British Microbiology Research Journal
11(1): 1-9, 2016, Article no.BMRJ.21427
ISSN: 2231-0886, NLM ID: 101608140

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Screening, Identification and Antibiotic Susceptibility Pattern of Bacteriocin-producing Lactic Acid Bacteria Isolated from Selected Traditionally Fermented Products

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Authors' contributions

This work was carried out in collaboration between all authors. Author IAA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors AOO and DJA managed the analyses of the study. Author IAA managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BMRJ/2016/21427
Editor(s):
(1) Marcin Lukaszewicz, Department of Biotransformation, Faculty of Biotechnology, University of Wroclaw, Wroclaw, Poland And Division of Chemistry and Technology Fuels, Wroclaw University of Technology, Wroclaw, Poland.
Reviewers:
(1) Arimah, Babatunde David, Igbinedion University, Okada, Edo state, Nigeria.
(2) M. Angeles Calvo Torras, Universidad Autónoma de Barcelona, Bellaterra, Spain.
Complete Peer review History: http://sciencedomain.org/review-history/11798

Original Research Article

Received 16th August 2015
Accepted 5th September 2015
Published 10th October 2015

ABSTRACT

Aims: To isolate and identify bacteriocin-producing lactic acid bacteria from traditionally fermented products and determine their antibiotic susceptibility pattern.
Place and Duration of Study: Department of Microbiology, Federal University of Technology, Akure, Nigeria between October, 2012 – March, 2013.
Methodology: Lactic acid bacteria (LAB) isolates from samples of traditionally fermented products ("burukutu", "pito", yoghurt, "wara" and "iru") were screened for bacteriocin production. Bacteriocin screening was performed both by the agar spot test and well diffusion assay. Four reference strains (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) were used as indicator strains to check sensitivity to the antimicrobial substances produced by the LAB isolates. Carbohydrate

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fermentation profiles of selected bacteriocin-producing LAB strains were determined using API 50CH kits to identify them up to the species level. Antibiogram of LAB isolates were determined by antibiotic sensitivity discs.

Results: A total of sixty-three (63) lactic acid bacteria (LAB) strains obtained from the fermented products were screened for bacteriocin production. Thirty-seven isolates (59%) of these LAB strains showed antimicrobial activity against two or more of the reference varieties used as indicator strains (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853). After excluding inhibition due to organic acids and hydrogen peroxide from the cell-free culture supernatants of these 37 LAB isolates, only 6 (16%) of the 37 selected LAB isolates (10% of the 63 initial LAB isolates) continued to show antimicrobial activity against three of the reference strains. The inhibitory effects of these six (6) LAB strains showed the presence of bacteriocins, hence potent bacteriocin producers. The selected bacteriocin-producing LAB strains (BE1, BO2, IO1, PO4, PO9 and YO7) were identified as *Lactobacillus cellobiosus*, *Lactobacillus brevis*, *Pediococcus pentosaceus*, *Lactobacillus rhamnosus*, *Tetragenococcus halophilus*, and *Lactobacillus fermentum* respectively. All the bacteriocin-producing LAB isolates were susceptible to erythromycin and zinnacef but were resistant to streptomycin and pefloxacin.

Conclusion: These results reveal six LAB isolates from traditional fermented products that were capable of producing bacteriocins which could have a potential for food applications as biopreservatives.

Keywords: Lactic acid bacteria; bacteriocin; fermented products; antibiotic; susceptibility.

1. INTRODUCTION

Lactic acid bacteria (LAB) encompass a heterogeneous group of Gram-positive, non-spore forming, non-motile, aerotolerant, rod and coccus-shaped organisms, which produce lactic acid as a major end product during carbohydrate fermentation. Early taxonomy defined four main 'core genera' involved in food fermentations, namely *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* [1,2]. Bacteriocin production is a desirable trait among LAB from the perspective of controlling microbial populations in fermented foods in order to extend product shelf-life and safety [3].

Bacteriocins are ribosomally-synthesized peptides or proteins with antimicrobial activity, produced by different groups of bacteria. Although bacteriocins are produced by many Gram-positive and Gram-negative species, those produced by LAB are of particular interest to the food industry, since these bacteria have generally been regarded as safe. Many lactic acid bacteria produce bacteriocins with rather broad spectra of inhibition [4,5]

Consumers consistently have been concerned about possible adverse health effects from the presence of chemical additives and preservatives in their foods. This has resulted in consumers' attention to natural and 'fresher' foods with no chemical preservatives added. This perception, coupled with the increasing demand for minimally processed foods with longer shelf life and convenience, has stimulated research interests in finding natural but effective preservatives. Bacteriocins, produced by LAB, may be considered natural preservatives or biopreservatives that fulfill these requirements [6]. Therefore, the objectives of this study were to isolate and identify bacteriocin-producing lactic acid bacteria from traditionally fermented products and determine their antibiotic susceptibility pattern.

2. MATERIALS AND METHODS

2.1 Isolation of Lactic Acid Bacteria

Lactic acid bacteria (LAB) were isolated from different samples of locally fermented cereal-based products ("burukutu" and "pito"), dairy products (yoghurt and "wara") and African locust beans ("iru") that were procured from sellers in various locations in Ondo and Edo States, Nigeria. Samples were kept at refrigerated condition until analysis.

Isolation of LAB was carried out using the method as described by Bhattacharya and Das [7]. Serial dilutions (10^{-3}, 10^{-6}, and 10^{-9}) of the samples were inoculated into de Man Rogosa Sharpe (MRS) agar (Oxoid, Basingstoke, UK) by pour plate method and incubated in anaerobic condition at 30℃ for 48 h for the colonies to

develop. Following incubation, different colonies for each sample were randomly selected from the MRS agar plates. The colonies were sub-cultured and purified by repeated streaking on MRS agar to obtain pure cultures. Pure isolates were then cultured on MRS agar slants and broths (in duplicates) and stored at 4℃ until used. Gram's staining and catalase test were carried out on each isolates.

2.2 Detection of Antimicrobial Activity and Assay for Bacteriocin Activity

Agar spot tests and agar well diffusion assays were used to study the bacteriocin activity of the LAB strains isolated on MRS agar (Oxoid) from the fermented products. For this purpose, four reference strains were used to check sensitivity to the antimicrobial substances produced by the LAB, these being *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, and *Pseudomonas aeruginosa* ATCC 27853, as described by Gonzalez et al. [8] and Khay et al. [9] with some modifications. LAB strains were grown in MRS broth (Oxoid) at 30℃ for 24 h. The indicator strains (reference strains) were cultured in Tryptone Soya Broth (TSB, Oxoid) at 37℃ for 24 h.

For the agar spot test, volumes of 3 µl of overnight cultures of LAB strains were spotted onto the surface of MRS agar (Oxoid) plates and incubated at 30℃ for 24 h to allow the development of colonies. Volumes of 100 µl of the indicator strains, with a total approximately 5 x 10^5 cfu/ml were inoculated into 7 ml of semi-solid TSA (broth plus 0.75% bacteriological agar), kept at a temperature of 45℃, then after agitation were poured over the plates of MRS agar on which the LAB strains under test had grown. The plates were incubated at 37℃ for 24 h and checked for inhibition zones. Inhibition was considered positive when the inhibition halo of the indicator strain above the LAB colonies was more than 2 mm.

For the well diffusion assay, an overnight culture of indicator strains grown in TSB broth at 37℃ was diluted to a turbidity equivalent to that of a 0.5 MacFarland standard. A lawn of an indicator strain was made by spreading the cell suspension over the surface of Mueller Hinton agar (MHA, LabM, Lancashire, UK) plates with a cotton swab. The plates were allowed to dry and a sterile cork borer of diameter 6.0 mm was used to cut uniform wells in the agar pates. LAB strains were grown in MRS broth at 30℃ for 48 h. Cultures were centrifuged at 4000 g for 20 min at 4℃, the cell-free supernatants (CFS) were collected, adjusted to pH 6.5 with 1 M NaOH to remove organic acid, then, catalase (1mg/ml) was added to remove hydrogen peroxide, and thereafter filtered through a 0.45 µm pore-size membrane (Millipore Corporation, USA). Each well in the MHA plates was filled with 80 µl of filter-sterilized cell-free supernatants of the potential producer strains. The plates were kept at 4℃ for 2 h, to ensure diffusion of the supernatant fluid into the agar, and then incubated at 37℃ for 24 h. The antimicrobial activity was determined by measuring the diameter of the inhibition zone around the wells. If inhibition was found present when this was done, it was deemed to be due to the production of bacteriocins or bacteriocin-like compounds.

2.3 Identification of the Bacteriocin-producing LAB Isolates

The selected LAB strains were further identified and characterized by API 50 CHL test kit (bioMerieux, France), as recommended by the manufacturer. The APIWEB software version 5.1 (bioMerieux) was used to analyze the fermentation profiles obtained with the identification strips.

2.4 Antibiotic Susceptibility Test (Antibiogram) of Bacteriocin-producing LAB Isolates

The selected LAB isolates were inoculated into MRS broth individually and incubated for 24 h. About 25 ml of solidified MRS agar plates were swabbed with standardized culture suspensions (0.5 MacFarland standard, equivalent to cell density of 10^8 cfu/ml) using a sterile cotton swab. DECA-discs (10 antibiotics in a single ring) were placed upside down, pressed on the top of the agar plates and kept at 4℃ for 1 h. The plates were incubated anaerobically at 30℃ overnight. Inhibition zone diameter was measured and the isolates were classified as sensitive, intermediate, and resistant [10,11].

2.5 Statistical Analysis

Data on the zone of inhibition obtained against the different bacterial pathogens used as indicator strains were expressed as mean ± standard deviation (SD) which was plotted as a graph using SPSS 16.0 for Windows.

3. RESULTS

3.1 Isolation of Potential LAB and Screening for Bacteriocin-producing LAB

A total of 120 bacterial strains were isolated from different samples of five types of fermented products ("burukutu", "pito", yoghurt, "wara", and "iru") using de Man Rogosa Sharpe (MRS) agar. Of these, 63 isolates were Gram positive and catalase negative, and were cocci or rod in shape, which indicated the typical basic characteristics of lactic acid bacteria.

These 63 LAB isolates were screened for bacteriocin production. 37 isolates (59%) of the 63 LAB isolates showed antimicrobial activity against two or more of the reference varieties used as indicator strains (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853) when the method used was agar spot test. Table 1 shows the inhibition of indicator strains by selected LAB isolates using agar spot test method.

When agar well diffusion assay was used to check the antimicrobial activity of the cell-free culture supernatants of these 37 LAB isolates after excluding inhibition due to organic acids and hydrogen peroxide, only 6 (16%) of the 37 selected LAB isolates (10% of the 63 initial LAB isolates) continued to show antimicrobial activity and only against three of the reference strains (*S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, and *E. coli* ATCC 25922) and three strains also inhibited *Bacillus cereus* (Fig. 1). The inhibitory effects that these 6 LAB strains (BE1, BO2, IO1, PO4, PO9, and YO7) demonstrated are due to the presence of bacteriocins which made them potent bacteriocin producers. *S. aureus* ATCC 25923 was observed to be the most sensitive indicator to bacteriocins produced by the 6 selected LAB strains while *P. aeruginosa* ATCC 27853 was resistant to them.

Table 1. Inhibition of indicator strains by selected lactic acid bacterial isolates using agar spot test method

LAB isolates (producer strains)	Sources	Inhibition of indicator strains			
		***S. aureus* (ATCC 25923)**	***E. faecalis* (ATCC 29212)**	***E. coli* (ATCC 25922)**	***P. aeruginosa* (ATCC 27853)**
BE1	Burukutu	++	++	++	(+)
BE2	Burukutu	++	++	++	-
BE3	Burukutu	++	++	+	-
BO2	Burukutu	++	+	++	+
PO2	Pito	+	+	(+)	-
PO3	Pito	+	++	++	-
PO4	Pito	++	++	++	-
PO8	Pito	++	+	+	-
PO9	Pito	++	+	+	+
YO2	Yoghurt	++	++	++	-
YO4	Yoghurt	+	++	(+)	-
YO5	Yoghurt	+	++	+	+
YO6	Yoghurt	++	++	++	-
YO7	Yoghurt	++	++	++	+
YO9	Yoghurt	++	+	+	-
IO1	Iru	++	++	+	+
IO2	Iru	++	+	+	(+)
WO1	Wara	+	(+)	-	-
BR3	Burukutu	+	+	+	-
BR4	Burukutu	+	+	(+)	-

Symbols: -, no detectable inhibition; (+), Small inhibition zone (<2 mm); +, inhibition zone of 2 to 6 mm; ++, inhibition zone > 6 mm

3.2 Identification of Bacteriocin-producing LAB Strains

Identification of bacteriocin-producing LAB strains BE1, BO2, IO1, PO4, PO9, and YO7 was carried out up to species level by using API 50 CHL system (bioMerieux, France). All of the selected strains showed positive results (i.e. were able to ferment) for D-Galactose, D-Glucose, Esculin ferric citrate and were negative for Glycerol, Erythritol, D-Arabinose, L-Xylose, D-Adonitol, Methyl-βD-Xylopyranoside, Dulcitol, Inositol, Methyl-αD-Mannopyranoside, Methy-αD-Glucopyranoside, Inulin, Amidon, Xylitol, D-Turanose, D-Lyxose, D-Fucose, L-Fucose, D-Arabitol, L-Arabitol, Potassium 2-KetoGluconate, and Potassium 5-KetoGluconate (Table 2a and 2b). LAB strains BE1, IO1, PO9, BO2 and PO4 were positive for D-Ribose, D-Fructose, D-Mannose, D-Maltose, and D-Trehalose but YO7 showed negative results. Also LAB strains IO1, PO9, and PO4 were positive for N-AcetylGlucosamine, Amygdalin, Arbutin, Salicin, and D-Celiobiose, but BE1, YO7 and BO2 showed negative results (Tables 2a and 2b).

Identification using apiweb^{TM} software version 5.1 showed that LAB strains BE1, BO2, IO1, PO4, PO9 and YO7 were identified as *Lactobacillus cellobiosus*, *Lactobacillus brevis*, *Pediococcus pentosaceus*, *Lactobacillus rhamnosus*, *Tetragenococcus halophilus*, and *Lactobacillus fermentum* respectively (Table 3).

3.3 Antibiotic Susceptibility Test (Antibiogram)

The results of sensitivity against antibiotics used are given in Table 4, which shows that all the bacteriocin-producing LAB isolates were sensitive to Erythromycin and Zinnacef but were resistant to streptomycin and pefloxacin. The susceptibility to other antibiotics was variable and depending on the strains.

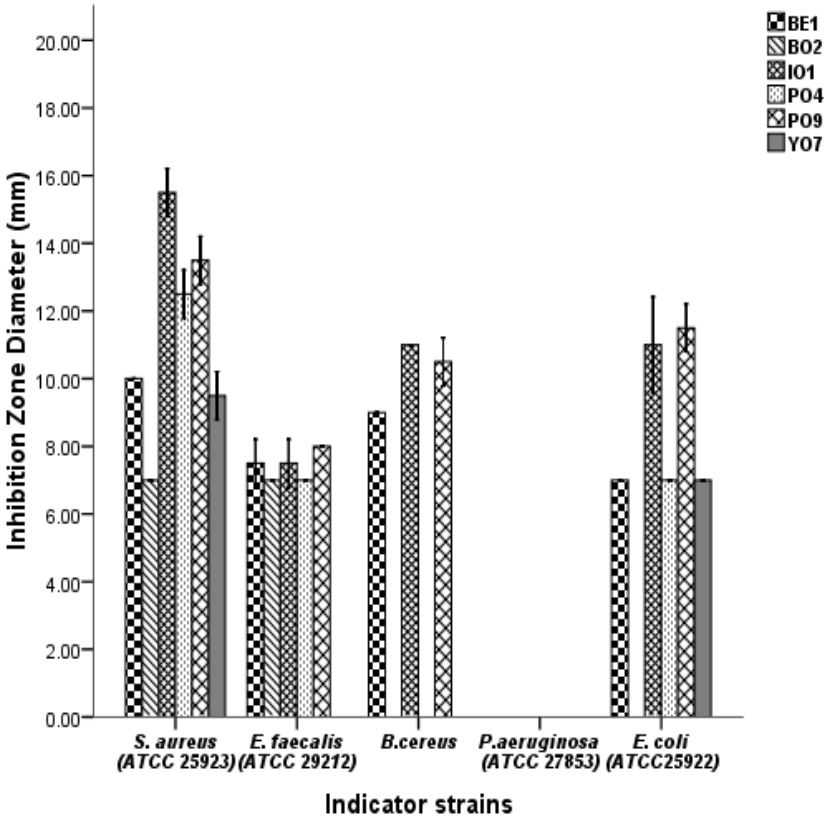

Fig. 1. Bacteriocin activity assay of six selected LAB strains using agar well diffusion

Table 2a. Carbohydrate fermentation profiles of selected bacteriocin-producing lactic acid bacteria by API 50 CH kit grown at 30℃

S/N	Substrates	LAB strains					
		BE1	IO1	PO9	YO7	BO2	PO4
0	Control	-	-	-	-	-	-
1	Glycerol	-	-	-	-	-	-
2	Erythritol	-	-	-	-	-	-
3	D-Arabinose	-	-	-	-	-	-
4	L-Arabinose	+	+	-	-	+	-
5	D-Ribose	+	+	+	-	+	+
6	D-Xylose	+	-	+	-	+	-
7	L-Xylose	-	-	-	-	-	-
8	D-Adonitol	-	-	-	-	-	-
9	Methyl-ßD-Xylopyranoside	-	-	-	-	-	-
10	D-Galactose	+	+	+	+	+	+
11	D-Glucose	+	+	+	+	+	+
12	D-Fructose	+	+	+	-	+	+
13	D-Mannose	+	+	+	-	+	+
14	L-Sorbose	-	-	-	-	-	+
15	L-Rhamnose	-	-	-	-	-	+
16	Dulcitol	-	-	-	-	-	-
17	Inositol	-	-	-	-	-	-
18	D-Mannitol	-	-	-	-	-	+
19	D-Sorbitol	-	-	-	-	-	+
20	Methyl-αD-Mannopyranoside	-	-	-	-	-	-
21	Methyl-αD-Glucopyranoside	-	-	-	-	-	-
22	N-AcetylGlucosamine	-	+	+	-	-	+
23	Amygdalin	-	+	+	-	-	+
24	Arbutin	-	+	+	-	-	+

Table 2b. Carbohydrate fermentation profiles of selected bacteriocin-producing lactic acid bacteria by API 50 CH kit grown at 30℃

S/N	Substrates	LAB strains					
		BE1	IO1	PO9	YO7	BO2	PO4
25	Esculin ferric citrate	+	+	+	+	+	+
26	Salicin	-	+	+	-	-	+
27	D-Celiobiose	-	+	+	-	-	+
28	D-Maltose	+	+	+	-	+	+
29	D-Lactose	+	-	+	+	+	+
30	D-Melibiose	+	+	-	+	+	+
31	D-Saccharose	+	-	-	+	+	-
32	D-Trehalose	+	+	+	-	+	+
33	Inulin	-	-	-	-	-	-
34	D-Melezitose	-	-	-	-	-	+
35	D-Raffinose	+	-	-	+	+	+
36	Amidon	-	-	-	-	-	-
37	Glycogen	-	-	-	-	-	+
38	Xylitol	-	-	-	-	-	-
39	Gentiobiose	+	+	+	-	-	-
40	D-Turanose	-	-	-	-	-	-
41	D-Lyxose	-	-	-	-	-	-

S/N	Substrates	LAB strains					
		BE1	IO1	PO9	YO7	BO2	PO4
42	D-Tagatose	+	+	+	-	-	+
43	D-Fucose	-	-	-	-	-	-
44	L-Fucose	-	-	-	-	-	-
45	D-Arabitol	-	-	-	-	-	-
46	L-Arabitol	-	-	-	-	-	-
47	Potassium Gluconate	-	-	-	-	+	+
48	Potassium 2-KetoGluconate	-	-	-	-	-	-
49	Potassium 5-KetoGluconate	-	-	-	-	-	-

4. DISCUSSION

Isolation and screening of microorganisms from natural sources has always been the most powerful means for obtaining useful and genetically stable strains for industrially important products [12]. This certainly holds true for lactic acid bacteria (LAB), which are used throughout the world for manufacture of a wide variety of traditional fermented foods [13].

Table 3. Identification using apiweb software v. 5.1

LAB isolate	Source	Identified as
BEI	Burukutu	*Lactobacillus cellobiosus*
IO1	Iru	*Pediococcus pentosaceus*
PO9	Pito	*Tetragenococcus halophilus*
YO7	Yoghurt	*Lactobacillus fermentum*
BO2	Burukutu	*Lactobacillus brevis*
PO4	Pito	*Lactobacillus rhamnosus*

In the present study, 63 LAB strains were isolated from different samples of five fermented products ("burukutu", "pito", yoghurt, "wara", and "iru") and screened for bacteriocin production. Out of the 63 LAB strains isolated, only 6 (10%) were identified as potent bacteriocin producers (*Lactobacillus cellobiosus* BE1, *Lactobacillus brevis* BO2, *Pediococcus pentosaceus* IO1, *Lactobacillus rhamnosus* PO4, *Tetragenococcus halophilus* PO9, and *Lactobacillus fermentum* YO7). Therefore, these results reflect the low frequency of LAB producing bacteriocins from the selected traditionally fermented products.

Similar results have been reported by other authors. Yang et al. [14] screened 138 LAB isolates from cheeses and yoghurts and found only 28 (20%) exhibited bacteriocin production, whereas Gonzalez et al. [8] reported detecting 24 (6%) bacteriocinogenic strains among 395 strains of lactic acid bacteria isolated from Genestoso cheese throughout manufacture and ripening.

The inhibitory effect observed for the other 31 LAB isolates when using the agar spot test could be attributed to the production of lactic and acetic acids and the consequent pH decrease or to the presence of hydrogen peroxide. Furthermore, false negative results in the well-diffusion assay for positive results in the agar spot test are possible due to aggregation, non-diffusible bacteriocins, protease inactivation and concentration effects [15,16].

Some of the lactic acid bacteria identified in this study have been reported to produce bacteriocins [17,13,18]. Therefore, it is likely that bacteriocins are responsible for the inhibitory effects observed for their cell-free culture supernatants.

Antibiotics are a major tool utilized by the health care industry to fight bacterial infections; however, bacteria are highly adaptable creatures and are capable of developing resistance to antibiotics. Resistance to common antibiotics by lactic acid bacteria could be intrinsic (naturally owned) or acquired [19].

Intrinsic resistance of lactic acid bacteria to many antibiotics may be considered as advantageous for those isolates with bacteriocinogenic/ probiotic potential. Such resistance could be helpful for sustainable utilization of the strains in human intestinal microflora during antibiotic therapy [20]. However, there is the danger of transferring multiple drug resistance to pathogens in the intestinal environment. The susceptibility of bacteriocinogenic LAB isolates

Table 4. Antibiogram of bacteriocin-producing LAB isolates determined by antibiotic sensitivity discs

Antibiotics	Concentration	LAB Isolates					
		BE1	BO2	IO1	PO4	PO9	YO7
Ciprofloxacin (CPX)	10	13 (I)	20 (S)	- (R)	20 (S)	- (R)	25 (S)
Streptomycin (S)	30	11 (R)	- (R)	- (R)	- (R)	- (R)	- (R)
Septrin (SXT)	30	11 (R)	12 (I)	- (R)	- (R)	- (R)	15 (I)
Erythromycin (E)	10	26 (S)	30 (S)	28 (S)	22 (S)	26 (S)	30 (S)
Pefloxacin (PEF)	10	- (R)	- (R)	- (R)	- (R)	- (R)	- (R)
Gentamycin (CN)	10	16 (I)	16 (I)	- (R)	- (R)	- (R)	19 (S)
Ampiclox (APX)	30	- (R)	- (R)	- (R)	- (R)	- (R)	12 (I)
Zinnacef (Z)	20	28 (S)	28 (S)	26 (S)	22 (S)	26 (S)	30 (S)
Amoxacillin (AM)	30	16 (S)	12 (I)	- (R)	- (R)	12 (I)	16 (S)
Rocephin (R)	25	- (R)	- (R)	- (R)	16 (S)	- (R)	- (R)

Note: Sensitive (S), Intermediate (I), Resistant (R)

studied to the clinically important antimicrobials, on the other hand, is beneficial as it minimizes the chances of disseminating resistance genes to pathogens both in the food matrix and/ or in the gastrointestinal tract. It could, thus, be concluded that these isolates are not reservoirs of transferable resistance genes for at least erythromycin and zinnacef, as all isolates were sensitive to these two antibiotics.

It is important to realize that bacteriocin-producing LAB have been part of our diet for as long as LAB-fermented food has existed. Most bacteriocinogenic LAB are actually isolated from food, and it is difficult to see how we can keep them away from our daily diet [21].

5. CONCLUSION

Traditional fermented products investigated provide an appropriate ecological habitat for bacteriocin-producing LAB, since the bacteriocin-producing LAB strains were found in a variety of fermented products indicating a high potential of their growth in different ecological complex environment. This study reveals six LAB isolates from traditional fermented products that were capable of producing bacteriocins which could have a potential for food applications as biopreservatives. Further purification, characterization, and technological application should be pursued, in order to propose potential uses as natural preservatives.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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