells	0.68	0.64	0.63	0.67	1.95	1.92	1.96	1.92
Phosphate buffer + bacteriocin + cells	0.65	0.62	0.60	0.64	3.32	3.32	3.46	3.46

FIGURE 2. Effect of the *L. lactis* subsp. *lactis* CCSUB202 bacteriocin on the resting cells of *L. lactis* subsp. *lactis* MTCC3038 at 37°C.



To find out any lysis or leakage of susceptible cells due to the activity of the bacteriocin, changes in the absorbance values of the cell suspension ( $5 \times 10^3$  cells) were monitored at two different wavelengths viz. 260 nm and 600 nm during the 4 hr incubation period at 37°C. It may be seen from Table 2 that optical density values of the bacteriocin in phosphate buffer (40,000 AU/ml) remained constant at both 0 and 4 h of incubation. The cell suspension in phosphate buffer also did not show either decrease at 600 nm or increase at 260 nm in the optical density values. The cell suspension in phosphate buffer containing bacteriocin showed an O.D. value at 600 nm of 0.65 in the beginning and 0.64 at the end of 4h incubation period. The O.D. values at 260 nm were 3.32 and 3.46 at start and end of the incubation period, respectively. Thus, there were no detectable changes in the O.D. 260 and O.D. 600 values of all the three samples during the incubation period for 4 h at 37°C.

The bactericidal action was observed within a few minutes (Figure 2) after the addition of the bacteriocin as revealed by a 99% reduction in the viable cell counts in the first 30 min. At the end of 4 hr incubation period, the viable cell counts in the bacteriocin added samples were 0.02-0.04% of the control samples. It was also observed that cell death was not accompanied by the lysis of the cells (Table 2) since there were no changes in the absorbance values at 260 and 600 nm at the end of 4 h incubation period even in the presence of very high concentrations (400,000 AU/ml) of *L. lactis* subsp. *lactis* CCSUB202 bacteriocin.

Except for a few exceptions, bacteriocins of LAB, in general, are bactericidal in their mode of action. In some strains, the bactericidal effect is associated with the lysis of cells. The bacteriocins of LAB exhibiting a bactericidal, non-bacteriolytic mode of action include Lactococin

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B (LcnB) (Venema *et al.*, 1993), Lactococcin 972 (Martínez *et al.*, 2000), Lactococcin MMT24 (Ghraiiri *et al.*, 2005), bacteriocin produced by *Pediococcus pentosaceus* K23-2 isolated from Kimchi (Shin *et al.*, 2008). Some bacteriocins such as leucocin B-Talla bring about killing effect by lysing the cells and thereby exhibiting a bactericidal, bacteriolytic effect.

#### CONCLUSION

It may be concluded that bacteriocin of *Lactococcus lactis* subsp. *lactis* CCSUB202 exhibit a bactericidal non-bacteriolytic mode of action. The indicator cells that survived the bacteriocin treatment were, however, found to be sensitive to the bacteriocin. Apart from providing fundamental insights into bacteriocin action, the result of this work is also of practical interest. Since the bacteriocin producer *L. lactis* subsp. *lactis* CCSUB202 can be used as an adjunct in cheese manufacture and the bacteriocin it produces has a broad spectrum of action, this strain and bacteriocin have both technological and preservative potentials.

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#### REFERENCES

Abee, T. (1995). Pore-forming bacteriocins of gram-positive bacteria and self-protection mechanisms of producer organisms. *FEMS Microbiology. Letter.* **129**:1-10.

Bierbaum, G. and H.-G. Sahl. (1987). Autolytic system of *Staphylococcus simulans* 22: influence of cationic peptides on activity of *N*-acetylmuramoyl-L-alanine amidase. *Journal of Bacteriology.* **169**:5452-5458.

Bruno, M. E. C. and T. J. Montville. (1993). Common mechanistic action of bacteriocins from lactic acid bacteria. *Applied and Environmental Microbiology.* **59**:3003-3010.

Collins, C.H. and Lyne, P.M. (1987). Microbiological methods. *Butterworths*. London.

Driessen, A. J., van der Hooven, H. W., Kuiper, W., van de Kamp, M., Sahl, H. G., Konings, R. N. & Konings, W. N. (1995). Mechanistic studies of lantibiotic induced permeabilization of phospholipid vesicles. *Biochemistry.* **34**: 1606-1614.

Ghraiiri, T., Frère, J., Berjeaud, J.M. and Manai, M. (2005). Lactococcin MMT24, a novel two-peptide bacteriocin produced by *Lactococcus lactis* isolated from *rigouta* cheese. *International Journal of Food Microbiology.* **105**: 389-398.

Harrigon, W.F. and Mc Cance, M.E., 1993. Laboratory Methods in food and dairy microbiology. Academic press, London.

Holt, J.G., Krig, N.R., Staley, J.T. and Williams, S.T. (1994). Gram-

positive cocci. *Bergey's Manual of Determinative Bacteriology.* 9<sup>th</sup> eds. Prestons street. Baltimore, Maryland 21202 USA, pp 528-540.

Klaenhammer, T.R. (1988). Bacteriocins of lactic acid bacteria. *Biochimie.* **70**:337-349.

Kumari Archana and Amar P. Grag (2007). A Bacteriocin from *Lactococcus lactis* CCSUB-94 isolated from milk and milk products. *Research Journal of Microbiology.* **2**: 375-380.

Kumari Archana, Amar P. Grag, Makeen, K., Mohan Lal, Charu Gupta and Swati Chandra. (2008). A Bacteriocin production on Soya Nutri Nuggets Extract Medium (SNNEM) by *Lactococcus lactis* subsp. *lactis* CCSUB202. *International Journal of Dairy Science.* **3**: 49-54.

Martínez, B., Rodríguez, A. and Suárez J.E. (2000). Lactococcin 972, a bacteriocin that inhibits septum formation in lactococci. *Microbiology.* **146**:949-955

Moll, G., H. Hildeng-Hauge, J. Nissen-Meyer, I. F. Nes, W. N. Konings, and A. J. M. Driessen. (1998). Mechanistic properties of the two-component bacteriocin lactococcin G. *Journal of Bacteriology.* **180**: 96-99.

Ojcius, D. M. & Young, J. D. (1991). Cytolytic pore forming proteins and peptides, is there a common structural motif? *Trends Biochemistry Science.* **16**: 225-229.

Piard, J.C., Muriana, P.M., Desmazeaud, M.J., Klaenhammer, T.R. (1992). Purification and Partial Characterization of Lacticin 481, a Lanthionine-Containing Bacteriocin Produced by *Lactococcus lactis* subsp. *lactis* CNRZ 481. *Applied and Environmental Microbiology.* **58**:279-284.

Schillinger, U. and Lucke, F.K. (1989). Antibacterial activity of *Lactobacillus sake* isolated from meat. *Applied and Environmental Microbiology.* **55**:1901-1906.

Shin, M.S., Han, S.K., Ryu, J.S., Kim, K.S. and Lee, W.K. (2008). Isolation and partial characterization of a bacteriocin produced by *Pediococcus pentosaceus* K23-2 isolated from Kimchi. *Journal of Applied Microbiology.* **105**: 331 - 339.

Shockman, G. D., and J.-V. Høltje. (1994). Microbial peptidoglycan (murein) hydrolases. *Comprehensive biochemistry*, p. 131-166. In J. M. Ghuyssen, and R. Hakenbeck (ed.), *Bacterial cell wall*. Elsevier, London, England.

Tagg, J.R., Dajani, A.S. and Wannamaker, L.W. (1976). Bacteriocins of gram-positive bacteria. *Bacteriol Rev.* **40**:722-756.

Venema, K., Abee, T., Haandrikman, A. J., Leenhouts, K. J., Kok, J., Konings, W. N. and G. Venema. (1993). Mode of Action of

Lactococcin B, a Thiol-Activated Bacteriocin from *Lactococcus lactis*. *Applied and Environmental Microbiology*. **59**: 1041-1048.

Zamfir, M., Callewaert, R., Cornea, P.C., Savu, L., Vatafu, I. and De Vuyst, L. (1999). Purification and characterization of a bacteriocin produced by *Lactobacillus acidophilus* IBB 801. *Journal of Applied Microbiology*. **87**: 923-931.

