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Characterization of a Bacteriocin-Like Inhibitory Substance Produced by *Lactobacillus plantarum* Isolated from Egyptian Home-Made Yogurt

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ABSTRACT: As demand for reduction of the use of chemical additives in food and for more natural and microbiologically safe food products is increasing, bacteriocins may have considerable potential for food preservation. This work aimed to isolate *Lactobacillus* strains of potential bioprotective application from Egyptian home-made yogurt. Out of 73 strains of isolated *Lactobacillus* strains, only four strains of *Lactobacillus plantarum* (AA110, AA125, AA135 and AA140) demonstrated production of antagonistic activity against foodborne pathogens. The antimicrobial agent excreted by *L. plantarum* AA135 was the most active against a wide range of Gram-positive and Gram-negative pathogens. Because of the interesting inhibition spectrum of agent AA135, it was selected for further characterization. The activity of the antimicrobial substance produced from *L. plantarum* AA135 was resistant to heat (121 °C for 30 min), catalase and lysozyme, but was destroyed by papain, trypsin and pepsin. The AA135 compound was produced during growth phase and, when the neutralized and concentrated supernatant was added to a culture of *Pseudomonas aeruginosa* in logarithmic phase, it produced a rapid decrease in optical density indicative of cell lysis. The AA135 agent could be extracted from the culture supernatant fluids with n-butanol. When 40% ammonium sulphate-precipitated agent was analysed by 10% SDS-PAGE, two peptides with high molecular sizes of ~45 kDa and <45 kDa were seen. *L. plantarum* AA135 does not contain any plasmid, which indicates that the antagonistic compound's production is encoded by chromosomal genes. The antibacterial agent was characterized as a bacteriocin-like inhibitory substance and designated as plantaricin AA135. The potential significance for improving the hygiene and safety of the food products is discussed.

KEYWORDS: *Lactobacillus plantarum*, plantaricin AA135, chromosome, protein patterns.

INTRODUCTION

Lactic acid bacteria have been widely used in the food industry as starter culture for fermentation. *Lactobacillus* species play a crucial role in foodstuffs, because of their fermentative ability and their health and nutritional benefits.[1] Lactic acid bacteria can produce many compounds such as organic acids, hydrogen peroxide, and bacteriocin during fermentation.[2,3] Bacteriocins are proteinaceous substances that display antimicrobial activity against species closely related to the producer strain and/or other bacteria.[4-11] A variety of antimicrobial agents that differ in their inhibitory spectra, mode of actions and biochemical characteristics is produced by *Lactobacillus* species. *L. plantarum* has been isolated from various habitats and several bacteriocins (antimicrobial peptides) have been described in strains from milk and cheese.[12,13] Production of an antimicrobial compound (lactolin) which resembled a bacteriocin from *L. plantarum* was firstly reported by Kodama.[14] Later, many other bacteriocins produced by different strains of *L. plantarum* were identified and characterized.[15-25] Because of the increasing demand for more natural and microbiologically safe food products, there is a need for biopreservation techniques. Bacteriocins have considerable potential for food preservation, as well as for human therapy as potential supplements or replacements for currently used antibiotics.[26,27] Therefore, the aim of this study was to screen a number of *L. plantarum* strains isolated from Egyptian home-made yogurt for antagonistic activity, in order to identify bacteriocins with broad inhibition spectra.

MATERIALS AND METHODS

Strains and Culture Conditions

Fifty samples of Egyptian home-made yogurt were collected for isolation of *Lactobacilus* strains on MRS agar[28] (Oxoid) at 37 °C for 48 hours anaerobically by using the BBL Gas-pak system. Colonies were taken

from the MRS plates, subcultured and maintained by bi-weekly transfer in MRS agar. Lactobacilli which produced antagonistic activity were identified according to the procedures described in the Bergey's Manual.[29] The different target organisms used to demonstrate antimicrobial activity, which are listed in Table 1, were grown aerobically at 37 °C in Luria Bertani Medium, LB medium[30] containing (g/l): tryptone, 10; yeast extract, 10; sodium chloride, 5; for pre-seeded agar plates of target strains.

Preparation of Crude Extracts

Cultures of lactobacilli were grown anaerobically (BBL Gas-pak system) in MRS broth at 37 °C for 48 hours. After incubation, the bacterial cells were removed by centrifugation at 10,000 x *g* for 5 min at 4 °C. The pelletted cells were kept at -20 °C for plasmid and protein pattern studies. The supernatant was adjusted to pH 6 with 1 M NaOH to eliminate inhibitory activity from acid and then treated with 5 mg/ml catalase to remove antagonistic activity from hydrogen peroxide. The supernatant was filter sterilized through a Millex-GV filter (0.22 mm pore size, Millipore) and stored at -20 °C until analysis.

Demonstration of Antagonistic Activity

Detection of antagonistic activity in culture extracts was performed by the method of Tag & McGiven[31] using the well diffusion agar assay. Indicator lawns were prepared by spreading 50 ml of each target strain (Table 1), grown in LB overnight at 37 °C, over the surface of prepoured LA plates (LB with 1.8% agar). Wells were cut in LA plates with a sterile 5-mm cork borer. Fifty microliters of pretreated extracts of the *Lactobacilli* strains were transferred to the wells in pre-seeded agar plates. After diffusion of the extract into the agar (6 hours at 25 °C), the plates were then incubated aerobically at 37 °C overnight. All antagonistic activity assays were conducted in duplicate. Finally, the plates were examined for the presence on inhibition zones and antimicrobial activity expressed as average size (mm) of inhibition zones.

Mode of Action of Antagonistic Compound AA135

In order to study the mode of action of the *L. plantarum* AA135 culture supernatant on sensitive cells, the concentrated supernatant fluids (concentrated using a disposable ultrafiltration device, Vivaspin, according to the manufacturer's instructions, Sartorius AG) were added to a fresh culture of *P. aeruginosa* at the mid-logarithmic growth phase in LB up to 10% (V/V). Protease-treated culture supernatant was used as a control. During growth at 37 °C and 200 rpm in an S 150 Orbital Incubator (Stuart Scientific), samples were taken and the optical densities (OD_{600}) of *P. aeruginosa* culture were measured every hour through the first three-hour period of incubation and every two hours for next eight-hour period.

Heat Resistance and Enzyme Sensitivity

Temperature stability was investigated by heating a 2 ml sample of *L. plantarum* AA135 culture supernatant

Table 1. Bacterial strains tested for inhibition by the antagonistic compounds produced by *L. plantarum* strains using the well diffusion assay. Inhibition zones higher than 10 mm are considered to indicate strong inhibitory activity.

Organisms *	Diameter (mm) of inhibition zone by culture extract from *Lactobacillus plantarum* strains			
	AA110	AA125	AA135	AA140
Gram-negative bacteria				
Salmonella paratyphi R46, Lab isolate	9	7	10	8
Pseudomonas aeruginosa ATCC 15442	20	18	21	19
Salmonella typhimurium ATCC 13311	8	7	9	7
Shigella sonnei ATCC 25931	7	11	12	6
*Shigella dysenteriae*A20, Lab isolate	12	11	13	10
Escherichia coli ATCC 11229	11	15	14	13
Shigella sp. A23, Lab. isolate	10	-¥	9	12
Gram-positive bacteria				
Micrococcus luteus A102, Lab isolate	10	11	13	7
Micrococcus roseus R76, Lab isolate	6	7	9	8
Staphylococcus epidermedis ATTC 12228	9	8	10	7
Streptococcus faecails ATCC 19433	7	9	11	8
Streptococcus pyogenes ATCC 19615	9	8	9	7
Staphylococcus aureus ATCC 9664	9	8	12	7
Listeria monocytogenes S39, Lab isolate	6	8	9	7
Bacillus subtilis R89, Lab isolate	7	8	9	7
Bacillus cereus ATCC1 1778	10	9	11	6

* Lab stock culture collection, ATCC; American Type Culture Collection.
¥ No inhibition zone, diameter of well is 5 mm.

at 20 °C, 37 °C, 60 °C, 80 °C, 100 °C and 121 °C for 30 min. The samples were then assayed for the remaining activity using the well diffusion assay. In order to test the sensitivity to enzymes, the crude extracts were treated with papain, trypsin, pepsin, catalase and lysozyme, each at a final concentration of 0.5 mg/ml, and then incubated at 37 °C for 2 hours. The enzymes were denatured by heating the samples at 95 °C for 10 min. Finally, the samples were filter-sterilized and residual antagonistic activity was determined by the well diffusion assay.

Stability of Crude Antagonistic Compound AA135 During Storage

L. plantarum AA135 was incubated anaerobically (BBL Gas-pak system) in MRS broth at 37 °C for 48 hours. The crude extract was separated and sterilized (as mentioned above).The sterile supernatant was stored in an incubator (37 °C), a refrigerator (4 °C) and a freezer (-20 °C). At different time intervals (every 10 days), samples were taken from the stored material for detection of antagonistic activity using the well diffusion assy.

Kinetics of Plantaricin AA135 Production

To study plantaricin AA135 production during growth, *L. plantarum* AA135 was incubated anaerobically (BBL Gas-pak system) in MRS broth at 37 °C. Optical densities (OD_{600}) of the culture were measured every 1-2 hours and culture extracts were prepared to determine their antagonistic activity against *P. aeruginosa* by the well diffusion assay.

Extraction of Plantaricin AA135 with Organic Solvents

Different organic solvents such as di-ethyl ether, n-hexane, n-butanol, chloroform, i-amylalcohol and n-propanol were added to culture supernatant fluids at a 1:1 ratio. Fifty grams per liter NaCl were added to the mixture only in the case of n-propanol extraction in order to obtain phase separation. The mixtures were thoroughly mixed and centrifuged at 10,000 x *g* at 4 °C for 2 min to achieve phase separation. The aqueous and organic phases were collected and the solvents were then removed by evaporation at 45 °C (using a rotary vacuum evaporator RVO 400 Ingos, Laboratory Instruments). The residue from the organic phase was resuspended in an amount of saline (8.5 g/l NaCl) equal to the initial volume of the original culture supernatant.[32] The antagonistic activities of two phases were detected by well diffusion agar assay.

Plasmid Isolation

Plasmid DNA was extracted and isolated from *L. plantarum* AA135 grown anaerobically in MRS broth at 37 °C for 48 hours at different growth stages (lag phase, early logarithmic phase, mid-logarithmic phase and stationary phase), by alkaline lysis using 10 mg/ml lysozyme according to the method described by Sambrook et al.[30] Plasmid DNA was separated on a 0.8% agarose mini horizontal slab gel (Bio-Rad).

Ammonium Sulphate Precipitation of Plantaricin AA135

Eight grams of ammonium sulphate were added to 20 ml of culture supernatant samples which were collected at lag phase, early logarithmic phase, mid-logarithmic phase and stationary phase of *L. plantarum* AA135, grown anaerobically in MRS at 37 °C. The mixtures were stirring overnight at 4 °C and then centrifuged at 10,000 x *g* at 4 °C for 20 min. Surface pellicles and bottom pellets were resuspended in 1 ml of 50 mM sodium phosphate, pH 7.0.

SDS-PAGE Analysis

The ammonium sulphate precipitated plantaricin AA135 samples (see above) and whole-cell protein extract of *L. plantarum* AA135 collected during growth phase were fractionated by polyacrylamid gel electrophoresis (10% SDS-PAGE) using a 1 mm-mini vertical slab gel (BioRad) as described by Laemmli.[33] The protein standards (Biorad) and their molecular sizes were: myosin; 199 kDa, β-galactosidase; 120 kDa, bovine serum albumin; 87 kDa and oval albumin; 48.1 kDa. Electrophoresis was conducted at a constant current of 30 mA for 45 min. The gel was stained with Coomassie brilliant blue-R (Sigma). A second gel was used for detection of the antagonistic activity. The gel was placed on an LA plate and subsequently overlaid with soft LA (LB plus 0.6% agar) which was seeded with *P. aeruginosa*. The plate was incubated overnight at 37 °C and examined for inhibition zones.

Results

Screening for Antagonistic Compounds

Seventy-three strains of lactobacilli isolated from Egyptian home-made yogurt were screened for antagonistic activity by the well diffusion assay on a number of selected Gram-positive and Gram-negative bacteria. Only four strains (AA110, AA125, AA135 and AA140) demonstrated inhibitory activity against both Gram-positive and Gram-negative strains (Table 1). The morphological, cultural, physiological and biochemical characteristics of the strains AA110, AA125, AA 135 and AA140 allowed identification as *L. planatrum*. The antagonistic compound from strain AA135 strongly inhibited Gram-positive foodborne pathogens, including *Staphylococcus epidermedis, Staphylococcus aureus,* and *Bacillus cereus,* whereas *Listeria monocytogenes* and *Bacillus subtilis* were weakly inhibited. Interestingly, several Gram-negative foodborne

pathogens were also strongly inhibited by the antagonistic agent from AA135, including *Salmonella paratyphi, P. aeruginosa, Shigella sonnei, Shigella dysenteriae* and *Escherichia coli*. However, *Salmonella typhimurium* and *Shigella sp.* were weakly inhibited. Surprisingly, *P. aeruginosa* was extremly sensitive to the antagonistic substance. Because of the interesting inhibition spectrum of the inhibitory agent from AA135, it was chosen for further studied.

Bacteriolytic Mode of Action

Exposure of *P. aeruginosa* to active culture supernatant of *L. plantarum* AA135 resulted in a strong decrease of the optical density (Fig. 1). Within four hours, the optical density (OD_{600}) declined from 1.4 to 0.22, indicative of cell lysis. According to the above mentioned observations, the inhibitory substance produced by *L. plantarum* AA135 was tentatively identified as a bacteriocin-like inhibitory substance and was designated as plantaricin AA135.

Properties of Crude Plantaricin AA135

A crude extract of plantaricin AA135 was heat stable at all studied temperatures. Plantaricin AA135 retained full activity after 30 min at 121 °C. Plantaricin AA135 was stable to catalase treatment, suggesting no involvement of hydrogen peroxide in inhibition. However, it was completely destroyed by treatment with pepsin, trypsin and papain. The plantaricin AA135 could be stored at – 20 °C or 4 °C for at least 100 days without substantial loss of its activity (Fig. 2). However, storage at 37 °C caused some loss of activity, possibly due to the action of proteolytic enzymes which might be found in the culture supernatant. The fact that plantaricin AA135 is heat-stable and strongly sensitive to proteolytic enzymes indicates a bacteriocin nature. Plantaricin AA135 was continuously produced during anaerobic growth of *L. plantarum* AA135 in MRS broth at 37 °C (Fig. 3). The greatest inhibition activity of the culture supernatant was detected during the mid-logarithmic phase and at the beginning of stationary phase, whereas activity of the culture supernatant during lag phase was not detectable.

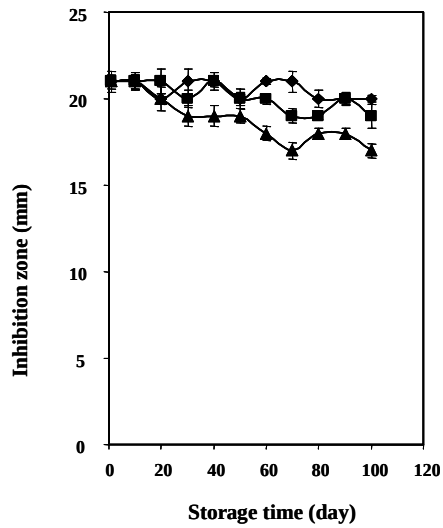

Fig 2. Effect of time on plantaricin AA135 activity when stored at -20 °C (♦), 4 °C (■) or 37 °C (▲). Antagonistic activity was detected by the well diffusion method using *P. aeruginosa* as a target organism.

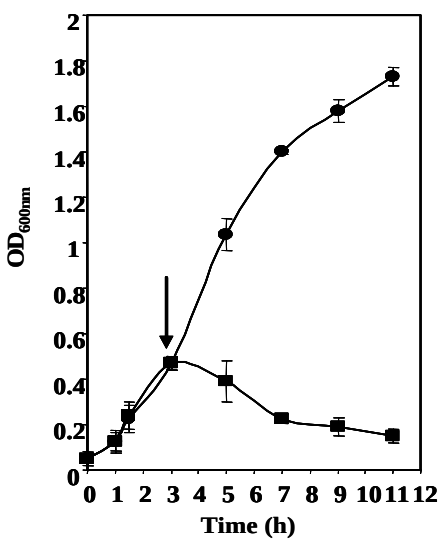

Fig 1. Bacteriolytic effect of plantaricin AA135 on the growth of *P. aeruginosa*. The time of addition of cell-free supernatant fluids to 10% is indicated by an arrow. Growth was aerobic in LB with bacteriocin (■) and without bacteriocin (•) at 37 °C.

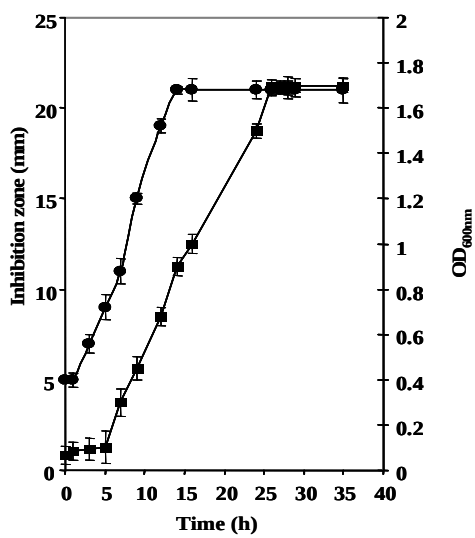

Fig 3. Kinetics of plantaricin AA135 production by *L. plantarum* AA135 grown anaerobically in MRS broth at 37 °C. At various time intervals taken for measurement of the optical density, $OD_{600\ nm}$ (■) and for detection of bacteriocin activity against *P. aeruginosa* (•).

Table 2. Extraction of plantaricin AA135 from culture supernatant of *L. plantarum* AA135 with various organic solvents. Data were obtained with the agar diffusion assay using *P. aeruginosa* as an indicator strain.

Organic solvents	Inhibition zone (mm)	
	Organic phase	Aqueous phase
None		21
Di-ethyl ether	-*	19
n-Hexan	-	20
n-Butanol	21	8
Chloroform	6	6
i-Amylalcohol	12	8
n-propanol	11	9

* No inhibition zone, diameter of well is 5 mm.

Extraction of Plantaricin AA135 with Organic Solvents

Extraction of plantaricin AA135 from the culture supernatant of *L. plantarum* AA135 was investigated using different organic solvents (Table 2). Plantaricin AA135 was not removed from the aqueous phase with very apolar solvents such as hexane and di-ethyl ether. Inhibitory activity of the bacteriocin was almost completely destroyed by chloroform. However, when various alcohols were used in the extraction method, plantaricin AA135 was removed from the aqueous phase and could be recovered from the organic phase. Butanol extraction exhibited complete recovery of plantaricin AA135 activity suggesting that at least part of the plantaricin AA135 molecule has a hydrophobic character. A low activity remaining in the aqueous phase with butanol extraction would have to do with the behaviour of the substance during partitioning and no due to an artefact.

Involvement of Chromosomal Genes in Plantaricin AA135 Production

In order to detect whether the genes responsible for plantaricin AA135 production are located on a plasmid or the chromosome, samples of *L. plantarum* AA135 culture grown anaerobically in MRS broth at 37 °C were harvested during growth phase for plasmid extraction. Electrophoretic analysis of plasmid preparations demonstrated that *L. plantarum* AA135 does not contain plasmids at any stage of bacterial growth, suggesting that the genes encoding bacteriocin production are located on the chromosome.

Determination of Molecular Size of Plantaricin AA135

In order to determine the molecular size of plantaricin AA135, 40% ammonium sulphate precipitated samples were prepared from the culture supernatant of *L. plantarum* AA135 growing anaerobically in MRS broth. The precipitated samples were subjected to SDS-PAGE (10%) analysis and subsequently the gel was stained with Coomassie blue. As shown in Fig. 4A, two major protein bands with molecular masses of approximately ~45 kDa and <45 kDa were detected in all samples. The activity of plantaricin AA135 is associated with those two protein bands (Fig. 4B). These two bands were also detected in whole-cell proteins. However, these two bands were produced more abundantly at mid-logarithmic phase than in other phases, supporting the conclusion that the maximum level of bacteriocin production was achieved during mid-logarithmic phase.

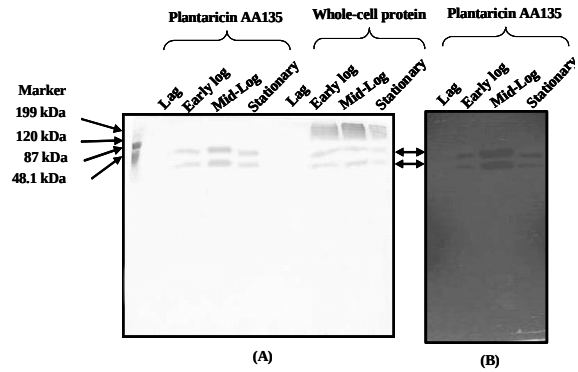

Fig 4. Coomassie blue-stained SDS-PAGE gel (10%) of ammonium sulphate-precipitated plantaricin AA135 and whole-cell protein extracts of *Lactobacillus plantarum* AA135 growing anaerobically in MRS medium at 37 °C during various phases of growth (A) and antagonistic activity of the bacteriocin bands (B). A) Protein standards and their molecular weights were: myosin; 199 kDa, B-galactosidase; 120 kDa, bovine serum albumin; 87 kDa and oval albumin; 48.1 kDa. The arrows indicate respective bacteriocin bands. B) Zones of growth inhibition, corresponding to the positions of the peptide bands in (A).

Discussion

Lactic acid bacteria can produce antagonistic compounds that vary in their spectra of activity. In this study, four strains of *L. plantarum* isolated from Egyptian home-made yogurt and designated as AA110, AA125, AA135 and AA140 were found to produce antimicrobial compounds. Similar observations were reported by Schillinger and Lucke[9] with lactobacilli. The antimicrobial agent from strain AA135 demonstrated a wide range and strong antimicrobial activity against both Gram-positive and Gram-negative bacteria. The antimicrobial agent produced by *L. plantarum* AA135 is heat-stable while it retains its activity even after autoclaving at 121 °C for 30 min, similar to the antibacterial substance produced by *Lactobacillus*

plantarum F1[27] (121 °C for 10 min), bacteriocins ST28MS & ST26MS[22] (121 °C after 20 min) and bacteriocin AMA-K[34] (20 min at 121 °C). Other reported bacteriocins are less heat-stable, such as lactacin B[35] (121 °C for 3 min), plantaricins A[16] (100 °C after 30 min) and S[17] (60 min at 100 °C). Therefore, this agent could maintain its activity in heat processed food-stuffs. Moreover, this antimicrobial substance has a proteinaceeous nature, as it was resistant to lysozyme and catalase but completely destroyed by proteases such as pepsin, trypsin and papain. The inactivation of this agent by proteases indicates a typical bacteriocin. Similarly, the inhibitory substance produced from *Lactobacillus plantarum* ATCC 8014[20], bacteriocins ST28MS and ST26MS[22] and plantaricin KW30[19] were also susceptible to digestion by various proteases. Previous other studies reported that many of the antimicrobial compounds produced by lactic acid bacteria are bacteriocins with a proteinaceous nature, while other non-protein agents are also produced.[36] Furthermore, the antimicrobial agent demonstrated a bacteriolytic mode of action, as the immediate decrease in the optical density of *P. aeruginosa* indicated cell lysis. The general mechanism of bacteriocin action which has been suggested is disruption of the electrochemical gradient across the cytoplasm membrane by pore formation.[8] The bacteriolytic mode of action of the crude extract is also typical of bacteriocins. Other bacteriocins with bactericidal modes of action without cell lysis have also been reported.[19,26,35,37,38] Therefore, the antimicrobial agent produced by *L. plantarum* AA135 is designated as plantaricin AA135. Plantaricin AA135 could be extracted well with butanol indicating that it is a hydrophobic protein. This property is similar to most other bacteriocins.[8] Plantaricin AA135 was continuously produced during growth, especially during logarithmic phase when nutrients are available for metabolic activity.[11,24] Similarly, plantaricin 423, produced by *L. plantarum* 423, was reported to be produced during exponential growth and reached a maximum activity at the beginning of stationary phase.[24] A wide spectrum of activity has been shown only in a few bacteriocins of lactic acid bacteria. Some bacteriocins, such as pediocins and nisin, inhibit a broad range of Gram-positive bacteria.[39] On the other hand, plantaricin F[26], pediocins and nisin[8], bacteriocin ST194BZ[21], bacteriocin from *Lactobacillus plantarum* ATCC 8014[20], bacteriocins ST28MS and ST26MS[22], plantaricin AA135 were active against *Staphylococcus, Bacillus, Micrococcus, Pseudomonas, Salmonella, Escherichia, Shigella*, and *Listeria* species. Molecular weights of bacteriocins produced by lactic acid bacteria have been reported to fluctuate from 3.4-5.6 kDa to 10-45 kDa.[40-42] In the present study, two peptides with high molecular weights of ~45 kDa and <45 kDa were detected by SDS-PAGE analysis of ammonium sulphate-precipitated cell-free supernatant fluids. These two peptides could act synergistically, as reported for some two-peptide bacteriocins, such as lactacin F and lactococcin G[6], and bacteriocin ST194BZ[27]. Proteins with higher molecular weights, from 22 kDa to 105 kDa, exhibiting widely ranging effects on bacteria, have been reported to indicate chromosome encoded bacteriocins.[43] This is further supported by the fact that no plasmids were detected in the producing strain, *L. plantarum* AA135. This result is in agreement with those reported by Olasupo et al.[44] and Todorov and Dicks[22,27], which suggested that the genes encoding bacteriocin production are located on the genomes.

In conclusion, the potential of plantaricin AA135 to inhibit foodborne pathogenic bacteria, such as *Staphylococcus aureus*, *Bacillus* spp., *p. aeruginosa*, *Salmonella, Shigella*, *Listeria* and *E. coli*, is of crucial interest. Some of these bacteria can produce toxins resulting in human illness. In addition to the broad inhibition spectrum, its technological properties and especially heat and storage stability, indicate that plantaricin AA135 has potential for application as a biopreservative to control pathogens in processed foods.

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