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ORIGINAL ARTICLE

Characterisation of the bacteriocins produced by two probiotic *Lactobacillus* isolates from *idli* batter

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Abstract *Lactobacillus plantarum* JJ18 and *Lactobacillus plantarum* subsp. *plantarum* JJ60, probiotics from *idli* batter, produce bacteriocins JJ18 and JJ60 having a wide spectrum of activity. After optimising the environmental conditions for bacteriocin production the effect of various media components was determined. Maximum bacteriocin production was observed in MRS broth, pH 6.4 at 37 °C after 36 h. Tryptone (as nitrogen source) and glucose (as carbon source) are required for optimal production of bacteriocins JJ18 and JJ60. Activity was not affected by surfactants like Triton X-100, Tween 80 and Tween 20 or by treatment with NaCl, urea and EDTA. Protease treatment resulted in complete loss of activity of the partially purified bacteriocins JJ18 and JJ60, while lipase and α-amylase had no effect, indicating that the bacteriocin is a simple protein. Tris tricine SDS-PAGE electrophoresis depicted a single band of less than 3.5 kDa. However, the strain *Lactobacillus plantarum* JJ18 was inhibited by bacteriocin JJ60 and *Lactobacillus plantarum* JJ60 by bacteriocin JJ18, whereas no inhibition was observed against the respective producer strains, indicating that the two bacteriocins are different. The bacteriocins remained active over a wide range of pH and temperature. The bacteriocins were able to adsorb onto producer and target cells, *Lactobacillus plantarum* and *Listeria monocytogenes* and differentially in the presence of various surfactants, salts and solvents. A bactericidal mode of action was observed against *Listeria monocytogenes*. Given their wide spectrum of activity against various pathogens, the bacteriocins JJ18 and JJ60 can be applied as bio-preservatives in the food industry.

Keywords *Idli* batter · *Lactobacillus plantarum* · Bacteriocin JJ18 · Bacteriocin JJ60 · Characterisation

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Introduction

Food fermentation is one of the oldest methods of food processing and preservation. Fermentation adds safety and nutritional value and characteristic flavor to the food. It increases the shelf life and microbiological safety of food and makes it more digestible (Caplice and Fitzgerald 1999). Food safety is a major issue for both the food industry and consumers. The general public demands high quality, preservative-free, safe, and minimally processed foods with extended shelf-life. Among the proposed technologies, biopreservation is considered as a promising perspective (Malheiros et al. 2010). Lactic acid bacteria (LAB) are promising alternatives to chemical preservatives. LAB are a heterogeneous group of bacteria used as starter culture for fermentation of different foods such as dairy, meat, vegetables and cereals. Their preserving effect relates mainly to the production of antimicrobial compounds such as lactic acid, acetic acid, hydrogen peroxide, diacetyl, CO_2, and bacteriocin (El-Ghaisha et al. 2011). Furthermore, LAB are indigenous inhabitants of the human gastrointestinal tract, and are thought to be among the dominant colonists of the small intestine (Marco et al. 2006). The increase in bacterial resistance to various antibiotics has stimulated investigators around the world to improve disease control strategies, which has led to the discovery of new vaccines and non-specific immune-stimulants. Thus, there is a growing interest worldwide in the use of probiotic bacteria for their various

beneficial influences on animal and human health. *Lactobacillus plantarum* JJ18 and *Lactobacillus plantarum* subsp. *plantarum* JJ60, which are explored in this study, are potent isolates from *idli* batter having beneficial properties (Agaliya and Jeevaratnam 2012). *Lactobacillus plantarum* JJ18 also has good cholesterol-lowering capacity (Agaliya and Jeevaratnam 2012).

Idli is a natural yeast-lactic fermented product, used mainly as a breakfast snack in southern India. In the majority of fermented foods, particularly traditional foods of India, which are based on cereals and legumes, the nature of fermentation involves LAB (Agrawal et al. 2000). The bacteriocins from lactobacilli isolated from other traditional foods of India have proven its applicability in biopreservation (Jamuna and Jeevaratnam 2004; Jamuna et al. 2005). Bacteriocins of LAB are defined as ribosomally synthesised proteins or protein complexes usually antagonistic to genetically closely related organisms (Klaenhammer 1988; Nes and Johnsborg 2004). Since the majority of bacteriocinogenic LAB are natural food isolates, their antimicrobial peptides can be exploited by the food industry as a tool to control undesirable bacteria in a food-grade and natural manner (Cleveland et al. 2001). A number of bacteriocins have been described for *Lactobacillus plantarum* isolated from various food sources and fermented beverages (Powell et al. 2007).

The bacteriocins from LAB have attracted significant attention because of their potential use as non-toxic and safe additives for food preservation and prevention of food spoilage by food borne gram-positive pathogenic bacteria (Henderson et al. 1992; Morisset et al. 2004). Studies have been conducted on the effect of various media components on the production of bacteriocins (Todorov and Dicks 2005a; Todorov et al. 2011b). The effect of organic nitrogen and carbon on the production of plantaricins has been studied. Bacteriocin production is known to be altered in different environmental conditions and optimum production may require a specific combination of environmental parameters (Leal-Sánchez et al. 2002). The production is often regulated by microbial growth, pH and temperature (Aasen et al. 2000)

Bacteriocins are generally low molecular weight proteins that gain entry into target cells by binding to cell surface receptors. The modes of action of bacteriocins are generally via targeting of the cytoplasmic membrane. They dissipate the proton motive force through the formation of pores in the phospholipids bilayer. This action results in the inhibition of protein or nucleic acid biosynthesis and loss of ions (Bauer and Dicks 2005).

The present study is focussed on the optimisation of bacteriocin production, partial purification and characterisation of bacteriocins produced by two *Lactobacillus plantarum* from *idli* batter.

Materials and methods

Bacterial strains and culture conditions

The probiotic lactobacilli isolated from *idli* batter were characterised as *Lactobacillus plantarum* JJ18 (GenBank ID: JN573601) and *Lactobacillus plantarum* subsp. *plantarum* JJ60 (GenBank ID: JN573602) by 16S rRNA gene sequence analysis and species specific multiplex PCR using *rec*A primer. The *Lactobacillus* was maintained on semisolid De Man, Rogosa and Sharpe (MRS) agar and was grown in MRS broth at 37 °C. The indicator organisms, viz., *Listeria monocytogenes* (MTCC 657), *Staphylococcus aureus* subsp. *aureus* (MTCC 737), *Aeromonas hydrophila* subsp. *hydrophilia* (MTCC 1739) , *Pseudomonas aeruginosa* (MTCC 2295), *Micrococcus luteus* (MTCC 106), *Bacillus cereus* (MTCC 1272), *Vibrio parahaemolyticus* (MTCC 451), *Bacillus subtilis* (MTCC 619), *Escherichia coli* (MTCC 728) for antibacterial activity determination were procured from the Microbial Type Culture Collection (MTCC) at Institute of Microbial Technology, Chandigarh, India. All the bacteria cultures used in the study were preserved in 70 % glycerol. They were propagated subsequently for use in appropriate media and temperature.

Bacteriocin activity and production

Lactobacillus plantarum JJ18 and *Lactobacillus plantarum* subsp. *plantarum* JJ60 were grown in MRS broth at 37 °C using inoculums of an overnight culture at 1 % level. The changes in the pH and optical density (600 nm) were recorded for every 6 h over a period of 72 h for the two cultures. The cell-free supernatant (CFS) concentrated (10-fold) using a rotary evaporator was used to determine bacteriocin activity using the agar well diffusion assay (Jamuna and Jeevaratnam 2004) against the indicator strains *Listeria monocytogenes* MTCC 657 and *Staphylococcus aureus* MTCC 737 at the same time interval, and activity was expressed as arbitrary units per millilitre (AU/mL) (Todorov et al. 2011a). Bacteriocin production was checked by growing the culture in MRS broth of various initial pH values (4.5, 5.0, 5.5, 6.0, 6.5 and 7.0) and at various temperatures (4, 10, 15, 25, 30, 37, and 45 °C).

Effect of media components on bacteriocin production

Fresh overnight inoculums of *Lactobacillus plantarum* JJ18 and *Lactobacillus plantarum* subsp. *plantarum* JJ60 were added to the growth media MRS broth, pH 6.4 (without tryptone, meat and yeast extract), supplemented with tryptone (20.0 g/L), meat extract (20.0 g/L), yeast extract (20.0 g/L), tryptone (12.5 g/L) plus meat extract (7.5 g/L), tryptone (12.5 g/L) plus yeast extract (7.5 g/L), meat extract (10.0 g/L) plus yeast extract (10.0 g/L), and a combination of tryptone (10.0 g/L), meat extract (5.0 g/L) and yeast

extract (5.0 g/L), respectively; MRS broth, pH 6.4 (without glucose), supplemented with 20.0 g/L glucose, fructose, sucrose, mannose, maltose, lactose, respectively. MRS broth (pH 6.4) supplemented with different concentrations K_2HPO_4 and KH_2PO_4 (0.2–10.0 g/L); and MRS broth (pH 6.4) supplemented with glycerol (1.0, 5.0, 10.0, 20.0 and 50.0 g/L, respectively). MRS broth (pH 6.4) supplemented with Tri-ammonium citrate (0.0, 5.0 and 10 g/L), $MgSO_4$ (0.0 and 0.1 g/L), $MnSO_4$ (0.0 and 0.02 g/L) and Tween 80 (0.0, 1.0, 2.0 and 5.0 g/L). The effect of various vitamins like vitamin C, vitamin B1, vitamin B12 and DL-6,8-thioctic acid (1.0 g/L) were also checked (Todorov et al. 2011b).

Partial purification of the bacteriocins

Lactobacillus plantarum JJ18 and *Lactobacillus plantarum* subsp. *plantarum* JJ60 were grown in 500 mL optimised media and incubated at 37 °C for 36 h. The CFS concentrate maintained at <0 °C was used to obtain an acetone extract of bacteriocins by gently adding ice-cold acetone with constant stirring to a saturation of 50 %, which was further subjected to 75 % acetone precipitation, wherein the precipitate was dissolved in distilled water and the residual acetone removed by rotary vacuum evaporation (Jamuna and Jeevaratnam 2004). The dry precipitate was dissolved in 5 mM acetate buffer (pH 5.0) and stored at 4 °C. The precipitate was passed through Sephadex G 25 column. By checking the fractions for inhibitory effect against *Listeria monocytogenes* MTCC 657 and *Staphylococcus aureus* MTCC 737 the active fraction was obtained. The active fraction was again passed through a Sephadex G 25 (super fine) column to obtain a homogenous partially purified bacteriocin, which was examined by Tris-tricine (16 % gel) SDS-PAGE electrophoresis. One half of the gel was fixed with 5 % glutaraldehyde solution and stained with Coomassie Brilliant blue G 250 (Schägger and Von Jagow 1987). The other half of the gel was fixed with 20 % isopropanol and 10 % acetic acid for activity staining. After washing with distilled water, the gel was overlaid with the indicator *Listeria monocytogenes* MTCC 657 of 10^6 CFU/mL (Todorov et al. 2010).

Protein assay

The protein was determined by modified Biuret method of Gornall et al. (1949) with bovine serum albumin (BSA) as standard.

Antibacterial (spectrum) activity screening

The partially purified bacteriocins JJ18 and JJ60 were used to obtain the antibacterial (spectrum) activity against various LAB and pathogens by agar well diffusion method (Jamuna and Jeevaratnam 2004).

Effect of various treatments on activity of bacteriocin

The influence of various enzymes, temperature and pH on the antibacterial activity of partially purified bacteriocins was analysed. Bacteriocins JJ18 and JJ60 were treated with proteinase K, pepsin, protease, lipase and α-amylase at 1 mg/mL and incubated for 2 h at 37 °C (Todorov et al. 2011b). The bacteriocins were also treated with 1 % (w/v) of sodium dodecyl sulphate (SDS), Tween 20, Tween 80, urea, Triton X-100, bile and NaCl. Untreated bacteriocins and detergents at these respective concentrations in water were used as controls. All samples were incubated at 37 °C for 5 h and tested for antibacterial activity. The effect of pH on the activity of bacteriocins was tested by adjusting the pH from 2.0 to 12.0 (in increments of two pH units) with sterile 1 M NaOH or 1 M HCl. After 1 h of incubation at room temperature (28 °C) the antibacterial activity was determined by readjusting the samples to pH 6.0. The effect of temperature on the bacteriocin activity was also tested by incubating the bacteriocins JJ18 and JJ60 at pH 6.0 at 4, 10, 25, 30, 37, 60, 80 and 100 °C for 2 h, except for 121 °C (20 min) (Todorov et al. 2011b). The antibacterial activity of the partially purified bacteriocin after these treatments was tested against *Listeria monocytogenes* MTCC 657 and *Staphylococcus aureus* MTCC 737 (Jamuna and Jeevaratnam 2004).

Adsorption of bacteriocin to producer and target cells

Lactobacillus plantarum JJ18 and *Lactobacillus plantarum* subsp. *plantarum* JJ60 were cultured at 30 °C for 18 h. The pH of the culture was adjusted to 6.0 with 1 M NaOH to allow maximal adsorption of the bacteriocin to the producer cells (Yang et al. 1992). The cells were then harvested (12,000 *g*, 15 min, at 4 °C) and washed with sterile 0.1 M phosphate buffer (pH 6.5). The pellet was re-suspended in 10 mL 100 mM NaCl (pH 2.0) and stirred slowly for 1 h at 4 °C. The suspension was then centrifuged (12,000 *g*, 15 min, 4 °C), the CFS adjusted to pH 6.0 with sterile 1 M NaOH and was tested for bacteriocin activity. Adsorption of bacteriocins JJ18 and JJ60 to target cells was also studied against one resistant (*Lactobacillus plantarum* MTCC 6160) and one sensitive strain (*Listeria monocytogenes* MTCC 657). The early stationary phase target cells were harvested and washed twice with 5 mM phosphate buffer (pH 6.5) and its OD adjusted to 1.0. Equal volumes of bacteriocins JJ18 and JJ60 (adjusted to pH 6.0, 1,600 AU/mL) were then added to the resuspended cells and the mixture was incubated for 1 h at 37°C. Finally, the activity of unbound bacteriocin in the supernatant was tested (Todorov et al. 2011b). The target cells were treated with 1 % (m/v) Tween 20, Tween 80, NaCl, ascorbic acid, potassium sorbate and sodium nitrate along with the bacteriocins JJ18 and JJ60. The pH of all samples were adjusted to 6.5 with 1 M NaOH or 1 M HCl

and incubated for 1 h at 37 °C. The effect of various pH and temperature on the adsorption to the target cells was also determined. The activity of bacteriocins JJ18 and JJ60 in the CFS was tested (Todorov et al. 2011b). The effect of solvents like methanol, ethanol and chloroform was also studied (Todorov et al. 2007b).

Mode of action of the bacteriocins JJ18 and JJ60

Ten millilitres of filter-sterilised partially purified bacteriocins JJ18 and JJ60 were added to a 50-mL culture of *Listeria monocytogenes* MTCC 657 at early exponential phase and then incubated at 37 °C. Samples were taken hourly to determine the viable cells (CFU/mL) on TSYE agar plates. The optical density (OD) of the samples was also monitored for every hour. Culture without bacteriocin was used as control (Xie et al. 2011).

Results and discussion

Production of bacteriocins JJ18 and JJ60 commenced at the log phase (200 AU/mL at 6 h) as deduced by inhibition against *Listeria monocytogenes* MTCC 657 and *Staphylococcus aureus* MTCC 737. The maximum production (1,600 AU/mL) against *Listeria monocytogenes* (Fig. 1a,b) and *Staphylococcus aureus* (Fig. 1c,d) was observed during 30 to 36 h, i.e. in early stationary phase, while the pH of the culture decreased to 5.5 and 5.0, respectively (Fig. 1a,b), while a decline was observed after 48 h. The initial pH of 6.0, 6.5 and 7.0 of MRS broth resulted in maximum production of JJ18 and JJ60 of 1,600 AU/mL (Table 1), while pH5.0 and 5.5 with 800 AU/mL yielded much lower activity of 400 AU/mL at pH 4.5. The initial pH of the medium plays a crucial role in production of bacteriocins. An initial pH of at least 6.0 is optimal for production of bacteriocin. Other *Lactobacillus plantarum* strains showed similar results (Todorov et al. 2011b). The optimal temperature for production of bacteriocin JJ18 and JJ60 was 30 to 37 °C, while temperatures below 30 °C and above 37 °C showed decreased production (Table 1). Studies conducted on bacteriocins from other LAB have suggested that production is often regulated by growth, pH and temperature with a few exceptions (Aasen et al. 2000).

Among the nitrogen sources, tryptone in combination with yeast extract or meat extract yielded an activity of 1,600 AU/mL. The combination of meat extract and yeast extract (10.0 g/L and 10.0 g/L) yielded only 25 % of the activity. When single nitrogen source of meat extract or yeast extract was tested, only 12.5 % of activity was observed (Table 2). The results suggested that tryptone is required for optimal production of bacteriocins JJ18 and JJ60. The results are in agreement with those reported for plantaricins ST194BZ and ST16Pa (Todorov and Dicks 2005a; Todorov et al. 2011b).

Bacteriocins JJ18 and JJ60 were produced at 1,600 AU/mL when strain was grown in MRS broth supplemented with 20.0 g/L of either glucose or maltose, while a decreased activity was observed with lactose, galactose, mannose (800 AU/mL) and fructose (400 AU/mL). An increase in bacteriocin JJ18 production was recorded in the presence of 30.0 g/L glucose (3,200 AU/mL) but the activity decreased to 50 % with 50.0 g/L glucose. On the other hand, glucose at 5.0 g/L or 10.0 g/L reduced the production of bacteriocin considerably. This suggests that glucose acts as the primary carbon source. The activity of 800 AU/mL bacteriocins JJ18 and JJ60 from lactose and sucrose would be because of the glucose moiety. Similar observations were made in other bacteriocins like ST194BZ and ST16Pa (Todorov and Dicks 2005a ; Todorov et al. 2011b). Glucose was shown to be a favourable carbohydrate for production of plantaricin UG1 (Enan et al. 1996) and bacteriocins ST202Ch and ST216Ch (Todorov et al. 2010). However, much higher glucose concentration (50.0 g/L) had a repressive effect resulting in only 800 AU/mL (Table 2). A similar repression effect was observed in the production of bacteriocin ST341LD (Todorov and Dicks 2006a).

Little is known about the influence of potassium ions on the production of bacteriocins JJ18 and JJ60. Levels of 10.0 g/L and 20.0 g/L KH_2PO_4 increased the production of bacteriocins JJ18 and JJ60 (1,600 AU/mL), but 2.0 g/L of the same salt reduced bacteriocin production by 25 %. On the other hand, low levels of K_2HPO_4 (2.0 g/L and 5.0 g/L) resulted in JJ18 and JJ60 bacteriocin production of 1,600 AU/mL, whereas 10.0 g/L and 20.0 g/L K_2HPO_4 resulted in a decrease to 400 AU/mL (Table 2). This variation in production cannot be due to pH changes caused by higher or lower potassium levels, since all media were adjusted to pH 6.5 before inoculation as reported by Todorov et al. (2011b).

Addition of glycerol (1.0 or 2.0 g/L) to MRS medium had no effect on production of bacteriocins JJ18 and JJ60 (1,600 AU/mL). In contrast, a considerable loss of activity observed when the glycerol concentration increased to 5.0 g/L and above. A similar effect of glycerol was reported for bacteriocin ST202Ch, ST216Ch (Todorov et al. 2010) and bacteriocin ST16Pa (Todorov et al. 2011b). Bacteriocins JJ18 and JJ60 production was higher (1,600 AU/mL) in the absence of tri-ammonium citrate (Table 2), but lower in the presence of 10.0 g/L tri-ammonium citrate. A similar effect of tri-ammonium citrate was found for production of bacteriocins ST8KF (Powell et al. 2007) and ST16Pa (Todorov et al. 2011b).

The presence of $MgSO_4$ (0.1 g/L) and $MnSO_4$ (0.02 g/L) was essential for maximum bacteriocin JJ18 and JJ60 (1,600 AU/mL) as there was reduced production in the

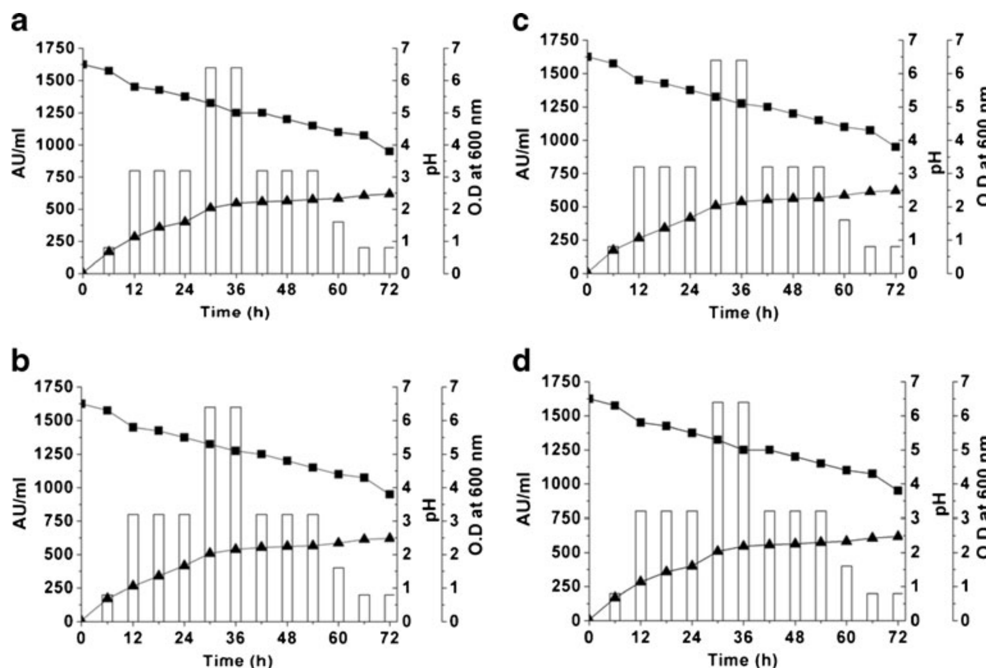

Fig. 1 **a**–**d** Effect of duration of incubation at 37 °C on the optical density at 600 nm (▲), pH (■) and antibacterial activity (*open bars*) of *Lactobacillus plantarum* JJ18 and *Lactobacillus plantarum* JJ60 in MRS broth medium. Antibacterial activity was evaluated against *Listeria monocytogenes* MTCC 657 (**a**, **b**) and *Staphylococcus aureus* MTCC 737(**c**, **d**)

absence of these salts (Table 2). Similar results were observed for bacteriocin ST16Pa (Todorov et al. 2011b). Omitting $MgSO_4$ from the medium formula had a negative effect on bacteriocin JJ18 and JJ60 production (Table 2). A similar negative effect on the production of both bacteriocin ST202Ch and bacteriocin ST216Ch was reported by Todorov et al. (2010). MRS medium supplemented with 1 g/L Tween 80 showed maximum production of bacteriocin JJ18; the surfactant Tween 80 probably facilitates the discharging of the bacteriocin from the cell surface of the producer strain (Todorov and Dicks 2005a). The absence of Tween 80 in the MRS medium had a positive effect on the production of bacteriocin JJ60, while increasing Tween 80 up to 5.0 g/L in MRS broth had no beneficial effect on bacteriocin production (Table 2). The presence of vitamins like thiamine, cyanocobalamin, vitamin C and DL-6, 8 thioctic acid did not stimulate the production of bacteriocins JJ18 and JJ60 but resulted in decreased activity (Table 2). A similar effect of vitamins on bacteriocin production was observed in bacteriocin ST23LD, ST341LD (Todorov and Dicks 2006a) and ST16Pa (Todorov et al. 2011b).

Table 1 Effect of pH and temperature on production of bacteriocins JJ18 and JJ60

	Activity (AU/mL)	
	JJ18	JJ60
pH		
4.5	400	400
5.0	800	800
5.5	800	800
6.0	1600	1,600
6.5	1,600	1,600
7.0	1,600	1,600
Temperature (°C)		
4	400	400
10	400	400
15	400	400
25	400	400
30	1,600	1,600
37	1,600	1,600
45	800	800

Partially purified bacteriocins JJ18 and JJ60 were obtained by passing the reconstituted 75 % cold-acetone precipitate through a gel permeation column—Sephadex G 25 initially and the pooled active fractions once again through Sephadex G 25 superfine. The Tris-tricine SDS PAGE electrophoresis of the partially purified bacteriocin preparation revealed a single protein band, with a molecular weight less than 3.5 kDa in one half of the gel stained with Coomassie Brilliant blue G 250 (Fig. 2), while the other half overlaid with *Listeria monocytogenes* MTCC 657 showed a zone of inhibition corresponding to the protein band (Fig. 2). A bacteriocin of approximately 2.9 kDa was reported in case of bac AMA-K (Todorov et al. 2007a), while that of bacteriocin LABB and LABP from appam batter were found to be 3.8 and 4.5 kDa, respectively (Jamuna and Jeevaratnam 2004). The molecular size of bacteriocins JJ18 and JJ60 are within the range of most bacteriocins reported for the genus *Lactobacillus* (Cintas et al. 2001). However, the strain *Lactobacillus plantarum* JJ18 was inhibited by bacteriocin JJ60 (Fig. 3a) and *Lactobacillus plantarum* JJ60 by

Table 2 Effect of various media components on the production of bacteriocins JJ18 and JJ60

Medium components	Activity (AU/ml)	
	JJ18	JJ60
Tryptone 20.0 g/L	800	800
Meat extract 20.0 g/L	200	200
Yeast extract 20.0 g/L	200	200
Tryptone+Meat extract 12.5 g/l+7.5 g/L	1,600	1,600
Tryptone+Yeast extract 12.5 g/L+7.5 g/L	1,600	1,600
Yeast extract+Meat extract 10 g/L+10 g/L	400	400
Tryptone+Meat extract+yeast extract 10 g/L +5 g/L+5 g/L	400	400
Glucose 20.0 g/L	1,600	1,600
Fructose 20.0 g/L	400	400
Galactose 20.0 g/L	800	800
Mannose 20.0 g/L	800	800
Lactose 20.0 g/L	800	800
Maltose 20.0 g/L	1,600	1,600
Sucrose 20.0 g/L	800	800
Glucose 5.0 g/L	200	200
Glucose 10.0 g/L	400	400
Glucose 20.0 g/L	1,600	1,600
Glucose 30.0 g/L	3,200	3,200
Glucose 50.0 g/L	800	800
KH_2PO_4 2.0 g/L	400	400
KH_2PO_4 5.0 g/L	400	400
KH_2PO_4 10.0 g/L	1,600	1,600
KH_2PO_4 20.0 g/L	1,600	1,600
K_2HPO_4 2.0 g/L	1,600	1,600
K_2HPO_4 5.0 g/L	1,600	1,600
K_2HPO_4 10.0 g/L	400	400
K_2HPO_4 20.0 g/L	400	400
Glycerol 1.0 g/L	1,600	1,600
Glycerol 2.0 g/L	1,600	1,600
Glycerol 5.0 g/L	400	400
Glycerol 10.0 g/L	400	400
Glycerol 20.0 g/L	200	200
Tri-ammonium citrate 0.0 g/L	1,600	1,600
Tri-ammonium citrate 5.0 g/L	1,600	1,600
Tri-ammonium citrate 10.0 g/L	400	400
$MgSO_4$ free	800	800
$MgSO_4$ 0.1 g/L	1,600	1,600
$MnSO_4$ free	400	400
$MnSO_4$ 0.02 g/L	1,600	1,600
Tween 80 free	800	1,600
Tween 80 1.0 g/L	1,600	1,600
Tween 80 2.0 g/L	400	400
Tween 80 5.0 g/L	400	400
Vitamin B1	400	400
Vitamin B 12	400	400
Vitamin C	400	400
DL-6,8-thioctic acid	400	400

bacteriocin JJ18 (Fig. 3b) whereas no inhibition was observed against the respective producer strains (Fig. 3a,b). This indicated that they are different even though they behaved similarly; however, further purification and sequencing of the bacteriocins would confirm these findings.

Bacteriocins JJ18 and JJ60 showed good antimicrobial activity against pathogens like *Bacillus cereus* and *Staphylococcus aureus*, which were earlier reported to be common contaminants in *idli* batter fermentation, and also showed anti-listerial activity—considered as a common food pathogen in food industry (Aymerich et al. 2000). The inhibition of other pathogens, such as *Aeromonas*, *Pseudomonas*, *Micrococcus* and *Bacillus*, indicate its application in biopharmaceuticals (Table 3).

Bacteriocins JJ18 and JJ60 were completely inactivated after treatment with proteolytic enzymes such as proteinase K, protease and pepsin, while bacteriocin activity remained stable after treatment with amylase and lipase (Table 4), confirming the proteinaceous nature of bacteriocins (Todorov and Dicks 2005b). Moreover, their activities were not affected by lipolytic or glycolytic enzymes, suggesting that the active moiety was not associated with a lipid or a glucan (Tomé et al. 2006). Bacteriocins JJ18 and JJ60 were not affected when treated with Tween 20, Tween 80, Triton X-100, and SDS at 1 % (w/v) or EDTA (0.1, 2, and 5 mM) (Table 3). Similar results were recorded for plantaricin C19 (Atrih et al. 2001) and bacteriocin AMA-K (Todorov et al. 2007a). The effect of

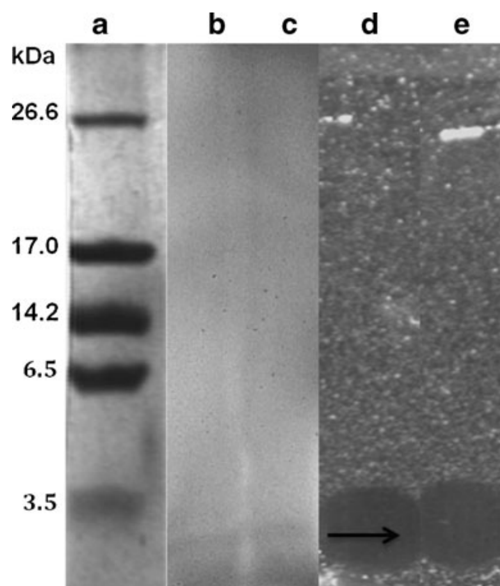

Fig. 2 Tris-tricine-SDS-PAGE of partially purified bacteriocins along with standard markers and the other half of the gel showing an inhibitory zone. Lanes: *a* Molecular weight marker, *b* protein band representing the bacteriocin JJ18, *c* protein band representing the bacteriocin JJ60, *d* zone of inhibition of *Listeria monocytogenes* by bacteriocin JJ60, *e* zone of inhibition of *Listeria monocytogenes* by bacteriocin JJ18

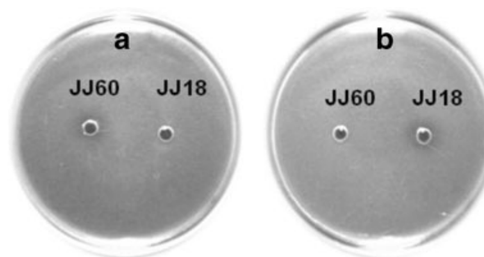

Fig. 3 Inhibition of **a** *Lactobacillus plantarum* JJ18 and **b** *Lactobacillus plantarum* JJ60 by bacteriocins JJ18 and JJ60

detergents on different bacteriocins provided information about the structure of the peptides. Anionic detergents often unfold proteins by complexion of the hydrophobic core of their native structure, which may affect their three-dimensional conformation (Todorov and Dicks 2005b). Antimicrobial activity was affected after treatment with either bile or urea (1 %w/v). Bacteriocins JJ18 and JJ60 were able to resist temperatures ranging from 4 °C to 60 °C. However, there was loss of 50 % activity from 80–100 °C after 2 h. The bacteriocins exhibited 50 % activity when subjected to 121 °C for 20 min (Table 4). Upon exposure to different pH values, bacteriocins JJ18 and JJ60 remained stable from pH 2 to 6, whereas at alkaline pH of 8 to 12, a loss of 25–50 % activity was observed (Table 4). This loss of activity might be ascribed to proteolytic degradation, protein aggregation or instability of proteins at this extreme pH (Aasen et al. 2000; Parente and Ricciardi 1994). In conclusion, bacteriocins JJ18 and JJ60 were thermostable, which is a very useful characteristic if they is to be used as a food preservative, because many food-processing procedures involve a heating step. The bacteriocins JJ18 and JJ60 were similar to a great extent to bacteriocin plantaricin MG (Gong et al. 2010), ST8KF (Powell et al. 2007) and AMA-K (Todorov et al. 2007a).

Table 3 Antibacterial activity against various pathogens

Indicator strain	Activity (AU/mL)	
	JJ18	JJ60
Listeria monocytogenes (MTCC 657)	1,600	1,600
Staphylococcus aureus subsp. *aureus* (MTCC 737)	1,600	1,600
Aeromonas hydrophila subsp. *hydrophilia* (MTCC 1739)	1,600	1,600
Pseudomonas aeruginosa (MTCC 2295)	1,600	1,600
Micrococcus luteus (MTCC 106)	1,600	1,600
Bacillus cereus (MTCC 1272)	1,600	1,600
Vibrio parahaemolyticus (MTCC 451)	1,600	1,600
Bacillus subtilis (MTCC 619)	1,600	1,600
Escherichia coli (MTCC 728)	1,600	1,600

Table 4 Effect of enzymes, chemicals, pH and temperature on activity of bacteriocins JJ18 and JJ60

	Activity of bacteriocin [AU/mL (%)]			
	Listeria monocytogenes		*Staphylococcus aureus*	
	MTCC 657		MTCC 737	
	JJ18	JJ60	JJ18	JJ60
Without treatment	1,600 (100 %)	1,600 (100 %)	1,600 (100 %)	1,600(100 %)
Effect of enzymes (1 mg/mL)				
Proteinase K, pepsin and protease	–	–	–	–
Amylase and lipase	1,600 (100 %)	1,600 (100 %)	1,600 (100 %)	1,600 (100 %)
Treatment with 1 % NaCl, SDS, Triton X100 and Tween 20	1,600 (100 %)	1,600 (100 %)	1,600 (100 %)	1,600 (100 %)
Tween 80	1,600 (100 %)	1,600 (100 %)	800 (50 %)	800 (50 %)
Urea	400 (25 %)	400 (25 %)	200 (12.5 %)	200 (12.5 %)
Bile	400 (25 %)	400 (25 %)	200 (12.5 %)	200 (12.5 %)
EDTA (0.1, 2 and 5 mM)	1,600 (100 %)	1,600 (100 %)	1,600 (100 %)	1,600 (100 %)
Effect of temperature (°C) after 2 h:				
4, 10, 25, 30, 37 and 60	1,600 (100 %)	1,600 (100 %)	1,600 (100 %)	1,600 (100 %)
80, 100 and 121(20 min)	800 (50 %)	800 (50 %)	800 (50 %)	800 (50 %)
Effect of pH after 1 h				
pH 2, 4 and 6	1,600 (100 %)	1,600 (100 %)	1,600 (100 %)	1,600 (100 %)
pH 8 and 10	800 (50 %)	800 (50 %)	800 (50 %)	800 (50 %)
pH 12	400 (25 %)	400 (25 %)	400 (25 %)	400 (25 %)

Bacteriocins JJ18 and JJ60 adsorbed at 50 % to cells of *Lactobacillus plantarum* (MTCC 6161) and 75 % to cells of *Listeria monocytogenes* (MTCC 657). Different levels of adsorption was observed in previous studies but, in general,

Table 5 Effect of temperature, pH, chemicals and solvents on adsorption to target cells

	Lactobacillus plantarum		*Listeria monocytogenes*	
	MTCC 6161[a]		MTCC 657[a]	
	JJ18	JJ60	JJ18	JJ60
Effect of temperature (°C)				
4 and 15	50	50	25	25
30	75	50	75	50
37 and 45	75	75	75	75
Effect of pH				
3.5, 5.5 and 7.0	50	50	50	50
Effect of chemicals				
Tween 80	50	50	25	25
Tween 20	50	50	50	50
Ascorbic acid	25	50	25	50
Potassium sorbate	87.5	87.5	75	75
Sodium nitrate	87.5	87.5	75	75
NaCl (0.5 and 1 %)	25	25	25	25
NaCl (1.5 and 2 %)	50	50	50	50
Effect of solvents				
80 % ethanol, chloroform, methanol	50	50	50	50

[a]Values are the residual (unbound) bacteriocin activity, mean of two independent experiments

the highest levels of adsorption were observed against sensitive strains compared to strains resistant to the bacteriocin (Todorov 2008). Bacteriocins JJ18 and JJ60 were adsorbed at 50 % to cells of *Listeria monocytogenes* and *Lactobacillus plantarum* at 4 °C and 15 °C. At 30 °C, adsorption was 75 % for *Listeria monocytogenes* and *Lactobacillus plantarum* for JJ18 and 50 % for JJ60. At 37 °C and 45 °C, the bacteriocins showed adsorption of 75 % against *Listeria monocytogenes* and *Lactobacillus plantarum*. Optimal adsorption of bacteriocins JJ18 and JJ60 at 50 % was recorded at pH 3.5, 5.5 and for *Listeria monocytogenes* and *Lactobacillus plantarum*. These results show the potential application of bacteriocin at neutral or moderately acidic pH (Table 5). Effect of various chemicals on adsorption was also checked; 50 % of adsorption was observed with chemicals like Tween 80 and Tween 20. Variation in level of adsorption for ascorbic acid was observed for bactericoins JJ18 and JJ60. Adsorption decreased with chemicals such as potassium sorbate and sodium nitrate (Table 5). Similar results were observed for bacteriocin ST16Pa (Todorov et al. 2011b). Adsorption of bacteriocins JJ18 and JJ60 to the cell surface decreased in the presence of sodium chloride, and the reduction was concentration-dependent. These inhibition patterns show that binding of bacteriocins JJ18 and JJ60 with cell surface receptors may be mediated by ionic interactions (Yildirim et al. 2002); 50 % adsorption was observed in 1.5 and 2 % NaCl, while increased adsorption was observed in 0.5 and 1 % NaCl. Fifty percent adsorption was also observed with different solvents like 80 % alcohol, methanol and chloroform (Table 5). From these results, we conclude that the adsorption of bacteriocins JJ18 and JJ60 to target cells is affected by environmental factors such as pH and temperature, and the presence of surfactants, inorganic salts and alcohols. From an industrial point of view, for instance the application of plantaricin as a food preservative, these are important factors that will have to be taken into account (Todorov and Dicks 2006b).

The addition of bacteriocin JJ18 and JJ60 to an exponentially growing culture of *Listeria monocytogenes* MTCC 657 did not alter the OD of the cells, indicating that there was no cell lysis (Fig. 4a,b). However, a drastic decrease in viable cell number (CFU) was observed within 3 h for bacteriocin JJ18 (Fig. 4c) and within 2 h for bacteriocin JJ60 (Fig. 4d). This suggested that the mode of action of bacteriocins JJ18 and JJ60 was bactericidal. A bactericidal effect was reported for bacteriocin AMA-K (Todorov et al. 2007a). Understanding the mode of action of a bacteriocin is important for its effective utilisation. Bactericidal mechanisms vary and may include pore formation, degradation of cellular DNA, disruption through specific cleavage of 16S rRNA, and inhibition of peptidoglycan synthesis (Heu et al. 2001).

In the present study, bacteriocins JJ18 and JJ60 were produced by *Lactobacillus plantarum* from *idli* batter. Various factors affecting the production of bacteriocins were studied. Partially purified bacteriocins of molecular weight less than 3.5 kDa were obtained. The bacteriocins were very stable to various pH and temperature conditions. The bacteriocins showed a broad spectrum of activity against various food spoilage pathogens like *Listeria monocytogenes* and

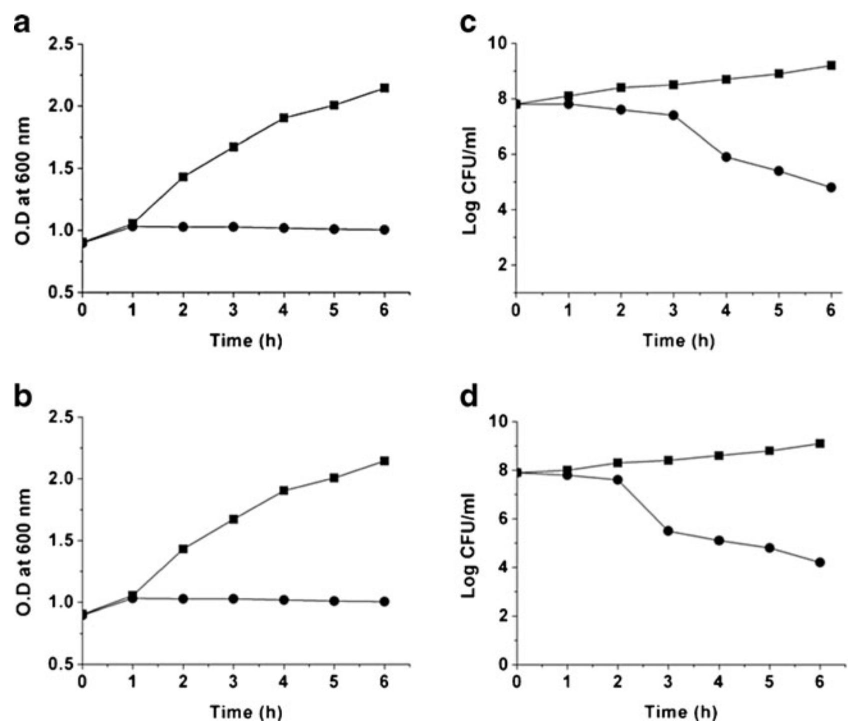

Fig. 4a–d Mode of action of bacteriocins JJ18 and JJ60against *Listeria monocytogenes* MTCC 657. **a**–**d** Optical density of cells measured at 600 nm treated with (■) and without (•) bacteriocin (**a**, **b**); and log CFU/mL counts of the indicator strain treated with (■) and without (•) bacteriocin (**c**, **d**)

Escherichia coli. According to recent trends in food preservation, the improvement of microbiological safety of foods can be attained with the help of bacteriocin-producing strains of LAB isolated from the same food that needs to be preserved. Moreover, *Lactobacillus plantarum* JJ18 and *Lactobacillus plantarum* subsp. *plantarum* JJ60 have probiotic properties. Thus the broad spectrum of activity of bacteriocins JJ18 and JJ60 could find a potential application in food as a biopreservative and in pharmaceuticals as antibacterial agents.

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