Journal of Agri-Food and Applied Sciences
Available online at jaas.blue-ap.org
©2015 JAAS Journal. Vol. 3(1), pp. 6-13, 28 February, 2015
E-ISSN: 2311-6730

Isolation and Characterization of Bacteriocin-Producing Lactic Acid Bacteria against Indicator Organisms

Nagwa B Elhag[1*], El Rakha B Babiker[2] and Ahmed A Mahdi[3]

1-Department of Food Hygiene and Safety, College of Puplic &environmental Health, Bahri University, Khartoum North, Sudan
2-Food Research Centre, Department of Microbiology and Biotechnology, Agricultural Research Corporation, Shambat, Sudan
3-Department of Botany and Agricultural Biotechnology, Faculty of Agriculture, University of Khartoum, Sudan

Corresponding Author: Nagwa B Elhag

Received: 09 December, 2014 *Accepted: 02 January, 2015* *Published: 28 February, 2015*

ABSTRACT

In the last recent years, bacteriocins produced by lactic acid bacteria (LAB) have received a considerable attention to be used as biopreservatives in different fields of food industry. LAB was isolated from Sudanese fresh sausages, intestines of different animals, saliva, cheese and cucumber in de Man Regosa and Sharp medium. In this study the antimicrobial effect of LAB producing-bacteriocins against *Staphylococcus aureus* ATCC 2818, *Bacillus cereus*, and *Escherichia coli* ATCC 29522 was studied. The agar well diffusion assay (AWDA) was followed to determine the antimicrobial effect. Characterization of bacteriocin produced by LAB was conducted by exposing bacteriocins to different heat treatment at 40, 60, 80, 100, and 121^{O}C for 0, 30, 60 or 90 minutes and after a week in a water bath set up at 37 ^{O}C, and adjusted to different pH levels at 2, 4, 6, 8, 10 and 12. Sensitivity to enzymes was assayed by treatment with α-amylase and the proteolytic enzyme pepsin. Only 16 LAB isolates of 30 produced clear inhibition zones. Only 3 LAB isolates of 16 exhibited the strongest performance against the indicator organisms. These LAB isolates were identified as *Enterococcus faecalis, Pediococcus pentosaceus,* and *Lactobacillus murinus.* Characterization of bacteriocins obtained revealed that these substances were found to be stable after heat treatment, at low pH and at pH 6-8. The proteinaceous nature of bacteriocins was completely lost after treatment with the proteolytic enzyme pepsin. Sudanese products of animal sources may be a rich source for bacteriocins-producing LAB which can be used as bio-preservatives

Keywords: *Bacteriocins, Characterization, Indicator organisms, Inhibitory activity, LAB.*

INTRODUCTION

Application of bio-preservatives has received great attention as a mean for preserving food (Holzapfel , 1995). Bacteriocins are ribosomal substances produced by bacteria. These compounds have a bacteriostatic or bacteriocidal effect on closely related species (Tagg 1976; Klaenhammer, 1988, Patil , 2007). Recently the definition has been extended to include similar substances that act on a wide range of species and contain other functional moieties, such as carbohydrates or lipids (Gray and Dykes, 1995). Bacteriocins can be produced by different variety of living organisms including prokaryotes and eukaryotes (Papagianni, 2003). These compounds can be produced by gram-positive and gram-negative bacteria, but bacteriocins produced by LAB are of particular concern due to their safety to be applied in food industry as bio-preservatives (Cleveland , 2001; O'Sullivan , 2002). Bacteriocins of LAB are heterogeneous group; therefore they differ in their antimicrobial activity. According to this there are two types of microbial activity of LAB, the classic type which have spectrum of activity only against homologues species while

the second one have a wide range on gram-positive bacteria (Prada 2007). Deegan (2006) claimed that some species of LAB have a narrow host range and effective against related bacteria with nutritive demands for the same scarce resources. Gram-negative bacteria are not affected due to the presence of outer barrier on their cells which prevent molecules of antibiotics, detergents and dyes to reach cell membrane (Steven, 1991). However some studies reported the effect of bacteriocin on gram-negative bacteria such as bacteriocin from *Lactobacillus plantarum* against *Salmonella typhimurium* (Shahlaa and Zahraa, 2010). Wala and Nibras (2013) found that bacteriocins from *Lactobacillus acidophilus* exhibited activity against *Serratia marcescens*.

Martins , (2003) reported that characterization of LAB bcateriocins revealed that these substances are active under certain ranges of temperature and pH and sensitivity to proteolytic enzymes confirmed their proteinaceous nature of these molecules Wala and Nibras (2013) found that bacteriocin of *Lactobacillus acidophilus* was stable at pH 4, 7, half of its activity was lost at pH 8 and whole activity was lost at other pH values. Bacteriocins from *Enterococcus faecium*, *Streptococcus thermophillus*, *Lactobacillus casi* and *Lactobacillus ca*si susp. *sakei* lose their proteinaceous character after treatment with the proteolytic enzymes protinase k, chymotrypsin and trypsin (Yang , 2012). Bacteriocins-like inhibitory substance produced by *Brevibacillus borstelensis* AG1 were heat stable at 80ºC and 100ºC and were sensitive to autoclaving at 121ºC (Sharma ; 2014). As reported by Parada , (2007), bacteriocins were resistant to heat and their antimicrobial activity was lost after 10 and 20 min at 100ºC. The thermo-tolerance feature might be referred to the structure of bacteriocin usually composed of small peptides without tertiary structure (Toro, 2005). This study was conducted to assess the antagonistic effect of bacteriocins produced by LAB against indicator organisms and to evaluate their sensitivity to heat treatment, enzymes and pH.

MATERIALS AND METHODS

Collection of samples

Nine different sources [sausages, intestines of chicken, pigeons, sheep and cattle, saliva, cheese, cleaned sheep intestines (locally called musarn mejrure) and cucumber] were collected and used as sources for the isolation of bactriocin-producing LAB. All samples were fresh and purchased from retail markets in Khartoum state except saliva. The collected samples were immediately transported ice-cooled in an insulated ice container to the laboratory for microbial analysis.

Indicator organisms

The selected indicator organisms included *Staphylococcus aureus*, *B. cereus* ATCC 2818, and *Escherichia coli* 29522. They were collected from Veterinary Microbiology Department, Faculty of Veterinary Sciences, University of Khartoum.

Screening for bacteriocin producing LAB.

Ten grams of each source mentioned above were aseptically weighed and hand mixed well in 100 ml of de Man Regosa and Sharp (MRS-Hi-media Laboratories Pvt. Ltd., India) broth for the enrichment of any resident LAB (de Man *et al*, 1960). The mixture was incubated at 37ºC for 24 hrs. The broth was then serially diluted in peptone water and LAB were enumerated by poured-plate method in MRS agar plates. The plates were anaerobically incubated at 37 ^{O}C for 48 hours using anaerobic jars with gas generating kits (Oxoid BR 0038b). Then the visible colonies on the plates were overlaid with MRS soft agar (0.75% agar) seeded with 0.5 ml of active broth of indicator organisms and was overlaid with the MRS soft agar medium followed by aerobic incubation at 37^{O}C for 24 hours to allow the colonies to develop. Colonies showing zones of inhibition were considered as potential bacteriocin producers.

The inhibitory activity of 30 LAB isolates was confirmed with the spot-on-lawn assay as described by Schillinger and Lücke (1989), Lewus (1991), and Van Reenen (1998). Overnight cultures of LAB isolates to be tested were spotted (5µl) onto the surface of MRS agar medium (4 spots in each) and incubated anaerobically (Gas kits, Oxoid) at 37^{O}C for 24 h to allow colonies to develop. Each plate was overlaid with 7 ml of soft agar (0.75%) seeded with 0.5 ml of overnight cultures of the indicator organisms. The plates were incubated at 37 ^{O}C for 24 h and clear zones around the spots were observed. The inhibitory reaction was scored as positive if the width of the clear zone around the colonies of LAB isolates were 5 mm or larger (Ogunbanwo, 2003; Lade, 2005).

Presumptive identification of bacteriocin producers:

The 3 LAB isolates which produced clear inhibition zone with diameter 5mm or larger were isolated, purified and identified depending on their morphological, cultural and biochemical characterization (Sneath, 1986, Harrigan, 1998; Holt 1998; Barrow and Fealthman, 1993; Axelesson, 2004 and Salminen 2004).

Antagonistic activity of bacteriocins

The agar well diffusion assay (AWDA) was used to determine the antagonistic effect on the indicator organisms (Schillinger and Lücke, 1989, and Takahiro 1991). Nutrient agar medium was used for culturing the indicator organisms. Wells of uniform diameter (8 mm) were bored in the agar using sterile micropipette tips. Then two-fold serial dilutions in sterile distilled water

were conducted to dilute supernatants of each isolate (Graciela 1995). Aliquots of 50µl from each bacteriocin dilution were placed in the wells in the plates already inoculated with active culture of indicator organisms. The plates were incubated (aerobically) overnight at 37^{O}C, and the diameters of inhibition zones around the wells were determined (Rammelsberg and Radler, 1990).

Bacteriocin Extraction

Lactic acid bacteria isolates which produced inhibition zone round the colonies (16 isolates) were inoculated in 100 ml MRS broth individually and incubated anaerobiacally (Oxoid gas generating kits) at 30ºC for 72 h. Then the broth was heated at 80ºC for 20 minutes in a water bath to kill living cells, and were then desorbed at pH 2-2.5 using sterile 0.1 N NaCl (Yang, 1992, Vera Pingitore, 2007). The cultures were then centrifuged at 10000 rpm for 20 min to obtain supernatants of crude bacteriocins. This was followed by filtration of the supernatants through 0.2 µm pore-size cellulose acetate filter. The obtained crude bacteriocins were treated with 40 % ammonium sulphate $(NH4)_2 SO_4$), and the mixtures were stirred for 1 hour at 4^{O}C, and were then centrifuged at 10,000 rpm for 20 minutes (Vera Pringitore, 2007). The supernatants were decanted, the pellets and surface pellicle were then dissolved in 10 ml sterile ultra pure water.

Characterization of bacteriocin samples

The most active bacteriocin producers were selected for this purpose. Dissolved pellets and surface pellicle (bacteriocins) obtained from *Enterococcus faecalis*, *Pediococcus pentosaceus* and *Lactobacillus murinus* were characterized with respect to thermal, pH stability and susceptibility to denaturation by enzymes. Bacteriocins produced by the three mentioned isolates were exposed to various heat treatments: 40, 60, 80, 100, and 121^{O}C in 50 ml MRS broth. Aliquots of each were then removed after 0, 30, 60 or 90 minutes and after a week in a water bath set up at 37 ^{O}C (Brink , 1994), and were assayed by the agar well-diffusion assay.

Sensitivity to enzymes was assayed by treatment with the proteolytic enzyme pepsin and α-amylase. Pepsin (Merk-7189) in sterile 0.002N HCl, and α-amylase (Sigma, Milan, Italy) at a final concentration of 0.2 mg /ml were used. The samples and controls (bacteriocin samples without enzyme solutions) were incubated at 37 ^{O}C for 2 hours and heated in a boiling water bath for 5 minutes to inactivate the enzymes. The remaining bacteriocin activity was determined by the agar well-diffusion assay (Bromberg , 2004).

Sensitivity to different pH values was investigated by adjustment of the pH of bacteriocins samples to pH 2, 4, 6, 8, 10 and 12 (with sterile 1M hydrochloric acid or 1M sodium hydroxide) and incubated for 4 hours at room temperature (Brink 1994). The samples were then re-adjusted to pH 6.5 using sterile NaOH and HCl. *Bacillus cereus* was used as an indicator organism for antimicrobial activity for all the tests.

RESULTS AND DISCUSSION

Thirty presumptive LAB bacteriocin producers were obtained from the investigated sausage samples and other sources. Sixteen isolates out of 30 isolates produced clear zones of inhibition against the indicator organisms. Only 3 isolates out of 16 were the strongest bacteriocin producers and were therefore selected for further tests. The three bacteriocin-producing LAB were identified as Enterococcus faecalis, Pediococcus pentosaceus and Lactobacillus murinus (Plates 1, 2, 3, 4). Lactobacillus, Pediococcus, and Enterococcus spp. have the ability to produce bacteriocins that inhibit or kill gram-positive and gram-negative bacteria (Swetwiwathana 2008). Bacteriocins from Enterococcus faecalis and other enterococci species can be used as biopreservatives of food or as probiotics (Callewaert 2000). Enterococci sp. are capable to produce a variety of bacteriocins with antimicrobial activity against Listeria monocytogenes, Staphylococcus aureus and Clostridium sp. (Gelsomino 2001) and were found to be suitable for improving food safety (Salminen , 1998). Pediococcus pentosaceus TISTR536, Pediococcus pentosaceus M13 and Lactobacillus plantarum RS49 and RS54, isolated from Thai traditional fermented beef products could produce bacteriocins at temperatures higher than 40ºC (Swetwiwathana, 2006). Pinto (2007) reported that bacteriocins of Pediococcus pentosaceus and Enterococcuss faecium isolated from ready-to-eat seafood have an active bacteriostatic inhibition on Listeria monocytogenes. Bacteriocins from Pediococcus sp. have an attractive interest to be used as biopreservative in food industry (Turcotte, 2004). Different types of

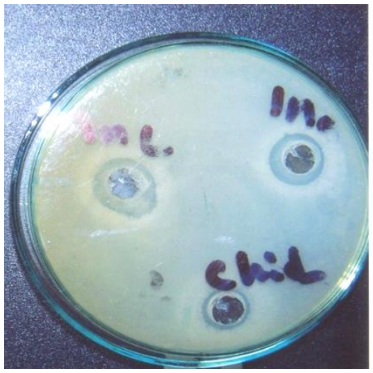

Plate 1. Antimicrobial activity of different (crude) bacteriocins on Staphylococcus aureus produced by: Inl, from Enterococcus faecalis; Ins, from Pediococcus pentosaceus; Chil, from Lactobacillus murinus

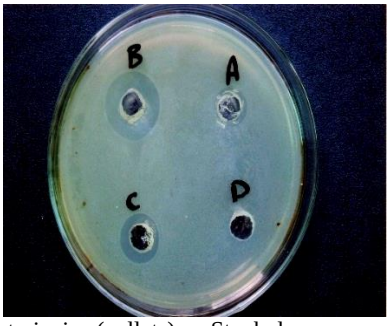

Plate 2. Antimicrobial activity of different bacteriocins (pellets) on Staphylococcus aureus. A, from Lactobacillus murinus; B, from Enterococcus faecalis; C, from Pediococcus pentosaceus; D, no bacteriocin added (control)

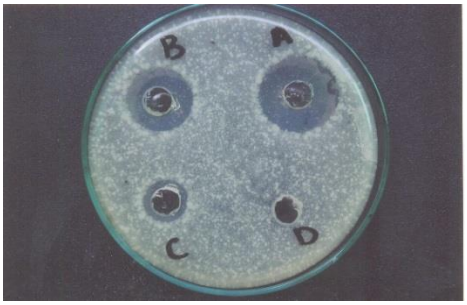

Plate 3. Antimicrobial activity of different bacteriocins (pellets) on Bacillus cereus. A, from Enterococcus faecalis; B, from Pediococcus pentosaceus; C, from Lactobacillus murinus; D, no bacteriocin added (control)

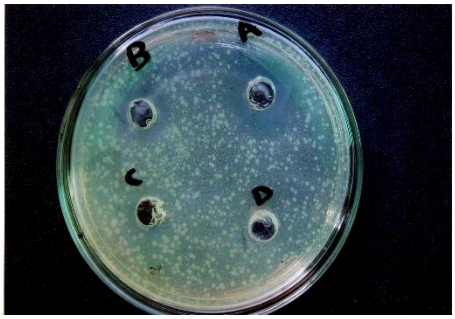

Plate 4. Antimicrobial activity of different bacteriocins (pellets) on Escherichia coli. A, from Enterococcus faecalis; B, from Pediococcus pentosaceus; C, from Lactobacillus murinus; D, no bacteriocin added (control)

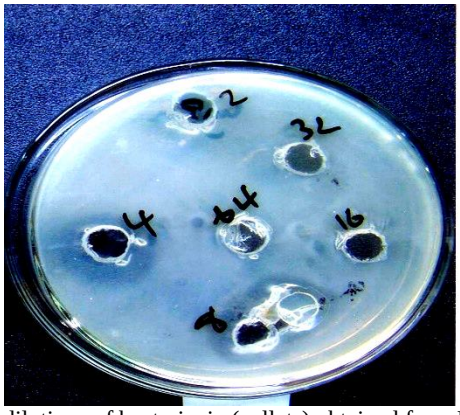

Plate 5. Antimicrobial activity of two-fold dilutions of bacteriocin (pellets) obtained from Pediococcus pentosaceus on Staphylococcus aureus

bacteriocins can be produced by Pediococcus spp. such as pediocin AcH obtained from Pediococcus acidilactici (Kim 1992) and pediocin N5 by Pediococcus pentosaceous (Strässer de Saad 1995). Pediococcus damonosus has been also reported to produce a pediocin PD-1(Green 1997) and it is bactericidal (Anastasiadou 2008). Soumya (2012) reported that 4 strains of Lactobacilli were having activity against S. aureus, and Pseudomonas aeruginosa and none of them have inhibitory effect on E.coli, Enterococcus faecalis and Klebsiella pneumoniae. Nardi (2005) found that Lactobacillus murinus produces at least two low molecular weight compounds active against B. cereus and Shigella sonnei. Also they reported that bacteriocin of this bacterium inhibits various pathogenic and food spoilage bacteria without affecting other lactobacilli.

The inhibitory effect produced by the test isolates Enterococcus faecalis, P. pentosaceus and Lactobacillus murinus against Bacillus cereus is considered to be heat stable after heating to 40, 60, 80,100 and 121^{o}C and incubation in a water bath at 37^{o}C for 7 days (Fig.1). Results obtained in this study are in accordance with that of Cocolin (2007) who reported that the effect of heat on the activity of bacteriocins produced by two strains of E .faecium isolated from Italian goat milk was not abolished when subjected to high temperature (100^{o}C for 10 min and 60^{o}C for 10 min). Those authors reported that the stability was also observed in the case of storage at 30, 4, - 20, and - 80^{o}C up to 7 days. Similar results were obtained by Ogunbanwo (2003) who recorded that bacterioins produced by L. brevis OG1 were considered to be the most heat stable as there was no reduction in activity after heating at 121^{o}C for 60 min, while bacteriocin produced by L. plantarum F1 was able to exhibit full activity after heating at 121^{o}C for only 10 min. Another study was conducted by Pinto (2007) who found that bacteriocins from E. faceium and P. pentosaceus were heat stable over a wide range of temperatures (4 – 100^{o}C) after 1h and 2 h incubation. The thermo-tolerance feature observed in this study might be referred to the molecular structure of the bacteriocins which are usually composed of small peptides without tertiary structure (Toro, 2005). Also heat stability may be due to the formation of small globular structures and the occurrence of strongly hydrophobic regions of stable cross-linkages, and high glycine content (de Vuyst and Vandamme, 1994). This heat stability would be a very useful characteristic if the bacteriocin was to be used as a food preservative, because many food-processing producers involve a heating step. Therefore, the bacteriocins produced by Enterococcu faecalis, Pediococcus pentosaceus, and Lactobacillus murinus would be useful as food preservatives in pasteurized or canned foods.

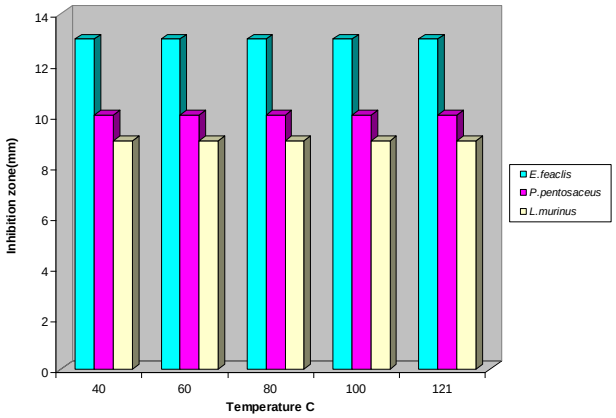

Fig.1. Effect of temperature on activity of bacteriocins (inhibition zone diameter) from three isolates on *B.cereus* as indicator organism

The antimicrobial activity of bacteriocins was lost after treatment with the proteolytic enzyme pepsin, whereas the treatment with amylase did not affect the activity of the bacteriocins obtained (Fig. 2).These results confirmed the proteinaceous nature of the supernatants. Many studies revealed that bacteriocins of different LAB species lost their proteinaceous nature when treated with proteolytic enzymes and were not affected by other non-proteolytic enzymes (De Martinis , 2003; Cocolin , 2007; Pinto , 2007). According to Fricourt (1994) lactic acid bacteria synthesize bactericidal agents that vary in their spectra of activity. Many of these agents are bacteriocins with proteinaceous active moiety, while others are non- protein agents (Paird and Desmazeaud, 1991, 1992).

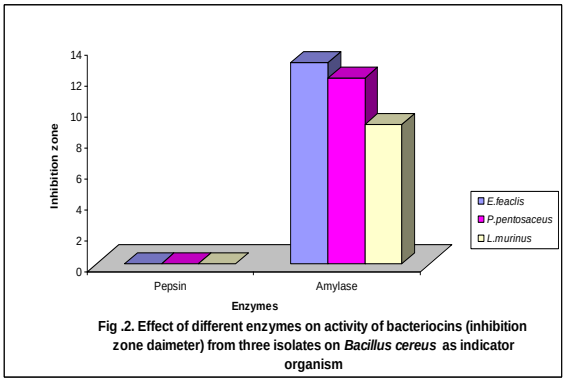

Fig .2. Effect of different enzymes on activity of bacteriocins (inhibition zone daimeter) from three isolates on *Bacillus cereus* as indicator organism

It was observed that bacteriocin activity of E. faecalis was stable at pH 6-8 and low pH (Fig. 3), while the activity of extracts from P. pentosaceus and L. murinus was almost stable at 2-6. However, fluctuation in activity was observed at alkaline pH, and almost stable at low and neutral pH values (Fig.3). With respect to bacteriocins characterization, the antimicrobial activity of pellets was observed at low, neutral, and high pH. The activity at neutral and high pH values excludes any effect due to acids. These bacteriocins were almost stable at acidic and neutral pH values. Bacteriocin ST28 MS produced by Lactobacillus plantarum remained stable after incubation for 2 h at pH values between 2.0 and 12.0 (Todorov and Dicks, 2005). Stability was greater at low pH rather than at high pH (Carolissen-Mackay 1997). Lade (2006) found that the antimicrobial activity of bacteriocin extracted from Lactobacillus lactis against Escherichia coli, Micrococcus lutae MTCC 106, Lactobacillus acidophilus, and Candida albicans was stable at pH 4 to 7 but it became inactivated in the alkaline range, whereas bacteriocins of Lactobacillus plantarum remained active only in the acidic pH from 4.0 to 6.o. Bacteriocins of P. pentosaceus and E. faecium were mostly active against L. mononcytogenes in a pH range of 2.0 – 8.0 and were partially inactivated at pH 10.0 -12.0 (Pinto , 2007). According to Jack (1995), bacteriocin antimicrobial activity at low pH may be due to aggregation of more molecules on sensitive cells. The stability of a bacteriocin, in particular at neutral and basic pHs, is considered as an advantage for its use as an additive in non-acidic foods.

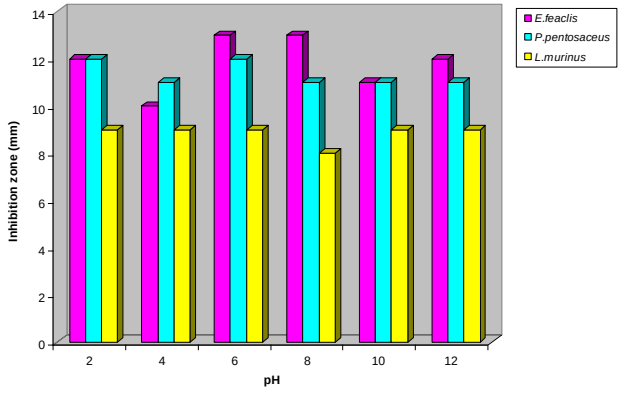

Fig.3. Effect of pH on activity of bacteriocins (inhibition zone diameter) from three isolates on *Bacillus cereus* as indicator organism

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