Basrah Journal of Veterinary Research,Vol.15, No.3,2016
Proceeding of 5th International Scientific Conference,College of Veterinary Medicine
University of Basrah,Iraq

PHENOTYPIC AND GENOTYPIC IDENTIFICATION OF BACTERIOCIN PRODUCER LACTIC ACID BACTERIA ISOLATED FROM COWS MILK

Hibbat Al Rahman, R.* , Fawziah A. Abdullah*

*Department of Microbiology, Collage of Veterinary Medicine, University of Basrah, Basrah,Iraq.

Keywords; Cowraw milk , bacteriocinogenic LAB

ABSTRACT

The Phenotypic identification results revealed that Lactic Acid Bacteria (LAB)isolates which is characterized by both Gram positive and catalase negative reactions was observed in 51 % of cow raw milk bacterial isolates with 51% an overall ratio.The higher results(**58.5**%.) of conventional bacteriological analysis were observed in raw milk bacterial isolates of cows at first age group(**≥3 - < 9** year) , There was significant effect (p< 0.05)for the cows age on the raw milk bacterial isolates distribution .The number of parturition affect significantly (p< 0.05) on the raw milk bacterial isolates distribution and high ratio(**52%)**of LAB isolates was observed in cows with ≥6-<12 number of parturition

Depending on genotypic identification results the high ratio (100%) of positive results of 16S rDNA based PCR appeared in all of raw milk bacterial isolates of cows at second age group(≥9 - <15 year) , and the difference between the two age groups stastically was not considered significant (P>0.05)**.**.The number of parturition had high significant effect (p< 0.001) on16S rDNA based PCR positive results and high ratio**(50%)** of these results was observed in cows with 1 - < 6 number of parturition**.**Subsequent bacteriocin encoding genes based PCR analysis of 16S rDNA genes based PCR positive LAB revealed that higher ratio of PCR positive results (**60.9%**) was observed in *Ent B* followed by *Nis* encoding genes(30.4%) . High significant difference(P<0.01) was observed among the *Nis, Ent A* and *Ent B* encoding genes based PCR positive results**.**

INTRODUCTION

Cow's milk has been a staple diet ever since the medical community publicized its nutritional benefits in the 1920s (1). The microflora of raw milk often contains lactic acid bacteria (LAB) (2). Food safety is one of the major concerns in public health due to outbreaks of food-borne diseases (3).LAB produce various antimicrobial compounds, which can be classified as low molecular mass compounds such as hydrogen peroxide, carbon dioxide, diacetyl, uncharacterized compounds, and high molecular mass compounds like bacteriocins (4).

Most of LAB bacteriocins are small thermostable or large thermolabile proteins or protein complexes that display antimicrobial properties against other bacteria often closely related gram positive bacteria, whereas producer cells are immune to their own bacteriocin(s) (5). During the last decade, a great number of LAB bacteriocins have been identified and their potential application as biopreservatives in foods or food products has been explored (6).

LAB displaying antimicrobial activities could be used as natural biopreservatives to prevent or inhibit the growth of pathogenic and spoilage bacteria and fungi. LAB also preserves the nutritive qualities of various foods (7). This century has been a major effect in describing, cataloging, and characterizing the wide variety of antagonistic compounds produced by LAB (8). The preservative effect of LAB is due to the production of one or more active metabolites, such as bacteriocins (nisin, reuterin, reutericyclin, pediocin, lacticin, enterocin and others) and bacteriocin-like inhibitory substances-BLIS (9)

Although bacteriocins, in a sense, can be considered as antibiotics, they differ from conventional antibiotics in numerous aspects (10). Bacteriocins are inherently tolerant to higher thermal stress and are more active at a wider pH range than conventional antibiotics. Development of resistant strains among their target bacteria is unlikely as they have fast-acting antimicrobial mechanisms that are highly potent even at very low concentrations. Furthermore, their proteinaceous nature minimizes resistance development as they are easily degraded by proteolytic enzymes, thus lessening the chances of target strains developing any resistance machinery. (10).This study aimed to identify the lactic acid bacteria that compose the microbiota of raw cow milk and their

bacteriocinogenic potential.And determine specific genes related to their bacteriocins production.

MATERIALS AND METHODS

Samples collection and bacterial isolation

All studied samples were collected through period extended from November 2015 to January 2016, including different animals farms in Basrah province .One hundred raw cow's milk samples were collected randomly from 100 healthy cows, All sample were placed in to sterilized test tubes and transported on ice in cooler boxer to the laboratory for subsequent analysis.One ml of milk transferred to 9 ml of MRS broth, Then 0.1 ml was streaked on the surface of MRS agar. The MRS agar culture plates were incubated at 37 °C for 2 days under anaerobic condition (11).

Identification of LA

Phenotypic and genotypic identification of LAB from other bacteria. was done according to the (11) .

Phenotypic identification

Phenotypic identification depend on Gram stain and catalase test

Genotypic identification

Genotypic identification was performed by PCR amplification of LAB16s rRNA and bacteriocins encoding gens using specific primers(Table 1).The DNA of raw milk bacterial isolates was extracted by using Wizard genomic DNA extraction and purification kit(Qiagen)according to the manufacturer'sinstructions.

Amplification genes encoded for LAB and its bacteriocin production and their PCR prereaction mix were displayed in table (2).The PCR tubes were transferred to the thermalcycler(Techne/UK) tostart the amplification reaction according to specific program(Tables 3,4,5,6) for each gene (11).

Table (1): Primer sequence used in PCR detection of bacteriocinogenic LAB

Primer set	Oligonucleotide sequence	Predicted size	References
16s rRNA-F 16s rRNA-R	GCGGCGTGCCTAATACATGC ATCTACGCATTTCACCGCTAC	700 bp	Klijn *et al*., (12)
Nis-F Nis-R	GGATAGTATCCATGTCTG CAATGATTTCGTTCGAAG	250 bp	Perin and Nero(11)
enti A –F enti A-R	CATCATCCATAACTATATTTG AAATATTATGGAAATGGAGTGTAT	126 bp	Toit *et al*., (13)
entB B–F entB B–R	AAATATTATGGAAATGGAGTGTAT GAAAATGATCACAGAATGCCTA	162 bp	Toit *et al*., (13)

Table(2) The prereaction mix (25 µl) for each16s rRNA, *Nisin, enti A, enti B*.

Material	Size
DNA template	5 µl
Master mix	12.5 µl
Primer forward	1 µl
Primer reverse	1 µl
Nuclease free water	5.5 µl

Table (3) : PCR condition for 16s rRNA .

Stage	Setps	Temperature	Time	No. of cycles
First	Denaturation 1	95C°	5 minuts	1
Second	Denaturation 2	95C°	1 minut	30
	Annealing	42	1 minut	
	Extension 1	72C°	1 minut	
Third	Extension 2	72C°	10 min.	1

Table(4) : PCR condition for *Nisin*.

Stage	Setps	Temperature	Time	No. of cycles
First	Denaturation 1	95C°	5 minuts	1
Second	Denaturation2	95C°	1 minut	30
	Annealing	55 C°	1 minut	
	Extension 1	72C°	1 minut	
Third	Extension 2	72C°	10 min.	1

Table (5) : PCR condition for *ent A* .

Stage	Setps	Temperature	Time	No. of cycles
First	Denaturation 1	95C°	5 minuts	1
Second	Denaturation 2	95C°	1 minut	30
	Annealing	56 C°	1 minut	
	Extension 1	72C°	1 minut	
Third	Extension 2	72C°	10 min.	1

Table(6) : PCRcondition for *ent B* .

Stage	Setps	Temperature	Time	No. of cycles
First	Denaturation 1	95C°	5 minuts	1
Second	Denaturation 2	95C°	1 minut	30
	Annealing	58 C°	1 minut	
	Extension 1	72C°	1 minut	
Third	Extension 2	72C°	10 min.	1

PCR result detection

The results of the PCR were performed in post amplification from amplification samples was loaded in a 1.5 % agarose gel containing 0.5 μl /25ml ethidium bromide the gel was run at 70 V. the products were visualized by UV transillumination .

Statistical analysis

To demonstrate any association between results, the exact Fisher test and Pearson'schi-squaredtestwith Yates correction were used with the limit of significance being set at 5%. Statistical analysis is done by using SPSS software version 11.

RESULTS

Phenotypic identification results

The LAB isolates which is characterizedby both Gram positive and catalase negative reactions was observed in 51 % of cowsraw milk bacterial isolates with 51% an overall ratio.The higher results of conventional bacteriological analysis were observed in raw milk bacterial isolates of cows at first age group(**≥3 - < 9** year) , particularly Gram positive catalase negative LAB isolates which were appeare in a ratio of **58.5**%.There was significant effect ($p< 0.05$)for the cows age on the raw milk bacterial isolates distribution .The number of parturition affect significantly ($p< 0.05$) on the raw milk bacterial isolates distribution and high ratio(**52%)**of LAB isolates was observed in cows with ≥6-<12 number of parturition(table7)

.

Table(7). Distribution of LAB isolates in cows raw milk according to conventional bacteriological analysis.

Variables		Conventional bacteriological analysis				
		Tested isolates	Gram Positive, cocci N(%)	Catalase negative	Gram positive catalase nagative	Statisticall analysis
Age groups (year)	≥3 - < 9	70	53(75.7)	47(67.1)	41(58.5)	X^2 18.437;DF: 5;P; 0.0024
	≥9 - <15	30	18(60)	13(43.3)	10(33.3)	
Total		100	71	60	51	
Parturition number	1 - < 6	75	55(73.3)	40(53.3)	38(50.7)	X^2:12.29;DF:5;P; 0.0309
	≥6-<12	25	16(64)	20(80)	13(52)	
Total		100	71	60	51	

Genotypic identification results

Amplification of 16S rDNA Region:

After DNA isolation the 16S rDNA region was amplified by PCR protocole. Then the PCR products were visulized by agarosegel electrophoresis under UV light. The length of amplification products was700 bp (Figure 1).

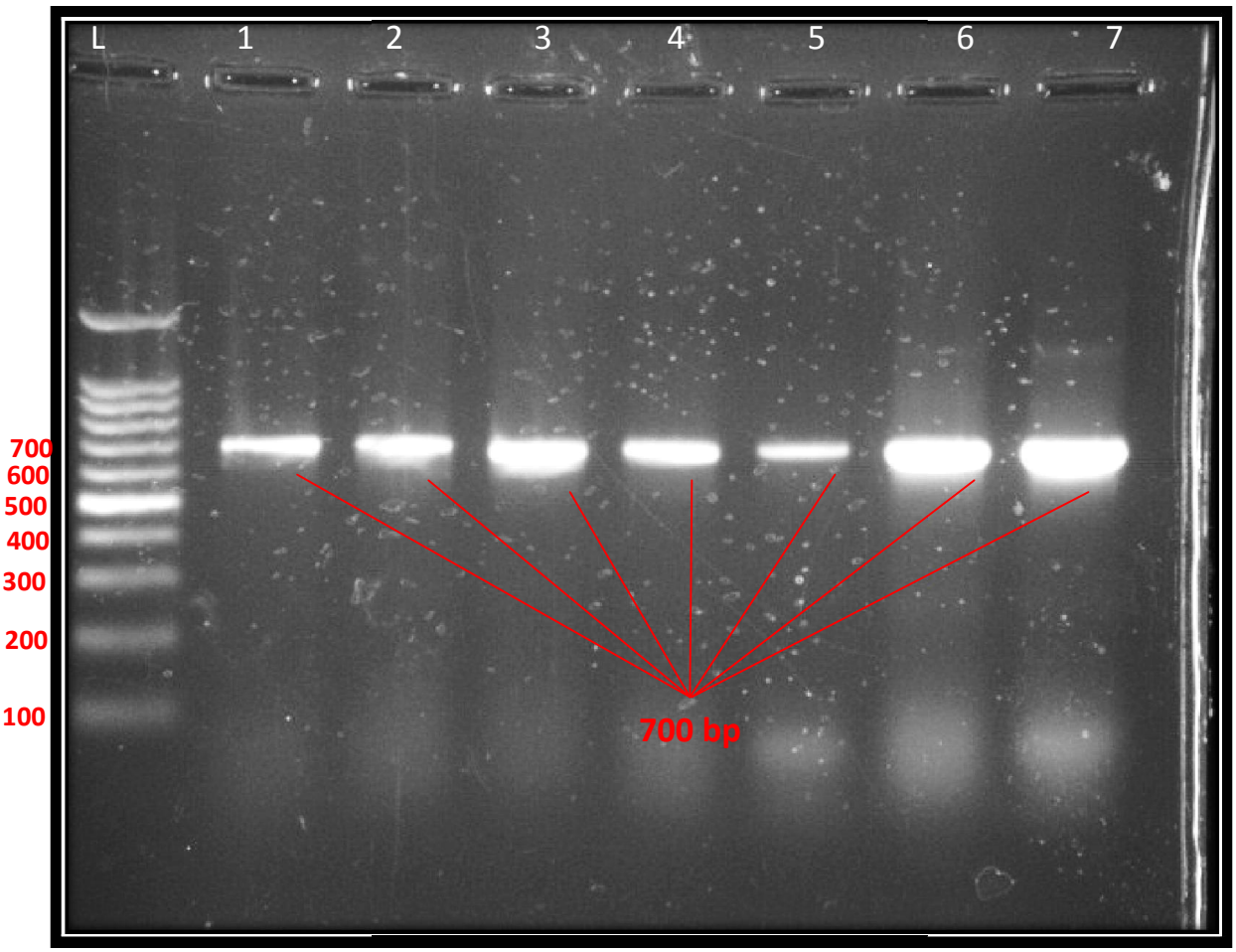

Figure (1). 16S Amplification products of cows raw milk LAB isolates lane (1) is 100 bp DNA marker, lane (2 -8) are positive LAB Isolates(700 bp).

Distribution of16S rDNA based PCR positive results

The high frequency(100%) of positive results of 16S rDNA based PCR appered in all of raw milk bacterial isolates of cows at second age group(≥9 - <15 year) , and the difference between the two age groups stastically was not considered significant (P>0.05)..The number of parturition had extremily significant effect (p< 0.001) on16S rDNA based PCR positive results of the raw milk LAB and high ratio(**50%)** was observed in cows with 1 - < 6 number of parturition(table 8).

Table (8)**: Distribution of 16S rDNA basedPCR positive results according to age and Parturition of cows:**

Variables		16S rDNA genes based PCR n.(%)			
		LAB Isolates n.	**Positive 16s rRNA**	**Negative 16s rRNA**	**Statisticall analysis**
Age groups (year)	**>3 - < 9**	**41**	**13(31.7)**	**28(68.3)**	X^2:0.348; df:1; P:0.55
	≥9 - <15	**10**	**10(100)**	**0**	
Total		**51**	**23(45.1)**	**28(54.9)**	
Parturition number	**1 - < 6**	**38**	**19(50)**	**19(50)**	X^2:17.043; df:1;P:0.00003654
	≥ 6-<12	**13**	**4(30.8)**	**9(69.2)**	
Total		**51**	**23(45.1)**	**28(54.9)**	

Bacteriocinen coding genes:

Subsequent bacteriocin encoding genes based PCR analysis of 16S rDNA genes positive LAB revealed that higher ratio of PCR positivity(**60.9%**) was observed in Ent Bfollowed byNis encoding genes(30.4%) .High significant difference(P<0.01) was observed among the Nis, Ent A and Ent B encoding genes based PCR positive results .Table 9 and figures 2,3,4 present the results for bacteriocin encoding genes in the LAB isolates of cows.

Table (9): Bacteriocin encoding genes based PCR results in LAB isolates of cows raw milk .

Bacteriocin encoding genes based PCR analysis	**16SrDNA based PCR positive LAB isolates**		
	Examined n.(%)	**Positive n.**(%)	Negative n.(%)
Nis	**23**	**7(30.4)**	**16(69.6)**
Ent A	**23**	**2(8.7)**	**21(91.3)**
Ent B	**23**	**14(60.9)**	**9(39.1)**
Test of significance	X^2:11.886;DF:2;P; 0.0026		

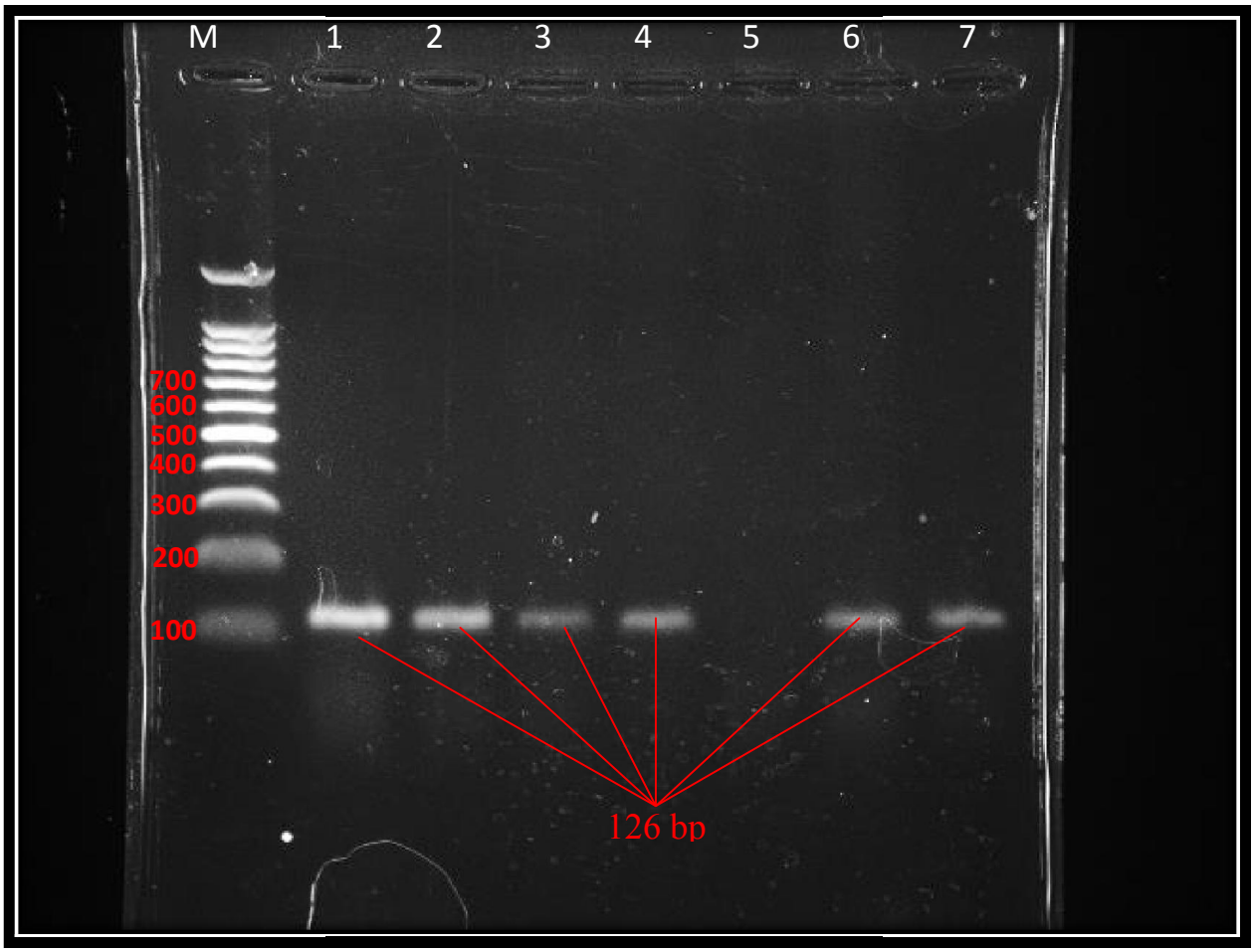

Figure 2. Enti A amplification products of cows raw milk LAB Isolates

Lane (1) is 100 bp DNA marker, lane (2 -5,7,8) are positive *enti A*(126 bp)

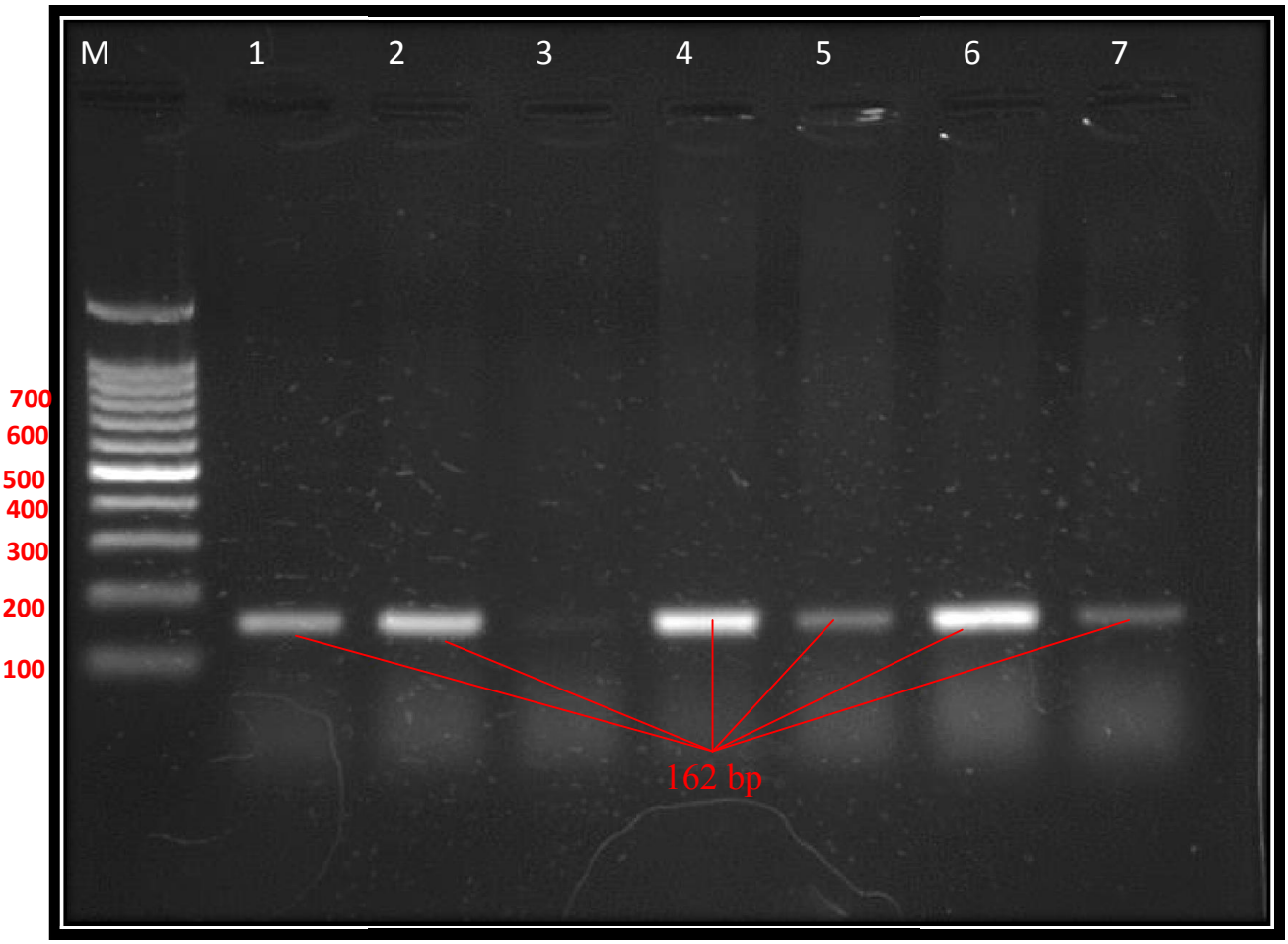

Figure 3. Enti B amplification Products of cows raw milk LAB Isolates lane (1) is 100 bp DNA marker, lane (2,3,5-8) are positive *Enti B*(162 bp)

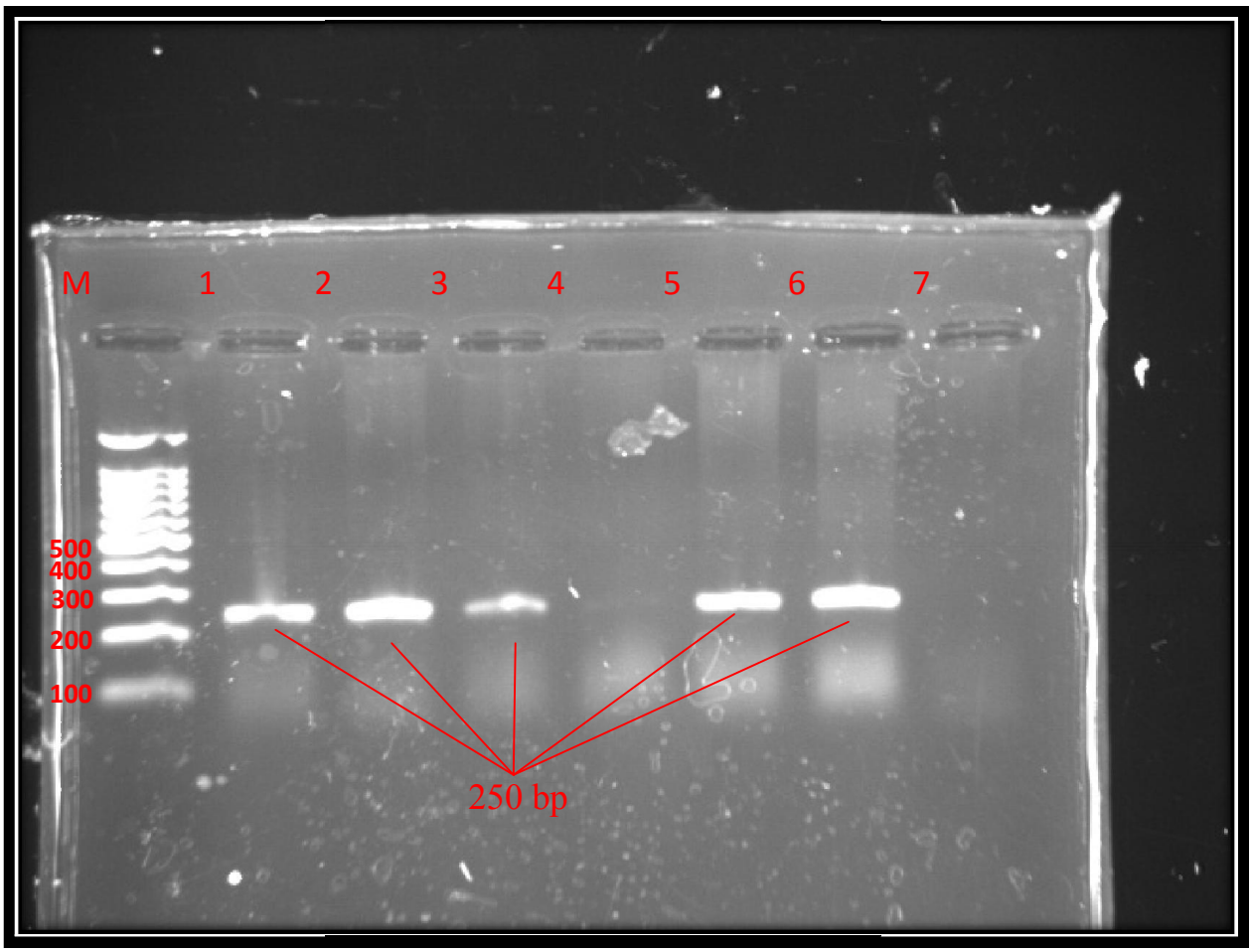

Figure 4. Nis amplification products of cows raw milk LAB Isolates Lane (1) is 100 bp DNA marker, lane (2,3, 4,7,8) are positive *Nis* (250 bp)

DISCUSSION

Modern applications of LABs have a long history in developed countries. In the past two decades, importance of these bacteria in industry and health improvement has encouraged other countries to make serious efforts to isolate and identify their local LABs, and optimize them for industrial applications..(14) in Malaysia ,(15) in Egypt and many other scientists around the world have been recently working on LABs. In the present study, the first important goal was to achieve a primary identification of local LABs present in raw milk of local cows and evaluate their bacteriocinogenic potentials.

The results of isolation of cow raw milk using MRS medium had showed that out of 100 bacterial isolates. 51 isolates. were considered as LAB characterized by Gram , negative positive catalase, and able to live in anaerobic condition., Abdelgadir *et al.,* (16); Savadog *et al.,* (17)supported the present result by their identification of LAB isolates in fermented cow and lamb milk and they observed that the most dominant bacteria were those from genus *Lactobacillus.*

PCR identification of LAB and bacteriocinogenic activity of isolates

Many studies highlighted the absence of adequate selectivity in the employed culture media, even for LAB Carr *et al.,* (18); Perin and Nero. (11)).Accordingly genomic DNAs of isolates were isolated using the method offered by DNA extraction kit manufacturer information then isolated DNAs were visulized by agarose gel electrophoresis under UV light. Then they were taken to the PCR step, All 51 isolates of cows that presented positive Gram and negative catalase reactivity was subjected to *16S rRNA* based PCR identification.Twenty three cows raw milk isolates were identified as LAB.The employement of *16S rRNA* (700bp) in the identification of raw milk LAB isolates by PCR was in agreement with Perin and Nero (11); Klijn *et al.,* (12) who observed that sequencing of the V1 region (90 bp) of the 16S rRNA gene was sufficient to provide a proper and reliable identification of the isolates, with variations that allowed differentiation of their species and subspecies. However, sequencing of the same region in *Enterococcus* spp. isolates was not enough to provide a reliable identification at the species level, as observed in previous studies (19-21);

All cows raw milk isolates presented at least one of the tested bacteriocin encoding genes ; no isolates presented *Ent A, Ent B* and *Nis* genes simultaneously.This finding was in agreement with study of Perin and Nero(11) in which 30 *Enterococcus* isolates presented at least one of the tested lantibiotic genes and no isolates presented *lanB, lanC* and *lanM* simultaneously**.** In the current study, presense of one bacteriocin gene in raw milk bacterial isolates was supported by previous studies which was reported that antimicrobial potential of the isolates was not affected by, the presence of at least one of the tested genes,as one gene would be sufficient for lantibiotic production (22,

23).Inconclusion, Phenotypic and genotypic identifications were effectively identified the LAB and the Phenotyic identifications support the genotypic characterization results and bacteriocinogenic properties of isolated bacteria were determined by PCR.

التشخيص المظهري و الوراثي لبكتريا حامض اللاكتك المنتجه للبكتريوسين المعزوله من حليب الابقار

هبة الرحمن رياض ،فوزيه علي عبدالله

فرع الاحياء المجهريه،كلية الطب البيطري،جامعة البصره،البصره،العراق.

الخلاصه

اظهرت نتائج التشخيص المظهري ان بكتريا حامض اللاكتك الموجبة الكرام وغير المنتجه لانزيم الكتليز تواجدها لوحظ في51 نموذج من حليب الابقار الخام وكانت نسبتها الكليه 51%. ولوحظت اعلى نتائج (58.5%.)للاختبارات البكتريلوجيه التشخيصيه التقليديه من العزلات البكتريه المأخوذه من حليب الابقار التي تتراوح اعمارها بين (9 > - 3≤) سنه، وكان لعمر الابقار تاثير احصائي معنوي (p< 0.05)على توزيع عزلات البكتريا للحليب الخام، كذلك كان لعدد الولادات تأثير احصائي معنوي(p< 0.05) على عزلات بكتريا حليب الابقار الخام، حيث لوحظت اعلى نسبه (52%)لعزلات بكتريا حامض اللاكتك في الابقار التي تكون عدد الولادات فيها 12>-6≤

اعتمادا على نتائج التشخيص الوراثي كانت اعلى نسبه(100%) لنتائج PCR المعتمد على *16S rDNA*ا لموجبه في جميع عزلات الحليب الخام للابقار من الفئه العمريه الثانيه(year 15> - 9≤) وان الفرق بين الفئتين العمريتين لم يعتبر ذو معنويه احصائيه.(P>0.05). عدد الولادات كان له تاثير عالي المعنويه (p< 0.001) على نتائج PCR المعتمده على*16S rDNA* وا *ن* اعلى نسبه(50%) لهذه النتائج لوحظت في الابقار التي لها عدد ولادات(6 > - 1). اظهرت نتائج تحليل PCR المعتمد على الجينات المشفره للبكتريوسين الذي اجري لاحقا على جراثيم حامض اللاكتك ذات نتائج PCR الموجبه المعتمده على *16S rDNA* ان اعلى نسبه(60.9%) لنتائج PCRالموجبه قد لوحظت في جينات*Ent B*وتلتها نسبة (30.4%) الجينات المشفره للنياسين.لوحظ فرق احصائي عالي المعنويه بين نتائج PCR الموجبه المعتمده على الجينات المشفره للنياسين و*Ent A*و*Ent B*.

.REFERENCES

1-Mendelson A. (2011). "In bacteria land": The battle over raw milk. Gastronomica : the journal of food and culture 11:35-43

2- Hickey, C.D., Sheehan, J.J., Wilkinson, M.G. and Auty, M.A.E. (2015). Growth and location of bacterial colonies within dairy foods using microscopy techniques: a review. Frontiers in microbiology, vol. 6: 99.

3- Gyawali R and Ibrahim SA. Natural products as antimicrobial agents. Food Control 2014; 46: 412-429.

4- Mobolaji OA and Wuraola FO. Assesment of the antimicrobial activity of lactic acid bacteria isolated from two fermented maize products-ogi and kunni-zaki. Malaysian Journal of Microbiology 2011; 7(3): 124-128.

5- Zacharof MP and Lovitt RW. Bacteriocins produced by lactic acid bacteria. APCBEE Procedia 2012; 2:50-56.

6- Cintas LM, Casaus MP, Herranz C, Nes I F and Hernández PZ. Review: Bacteriocins of lactic acid bacteria. Food Sci. Tech. Int. 2001;7(4):281-305.

7- Ahmadova A, Todorov SD, Hadji-Sfaxi I, Choiset Y, Rabesona H, Messaoudi S,et al. Antimicrobial and antifungal activities of *Lactobacillus curvatus* strain isolated from homemade azerbaijani cheese', Anaerobe 2013;20: 42-47.

8- Liu SQ. Practical implications of lactate and pyruvate metabolism by lactic acid bacteria in food and beverage fermentations. International Journal of Food Microbiology 2003; 83: 115-131.

9- Favaro L, Penna AL and Todorov DD. Bacteriocinogenic lab from cheeses - Application in biopreservation. Trends in Food Science Technology 2015; 41: 37-48.

10- Perez RH, Zendo T and Sonomoto K. Novel bacteriocins from lactic acid bacteria with various structures and applications. Microbial Cell Factories 2014;13(Suppl1): S3.

11- Perin L M and Nero L A. Antagonistic lactic acid bacteria isolated from goat milk and identification of a novel nisin variant *Lactococcus lactis* . BMC Microbiology 2014; 14:36.

12-Klijn N, Weerkamp AH and de Vos WM. Identification of mesophilic lactic acid bacteria by using polymerase chain reaction-amplified variable regions of 16S rRNA and specific DNA probes. Appl. Environ Microbiol 1991; 57(11):3390–3393.

13-Toit MD, Franz CMAP, Dicks LMT and Holzapfel WH. Preliminary characterization of bacteriocins produced by *Enterococcus faecium* and *Enterococcus faecalis* isolated from pig faeces. J. Appl. Microbiol 2001; 88(3):482–494.

14-Tannaz Jalilsood1, Ali Baradaran1, Adelene Ai‑Lian Song1, Hooi Ling Foo2,3, Shuhaimi Mustafa4,5, Wan Zuhainis Saad4,6, Khatijah Yusoff3,4 and Raha Abdul Rahim1. Inhibition of pathogenic and spoilage bacteria by a novel biofilm‑forming*Lactobacillus* isolate: a potential host for the expression of heterologous proteins. Jalilsood et al.Microb Cell Fact 2015; 14:96.

15-Eman N A and Asmaa B M B T. Identification of Lactic Acid Bacteria in Raw Milk and Kariesh Cheese with Special Reference to *Lactococcus garvieae*. Journal of Food and Nutrition Sciences 2015; 3(6): 203-208.

16-Abdelgadir W S, Hamed S , Moller S, Jakobsen P L. Characteristics of The Dominant Microbiota of Sudanese Fermented Milk Rob. Int. Dairy Journal 2001; Vol 11 : 63-70.

17-Savadogo A, Quanttara C A T, Savadogo P W, Quatta A S, Barro N and Traore A S. Microorganisms involved in Fulani Traditional fermented milk in Burkania Faso. Pakistan Journal of Nutrition2004; 3 (2): 134- 139.

18-Carr FJ, Chill D and Maida N. The lactic acid bacteria: a literature survey. Crit. Rev. Microbiol 2002; 28(4):281–370.

19-Campos CA, Rodríguez Ó, Calo-Mata P, Prado M and Barros-Velázquez J. Preliminary characterization of bacteriocins from *Lactococcus lactis*, *Enterococcus faecium* and *Enterococcusmundtii* strains isolated from turbot (Psetta maxima). Food Res. Int. 2006; 39(3):356–364.

20-Giraffa G and Neviani E. DNA-based, culture-independent strategies for evaluating microbial communities in food-associated ecosystems. Int. J. Food Microbiol 2001; 67(1):19–34.

21-Moraes PM, Perin LM, Silva A Jr and Nero LA. Comparison of phenotypic and molecular tests to identify lactic acid bacteria. Braz. J. Microbiol 2013; 44(1):109–112.

22-Hyink O, Balakrishnan M and Tagg JR. *Streptococcus rattus* strain BHT produces both a class I two-component lantibiotic and a class II bacteriocin. FEMS Microbiol Lett. 2006; 252(2):235–241.

23-Wirawan RE, Klesse NA, Jack RW and Tagg JR. Molecular and genetic characterization of a novel nisin variant produced by *Streptococcus uberis*. Appl Environ Microbiol 2006; 72(2):1148–1156.