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Antimicrobial Activity of Bacteriocin Produced by Lactic Acid Bacteria Isolated from Milk Products

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Lactic acid bacteria (LAB) commonly used as starter cultures in food are known to produce antimicrobial substances such as bacteriocins, having great potential as food biopreservatives. The present study was aimed at isolating bacteriocinogenic LAB from traditional milk product such as Curd, Cheese, Butter, whey and Ghee. The isolates were identified, based on characteristics of the strains of *Lactococcus* spp. as present in Bergey's manual of determinative bacteriology, the metabolite bacteriocin was extracted from the isolated *Lactococcus* spp. and the antibacterial activity was evaluated against bacterial pathogens. The strain C3 of bacteriocin producing *Lactococcus* spp. exhibited the highest inhibitiory activity against *Staphylococcus aureus* whereas *Escherichia coli* was found resistant to the different concentrations of crude bacteriocin. This study revealed the possibility of using bacteriocin as food biopreservative to control food spoilage and pathogenic bacteria.

Key words: Bacteriocin, Lactic acid bacteria, Probiotics, Antimicrobial activity.

Lactic acid bacteria, particularly those belonging to beneficial and non-pathogenic genera (*Lactobacillus, Lactococcus, Streptococcus, Pediococcus and Leuconostoc*) are widely used in food industry. Among lactic acid bacteria; *Lactococcus* are the most important group and are gaining increasing attention in food fermentation industry because of their potential biotechnological interest. This organism prevents the growth of pathogenic bacteria in different ecosystems by production of antimicrobial substance such as organic acids, hydrogen peroxide and bacteriocins[1]. Bacteriocins are small proteins with bactericidal or bacteriostatic activity[2]. There is a growing consumer demand for processed dairy products containing no chemical preservatives, leading to indigenous studies in the field of screening bacteriocin as food preservatives which are isolated from certain traditional dairy products.

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Depending on the producer organism and classification criteria, bacteriocins can be classified into several groups[3-4] in which classes I and II are the most thoroughly studied. Class I, termed lantibiotics, constitutes a group of small peptides that are characterized by their content of several unusual amino acids[5]. The class II bacteriocins are small, non modified, heat stable peptides[6]. Many bacteriocins are active against food borne pathogens[7].

Bacteriocins from lactic acid bacteria (LAB) are natural antimicrobial peptides or proteins with interesting potential applications in food preservation and health care. Nisin, the best-known LAB bacteriocin, has been repeatedly shown to be safe and effective for use in foods over the past 30 years Pediocin is another well-studied bacteriocin that will likely be the second LAB bacteriocin to be widely used in the food industry. This present study was conducted to evaluate the antibacterial activity of bacteriocin producing *Lactococcus* spp. isolated from traditional milk products.

MATERIALS AND METHODS

Collection of dairy products

Five different dairy products viz., Curd ,cheese, whey, Ghee and Butter were collected from local market in Solan (Himachal Pradesh) India.

Isolation of bacteriocin producing isolates

Isolation of bacteriocin producing *Lactococcus* spp. The bacteriocin producers from natural traditional milk products were isolated by Pour plate technique as per the conventional method using MRS (Man Sharpe Rogosa) agar, and the plates were incubated at 37°C for 24 h, the typical colonies were purified and sub-cultured.

Screening of Bacteriocin producing strains

The isolated colonies were picked up and transferred to MRS broth. The enriched broth was screened for antimicrobial activity by swab paper disc method[8]. In this method supernatant (20ìl) of bacterial isolates, was suspended in the sterile paper disc against the indicator organisms. The bacteriocin sensitive test strain *Lactococci lactis subsp.lactis. (*MTCC3038) strain was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology Chandigarh, India. All the plates were incubated at 37°C for 24 hours. Colonies showing zone of inhibition were considered as bacteriocin producers. The bacteriocin producing bacterial strains were subjected to morphological characterization. Five efficient bacteriocin producing isolates one each from five milk product samples (curd, cheese, whey, ghee and butter) were further subjected to morphological and biochemical identification.

Production of crude bacteriocin

Amongst the five most efficient bacteriocin producing strains only one strain which showed highest bacteriocin activity was used for further studies related to antibacterial activity. The isolated strain was grown in MRS broth (Hi Media Laboratory Pvt. Ltd. India) seeded with 5% inoculum of overnight culture and maintained aerobically at 37°C for 24-48 h after incubation, cells were removed from the growth medium by centrifugation 12000 rpm for 15 min at 4°C.The cell free supernatant was used as a crude Bacteriocin and stored in refrigerator at 4°C [9].

Pathogenic bacteria cultures

Standard pathogenic bacteria cultures of *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhi, Shiegella flexneri,* were procured from Shoolini Institute of life sciences & Business Management (SILB) Solan (HP), and sub-cultured at regular intervals.

Antibacterial effect of Bacteriocin against different pathogenic bacterial strains

Antimicrobial activity of the most efficient bacteriocin producing bacterial isolates against the pathogenic microorganisms was tested by disc diffusion method. MHA plates were prepared with lawn of pathogenic strains & discs were placed on these plates at appropriate distance. Different volume of crude Bacteriocin (20, 40, 60 µl) were dispended on filter paper discs . Plates were kept in refrigerator for two hours and incubated at 37°C for 24 h. The antimicrobial activity was determined by measuring the diameter of inhibition zone around the disc. The percent bacteriocin activity was calculated using the following formula:

$$\text{Bacteriocin activity } (\%) = \frac{B-A}{A} \times 100$$

A = Disc diameter
B = Inhibition zone diameter

RESULTS AND DISCUSSION

Lactic acid bacteria (LAB), particularly those belonging to beneficial and non-pathogenic bacteria (*Lactococus, Lactobacillus, Leuconostoc, and Streptococcus*) play an essential role in the dairy industry due to the tremendous level of human consumption of several important fermented products, mainly cheese and acidified or fermented milks[11]. Present studies were carried out to isolate and identify bacteriocin producing LAB from different milk products such as curd, cheese, whey, butter and ghee. In the present studies *Lactococcus* spp, isolated from different milk products such as curd, cheese, whey, butter and ghee were characterized and identified. The total microbial populations of different milk products were enumerated and the results are presented in Table 1. Highest diversity of microorganism were found in curd sample ($42x\ 10^7$) this was closely followed by the butter sample (31×10^7) where's minimum diversity was found in cheese sample ($24\ x\ 10^5$). The incidence of different *Lactococc*i spp. in traditional sardinian dairy products[12],

Table 1. Microbial viable counts

Sample	Cfu/ml
Whey	24×10^6
Ghee	20×10^7
Cheese	17×10^5
Curd	42×10^7
Butter	31×10^7

Lactobacillus in curd[13] , Nisin producing *Lactococcus lactis subsp. lactis* from raw milk[14-15] has also been reported earlier. The results of present investigations are in line with the earlier findings[16] who reported a high incidence of microbial load in curd sample, when compared with other dairy products like milk ,peda and cheese.

Screening of bacteriocin producing Isolates

The Screening of bacteriocin producing isolates was done by swab disc method. Out of 134 isolates only 72 isolates showed zone of inhibition against bacteriocin sensitive strain (*L. lactis).* Only fifty two isolates were identified as *Lactococci* spp. on the basis of morphological characters and biochemical studies (Table 2 and 3). All the five isolates/strains of *Lactococci* spp. (C1, C2, C3, C4 & C5) one each from curd, cheese, whey, butter and ghee were found to be gram positive, catalase, oxidase, indole and MR - negative and cocci in shape. Strain C3 showed highest antibacterial activity against test strain *L. lactis subsp.lactis* MTCC 3038 so this strain was selected for detailed study. All isolates were identified compared to the Bergey's manual of systematic bacteriology[10]. Bacteriocin producing strain C3 belonged to the species *L. lactis subsp.lactis* (Fig. 3). The strain (C3) was Gram-positive cocci in pairs or small chains (Fig. 4). The strain was found to be catalase negative, therefore, couldn't be the species of aperture for *Staphylococcus* or *Micrococcus,* which are

Table 2. Morphological characteristics of the isolated bacterial strains

Isolate	Gram reaction	Shape	Colony Morphology on MRS Agar
C1	Gram positive	Cocci	Creamy, little sticks smooth round colonies.
C2	Gram positive	Cocci	Small, slightly raised Colonies with smooth margins.
C3	Gram positive	Cocci	Large, slightly raised colonies with smooth margins.
C4	Gram positive	Cocci	Small, flat colonies with irregular margins.
C5	Gram positive	Cocci	Large colonies with smooth round margins.

Table 3. Biochemical characterization of bacterial isolates

BiochemicalTest	C1	C2	C3	C4	C5
Catalase	–	–	–	–	–
Oxidase	–	–	–	-	-
Indole	–	–	–	–	–
M R	–	–	–	–	–
V P	+	+	+	+	+
Citrate	–	–	–	–	–
Lactose	+/-	+/-	+/-	+/-	+/-
Glucose	+/-	+/-	+/-	+	+
Sucrose	+/-	+/-	+/-	–	–
Mannitol	–	+	–	–	–
Urease	+	+	+	–	–
Nitrate	–	–	–	–	–
Motility	–	–	–	–	–
T S I	Acidic butt and acidic slant	Acidic butt and acidic slant	Acidic butt and acidic slant	Alkaline butt and alkaline slant	Alkaline butt and alkaline slant

-: negative result + : positive result +/- : Acid/No gas +/+ : Acid/ Gas

catalase positive. The optimum temperature for growth of the organism is 35 to 38°C, therefore, it cannot be a member of the pathogenic group of *Streptococci* i.e., *Streptococcus* Lancefield group A, B, C, D. viridans group or *Streptococcus pneumonia*. The organism is lactose positive therefore, may not be an *Enterococci*, which are lactose negative. Taking the above facts into consideration, it was concluded that the organism was a species of *Lactococcus.*

Antibacterial activity of crude bacteriocin produced strain (C3) against different pathogenic bacterial strains (*E. coli, S. aureus, P. aeruginosa, S. typhi, S. flexneri*) was determined by discs diffusion method at different concentrations (20,40 and 60 µl) (Figure1 and 2). Isolate C3 of *Lactococcus* spp. at 20µl concentration showed inhibitory activity against *S. aureus, P. aeruginosa, S. typhi, S. flexneri* (Table 4). Maximum inhibitory activity was observed against *S. aureus,* (154.5%) followed by *S. typhi* (118.2%) where as minimum activity was observed against *P. aeruginosa* (63.6%). *E.coli* was found to be resistant. At 40µl and 60 µl concentrations strain (C3) of

Table 4. Antibacterial activity of crude Bacteriocin (20µl) against different pathogenic bacterial strains

Pathogenic strains	Disk diameter (in mm) A	Diameter of zone of inhibition (in mm) B	Bacteriocin activity
P.aeruginosa	11	18	63.6%
S.aureus	11	28	154.5%
S. typhi	11	24	118.2%
Shiegella flexneri	11	21	90.9%
E.coli	11	-	-

- =No growth

Table 5. Antibacterial activity of crude Bacteriocin (40µl) against different pathogenic bacterial strains

Pathogenic strains	Disk diameter (in mm) A	Diameter of zone of inhibition (in mm) B	Bacteriocin activity
P.aeruginosa	11	20	81.8%
S.aureus	11	29	163.6%
Salmonella typhi	11	26	136.6%
Shiegella flexneri	11	24	118.2%
E.coli	11	-	-

- =No growth

Table 6. Antibacterial activity of crude Bacteriocin (60µl) against different pathogenic bacterial strains

Pathogenic strains	Disk diameter (in mm) A	Diameter of zone of inhibition (in mm) B	Bacteriocin activity
P.aeruginosa	11	23	109.1%
S.aureus	11	32	190.9%
Salmonella typhi	11	28	154.5%
Shiegella flexneri	11	26	136.4%
E.coli	11	-	-

- =No growth

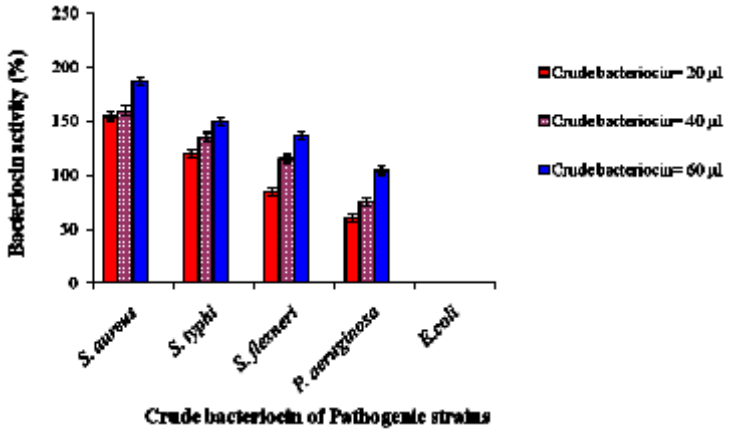

Fig. 1. Inhibitory activity of crude bacteriocin at different concentrations

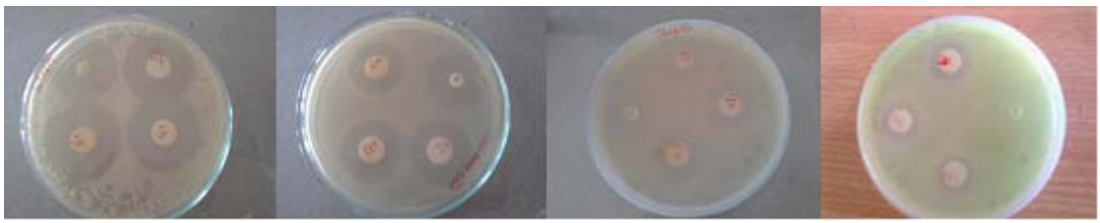

Fig. 2. Inhibitory activity of crude bacteriocin at different volumes against *S.aureus, S.typhi, S flexneri and P. Aeruginosa*

Fig. 3. *Lactococci* Colonies on MRS Agar Plate

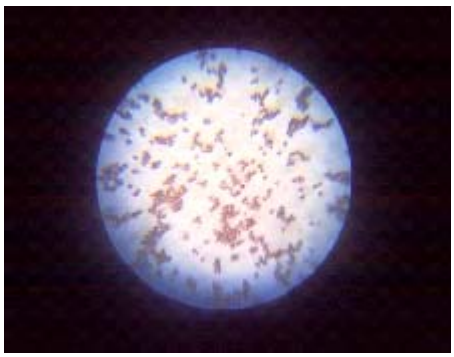

Fig. 4. Gram positive cocci

Lactococcus spp. showed inhibitory activity against all the tested pathogenic strains , except *E. coli.* (Table 5 & 6). Maximum inhibitory activity was observed against *S. aureus*, (163.6%, 190.9%) and minimum activity was observed against *P. aeruginosa (* 81.6%, 109.1%) at 40µl and 60 µl concentrations respectively. No inhibitory activity was recorded against *E. coli* . Bacteriocins have been reported to be inhibitory against several other bacteria[17- 19]. The results of our present study are in line with the earlier findings of other workers[20] who reported highest inhibitory activity of bacteriocin producing *Lactobacillus* LBC against *S. aureus* Possession of bacteriocin by *Lactococci spp.* is an indication that the bacteria can be used as Probiotics and as biopreservative.

CONCLUSION

In the present study bacteriocin producing strain were isolated from different curd samples. . was found to be *Lactococci spp.(C3).* The crude bacteriocin produced by the most efficient bacteriocin producing strain showed highest inhibitory activity against *Staphylococcus aureus, followed by Salmonella typhi, Shiegella*

flexneri and *Pseudomonas aeruginosa* while *Escherichia coli* was found resistant at all the concentrations of bacteriocin used. Inhibitory effect demonstrated by *Lactococci*. sp*p. C3* against these bacteria is an indication of possession of antibacterial activity. So, it could be concluded from the present study that bacteriocin produced by *Lactococci spp. (C3)* can be utilize as biopreservative in food.

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