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Isolation and characterization of bacteriocin produced by *Lactobacillus fermentum* LBC 1

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Abstract
The lactic acid bacteria are used industrially for the production of Yoghurt, Cheese, sauerkraut, pickles, beer, wine, cider, kimichi, chocholate and other fermented foods, as well as animal foods, such as silage. In the present study the bacteriocin isolated from *Lactobacillus fermentum* LBC1 was protein in nature with antimicrobial effects on some important food borne pathogens. This strain was isolated from the cheese sample and they were cultivated on MRS agar and identified using biochemical methods. The extracted crude bacteriocin was also tested for some physiological conditions heat, pH and enzymes. The crude bacteriocin of *Lactobacillus fermentum* LBC1 exhibited no effect on the proteinase-K and Trypsin. and active at 100 °C. The crude proteins showed the residual activity remain at pH 3.0 to 9.0 ranges.

Keywords: *Lactobacillus fermentum*, lactic acid bacteria, cheese, bacteriocin

Introduction
The lactic acid bacteria (LAB) are a group of Gram positive bacteria, non-respiring, non-spore forming, cocci or rods, which produce lactic acid as the major end product of the fermentation of carbohydrates. They are the most important bacteria in desirable food fermentations, being responsible for the fermentation of sour dough bread, sorghum beer, all fermented milk, cassava (to produce garri and fufu) and most "pickled" (fermented) vegetables (Savadogo *et al*., 2006; De Vuyst *et al*., 2007) [15, 4]. Lactic acid bacteria occur naturally in several raw materials like milk, meat and flour used to produce foods. LAB is used as natural or selected starter cultures in food fermentations in which they perform acidification due to production of lactic acids. Protection of food from spoilage and pathogenic microorganisms by LAB is through producing organic acids (Ross, 2002) [13]. The LAB produces an array of antimicrobial substances (such as organic acids, diacetyl, acetone, hydrogen peroxide, reuterin, anti-fungal peptides and bacteriocins (El-Ziney *et al*., 2000) [5].
Bacteriocins of LAB are considered as safe natural preservatives or biopreservatives as it is assumed that they are degraded by the proteases in gastrointestinal tract (Cleveland *et al*., 2001) [2]. Bacteriocins are extracellularly released peptides or protein molecules, with a bactericidal or bacteriostatic mode of action against closely related species. The inhibitory spectrum of some bacteriocins also includes food spoilage and/or food-borne pathogenic microorganisms (Schillinger *et al*., 1996) [16]. The discovery of nisin, the first bacteriocin used on a commercial scale as a food preservative dates back to the first half of last century but research on bacteriocins of LAB has expanded in the last two decades, searching for novel bacteriocin producing strains from dairy, meat and plant products, as well as traditional fermented products. Many bacteriocins have been isolated and characterized (Cleveland *et al*., 2001) [2]. In the Present investigation reports on the isolation and characterization of the other bacteriocin producing Lactic acid bacteria from cheese.

Materials and Methods
Isolation of bacteriocin producing microorganism
Ten gram of cheese samples was weighed aseptically. This was macerated individually with 100 ml of alkaline peptone water in a surface sterilized pestle and mortar. This macerate was serially diluted up to 10^{-6} dilution. About 1 ml of appropriate dilution of the sample was pipette into sterile petridishes. MRS agar media were poured and incubate at room temperature for 48 hrs.
The LAB was identified on the basis of growth on selective MRS agar (pH 5.2), cell morphology, gram staining, catalase activity and biochemical identification of LAB. Further identification of the species of this LAB was performed according to carbohydrate fermentation patterns and growth on MRS broth (HI Media) as described in Bergey's manual of systematic bacteriology. The isolated LAB were sub cultured and the purified cultures

maintained at MRS agar slants.

Test microorganisms
Nutrient broth was prepared and sterilized. Four pathogenic organisms such as *Staphylococcus aureus*, *Bacillus subtilis*, *P. aeruginosa* and *E. coli*, were inoculated separately and kept for incubation for further use.

Preparation of culture supernatants
The bacteriocin producing strain was grown in MRS broth (pH 5.5) at 37 °C for 24-30 hrs. The isolated LAB culture centrifuged at 10000 rpm for 5 min. and then the supernatant was adjusted to pH 6.5-7.0 with 1 N NaOH (Anonymous, 1992) [1].

Extraction of crude protein
The cell free supernatant from *Lactobacillus fermentum* LBC1culture was treated with solid ammonium sulphate to 40, 50 and 60 per cent saturation. The mixture was stirred for 2 h at 4 °C and centrifuged at 20000xg for 1 h (4 °C). The precipitate was resuspended in 5 ml of sodium phosphate buffer (50 mM; pH 7.0) then the crude extract was stored at -20 °C.

Bacteriocin assay
The antimicrobial activity was determined by agar well diffusion method. Muller Hinton agar plates were overlaid with 10 ml Muller Hinton soft agar (0.75% agar) lawn containing an indicator bacterial strain. The indicator lawns prepared by adding 0.25 ml of a 10^{-1} dilution overnight cultures of test organisms. Wells 8 mm in diameter were cut into agar using sterile cork borer. Then 100 µl of culture supernatant fluids of *Lactobacillus fermentum* LBC1 was placed into each well. The plates were incubated at 37 °C for 24 h and examined for zones of inhibition.

Sensitivity of crude protein to enzymes, heat and pH
The sensitivity of crude protein to proteolytic and other enzymes were tested on crude protein (pH 7.0) of 24 h cultures incubated at 37 °C. Samples of 100 µl were treated for 2 h with 1 mg ml^{-1} of Trypsin, proteinase-K, α-amylase and lipase. All the samples and controls were incubated at 37 °C for 5 h and tested for activity. The sensitivity of crude protein to different pH was estimated by adjusting the pH of crude bacteriocin of *Lactobacillus fermentum* LBC1 to pH 2, 3, 4, 5, 6, 7, 8, 9 and 10 with NaOH or HCl and testing against the indicator strain after 2 h incubation. The sensitivity to heat was tested by heating crude bacteriocin of *Lactobacillus fermentum* LBC1 to 37, 50, 70, 90, 100 °C for 30min. and 121 °C for 10 min. and testing the residual activity after the treatment by well diffusion assay.

Optimum conditions for bacteriocin production
The optimum conditions are tested in MRS medium which can replaced the different types of carbon and nitrogen sources. Production medium contains peptone -10g, carbon source -20g, potassium phosphate -2g, sodium acetate -5g, MgSO4 -0.2g, MnSO4-0.05 g with 1 litre distilled water.

Effect of carbon and nitrogen sources on bacteriocin production
The study of bacteriocin production with various carbon sources in the production medium supplemented with different types of carbon sources like sucrose, glucose, maltose lactose and fructose. Nitrogen sources like peptone, tryptone, yeast extract. The sterilized medium flasks were inoculated with 0.1% inoculums and incubated on rotary shaker at 120 rpm for 2 days after the incubation period microbial cells were removed by centrifugation at 3000 rpm for 15 min. Antimicrobial activity of crude protein was estimated by well diffusion method.

Results and Discussion
Bacteriocins are proteinaceous antibacterial compounds and exhibit bactericidal activity against species closely related to the producer strain. In this study, lactic acid bacteria were isolated from cheese. Microscopic identification of the isolate could determine the rod shaped cells, gram positive, catalase negative, non-motile rods and oxidase negative which indicated the typical basic characteristics of Lactobacilli. Based on the carbohydrate utilization pattern of bacterial isolates were identified as *Lactobacillus fermentum* LBC1. The results are tabulated in Table 1. Similar characters for lactic acid bacteria observed earlier by Kandler and Weiss (1986) [9]. Seema Nair and Surendran (2009) in their studies isolated and characterized the lactic acid bacteria from fish and prawn, cheeses (Tulumoğlu *et al*., 2014) [18].
The lactic acid bacterial isolate was tested for their inhibitory activity over some food borne pathogens, *Staphlococcus aureus*, *Bacillus subtilis*, *P. aeruginosa* and *E. coli*. Almost all pathogens were inhibited by bacteriocin producer. The results are tabulated in Table 2.
The effect of various enzymes on the crude protein of *Lactobacillus fermentum* LBC1was studied, inhibitory activity of crude bacteriocin of *Lactobacillus fermentum* LBC1 was completely inactivated by proteolytic enzymes such as proteinase k and trypsin, but not affected by non-proteolytic enzymes such as α-amylase, lipase. Thus, it can be inferred that the bacteriocin is proteinaceous in nature and does not require a carbohydrate or lipid moiety for the activity (De Martinis *et al*., 2001) [3]. According to Fricourt *et al*. (1994) [6], lactic acid bacteria synthesize bactericidal agents that vary in their spectra of activity. Many of these agents are bacteriocins with a proteinaceous active moiety while others are non-protein agents (Piard and Desmazeaud, 1991) [12].
The bacteriocins were shown to be stable over a broad pH range with all peptides maintaining some antimicrobial activity within the pH range of pH 3 to 10. According to Tagg *et al*. (1976) [17], bacteriocins differ greatly with respect to sensitivity to pH and Tween 80. Many of them considerably more tolerant of acid than alkaline pH values. In the present study, crude bacteriocin of *Lactobacillus fermentum* LBC1 exhibited inhibitory activity at pH values between 2 to. Highest inhibitory activity was recorded at pH 6.0. These results surely support the view expressed by Natthida and Piyawan (2011) [10] who found that the crude bacteriocin of *Lactococcus lactis* NN-MD1-7 showed the residual activity remained at pH 3.0 to 9.0 ranges mean while declined activity when treated with those exterior ranges. The highest residual activity was found in the ranges of 93 to 100% at pH 5.0 to 7.0 and maximum activity was shown at pH 6.0.
The crude bacteriocin of *Lactobacillus fermentum* LBC1was relatively stable during heat treatments at 37, 50, 70, 90, 100 °C for 30 min and 121 °C for 10 min. Among the different heat treatments, the highest inhibitory was recorded at 37 °C. Residual activity of bacteriocin did not show significant difference from the control. The bacteriocin produced by the isolate was considered to be most heat stable as the activity

remain after heating at 121 °C. The results were in accordance with Ogunbanwo *et al*., (2003) [11] who observed that activity of bacteriocin produced by *L. brevis* remained after heat at 121 °C for 16 min. Considering the harsh conditions of food processing, high thermal and wide range of pH stability are major criteria in the selection of candidate bacteriocins for biopreservation of processed foods (Yi *et al*., 2016; Hemu *et al*., 2016) [19, 7].

Different carbon sources were used for the bacteriocin production, when sucrose was used as carbon source it recorded highest growth compared to remaining carbohydrates such as maltose, glucose, fructose and lactose respectively. The results are shown in Fig. 1. These results surely support the view expressed by Huot *et al*., (1996) [8] who reported that sucrose is the best carbon source for a bacteriocinogenic strain of *Lactococcus lactis.* In case of nitrogen sources, the highest growth was observed in tryptone, followed by yeast extract, peptone (Fig 2) The results obtained from this study isolated *Lactobacillus fermentum* isolate potent bacteriocin producer and antimicrobial properties that exerts in the usage of this compound as a preservative for maintaining hygiene of fermented foods.

Table 1: Morphological and biochemical characteristics of lactic acid bacterial strain

Morphological Characters	**LBC1**
Gram reaction	+
Spores	-
Shape	Rods
Size	0.5µm x 0.8 µm
Motility	-
Biochemical Characters	
Catalase test	-
Oxidase test	-
NH3 from arginine	+
Gas production from glucose	+
Carbohydrates	
Arabinose	D
Cellobiose	D
Esculin	-
Fructose	+
Galactose	+
Glucose	+
Lactose	+
Maltose	+
Mannitol	-
Mannose	+
Melezitose	-
Melibiose	+
Raffinose	+
Rhamnose	-
Ribose	+
Salicin	-
Sorbitol	-
Sucrose	+
Trehalose	D
Xylose	D
Indentified as	*Lactobacillus fermentum*

+ Positive; -negative; D-delayed reaction

Table 2: Inhibitory spectrum of crude bacteriocin of *lactobacillus fermentum* LBC1 against food borne pathogens

Food Borne Pathogens	**Inhibition Zone (in mm)**
Staphylococcus aureus	12
Pseudomonas aeruginosa	10
Bacillus subtilis	14
Escherichia coli	8

Table 3: Effect of enzymes, pH and heat treatment on inhibitory activity of crude bacteriocin of *Lactobacillus fermentum* LBC1

S. No	**Treatment**	**Inhibition Zone (in mm)**
1	Crude bacteriocin (control)	14.0
2	Trypsin	-
3	Proteinase K	-
4	α-amylase	14.0
5	Lipase	13.5
	pH	
6	2	3.5
7	3	5.3

8	4	9.5
9	5	12.3
10	6	14.2
11	7	11.6
12	8	8.6
13	9	5.4
14	10	3.6
	Temperature	
15	37 °C	14.2
16	50 °C	12.3
17	70 °C	11.0
18	90 °C	8.3
19	100 °C	7.5
20	121 °C	6.3

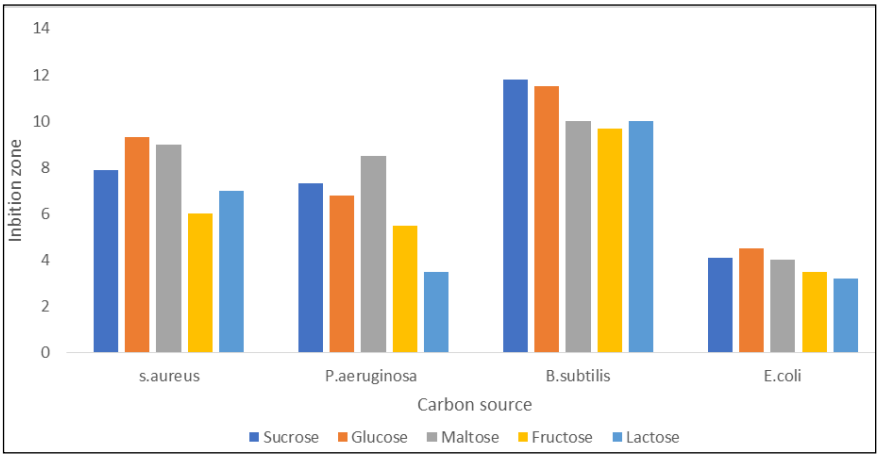

Fig 1: Effect of carbon sources on bacteriocin production

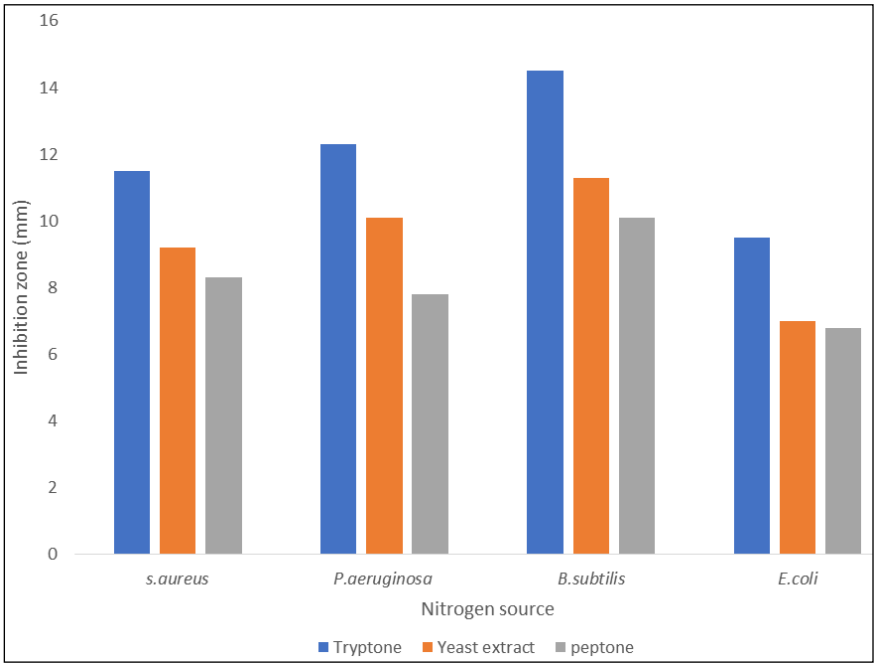

Fig 2: Effect of nitrogen sources on bacteriocin production

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