Short communication

Identification of vancomycin sensitive and heat stable anti-listerial bacteriocin producing *Enterococcus faecium* isolated from idli batter

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Abstract Lactic acid bacteria (LAB) are known to produce various types of bacteriocins, ribosomally synthesized polypeptides, which have antibacterial spectrum against many food borne pathogens. *Listeria monocytogenes*, a pathogenic bacterium, is of particular concern to the food industry because of its ability to grow even at refrigeration temperatures and its tolerance to preservative agents. Some of the bacteriocins of LAB are known to have anti-listerial property. In the present study, vancomycin sensitive *Enterococcus faecium* E1 and J4 were isolated from idly batter samples and identified by both biochemical and molecular methods. The isolates found to tolerate high temperatures of 60°C for 15 and 30 min and 70°C for 15 min. It produced heat stable anti-listerial bacteriocin, which has not lost anti-listerial activity when treated at 100°C for 30 min or at 121°C for 15 min. The bacteriocin lost its antimicrobial activity when treated with trypsin, protinase-K, protease, and peptidase. The mutants obtained by treating the wild strains with novobiocin did not show antibacterial activity.

Keywords anti-listerial, bacteriocin, *Enterococcus*, identification, heat stable

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Introduction

Consumers are concerned about the synthetic chemicals used as preservatives in food and this is resulting in a trend towards use of less processed food. As a result, there has been great interest and research on naturally produced antimicrobials, such as ribosomal peptides, synthesized by lactic acid bacteria (LAB), named bacteriocins. Bacteriocins are ribosomally synthesized polypeptides possessing bactericidal activity[1]. LAB are known to produce various types of bacteriocins, which have antibacterial spectrum against many food-borne pathogens. LAB occupies a central role in fermented processes and has a long history of application and consumption in the production of fermented foods and beverages[2]. The use of bacteriocin is among the new approaches, which may further contribute to reduce risks of food borne disease outbreaks and increase food quality. LAB has a major potential for use in biopreservation because they are safe to consume and during storage they naturally dominate the natural micro flora of many foods. LAB are a group of fastidious group of aero-tolerant, Gram-positive, non-spore forming, non-motile, catalase negative and carbohydrate fermenting food grade GRAS (generally regarded as safe) state microorganisms[3-4].

Enterococcus is an important genus of LAB involved in food fermentation and preservation[5]. Production of anti-listerial bacteriocins known as enterocins by enteroccci is well documented[4,6-9]. Besides typical bacteriocins, production of atypical bacteriocins by enterococci is also reported[7,10]. Several *Enterococcus* species have been isolated from both foods, such as fermented Spanish sausages[11], cheese[12-13], Japanese rice bran paste-*Nuka*[14], milk[15] and fermented meat[8] and non-food sources like pig faeces[16], infant[17], gut of chicken[18] and vaginal secretions of HIV infected children[19]. This suggests the wide spread distribution of enterococci in different habitats. Enterocin produced by *Ent. faecium* has abroad spectrum of activity towards food-borne pathogens like *Listeria* and clostridia, indicating its application in foods as a co-culture or as an additive[20]. *Listeria monocytogenes*, a food-born pathogen, is of particular concern to the food industry because of its ability to grow at refrigeration temperatures and its tolerance to preservative agents. Anti-listerial property of the enterocin produced by *Ent. faecium* was recorded[11, 21-22].

Idli is one of the widely used fermented foods of India. It is prepared from rice (*Oryiza sativa*) and black gram dhal (*Phaseolus mungo*), a legume. The ingredients are washed, soaked in water separately, ground, mixed and finally allowed to ferment overnight[23]. It is cooked by steaming when the batter has

been raised sufficiently and served hot. The product has a very soft and spongy texture and a desirably sour flavor and taste. During the process of idli batter fermentation, large number of LAB are known to be involved in the fermentation. Among them *Leuconostoc mesenteriods* is the microorganism essential for leavening the batter and also responsible along with *Enterococcus*[24]. It is therefore planned to isolate native strains of *Enterococcus* from idli batter that are producing anti-listerial bacteriocin. The present paper mainly deals with the isolation and identification of the bacteriocin producing *Enterococcus* spp. and the stability of its bacteriocin to different temperatures and enzymes.

Materials and methods

Isolation of bacteriocin producing cultures

The idli batter samples (50) were collected from various places of Southern India like Mysore, Coimbatore and Udumalpet. The samples were then serially diluted with 0.85% saline and appropriate dilutions were pour plated individually using *Lactobacillus* deMan, Rogosa and Sharpe (MRS) agar medium (Hi-media Labs, India). The plates showing well isolated colonies were overlaid with 10 ml of BHI soft agar seeded with 10 µl of freshly grown (10^6 cells/ml) indicator strain *Listeria monocytogenes* Scot-A, grown in BHI broth at 37°C for overnight under shaking condition. The plates were then incubated at 37°C for 24 h and observed for the zone of inhibition. The colonies showing maximum zone of inhibition were transferred aseptically to MRS broth and then incubated at 37°C for 24 h under static condition. Purity of the isolates was confirmed by streaking on to MRS agar plates.

Identification of the isolates

The morphology of the isolates was determined after Gram's staining the smears of freshly grown cultures and observing under light microscope and scanning electron microscopy (SEM). For SEM, the isolates were grown in MRS broth for 10 h at 37°C and the cells were harvested by centrifugation at 10,000 rpm for 10 min. The cells were washed twice with 1x PBS buffer and fixed with 2% glutaraldehyde solution and dehydrated with ethanol gradient of 10 to100%. The dried cells were subjected to gold plating (Cool Sputter coat system, England) and subjected to SEM (435 V.P., Leo Electron Microscopy, UK). Motility of the cultures was determined by hanging drop method. For identification, the isolates were subjected to biochemical characterization using tests such as production of catalase and oxidase; citrate utilization; gelatin and starch hydrolysis; curdling of milk and growth in 0.5% bile salts. The isolates were

also identified by performing polymerase chain reaction (PCR) with known primer sequences of *Enterococcus* sp. The PCR sequences [25] used were as follows:

BS F 5' GAG TTT GAT CCT GGC TCA GG 3'

BS R 5' TCA TCT GTC GTC CCA CCT TCGGC 3'

The reaction was carried out in the Thermocycler Gene Amp PCR system 9700 (Perkin Elmer, USA) using standard protocol[26]. The PCR conditions were as follows: one cycle of denaturation for 3 min at 95°C, primer annealing for 40 sec at 48°C and extension for 2 min at 72°C followed by 34 cycles of denaturation for 40 sec at 94°C, primer annealing for 20 sec at 48°C, extension for 2 min at 72°C and final extension for 15 min at 72°C. The PCR product was analyzed by using 1.5% agarose gel electrophoresis.

Screening the isolates for antibacterial activity

Agar well diffusion assay was used for testing the antimicrobial activity of the culture filtrate (CF) of the bacteriocin producing isolates[3]. The isolates were freshly grown in MRS broth for 15 h and the cells were removed by centrifugation (R-24 model, Remi Instruments, Mumbai) at 10,000 rpm for 10 min. The supernatants were collected in sterile eppendorf tubes. The CFs were neutralized to pH 7.0 and used to check the antimicrobial activity. The assay plates were prepared with MRS agar in which requisite numbers of wells of 6 mm diameter were made with a suction borer. Forty µl of the neutralized culture filtrate was added to the labeled wells. The plates were kept at 4°C for 1 h to allow the filtrate to diffuse into the agar and then the plates were kept at 37°C for 20-30 min for drying. These plates were overlaid with BHI soft agar seeded with indicator organisms such as *L. monocytogens* Scot-A, *L. greyi* B3, *L. innova* B2, *L. murray* B5 or MRS soft agar seeded with *Pediococcus acidilactici* B 1153, *P. acidilactici* K7, *Ent. faecium* MTCC 5153 and *Ent. faecium* DPC, individually. The overlaid plates were kept for overnight incubation at 37°C. These plates were observed for zone of inhibition around the wells. An inhibition zone of ≥8mm was considered as positive.

Antibiogram of the isolates

The antibiotic sensitivity of the isolates was determined to differentiate the isolates. For this, MRS agar plates overlaid with 10 ml of MRS soft agar (0.8%) seeded with 10 µl of freshly grown isolates were used. The plates were incubated for 1 h at 4°C and octa disc (containing 8 antibiotics individually in

each arm, Hi-media Labs, Mumbai, India) was placed in the center of the plate and incubated at 37°C for overnight. The pattern of growth inhibition of the isolates by each antibiotic was observed.

Immunity assay

MRS agar plates overlaid with 10 ml of MRS soft agar (0.8%) seeded with 10 µl of freshly grown isolates were used. Forty µl of the CF of the *Ent. faecium* DPC, MTCC 5153, *Pediococcus acidilactici* PAC, 10 & K7 was added to the wells individually and allowed to diffuse at 4°C for 1 h. The plates were kept for incubation at 37°C for 24 h and the zone of inhibition around each well was measured.

Susceptibility of the bacteriocin to proteolytic enzymes

The susceptibility of the CF of the isolates was checked against various proteolytic enzymes like trypsin (type, bovine pancreas, 1000 BAEE units/mg of protein, Sigma USA), proteinase-K, papain, lysozyme, aminoglycosidase, protease, peptidase, and amylase to confirm the compound responsible for antimicrobial activity is a protein. A 100 µl of stock solutions of all the enzymes (1 mg/1 ml) were added individually to 400 µl of CF and allowed to react at 37°C for 1 h. Treated and untreated samples (40 µl each) were added into the labeled wells of MRS agar plates individually and the anti-listerial activity was carried out as indicated earlier.

Heat tolerance of bacteriocins

To know the heat tolerance of the bacteriocins, the freshly collected CF of the isolates was heated at 100°C for 30 min and at 121°C for 15 min and checked for the antimicrobial activity. The heat-treated CF samples were analyzed for anti-listerial activity using agar well assay as indicted earlier.

Isolation of bacteriocin defective mutant

TGE broth containing 10µl ml^{-1} novobiocin was inoculated with 1% inoculum of the isolate. The culture was transferred in the same media on successive days over the course of one week. At the end of the treatment period, the culture was plated on MRS agar plates and screened against *L. monocytogenes* Scot A, to identify the non- bacteriocin producers that failed to form inhibition zones. The mutant colonies were purified by streaking on MRS agar plate. Wild type and cured strains were grown in TGE broth and the CFs were prepared and spotted on MRS agar plates seeded with *L. monocytogenes* to test for bacteriocin production.

Results and Discussion

Isolation of bacteriocin producing LAB

Idli is a natural yeast-lactic fermented product, mainly used as a break fast snack in southern India. Now-a-days, consumers prefer to have a natural or near natural preserved foods than the processed foods that contain lot of chemical preservatives. Therefore, search is on all over the world to isolate microorganisms that possess antimicrobial activity by way of producing bacteriocins, especially effective against food borne pathogen like *Listeria monocytogenes*. Fermented idli batter samples collected from various places of southern India were subjected to screening for LAB cultures producing anti-listerial bacteriocin using MRS agar as a selective medium. The isolates showing antagonistic activity by prominent zone of inhibition (> 8 mm) against the *L. monocytogenes* Scot -A were picked (Fig.1) for identification.

Identification of the isolates

Morphological, biochemical and physiological tests were conducted for preliminary identification of the native isolates based on Bergey's manual[27]and that of Stiles and Holzapfel[28]. The isolates were found to be non-motile, Gram positive cocci and negative to catalase and oxidase reactions. Both the isolates did not hydrolyze starch, arginine, casein and gelatin. They have grown in 0.5% bile salts. They found to have good curdling action through the skimmed milk test. Carbohydrate fermentation test (glucose) was carried out to know whether the isolates are homo-fermentative or hetero- fermentative LAB. Both the native isolates produced acid without gas production, indicating that both the isolates are homo-fermentative cultures. The isolates were found to utilize lactose, maltose, sucrose, glucose and raffinose, but not glycerol. A specific test of growth at 10°C was also carried out, which is very helpful in differentiating *Enterococcus* and *Pediococcus*. Both the isolates were found to survive at high temperatures like 45°C, 50°C. However, they could not grow at 10°C. The isolates could grow at high pH (9.6) and with 6.5 and 8% of NaCl. The isolates were found to tolerate high temperatures of 60°C for 15 and 30 min and 70°C for 15 min. Accordingly, both the native isolates were identified to be *Enterococcus* spp[29].

Both the isolates were positive for growth with 0.1% methylene blue or 0.04% sodium azide. The isolates could not reduce potassium tellurite, indicating that these isolates belong to *Ent. faecium*[30]. Therefore, both the isolates were found to belong to the genus *Enterococcus*. Polymerase chain reaction was carried out with specific primers of *Ent. faecium* to identify the isolates. The amplified fragments of

native isolates showed specific banding pattern as of the positive control. These results confirmed that the native isolates of idli batter are strains of *Ent. faecium* and these have been named as E1 and J4. Similarly, Cheng et al[31] also used a PCR assay for the identification of *Ent. faecium.*

Sensitivity to antibiotics

The response of various bacteriocinogenic isolates to a wide range of antibiotics is very helpful in differentiation of strains. Study with antibiogram indicated that the native isolates were sensitive to vancomycin, amikacin, cephataxime, netlmicin and ceftriaxone at 30 µg concentration and lincomycin and ofloxacin at 2 µg concentration. It showed resistance only to ceftazidime at the same concentration., Hence, the antibiotic markers could be used as a tool for identification of the bacteria. Similarly, isolation of vancomycin sensitive enterococci from meat [32] and dairy products and human faecal samples[33] have been reported. However, reports on isolation of vancomycin resistant *Enterococcus* strains are also available[5, 34-35].

Properties of the bacteriocin

To differentiate between the antibacterial activity of acid and the bacteriocin, the pH of the CF was neutralized and the antibacterial activity was checked[16]. Both untreated and neutralized CFs showed similar strong inhibition of *Listeria*, depicting that the antimicrobial activity of the CFs is not due to the acid production by the isolates. The CF after heat treatment at 100°C for 30 min and autoclaving at 121°C for 15 minutes was found to be resistant to heat and the antibacterial activity against *Listeria* was not reduced after heat treatment (data not shown), indicating that the antimicrobial compound is heat stable. Based on these experiments, the antimicrobial compounds are assumed as anti-listerial heat stable peptides. These characters belong to the class II pediocin type bacteriocins[11,36-37].

To determine the whether the isolates are pediocin PA-1 or enterococcin producers, the activity of the CF was checked against different producing cultures and the CF of native isolates showed antimicrobial activity against pediocin producer like *Ped. acidilactici* K7/ PAC. The CF showed strong antimicrobial activity against several species of *Listeria* like *L. monocytogens* Scot-A, *L. monocytogens* B1, *L. greyi* B3, *L. innova* B2 *and L. murray* B5. The culture filtrate of native isolates did not show inhibition against *lactics* such as *Lactococcus lactis* sub sp *lactis, Lc. lactis* sub sp. *cremoris, Lactobacillus acidophilus, Lb. casei, Lb. amylovorus* B4552, *Lb. helverticus* B4526, *Lb. acidophilus* B4496 and *Lb. casei* B1922; and the food

borne pathogens like *Salmonella typi, Staph. aureus, Enterobacter* sp., *E. coli* and *Yersinia enterocolitica.* Based on these results the antimicrobial peptides produced by both the isolates of this study are characterized as heat stable narrow spectrum anti-listerial bacteriocins.

The CFs of the both native isolates had shown resistance to lysozyme and papain and carbohydrate cleaving enzyme like aminoglycosidase. This confirms that the antimicrobial compound is not a carbohydrate molecule. The CF of both the native isolates was sensitive against trypsin, protinase-k, protease, and peptidase and lost their antimicrobial activity, confirming that the antimicrobial activity of CF is due to the proteins or peptides that are cleaved by proteolytic enzymes. Similar results were observed in *Ent. faecium*[16]. Unlike the wild cultures, the mutants obtained after treating the isolates with novobiocin did not show antibacterial activity and was found to be sensitive to the bacteriocin produced by the wild cultures.

Conclusions

We could isolate two enterococcal isolates from idli batter samples, which have shown anti-listerial property, besides activity against other pathogens and LAB cultures. Because of the vancomycin sensitivity of the isolates, they may be safely exploited as starter cultures, co-cultures or probiotic bacteria in the preparation of fermented products or in animal feed formulations.

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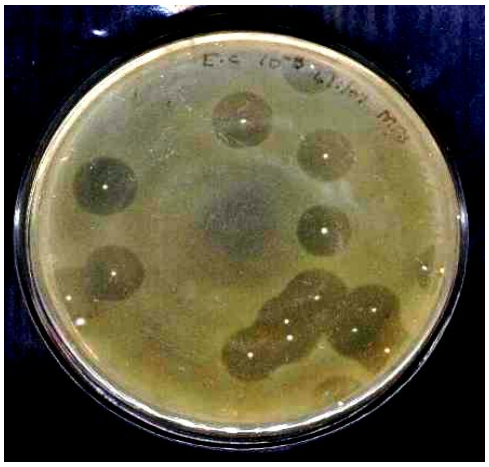
Figure legends:

Fig. 1 Antilisterial activity of the isolated *Enterococcus faecium* E1 (A) and J4 (B)

A



B