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Characterization of Partially Purified Bacteriocin Like Substance (BLIS) Produced by Probiotic *Lactobacillus* Strains

Saeed Ismail Khanian [1]; Naheed Mojgani [2,*]; Morteza Khan Ahmedi [1]

[1]Department of Animal Science, Isfahan Center for Research of Agricultural Science and Natural Resources, Isfahan, IR Iran
[2]Biotechnology Department, Razi Vaccine and Serum Research Institute, Karaj, IR Iran

*Corresponding author: Naheed Mojgani, Biotechnology Department, Razi Vaccine and Serum Research Institute, Karaj, IR Iran. Tel.: +98-264570038, Fax: +98-264570038, E-mail: dnmoj@yahoo.com

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Background: There is an increasing interest in search for antimicrobial peptides (bacteriocins and bacteriocin-like compounds) produced by lactic acid bacteria (LAB) because of their potential to be used as antimicrobial agents for improving the safety of food products.
Objectives: The main objective of study was to evaluate the antibacterial potential of locally isolated Lactic Acid bacteria (LAB) and determine their bacteriocin producing ability in in-vitro conditions.
Materials and Methods: The antibacterial activity of 77 isolated LAB strains was tested against a number of pathogens by well-diffusion method. The isolates demonstrating antimicrobial potential were selected and tested for the production of bacteriocin or bacteriocin like substance. The bacteriocin produced by two of the isolates were partially purified and characterized.
Results: The results indicated the neutralized supernatant fluid of two of the isolates identified as *L. brevis* LB32 and *L. pentosus* LP05, were active against the growth of *Listeria monocytogenes*, *Salmonella enteritidis*, *Shigella dysenteriae*, *Staphylococcus aureus* and *Streptococcus pneumoniae*. Additionally, *L. brevis* LB32 was able to inhibit the growth of *Salmonella typhi* and *Klebsiella pneumoniae*, while, *S. pnuemoniae* and *L. monocytogenes* appeared to be the most sensitive strain as apparent by highest zone of inhibition against these pathogens, respectively. The antimicrobial activity in the supernatant fluids of the mentioned strains remained unaffected after treating with enzymes catalase, lipase and lysozyme, while were strongly sensitive to the action of proteolytic enzymes, suggesting the presence of bacteriocin like inhibitory substance (BLIS) in the two isolates. The inhibitory substance produced by the two isolates appeared heat resistant and tolerated 100 °C and 121 °C for 55 minutes and 20 minutes, respectively. Partial purification of the concentrated culture supernatant fluids of *L. brevis* LB32 and *L. pentosus* LP05 by ammonium sulphate (80%) and DEAE cellulose columns resulted in an enhanced activity (AU/mL) and yield. Using different pore size ultra filter membranes and SDS-PAGE analysis, the approximate molecular weight of the BLIS produced by *L. brevis* LB32 and, *L. pentosus* LP05 appeared to be approximately 4.5 and 6 KDa, respectively. In contrast to *L. brevis* LB32, *L. pentosus* LP05 harbored an 18Kb plasmid DNA which appeared to be carrying the bacteriocin gene as evident by plasmid curing experiments. All the mutants retained their host immunity and were resistant to the bacteriocin produced by the parent strain.
Conclusions: In conclusion, the antibacterial activity possessed by these isolates might be used for the control of unwanted pathogens mainly in dairy products, and could be investigated further for using in fermented dairy products.

Keywords: Lactobacillus Strains; Probiotics; Pathogens

1. Background

The beneficial role of LABs and their safety in food fermentation is well documented. These bacteria are well recognized for their extensive use in food processing, such as in dairy and meat fermented products for improving shelf life, texture, and organoleptic properties (1, 2). Additionally, they play an essential role in food preservation due to their ability to produce a variety of antimicrobial agents, including organic acids like lactic and acetic acid, ethanol, carbon dioxide, diacetyl, hydrogen peroxide, and bacteriocins or bacteriocin like inhibitory substance (3-5). Bacteriocins are defined as a heterogenous group of anti-bacterial proteins that vary in the spectrum of activity, mode of action, molecular weight, genetic origin and biochemical properties (6, 7). The bacteriocin producing ability of lactic acid bacteria (LAB) is often proposed as a beneficial characteristic of probiotic bacteria (8, 9). It may contribute to the colonization resistance of the host and thus is protective against gastrointestinal pathogens (10). Currently, artificial chemical preservatives are employed to limit the number of microorganisms capable of growing within foods, but increasing consumer awareness of potential health risks associated with some of these substances has led researchers to examine the possibility of using bacteriocins produced

Implication for health policy/practice/research/medical education:
A number of Probiotic Lactic Acid Bacteria Produce bacteriocins which could be used in pharmaceutical industry as an alternative to antibiotics and in food industry as an alternative to chemical preservatives.

by LAB as bio-preservatives (11, 12). In this study, the bacteriocin producing ability of two *Lactobacillus* strains isolated previously from Ewe milk was investigated. The inhibitory agent produced by the isolates was characterized after partial purification and the bacteriocin genes were identified.

2. Objectives

The use of bacteriocinogenic lactic acid bacterial strains as starter or co-cultures is a promising alternative in food fermentation processes like cheese manufacturing, both to prevent late blowing and to combat *Listeria* spp. (13). In this study, the bacteriocin producing ability of locally isolated LAB strains was determined. The bacteriocins were then isolated, partially purified and characterized in detail.

3. Materials and Methods

3.1. Bacterial Strains and Culture Conditions

The LAB isolates used in this study were isolated previously from Ewe milk and traditionally made yoghurt and buttermilk samples collected from Myaneh and Hashrood cities in north-west of Iran (14). All isolated LAB strains were grown in MRS broth (HiMedia India) at 37˚C for 24-48 hours, while the gram negative pathogens used in study were grown on chocolate agar. All gram positive pathogens used were grown in BHI (HiMedia-India) at 37˚C for 18-24 hours under aerobic condition. The strains were maintained at 4˚C and renewed every week for short-term preservation. The long-term conservation of the purified isolates was carried out in MRS broth with sterile glycerol (20%) and stored at -70˚C.

3.2. Inhibitory Spectrum

The antimicrobial effects of selected LAB against Gram positive and negative pathogens were examined by agar well-diffusion method described earlier (15). A number of gram positive and gram negative pathogens were used as indicator culture in the study (Table 1). The antimicrobial activity was recorded as appearance of clear zone around the wells and the zone diameter (mm) was recorded.

3.3. Titer of Inhibitory Activity

The extent of antibacterial activity demonstrated by the two selected isolates against the mentioned gram positive and negative pathogens was determined by the critical dilution assay described previously (4). A serial two-fold dilution of neutralized supernatant fluid was made in fresh MRS broth and the activity of each concentration was determined by agar well diffusion assay. The highest dilution resulting in a clear zone of inhibition was considered to contain one unit of bacteriocin per ml. This activity was expressed in arbitrary units per milliliter (AU/mL), defined as the reciprocal of highest dilution showing discernible activity.

3.4. Characterization of Antimicrobial Agent

The cell free supernatants of the two selected LAB strains demonstrating maximum antibacterial activity against a number of tested pathogens was further studied for following traits.

3.4.1. Sensitivity to pH, Enzymes and Heat Treatments

Overnight grown cultures of the tested isolates were centrifuged and the obtained supernatant fluids were adjusted to pH = 6.5 with 1 N NaOH followed by filtration through a 0.22 μm pore size filters. The remaining activity was determined by agar well diffusion assay using *L. monocytogenes* as indicator strain. The supernatant fluids of the isolates were treated with catalase, lysozyme, lipase, pronase E, trypsin and proteinase K (Sigma) at final concentrations of 1 mg/ml in phosphate buffer (pH = 7.0). The remaining activity was determined as described earlier, after two hours of incubation of the treated samples at 37˚C. The thermostability of the antagonistic compound was evaluated by heating the supernatant fluid at 100 and 121˚C and determining the residual activity after every ten minutes for maximum of two hours by the method described earlier.

3.4.2. Adsorption Assay

Equal volumes of culture supernatant fluids of the test strains and the sensitive indicator cells (*S. pneumonaie*) were mixed and placed in water bath at 37˚C. After every five minutes the samples were chilled at 4˚C and centrifuged at 6000 rpm for ten minutes and the remaining antibacterial activity in the supernatant fluid was determined after neutralization.

3.4.3. Concentration and Partial Purification of the Antagonistic Agent

The antagonistic substance produced by the selected isolates was partially purified by subjecting the supernatant fluid (pH = 6.5) of the producing strains to ammonium sulfate precipitation at final concentrations of 80% w/v (16) with slight modifications. Prior to salt saturation, the neutralized supernatant fluids were vacuum dried to one tenth of their original volumes. The precipitate at the final round was recovered by centrifugation (11,200 ×g, 15 minutes, 4˚C), resuspended in sterile MilliQ water and dialyzed against sterile water at 8 ˚C for 24 hours, using Sephadex G-25 (coarse) column (2×5 cm; Bio-Rad) equilibrated with 50 mM phosphate buffer pH 7.0. The pH of the desalted sample was adjusted to 6.5 with 0.1 N NaOH, and subsequently applied to DEAE cellulose chromatography using column (5 × 20 cm diameter) equilibrated with 10 mM sodium phosphate buffer (pH = 6.5). The

columns had a bottom layer of 0.5 to 1 cm Sephadex G25 to improve the flow rate. The columns were equilibrated and washed with two bed volumes of 50 mM phosphate buffer pH = 7.0. The bounded protein (light brown color) was eluted with 1 bed volume of the column buffer containing 100 mM NaCl. The fractions were frozen at -20˚C and freeze dried by vacuum evaporation to obtain dry powder form. Main drying cycle was performed at 15˚C for 15 hours at a pressure of 22 Pa. The antibacterial activity (AU/mL) and the protein concentrations of the fractions were determined by Lowry method (17) with 1% BSA as standard.

3.4.4. Molecular Size Estimation of Partially Purified Bacteriocin

Approximate molecular weight of the antagonistic peptides produced by the two selected isolates were determined by ultrafilteration and SDS-PAGE analysis. A 2 mL culture free supernatant fluid was ultrafiltered through cellulose membranes with 5, 10 and 30 KDa exclusion units (Centricon, Micro concentrations, USA). Bacteriocin activity in the retained and eluted fractions were determined by well-diffusion agar.

Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) was carried out as described previously (18), using 7.5 and 12.0% Polyacrylamide concentrations in the stacking and the separating gels, respectively. The samples were loaded in duplicate and electrophoresis was performed at a constant voltage of 60 V for one hour. The gel was cut into two halves and one part was stained with Coomassie brilliant blue R 250 (Sigma-Aldrich, Belgium). The other half was washed extensively with pure water to reduce the SDS content and to avoid inhibition of the sensitive strains by the detergent, and then was overlaid with the indicator strain. Ultralow range molecular weight marker (Sigma, UK) was used for approximate molecular weight estimation.

3.4.5. Locus Identification of Bacteriocin Genes

The presence of plasmid in the wild strains was analyzed by Echardt gel electrophoresis (19) and analysis on 0.9% agarose gel. Plasmid curing was performed by exposing the producer cells to different concentrations of ethidium bromide (50-120ug/mL) and selecting the colonies lacking antibacterial activity. The bacteriocin negative (bac-) colonies were then screened for the presence or absence of plasmid by the above method.

4. Results

Seventy-seven bacterial isolates from the traditionally made dairy samples including yogurt and butter milk were identified as LAB. Initially all the isolates were screened for antibacterial activity against each other. According to the results, most of the bacteria isolated from milk or yoghurt exhibited activity against indicators that were isolated from the same products. Moreover, the antagonistic activity demonstrated by rod shape (bacilli) bacterial isolates was significantly higher than cocci isolates. All isolates showed different level of activity against the tested pathogens. Among these isolates, only two isolates demonstrating maximum activity against the growth of other closely related bacteria were selected for further studies. The two selected isolates were identified as *L. brevis* LB32 and *L. pentosus* LP05 based on their carbohydrate fermentation patterns (Iranmanesh et al. (14)) and 16 S rRNA gene sequencing (data not shown).

Table 1 depicts the inhibitory spectrum of the selected isolates against a number of other gram positive and negative pathogens. The selected isolates were able to inhibit the growth of *L. monocytogenes*, *S. enteritidis*, *Sh. dysenteriaea*, *S. aureus* and *S. pnuemoniae*. However, different level of inhibitory action against these pathogens was demonstrated by the two isolates as seen by their zone diameters. Compared to *L. pentosus* LB32, the spectrum of inhibition of *L. brevis* LB32 was wider as it also inhibited the growth of *Sh. dysenteriaea*, *S. typhi* and *K. pneumoniae*. Both the isolates were unable to inhibit the growth of *H. influenzae* b and *Ps. aerouginosa*, while *S. pnuemoniae* appeared to be the most sensitive strain as apparent by highest zone of inhibition against this pathogen.

Table 1. The Antagonistic Activity of Selected LAB Strains Against Pathogenic Bacteria [a]

Indicator Pathogens	*L. brevis* **LB32**	*L. pentosus* **LP05**
Haemophilusinfluenzae **(ATCC 10211)**	-	-
Klebsiellapneumoniae **(PTCC 1053)**	+++	-
Listeria monocytogenes **(RTCC 1298)**	+++	++
Pseudomonas aeruginosa **(local isolate)**	-	-
Salmonella enteritidis **(local isolate)**	+	+++
Salmonella typhi **(local isolate)**	+++	-
Shigelladysenteriae**a (local isolate)**	+++	-
Shigelladysenteriae**a (local isolate)**	+	-
Staphylococcus aureus **(RTCC 1112)**	+	+
Streptococcus pneumoniae **(local isolate)**	++++	++++

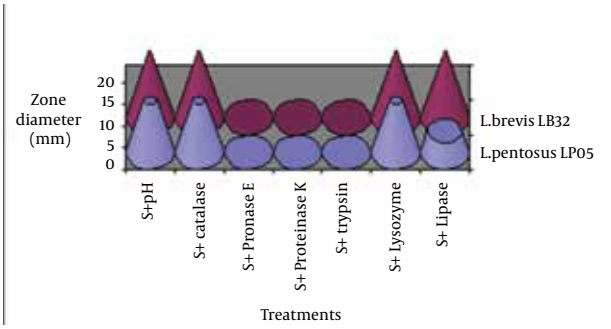
[a] +, zone of inhibition; -, no zone of inhibition; ++, 3 mm< zone; ++, 3 mm< zone <5 mm; +++, 5 mm< zone <7 mm.

The antibacterial activity of the two selected isolates appeared sensitive to the action of proteolytic enzymes and was completely diminished after treatment with the mentioned enzymes (Figure 1). However, lysozyme had no effect on the supernatant fluid of these isolates and their activity was retained after treatment with this enzyme. In contrast to *L. brevis* LB32, the antibacterial activity of *L. pentosus* LP05 appeared affected by the action of lipase and its activity was partially reduced after treatment with this enzyme indicating its lipoprotein nature.

The antibacterial peptide produced by *L. pentosus* LP05 was highly heat tolerant as it could resist 100˚C for 120 minutes and the autoclaving temperature of 121˚C for 20 minutes. The antibacterial substance produced by *L. brevis* LB32 was able to resist 100˚C for 15 minutes and lost its activity at 121˚C within ten minutes. Significant increase in the activity of the bacteriocins produced by *L. brevis* LB32 and *L. pentosus* LP05, was observed during purification at each subsequent stage (Figure 2).

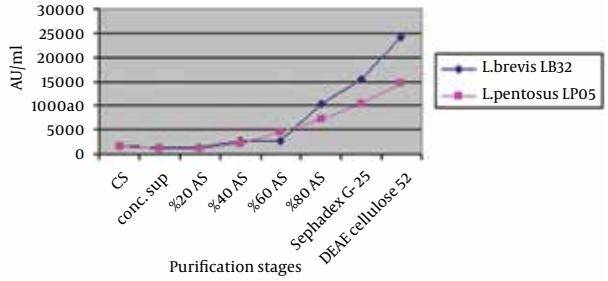
The overall yields and activities are summarized in Table 2. After the cationic separations, high purification fold of 169 and 177 was recorded for *L. brevis* LB32 and *L. pentosus* LP05, respectively. The fractions showing antibacterial activity corresponded to peptide molecules in the range of 5-10 KDa which was subsequently confirmed by subjecting the fractions to ultra filtration using filtron membranes with 5, 10 and 30 KDa molecular weight cut off and finally SDS-PAGE analysis. The antagonistic compound produced by both the isolates was able to pass through 10 and 30 KDa cellulose membranes, while was retained in 5KDa cutoffs which indicated peptide molecules of approximate range between 5 and 10 KDa.

Figure 1. Effect of Different Treatments to modify the Antibacterial Activity of Supernatant Fluids From *L. brevis* LB32 and *L. pentosus* LP05



S+pH, neutralized supernatant fluids; S+, treated supernatant fluids with the mentioned enzymes.

Figure 2. Increase in Activity (AU/mL) of Bacteriocins Produced by *L. brevis* LB32 and *L. pentosus* LP05 During Purification



CS, crude supernatant fluid; Conc.sup: concentrated supernatant fluids; 20, 40, 60, and 80 % AS, precipitate obtained during increasing concentrations of ammonium sulphate.

Table 2. Summary of the Purification Steps of Bacteriocin from the Culture Supernatant of *L. brevis* LB32 and *L. pentosus* LP05 [a, b]

Purification Stages	Volume, mL	Activity, AU/mL	Total Protein, mg	Specific Activity	Yield, %	Purification, fold
***L. brevis* LB32**						
CCS	100	1600	21.90	73.05	100	1.00
Conc.S	10	1280	9.91	129.16	80.0	1.76
80 % AS	10	10240	12.21	838.65	640	11.48
S-G25	05	9830	4.9	2006	614	27.46
e. D-C52	04	10400	0.84	12380	650	169
***L. pentosus* LP05**						
CCS	100	1600	23.14	69.14	100	1.00
Conc.S	15	960	12.11	79.27	60.0	1.14
80 % AS	07	7168	16.43	436.27	448	6.31
S-G25	04	10240	5.6	1828.57	640	26.44
D-C52	03	14700	1.2	12250	918	177

[a] Abbreviations: CCS, crude culture supernatant fluid; Conc.S, concentrated supernatant; D-C52, DEAE cellulose 52; S-G25: sephadex G25.
[b] Specific Activity, AU/mg = Total activity of the subsequent purification step/Total protein of the same step; Yield, % =Total activity of the subsequent purification step/Total activity in the crude culture supernatant; Purification, fold = Specific activity of the subsequent purification step/ specific activity of the crude culture supernatant.

SDS-PAGE analysis provided approximate molecular weights of the antibacterial peptide molecules produced by the two selected isolates. The antagonistic compound produced by *L. pentosus* LP05 and *L. brevis* LB32 were resolved on SDS-PAGE as very strong single bands of ~ 4.5 and ~6 KDa, respectively. The location of genes encoding these antagonistic compounds (BLIS) in both the selected isolates was evaluated using plasmid isolation and curing experiments. Based on plasmid curing experiments, no plasmids were detected in *L. brevis* LB32 while, the bacteriocinogenic genes in *L. pentosus* LP05 appeared to be located on a plasmid as bac negative mutants lacking plasmids and showing no antibacterial activity were isolated after curing with 120 ug/ml of ethidium bromide. A plasmid of 18 Kb was evident in the above mentioned isolate which was lost after treatment with the curing agent. However, the bacteriocin immunity genes in both of the isolates appeared to be chromosomally located as bacteriocin negative mutants were able to retain their resistance to the bacteriocin of parent strains.

5. Discussion

Among a number of traditional dairy products consumed in Iran, yoghurt is one of the most popular fermented milk products. Moreover, traditionally made dairy products are also highly popular in this region with people consuming traditionally made yogurts, butter and sour butter milk and similar products. Although the traditionally made dairy products are more easily available in smaller towns and villages but their health benefits are well known all around. The present investigation highlights the bacteriocinogenic potential of LAB strains isolated from traditionally made yogurt and sour butter milk made from Ewe's milk. Previously, a number of published reports have indicated the presence of *Lactobacillus* strains in sheep, goat and cow milk (20, 21). However, Skelin and his colleagues (22) reported isolation of a number of *Lactobacillus* strains including *L. brevis* and *L. pentosus* from traditional Istrian Ewe's cheese.

Numerous studies have shown the inhibitory activities of a number of LABs such as *L. brevis* (11, 23) and *L. acidophilus*,*L. sake*, *L. pentosus*etc (7, 24, 25). The antibacterial potential of these bacteria havebeen attributed to their ability to produce H_2O_2, acids, phages, bacteriocins and bacteriocin like substances (26). It is assumed that some of the bacteria in the intestinal tract produce bacteriocins as a means to achieve a competitive advantage, and bacteriocin-producing bacteria might be a desirable player in competitive exclusion preparations. Purified or partially purified bacteriocins could be used as preservatives or for the reduction or elimination of certain pathogens (4). The bacteriocins produced by LAB are considered potent bio-preservative agents and their application in food industry is currently the subject of extensive research (27). So far, nisin and pediocin PA-1 are bacteriocins licensed for use in certain foods as preservatives (28). In our investigations we were able to isolate and identify a number of LAB with ability to produce wide spectrum bacteriocin or bacteriocin like substance (BLIS). The spectrum of activity of these antagonistic compounds against certain important food borne pathogens for instance *S. aureus* and *L. monocytogenes* appeared very promising, as *S. aureus* is considered a potential public health risk due to its production of enterotoxins causing food poisoning (29), and food borne transmission of *L. monocytogenes* responsible for listeriosis in human (30).

Previous reports have indicated that bacteriocins might be either simple proteins or proteins linked to lipid or carbohydrate moieties. During the course of study it was observed that while carbohydrate moiety was not critical for activity in both isolates, the lipid component had an essential role in the bioactivity of the bacteriocin produced by *L. pentosus* LP05. This phenomenon might also define the reasons for higher heat stability of this bacteriocin compared to the one produced by *L. brevis* LB32. However, similar to sakacinA, plantacin B, lacticin A and B (24), the bacteriocin produced by *L. brevis* appeared as a simple protein molecule rather than a conjugated one. Bacteriocins from LAB are subdivided into class I, II and III based on their structural, physiochemical and molecular properties. Nonetheless, this classification is continuously being reviewed and it is evolving with the discovery of new bacteriocins and advancement of knowledge about them (31-33). The bacteriocins produced by both the isolates in our study appeared to share characteristics similar to Class IIa bacteriocins. As described by Fimland and his colleagues (33), Class IIa bacteriocins or pediocin-like bacteriocins are small, cationic, hydrophobic, heat-stable peptides with a strong anti-*Listeria*-l effect.

In conclusion, the traditional dairy products in our country are rich sources of LAB with probiotic properties. The antibacterial potential of these bacteria may be used in the food and healthcare industries with wide applications. This study proved the presence of viable bacteriocinogenic probiotic LAB micro flora in these (dairy) products.

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Authors' Contribution

All authors have participated in the study.

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