Molecular detection of bacteriocin producing Lactic acid bacteria from fermented milk in Alnajaf

الكشف الجزيئي عن بكتريا حامض اللاكتيك المنتجة للبكتريوسين المعزولة من الالبان في محافظة النجف

Dr. Nawfal Hussein Aldujaili
Department of Biology\ Faculty of Science\ University of Kufa
e-mail: nawfal_hm@yahoo.com

Abstract

Bacteriocins are ribosomal synthesized antimicrobial peptides produced by one bacterium that are active against other bacteria, either in the same species (narrow spectrum), or across genera (broad spectrum).

Thirty isolates of Lactic acid bacteria obtained from fermented milk and Yoghurt from various locations in Alnajaf were analyzed by PCR to rapid screening of pediocin, plantaricin and enterocin genes that present on the bacterial chromosome or on plasmids

Multiplex PCR method was used to detect the presence of bacteriocin gene by using specific primers to amplify a fragment from bacteriocin structural gene. Eighteen strains produced one of the PCR fragments with specific primers. Highest frequency of occurrence 11(61.1%) of isolates produced fragment of 428 bp indicate to presence of plantaricin gene, 5 (27.8%) produced fragment of 332 bp indicate to presence of pediocin gene whereas 2(11.1%) produced fragments of 412 bp indicate to presence of enterocin gene. The agar well diffusion methods was used to detect the selected strains with antibacterial activities against *P. aeruginosa* . The supernatant of the thirty LAB screened for bacteriocin production, eighteen (60%) were gave inhibition zones (12-20mm) onto the indicator pathogenic strain. Among LAB isolates, strain M5 plantaricin producer the most effective antibacterial compounds against *P. aeruginosa.* (20 mm as diameter of inhibition). The LAB isolates were: *Lactobacillus plantarum, Pediococcus and Enterococcus*. *L. plantarum* had the highest frequency of occurrence 11(61.1%), while, *Pediococcus and Enterococcus* had 5 (27.8%) and 2(11.1%) respectively.

Key words: lactic acid bacteria ,bacteriocins, Molecular detection

الخلاصة :

البكتريوسين هي ببتيدات رايبوسومية الصنع لها فعالية ضد الاحياء المجهرية تنتج من قبل بكتريا ولها تاثير فعال ضد بكتريا اخرى من نفس النوع (فعالية محددة) او ضد اجناس اخرى (فعالية واسعة) .

تم الحصول على ثلاثين عزلة من بكتريا حامض اللاكتيك من اللبن المتخمر والمعلب من مناطق متعددة في محافظة النجف .اجريت غربلة سريعة لمعرفة قابلية العزلات على انتاج البكتريوسين pediocin و plantaricin و enterocin من خلال الكشف عن جينات هذه البكتريوسنات باستعمال تقنية تفاعل البلمرة المتسلسل مع بادئات خاصة بها .اظهرت النتائج ان ثمانية عشر فقط من العزلات المدروسة اظهرت احدى الحزم الخاصة .اذ كانت 11 (61.1%) عزلة اظهرت حزمة بطول 428 زوج قاعدي والتي تشير الى وجود جين ال plantaricin وهي النسبة الاعلى .و 5(27.8 %) منها اظهرت حزم بطول 332 زوج قاعدي والتي تشير الى وجود جين ال pediocin . فيما كان 2 (11.1 %) منها اظهرت حزمة بطول 412 زوج قاعدي مما تشير الى وجود جين ال enterocin .كما استعملت طريقة الانتشار بحفر الاكار لتاكيد فعالية البكتريوسينات المنتجة من بكتريا حامض اللاكتيك ضد بكتريا ممرضة *Pseudomonas aeruginosa*

Introduction

The Lactic acid bacteria can produce antimicrobial substances able to inhibit the growth of pathogenic and spoilage microorganisms in foods. The primary antimicrobial effect exhibited by LAB is the production of lactic acid and reduction of pH (1) .In addition, LAB can produce various antimicrobial compounds which can be classified as low-molecular-carbon mass compounds such

hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl (2,3-butanedione), uncharacterized compounds and high-molecular-mass compounds like bacteriocins (2,3). Bacteriocins are ribosomal synthesized antimicrobial peptides produced by one bacterium that are active against other bacteria, either in the same species (narrow spectrum), or across genera (broad spectrum) and, as with host defense peptides, cell signaling mechanism can also be involved (4).

The bacteriocins of LAB are commonly classified into three classes :class I-the lantibiotics ,class II-the heat stable unmodified bacteriocins and class III the larger heat labile bacteriocins . Class II can subdivide into class IIa, class IIb and class IIc. class IIa bacteriocins are small (less than 10kD),heat-stable , unmodified peptides of 37-48 amino acids (5,6).It can inhibit growth of some food spoilage and pathogenic bacteria such as *Listeria monocytogenes*, *Bacillus cereus*,*Clostridium perfringenes*,*Staphylococcus aureus* and *E.coli* (7). It is the most investigated with respect to production,structure-function relationship, and have been considered as one of the most interesting and potential groups of antimicrobial peptides for use in food preservatives (8).

The location of bacteriocins genes fear to be chromosomal (9) or plasmid. Genes involved in the production of bacteriocins are generally organized as operon (10).

The objective of this study was to using molecular techniques (PCR) for rapid screening of lactic acid bacteria (isolated from fermented milk and Yoghurt from various locations in Alnajaf) producing class IIa bacteriocins ,as well as agar well diffusion assay to study the inhibitory activity.

Materials and Methods

Isolation of Lactic Acid Bacteria

The lactic acid bacteria isolates were obtained from fermented milk and Yoghurt from various locations in Alnajaf.

Ten grams of each sample were aseptically added into 90 ml of sterile 0.9% NaCl solution. Homogenized and serially diluted, 1 ml of the diluents was pour-plated on de Man, Rogosa and Sharpe (MRS) agar. Plates were incubated for 24 hrs at 30 °C under anaerobic condition (anaerobic generating Kit, Merck). Total of 30 representative colonies were randomly picked and sub-cultured to obtained pure culture. The isolates were maintained on MRS agar plates kept at 4°C. The stock cultures were stored at -20°C in MRS broth supplemented with 15% of glycerol for subsequent use.

The bacteria were characterized by microscopic morphological examination and by conventional biochemical and physiological tests. Gram staining, catalase activity, gas production from glucose, growth in NaCl (2-6.5%), growth at different temperature (10-45°C), and production of amino acid from arginine and the identification work were done (11,12).

Molecular screening of LAB producing bacteriocins by PCR: Thirty strains of LAB were analyzed by PCR to rapid screening of LAB producing bacteriocins genes that present on the bacterial chromosome or on plasmids. Total DNA was extracted from culture broth, 1.5 ml of culture broth pipetted into Eppendorf tubes then centrifuged at 4.300 x g for 5 min and the supernatant discarded; 200 ul of TE buffer was added, vortexes well, boiled for 10 min, and then put on ice immediately for 1 min; this was centrifuged again at 6,700 xg for 10 min and the supernatant was collected, which contains DNA for use as DNA template (13).

PCR amplifications: Supernatant (9.5 ul) was used as a template and 0.5 ul (10uM) of each specific primers (table1) were added to 12.5 ul of PCR mixture (2X KAPA2G Fast Multiplex Mix). The PCR mixture contains KAPA2G Fast HotStart DNA Polymerase (1 U per 25 µl reaction), KAPA2G Buffer A (1.5X at 1X), dNTPs (0.2 mM each dNTP at 1X), $MgCl_2$ (3.0 mM at 1X) and stabilizers. The amplification profile was initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing 51°C for 45 s, extension 72°C for 45 sec and final extension 72°C for 5 min,.

The presence of PCR products was determined by gel electrophoresis in 1.0 % agarose gel containing ethidium bromide. Electrophoresis in 1XTris-borate-EDTA was performed at 100 volts, and photographed under an Ultraviolet transilluminator.

Table (1) Primers sequences used for PCR amplification

Target gene	Primer sequence	Product size (bp)	Reference
Pediocin	F 5'-GGTAAGGCTACCACTTGCAT-3' R 5'-CTACTAACGCTTGGCTGGCA-3'	332	(13)
Enterocin	F 5'-GGGTACCACTCATAGTGGAA-3' R 5'-CCAGCAGTTCTTCCAATTTCA-3'	412	(13)
Plantaricin	F 5'-GGCATAGTTAAAATTCCCCCC-3' R 5'-CAGGTTGCCGCAAAAAAAG-3'	428	(14)

Screening for antibacterial activity. The agar well diffusion assay was used to determine antibacterial activities against *P. aeruginosa* . Single colony from MRS agar plate was inoculated in 5ml MRS broth and incubated at 30°C for 18 -24 hr under anaerobic condition.Cell-free supernatant was obtained by centrifugation at 10.000 xg for 10 min with adjustment of pH to 6 (2N NaOH) to eliminate the inhibitory effect of organic acids.

Inhibitory activity was detected by the agar-well diffusion method (15), with some modifications. Portions (100 µl) of cell free supernatant were added to wells (5mm) cut into the plate, which was inoculated with *P. aeruginosa (*as the indicator strain) and the plate was incubated for 18 hour at 37°C. The isolates that showed clear inhibition zones were purified by streaked from the broth and restreaked for single colony.

Results and Discussion

Molecular screening of bacteriocins genes by PCR: Thirty strains of LAB had were analyzed by PCR to rapid screening of LAB producing bacteriocins genes that present on the bacterial chromosome or on plasmids. The results of the specific PCR reactions are shown in figure 1.Eighteen (60%) strains produced one of the PCR fragments with specific primers. Fragment of 332 bp indicate to presence of pediocin , fragment of 428 bp indicate to presence of plantaricin, whereas fragment of 412 bp indicate to presence of enterocin. This techniques able to easily determine whether or not classIIa bacteriocin genes were present in lactic acid bacteria as well as this technique was proved to be useful for the rapid screening of classIIa bacteriocin producing bacteria and can be an alternative method to direct detection of bacteriocin production without sub culturing and more specific DNA extraction, especially when multiple species present in natural samples such as milk, faeces and fermented food (14).

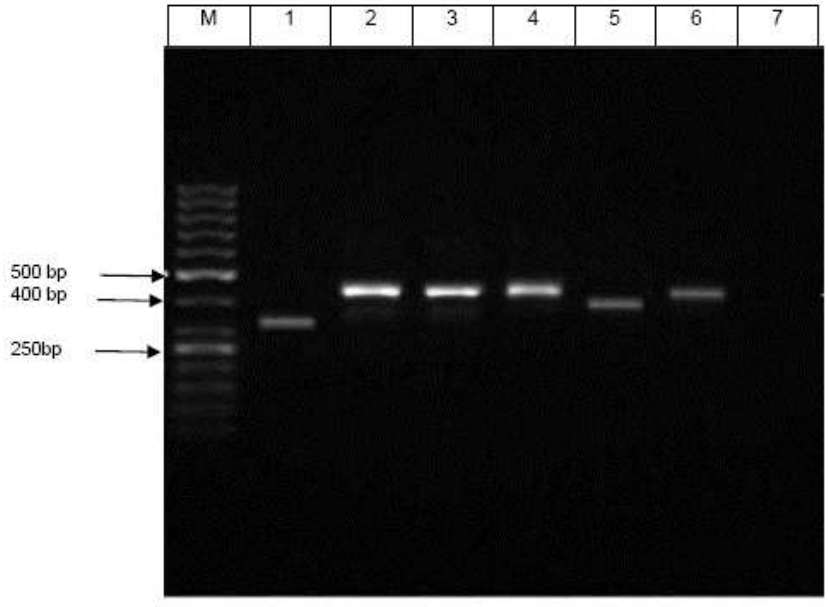

Figure (1) Agarose gel electrophoresis of PCR products from amplification of plantaricin (lane 2,3,4,6) ,pediocin (lane 1), enterocin (lane 5) genes,Lane 7 no PCR products detected. Lane M, DNA ladder; lanes 1–7 amplicons from DNA isolated from selected LAB colonies.

Antibacterial activities of selected lactic acid bacteria The agar well diffusion assay (AWDA) was used to detect strains with antibacterial activities against *P. aeruginosa* . The supernatant of strains of lactic acid bacteria gave inhibition zones (12-20mm) onto the indicator pathogenic strain (*P. aeruginosa*) tested (figure 2 and table 2). Of the thirty LAB screened for bacteriocin production, eighteen (60%) were potential bacteriocin producers. Among LAB isolates, strain M5 plantaricin producer the most effective antibacterial compounds against *P. aeruginosa. (*20 mm as diameter of inhibition). It was reported that LAB species can produce antimicrobial substances which can be assimilated to bacteriocins (16).

Figure (2) Inhibition zones around cell free supernatants of different tested isolates detected by using the agar well diffusion method, *Pseudomonas aeruginosa* was used as the indicator strain.

Among LAB species, the strain S5 produced most active inhibitory substance (18 mm of diameter of inhibition) (Fig 1). It has also been reported (17) that species can produce active antimicrobial substances like bacteriocins. The spectrum activity varies within the same genus. The difference observed in the spectra of activity of bacteriocins produced by the lactic acid bacteria strains could be due to the structural diversity of bacteriocins synthesized. In fact, the four classes of bacteriocins (18) known to date have different structures and different modes of action. The mode of action of bacteriocins is their "macroscopic" effect in a population of target bacteria. It is generally accepted that those bacteriocins produced by lactic acid bacteria act on target cells in two stages: adsorption of bacteriocin on the cell surface followed by a lethal effect (19). Physiological state of the target bacteria plays a role in the mode of action of a bacteriocin (20)**.** The combined effect of different bacteriocins can increase activity and spectrum of action, especially by combining bacteriocins belonging to different classes. The mechanism of action of the bactériocins breaks up into three stages: the first stage consists of the fixing of peptide on the membrane of the target cell. During this stage, peptide adopts a three- dimensional conformation enabling to express its activity. The second stage consists with the insertion of the bactériocin in the cytoplasmic membrane. During this stage, several antibacterial peptides are recruited to form a pore (18, 20). These results indicate that the lactic acid bacteria strains screened in the study are capable of synthesizing antimicrobial substances active on *P. aeruginosa.* Thus those antimicrobial substances produced by our lactic acid bacteria strains comprise a heterogeneous group of physicochemical diverse ribosomally – sythesesized peptides or proteins showing a narrow or broad antimicrobial activity spectrum against Gram-positive bacteria.

Further investigations of these strains to study the spectrum inhibitory activity against other pathogenic microorganisms to explore their potential as preservative in food industry.

Table (2): Antimicrobial producing isolates isolated from various samples.

Types of samples	Total isolates	Number of isolates that produced clear zone
Yoghurt	15	6
Fermented milk	15	12
Total	30	18

Biochemical, morphological and physiological characteristics of isolated strains

Thirty lactic acid bacteria were obtained from fermented milk and Yoghurt. The isolates were initially differentiated on the basis of their cultural and morphological studies after which they were subjected to various physiological and biochemical tests.The cell studies revealed medium short rods to relatively long rods. The isolates were Gram positive, non-sporing, non-motile, Catalase, Oxidase, methyl red, voges-proskauer and indole negative. They cannot produce H_2S gas and cannot hydrolyse starch. Fermentation tests reveal the isolates possessing the ability to ferment almost all sugars.

The LAB isolates were: *Lactobacillus plantarum*, *Pediococcus and Enterococcus*. *L. plantarum* had the highest frequency of occurrence 11(61.1%), while, *Pediococcus and Enterococcus* had 5 (27.8%) and 2(11.1%) respectively, this has being reported by various workers (21,22). The lactic acid bacteria constitute an important group of organisms, particularly in the food processing industry. Generally, the cultural and biochemical properties of the isolates agreed and confirmed with Bergey's Manual of systematic bacteriology (Sneath *et al*. 1986).

References

1. Daeschel, MA (1989). Antimicrobial substances from lactic acid bacteria for use as food preservatives. *Food Technol*. 43(1): 164-167.
2. Klaenhammer TR (1988). Bacteriocins of lactic acid bacteria. *Biochimie.* 70: 337 – 349.
3. Khay E,Idamar M,Castro LMP et al(2011). Antimicrobial activities of the bacteriocin –like substances produced by lactic acid bacteria isolated from Moroccan dromedary milk.*Afr J Biotechnol*.10(51):10447-10455.
4. Cotter PD,Hill C and Ross Rp(2005) .Bacteriocins :developing innate immunity for food. *Nat Rev Microbiol* .3:777-788.
5. Cleveland, J.; Montville, TJ.; Nes ,IF.; and Chikindas, ML.(2001). Bacteriocins: safe, natural antimicrobials for food preservation. *Int J Food Microbiol*. 71: 1-20.
6. Deegan,L.H;Cotter,P.D.;Hilla,C..and Ross, P(2006). Bacteriocins:Biological tools for bio-preservation and shelf-life extention .*International Dairy Journal*,16,1058-1071.
7. Rodriguez, E.; Calzada, J.,Arques, J.; Rodriguez, J.M.; Nunez, M.; and Medina, M. (2005). Antimicrobial activity of pediocin-producing *Lactococcus lactis* on *Listeria monocytogenes* .*S.aureus* and *E.coli* O157:H7 in cheese. *International Dairy Journal*.15,51-57.
8. Calo-Mata,P., Arlindo,S., Boehme,K., Miguel,T., Pascoal, A and Barros-Velazquez,J.(2008).Current applications and future trends of lactic acid bacteria and their bacteriocins for biopresevation of aquatic food products. *Food Bioprocess Technology*. 1,43-63.
9. Cintas, LM.; Casaus, P.; Herranz, C.; Nes, IF.;and Hernandez PE. (2001).Bacteriocins of Lactic Acid Bacteria.*Food SCi Tech Int*. 7:281-305.
10. Marrugg JD, Gonzales CF , Kunka BS et al . (1992) . Cloning ,expression , and nucleotide sequence genes involved in production of pediocin PA-1 and bacteriocin from Pediococcus acidilactica PAC1.0 . *Appl Environ Microbiol*. 58(8): 2360-2367.
11. Sneath, P. H. A., Mair, N. S., Sharpe, M. E. & Holt, J. G. (editors) (1986). Bergey's Manual of Systematic Bacteriology, vol. 2. Baltimore: Williams & Wilkins
12. Atlas, M.; Parks, C.; and Brown, A. (1995).Laboratory Manual of Experimental Microbiology.Mosby-Year-book, Inc., USA.

13. Suwanjinda,D, Eames ,C, and Panbangred W.(2007). Screening of Lactic Acid Bacteria for Bacteriocins by Microbiological and PCR Methods. *Biochemistry and molecular biology education*. 5, 364–369.
14. Yi,H.,Zhang,L ,Tuo,Y, Han,X and Du,M .(2010) .A novel method for rapid detection of class IIa bacteriocin –producing lactic acid bacteria .*Food control*. 21.426-430.
15. Tagg,J.R.and McCiven,A.R.(1971).Assay system for bacteriocins.*Appl.Microbiol*.21: 943- 948.
16. Lade, H. Chitanand M, Gyananath G et al. (2009). Studies on Some Properties of Bacteriocins produced B Lactobacillus species isolated from Agro-Based Waste. *The nternet Journal of Microbiology* .2:1.
17. Tuncer Y and Ozden B (2010). Partial biochemical characterization of nisin-like bacteriocin produced by *Lactococcus lactis subsp. lactis* YBD11 isolated from Boza, a traditional fermented Turkish beverage. *Rom Biotechnol Lett*. 15(1): 4940 – 4948.
18. Klaenhammer, TR. (1993) Genetics of bacteriocins produced by lactic acid bacteria *FEMS.Microbiolol Rev*. 12 (1-3) .39-85.
19. Bhunia AK, Johnson MC, Ray B *et al*. (1991). Mode of action of pediocin AcH from Pediococcus *acidilactici* H on sensitive bacterial strains. *J Appl Bacteriol*. 70: 25 – 33 33.
20. Martinez-Cuesta MC, Palaez C, Juarez M et al. (1997). Autolysis of *Lactococcus lactis ssp. lactis* and *Lactobacillus casei ssp. casei*. Cell lysis induced by a crude bacteriocin. *Int J Food Microbiol*. 38(2 – 3):125 – 131.
21. Steinkraus,K.H.,(1983).Handbook of Indigenous Fermented Foods.New York,USA:Marcel Dekker Inc.
22. Adebayo-tayo, BC. and Onilude, AA (2008). Screening of Lactic Acid Bacteria Strains Isolated from Some Nigerian Fermented Foods for EPS Production. *World Applied Sciences Journal* 4 (5): 741-747.