Characterization of a heat stable anti-listerial bacteriocin produced by vancomycin sensitive *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, the bacteriocin produced by vancomycin sensitive *Enterococcus faecium* E1 and J4 isolated from *idli* batter samples was characterized. The isolates were found to tolerate high temperatures of 60°C for 15 and 30 min and 70°C for 15 min. The bacteriocin was found to be heat stable and had anti-listerial activity. The bacteriocin did 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 after treating with trypsin, protinase-K, protease and peptidase.

Keywords Anti-listerial · Bacteriocin · *Enterococcus*, Identification · Heat stability

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. 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 microflora 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 [2].

Enterococcus is an important genus of LAB involved in food fermentation and preservation [3]. Production of anti-listerial bacteriocins known as enterocins by enterococci is well documented [4–5]. Several *Enterococcus* species have been isolated from both foods, such as fermented meat [5], cheese [6] and milk [7] and non-food sources like infant [8] and vaginal secretions of HIV infected children [9] suggesting the widespread distribution of enterococci. Enterocin produced by *Enterococcus faecium* has a broad-spectrum of activity towards food-borne pathogens like *Listeria* and clostridia, indicating its application in foods as a co-culture or as an additive [10]. *Listeria monocytogenes*, a food-born pathogen, is of particular concern to the food industry because of its ability to grow at refrigeration

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temperatures and its tolerance to preservative agents. Anti-listerial property of the enterocin produced by *E. faecium* was recorded [11].

Idli is one of the widely used fermented foods of India. It is prepared from rice (*Oryza sativa*) and black gram dal (*Phaseolus mungo*), a legume. The ingredients are washed, soaked in water separately, ground, mixed and finally allowed to ferment overnight [12]. 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. Large number of LAB are known to be involved in the *idli* batter fermentation. Among them *Leuconostoc mesenteriods* is essential for leavening the batter and *Enterococcus* species were also isolated from the batter [13]. 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 characterization of the bacteriocin produced by vancomycin sensitive *Enterococcus* species and the stability of the bacteriocin to different temperatures and enzymes.

The *idli* batter samples (50) were collected from domestic and commercial sources of Southern India like Mysore and Coimbatore. 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 brain heart infusion (BHI) soft agar seeded with 10 μ l of freshly grown (10^6 cells/ml) indicator strain *L. 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.

Agar well diffusion assay was used for testing the antimicrobial activity of the culture filtrate (CF) of the bacteriocin producing isolates [14]. The isolates were freshly grown in MRS broth for 15 h and the cells were removed by centrifugation (Remi Instruments, India) 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 microliter of the neutralized CF 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. monocytogenes* Scot-A, *L. greyi* B3, *L. innacova* FB21, *L. murrayi* FB69 or MRS soft agar seeded with *Pediococcus acidilactici* B1153, *P. acidilactici* K7, *E. faecium* MTCC 5153 and *E. 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 ≥ 8 mm was considered as positive.

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 μ l 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, 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. Besides this, effect of different antibiotics such as ampicillin, streptomycin, tetracycline, chloramphenicol and erythromycin, on the isolates was tested by determining the minimum inhibitory concentration (MIC) of these antibiotics [15].

To carry out immunity assay, MRS agar plates overlaid with 10 ml of MRS soft agar (0.8%) seeded with 10 μ l of freshly grown isolates were used. Forty microliter of the CF of the *E. faecium* DPC, MTCC 5153, *P. acidilactici* PAC, 10 and 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.

The susceptibility of the CF of the isolates to various proteolytic enzymes such as trypsin (type, bovine pancreas, 1,000 BAEE units/mg of protein), proteinase-K, papain, lysozyme, aminoglycosidase, protease, peptidase and amylase (all from Sigma, USA) was checked to confirm the compound responsible for antimicrobial activity is a protein. A 100 μ l of stock solutions of all the enzymes (1 mg/1 ml) were added individually to 400 μ l of CF and allowed to react at 37°C for 1 h. Treated and untreated samples (40 μ l each) were added into the labeled wells of MRS agar plates individually and the anti-listerial activity was carried out as indicated earlier.

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 indicated earlier.

Idli is a natural yeast-lactic fermented product, mainly used as a breakfast snack in southern India. Nowadays, 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 *L. 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. Two isolates showing antagonistic activity by prominent zone of inhibition (>8 mm) against the *L. monocytogenes* Scot-A were selected (Fig. 1) and identified up to genus level based on Bergey's manual [16]. The morphological, biochemical and physiological tests revealed that the isolates are *Enterococcus* species. 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 the isolates as *E. faecium* [17].

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, cephalaxime, netilmicin and ceftazidime at 30 μ g concentration and lincomycin and ofloxacin at 2 μ g 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 [18] and human fecal samples [19] have also been reported. The MIC values for ampicillin, streptomycin, tetracycline, chloramphenicol and erythromycin were determined to be 1, 256, 0.5, 8 and 0.5 μ g/ml, respectively for *Enterococcus* E1.

To differentiate between the antibacterial activity of acid produced by the isolate and the bacteriocin, the pH of the CF was neutralized and the antibacterial activity was checked. Both untreated and neutralized CFs showed similar strong inhibition of several *Listeria* strains as indicated in materials and methods section, indicating 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 min 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 characteristic features belong to the class II pediocin type bacteriocins [20–22].

To determine the whether the isolates are pediocin PA-1 or enterococin 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 *P. acidilactici* K7/ PAC. The CF showed strong antimicrobial activity against several species of *Listeria* like *L. monocytogenes* Scot-A, *L. monocytogenes*

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

B1, *L. greyi* B3, *L. innacova* FB21 and *L. murrayi* FB69. The culture filtrate of native isolates did not show inhibition against lactics such as *Lactococcus lactis* subspecies *lactis*, *L. lactis* subspecies *cremoris*, *Lactobacillus acidophilus*, *L. casei*, *L. amylovorus* B4552, *L. helveticus* B4526, *L. acidophilus* B4496 and *L. casei* B1922; and the food-borne pathogens like *Salmonella typhi*, *Staphylococcus aureus*, *Enterobacter* species, *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, proteinase-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.

In conclusion, 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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