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Culture-Dependent and Culture-Independent Qualitative Analysis of Dairy Products for Bacteriocin Production by Lactic Acid Bacteria

[1]Mahboubeh Mirhosseini, [2]Iraj Nahvi, [2]Giti Emtiazi and [2]Manouchehr Tavasoli

[1]Microbiology Group, Department of Biology, University of Isfahan, Iran
[2]Department of Biology, University of Isfahan, Iran

Abstract: This comprises a module to screen foods for bacteriocin producing Lactic Acid Bacteria (LAB). Bacteriocin-producing LAB were isolated from dairy products. Subsequently, possible LAB colonies were picked from the medium and identified by means of PCR amplification of the 16srDNA genes and sequencing. LAB classified as *E. faecium*, was found most frequently. DNA was extracted by simple and rapid methods for PCR. Five pairs of specific primers (nisin, lacticin3147, enterocin A, enterocin P and enterocin As48) used for PCR to test for the presence or absence of various bacteriocin genes in the isolated LAB. In parallel, all products were also subjected to culture-independent analysis which involved a PCR step on total bacterial DNA extracted directly from the dairy products. The results show that out of 26 dairy samples only 6 strains from 79 isolated strains produced enterocin A bacteriocin. The results of PCR method show that all of 26 dairy samples only have enterocin A and other bacteriocins were absent. The Results obtained by PCR analysis were the same with culture dependent methods with this different that enterocin A was found in all dairy products. The PCR approach was found to have a much higher sensitivity for detection of bacteriocin producing strains in dairy products in a fast, reliable and reproducible manner.

Key words: Bacteriocin · Lactic acid bacteria · PCR

INTRODUCTION

Lactic acid bacteria (LAB) are widely used in food fermentation of dairy, meat, vegetable and bakery products. There are many kinds of fermented foods in world in which LAB can be found. LAB are known to produce a variety of antibacterial substances including bacteriocins that can inhibit the growth of undesirable bacteria such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus spp.* and *Clostridium spp*. Bacteriocins are produced by bacteria and comprise peptides or proteins that have antibacterial activity against other bacteria genetically related to the producer strain [1-4]. The bactericidal action appears to be membrane based, resulting in ion leakage and loss of proton motive force that ultimately lead to cell death. Depending on their structure, bacteriocins have been divided into three classes [3]. The lantibiotic bacteriocins in Class I are small (<5 kDa), heat-stable proteins that contain lanthionine formed by posttranslational side-chain modifications of a precursor peptide. One example of a bacteriocin in this class is nisin from *Lactococcus lactis* [4-5]. Class II contains small (<10 kDa), heat-stable bacteriocins that are formed directly, without posttranslational modification. Examples are pediocin from *Pediococcus pentosaceus* and *P. acidilactici* [1] and enterocin and plantaricin 423 from *Enterococcus facieum* [2,6,7] and *Lactobacillus plantarum* 423, respectively. Class III contains large bacteriocins (>30 kDa) that are heat-labile proteins. An example is helveticin J, produced by *Lc. helveticus* 481 [3]. Bacteriocins and especially those from LAB, have been considered for use as biological control agents in foods. They could provide healthier foods by allowing a reduction in the level of chemical additives or the physical treatments currently employed during food processing [2,3]. In this article, we screen milk and dairy products for the presence of LAB and a PCR to test the LAB for the presence of bacteriocins. The latter involves a simple DNA template isolation protocol and pairs of specific primers designed for detecting five bacteriocin genes, which encode lactocin, enterocin and nisin. The detection of bacteriocin genes in bacteria from dairy products has been selected because Iran has many traditional

Corresponding Author: M. Mirhosseini, Department of Biology, University of Isfahan, Hezar Jarib Street, Isfahan, Iran

fermented foods and because some bacteriocins (e.g. nisin and pediocin) are already used widely in the food industry. However, bacteriocin- producing bacteria can be found in other fermented foods such as cheeses, yogurts [1,3,5], so the module could be easily detected bacteriocin producing LAB in Iranian dairy products.

MATERIALS AND METHODS

Bacterial strains and media: Bacterial strains used in this study are as follow. *Listeria monocytogenes* PTCC19112, *Lactococcus lactis* ATCC19435 and ATCC11454 which were grown in tryptic soy broth (TSI) or Brain heart infusion (BHI, Pronadisa, Farmacopea, Spain) at 30°C. All LAB isolates and indicator strains were grown in MRS broth (Sharlua, Barcelona, Spain) at 30 or 37°C. All of experiments were performed in duplicate.

Isolation of Bacteriocin-producing Bacteria and Bacteriocin Assay: Bacteriocin-producing bacteria were isolated by the direct plating method. A 10% food sample in diluents (0.85% NaCl) was homogenized and 10-fold serially diluted. Pour plates of serial dilutions (1 mL aliquots) in media MRS were incubated under anaerobic conditions (anaerobic generation kit; Merck, Darmstadt, Germany) for 48 h at 30°C. Multiple plates of serial dilution (three to five plates providing a total of approximately 1000 colonies) were overlaid with a set of two indicators and incubated at 30°C for another 18–24 h. Two indicator strains used were *Lactococcus lactis*ATCC19435*, Listeria monocytogenes* [8,9,11,12]. Colonies suspected to be producing zones of growth inhibition in the indicator lawn were randomly selected and removed using a sterile Pasteur pipette. The agar plug (from Pasteur pipette) was inoculated into MRS broth media then incubated anaerobically for 48 h at 30°C. Culture supernatant was obtained by centrifugation at 12,000 rpm for 20 min at 12°C and then was adjusted to pH 6.5 with 5 M NaOH and filtered through a 0.45 μm filter. Inhibition was tested by spotting 10 μL of the supernatant onto soft agar lawn (0.6%) seeded with 0.1 mL of an overnight grown indicator strain and incubated overnight. Cultures producing an inhibitor in broth were then purified by streaking from the broth for single colony [5].

Identification of the Bacteriocin-producing Isolate: Only six isolate designated (a_9, a_{10}, a_{11}, a_{12}, a_{14}, a_{21}) of the 79 LAB isolated produced bacteriocin in broth media. Gram positive and catalase negative isolates were examined for cellular morphology by phase contrast microscopy on overnight cultures in MRS, grown after 7 day at 10°C after 2 day at 40°C and in the presence of 6.5% NaCl. Production of CO_2 from glucose was tested in MRS after 2 and 5 day at 30°C. Arginine hydrolysis was determined after 3 and 7 day at 30°C in MRS supplemented with L-arginine monochlorhydrate at 0.3% using Nessler reagent [2, 9, 10-12]. Growth in KF Streptococcus agar (Merck, Darmstadt, Germany) after 18 h at 45°C and in Bile aesculin agar (Oxoid, Hapshire, UK) supplemented with 20 gL^{-1} of bile-salt (Oxoid, Hapshire, UK) at 45ºC for 24 h were also examined. API 50 CHL strips (biomrieux, Lyon, France) were used for the characterization of some isolates [2]. Also genetic characterization of LAB was performed by a PCR method based on the detection of 16 S rRNA sequences using the set of primers RW01 and DG74 [7,13].

PCR Detection of Bacteriocin Structural Genes: PCR amplification of known structural genes of enterocin A, enterocin P and enterocin As 48 was performed with the specific primers listed in Table 1. The cycles used were 95°C for 5 min for the first cycle, 95°C for 30 sec, 58°C (for the primers of enterocin A) or 56°C (for the primers of the enterocins P and AS-48) for 30 sec and 72°C for 30 sec for the next 30 cycles; 72°C for 5 min were used after the last cycle [6]. PCR products were visualized on 2% agarose gel by ethidium bromide straining. *E. faecalis* PTCC1237 and *E. faecium* (enterocin A) strains were used as the negative and positive controls, respectively. There were no positive controls for enterocin P and AS48 [6].

DNA Extraction: The method used for extraction of total bacterial DNA was based on the method described by Masco *et al.* [14], with slight modifications. For dairy products, 1 mL of product was centrifuged for 10 min at 13,000 rpm in a centrifuge (Eppendorf, Hamburg, Germany); and the pellet was resuspended in 1 mL of Tris-EDTA (TE) buffer. All cell suspensions in TE buffer were centrifuged for 5 min at 13,000 rpm. In each case the supernatant was removed [14]. The cells were resuspended in 200 μL of Tris-EDTA (TE) buffer and then kept in a boiling water bath for 10 min. After cooling on ice for 5 min, the cells were removed by centrifugation [15]. In the case of DNA extraction from samples, was performed by phenol extraction methods. Finally, 8 μL of the DNA solution was mixed with 2 μL of loading dye (Fermentase, UE) and electrophoresis on a 1% (wt/vol) agarose gel in 1X TAE buffer for 30 min at 100 V to verify

Table 1: Specific primers for the PCR detection of bacteriocin structural genes

Bacteriocin	Forward primer 5′°°′-3′	Reverse primer 5′°°′-3′
Enterocin A	GGTACCACTCATAGTGGAAA	CCCTGGAATTGCTCCACCTAA
Enterocin P	GCTACG CGTTCATATGGTAAT	TCCTGCAATATTCTC TTT AGC
Enterocin AS48	GAGGAGTATCATGGTTAA AGA	ATATTG TTAAATTAC CAA
Nisin	AAGAATCTCTCATGAGT	CCATGTCTGAACTAACA
Lacticin3147	TACTGGGGAAATAACGG	TGGACAAGTATTGGTAC

the DNA extraction. The quality of the DNA samples was verified by spectrophotometric measurements at 260 and 280 nm [14].

PCR: PCR performed on these directly DNA extracted from dairy samples. The primers used were those described in Table 1. For control PCR amplification performed for bacterial 16S rDNA. -Forward primer DG74 and reverse primer RW01- [2, 6, 16]. The PCR was verified by mixing 8 µL of PCR product with 2 µL of loading dye and electrophoresing it on a 2% gel. For controls, inoculated milk by *E. feacalis, E. faecium* (enterocin A) and *L. lactis* (nisin) were used.

RESULTS

Culture-dependent Analysis of Milk and Dairy Products: A total of 26 samples were examined as sources for the isolation of LAB using *L. lactis* and *L. monocytogenes as* indicator strains. After the initial screening test, 39 colonies showing large inhibition zones on lawns of indicator organisms were selected. The production of bacteriocin-like inhibitory substances was confirmed in the neutralized supernatant of 39 isolates by spotting 10 µL of the supernatant onto soft agar lawn (0.6%) seeded with 0.1 mL of an overnight grown indicator strain and incubated overnight. Only 6 (a_9, $a_{10,}$ $a_{11,}$ a_{12}, a_{14}, a_{21}) isolates stably secreted inhibitory substance into culture broth. Phenotypic identification of the 6 selected isolates was carried out. These strains produced CO_2 and hydrolyzed arginine and grew in media containing NaCl at 6.5%, KF, Bile esculin agar media. Also these strainshave good growth on MRS broth at 10, 40°C. Based on these characteristics and the analysis of the carbohydrate fermentation pattern by the API CHL kit, these strains were potentially identified as *Enterococcus spp*. Furthermore, PCR identification by 16srDNA and sequencing suggested that *E. faecium* is predominant enterococcal species in dairy product. Enterocins A, P and AS48 were targeted by PCR using specific primers as already described by other authors [6]. The results obtained after gel electrophoresis are displayed in Fig. 1.

Fig. 1: PCR products of purified DNA from isolated strains amplified with primers for enterocin A, Lan 1& 4, *E. feacalis*; Lan2, 50bp ladder; Lan3, *E. faecium*; Lan 5, a_9; Lan6, $a_{10;}$ Lan7, a_{11}; Lan8, $a_{12;}$ Lan9, $a_{14;}$ Lan10 , a_{21}

As shown only the primers specific for enterocin A gave PCR products.

Culture-independent Analysis of Milk and Dairy Products: Total of 26 dairy samples were examined as sources for the extraction DNA. Then PCR analysis performed with 5 pair specific primers. Results of PCR method were showed that enterocin A only found in all of dairy samples. This result showed that enterocin A was predominate bacteriocin in all of dairy samples and other bacteriocins absent these dairy samples (Fig. 2).

DISCUSSION

LAB isolates for bacteriocin-producing LAB were obtained from a high number of raw milk samples and dairy products. After neutralization, bacteriocin activity was confirmed in 6 (7.59%) out of 79 isolates of LAB showing inhibitory activity in solid media, a higher percentage than those reported in previous study by Thuault, Beliard, Le Guern and Bourgeois [4]. Six selected

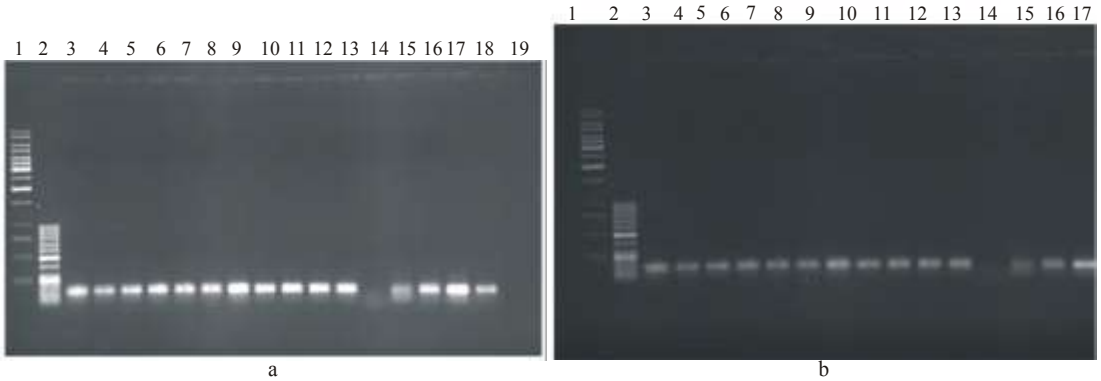

Fig. 2: PCR products from directly DNA extraction of dairy products amplified with primers for enterocinA, a: Lan1, 1kb ladder; Lan2, 50bp ladder; Lan3, *E. faecium*; Lan4, a_1; Lan5, a_2, Lan6, a_3; Lan7, a_4, Lan8, a_5, Lan9, a_6, Lan10, a_7; Lan11, a_8; Lan12, a_9, Lan13, a_{10}, Lan14 & 19, *E. faecalis*; Lan15, a_{11}, Lan16, a_{12}, Lan17, a_{13}, Lan18, a_{14}. b: Lan1, 1kb ladder; Lan2, 50bp ladder; Lan3, a_{14}; Lan4, a_{15}, Lan5, a_{16}; Lan6, a_{17}, Lan7, a_{18}, Lan8, a_{19}, Lan9, a_{20}; Lan10, a_{21}; Lan11, a_{22}, Lan13, a_{24}, Lan14, *E. faecalis*; Lan15, a_{25}, Lan16, a_{26}, Lan17, *E. faecium*.

bacteriocin-producing LAB from dairy products were identified at the spices level as *Enterococcus faecium*, this level found in raw milk and dairy products was much higher compare to other study [17]. Production of bacteriocins may constitute an ecological advantage allowing these to predominate in raw milk. PCR analysis using specific primers to enterocin P, enterocin A and enterocin AS-48 led to a fast identification of bacteriocins produced by whole selected isolates. This powerful tool to identify bacteriocins has been previously applied by [2,16,18]. In this study all 6 bacteriocin-producing enterococci strains produced only enterocin A according to PCR analysis. Results of directly DNA extraction of dairy product also were the same with dependent culture methods, but in all of dairy samples enterocin A gene were detected, while other bacteriocins genes were absent. These results showed that the PCR approach was found to have a much higher sensitivity for detection of bacteriocin producing strains in dairy products in a fast, reliable and reproducible manner. Our results confirm that enterocin production is a widely spread trait in raw milk and raw milk products, which this is differ from Martinez *et al*. [17], who detected the structural gene of nisin in 21 out of 23 bacteriocin-producing lactococci from homemade cheeses. Enterocin A is widely distributed among enterococci [18] and exhibits a strong antilisterial activity [18,19]. Our results confirm the high incidence of bacteriocin producing LAB in raw milk samples and dairy products, with inhibitory activity against both pathogenic and spoilage microorganisms. Also results were showed that enterocin-producing isolates were the most abundant in milk and dairy products. Bacteriocin-producing LAB isolated and characterized in the present work offering a useful protection against eventual contamination of milk or dairy products with pathogenic or spoilage microorganisms.

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