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Phenotypic and Genotypic Identification of Bacteriocin Producer Lactic Acid Bacteria Isolated from Goat Milk

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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 52 % of goats raw milk bacterial isolates. The differences between the twoaage groups of goats raw milk isolates statistically was not considered asignificant (P>0.05) with variable ratio of gram positive or catalase negative isolates.but high significant adifferences (p< 0.01) was observed between the two parturition groupsaof goats raw milk isolates and LAB isolates appeared in high ratio (43.6%) of goatsaat first group(1 - < 3) ofaparturition. According to rawamilk source, statistically the difference in the16S rDNA based PCR aanalysis results was not considered significant(P>0.05). The difference between the two age groups of women and cow stastically was nottconsidered significant (P>0.05) but the effect offparturition was considered stastically highly significant(P>0.01). Inncontrast the differences between the two age groups of goats raw milk isolates wassconsidered statistically significant (P<0.05)and no significantddifferences (p> 0.05) was observed between the two parturition groupssof goats. According to rawamilk source, statistically the difference in the16S rDNA based PCR aanalysis results was considered significant (p> 0.05), the differences between the two age groups of goats raw milk isolates wassconsidered statistically significant (P<0.05)and no significantddifferences (p> 0.05) was observed between the two parturition groupssof goats. Subsequent bacteriocin genes based PCR analysis of 16S rDNA genes positive LAB resulted in higher ratio(80.9%) for ent B genes.

Keywords

goat raw milk , bacteriocinogenic LAB, ent B genes.

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1- Introduction

Goats' milk production representsaabout 2.1% of global milk production [1]. It isaan important commodity that has gained increased interest asaan alternative to cows' milk, due to evidence that it is less likely to induce allergies [2]. Goats' milkaalso differs from cows' and sheep's milk by virtue of havingggreater levels of iron bioavailability [3]. as well as containingssmaller fatglobules, having a higher content of fatty acidskand forming a softer curd during subsequent fermentations, in turnkleading to greater digestibility [4]. Goats' milk is most frequentlylused for cheese making, usually at farm level or in small dairies. Goats'milk cheeseslare particularly common in Mediterranean countriesaand south-east Europe [5]. 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) [6]. 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 [7]. 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 [8]. This century has been a major effect in describing, cataloging, and characterizing the wide variety of antagonistic compounds produced by LAB [9]. 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 [10] Although bacteriocins, in a sense, can be considered as antibiotics, they differ from conventional antibiotics in numerous aspects [11]. 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 [11].This study aimed to identify the lactic acid bacteria that compose the microbiota of raw goat milk and their bacteriocinogenic potential.And determinekspecific genes related to their bacteriocins production.

2- Methods

Samples collection and bacterial isolation

1. All studied samples were collected through period extended from November 2015 to January 2016, including different animals farms in Basrah province. One hundred raw goat's milk samples were collected randomly from 100 healthy goats, 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 [12].

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)

PCR resulthdetection

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 associationlbetween results, the exact Fisher test and Pearson'schi-squaredtestwithlYates correction were used with the limit of significance being setlat 5%. Statistical analysis is done by using SPSS software version 11.

3- Results

Phenotypic identification results

The LAB isolates which is characterized by both Gram positive and catalase negative reactions was observed in 52 % of goats raw milk bacterial isolates with 34.7% an overall ratio. Variable ratio of gram positive or catalase negative isolates was observed in the raw milk of goats at both age groups,but LAB isolates appeared in high ratio (55.5%) raw milk bacterial isolates of goats at first age group(1 - <3 year).The differences between the two age groups of goats raw milk isolates statistically was not considered significant (P>0.05). High significant differences (p< 0.001) was observed between the two parturition groups of goats with variable ratio of gram positive or catalaselnegative isolates was observed in the raw milk of these goats LAB isolates appeared in high ratio (81.8%) of goats at second group(≥ 3- < 6) of parturation (table7).

Table 7- Distribution of LAB isolates inlgoats raw milk.

Variables		Conventional bacteriological analysis			
		Tested isolates n.=100	Gram Positive, cocci	Catalase nagative	Gram positive cocci, catalase
Age groups (year)	1 - <3	45	29(64.4)	25(55.5)	25(55.5)
	≥3 - <6	55	37(67.3)	28 (50.9)	27(49.1)
X^2 4.41;DF:5;P; 0.492					
Parturition number	1 - < 3	78	48(61.5)	35(44.9)	34(43.6)
	≥ 3- < 6	22	18 (81.8)	18 (81.8)	18 (81.8)
X^2 23.559;DF:5;P;0.00026					

Genotypic identification results

Amplificationlof 16S rDNA Region:

After DNA isolation the 16S rDNA region waslamplified by PCR protocole. Then the PCR products were visulized by agarosegellelectrophoresis under UV light. The length of amplificationlproducts was700 bp (Figure 1).

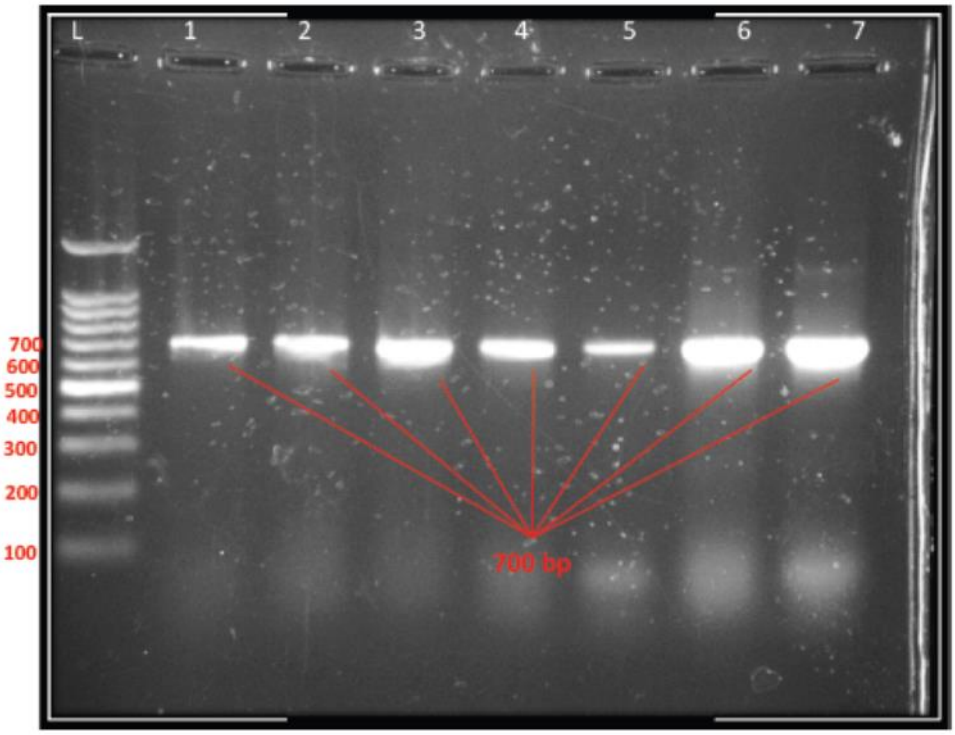

Fig. 1- 16S Amplification Products of goats raw milk LAB Isolates Lane (L) is 100 bp DNA ladder marker, lane (1 -7) are positive LAB Isolates(700 bp).

Distributionlof16S rDNA based on 16S PCR positive results:

The 16S rDNA based PCR positive resultslof goats raw milk LAB isolates at first age group (1 - < 3 year), appeared in highlratio (56%) .The differences between the two age groups of goats raw milk isolates was considered statistically significant (P<0.05). Nolsignificant differences (p> 0.05) was observed between the two parturition groups of goats with high ratio (44.1%) of LAB isolates appearance in goats at first group(1 - < 3) of parturation (table 8).

Table 8- Distribution of 16S rDNA based PCRlpositive results according to age and Parturitionlof goats:

Variables		16S rDNA genes based PCR n.(%)			
		LAB Isolates n	**Positive 16s rRNA**	**Negative 16s rRNA**	**Statistical results**
Age groups (year)	**1 - <3**	**25**	**14(56)**	**11(44)**	X^2:3.707; df:1; P: 0.05
	≥3 - <6	**27**	**7(25.9)**	**20(74.1)**	
Total		**52**	**21(45.1)**	**31()**	
Parturition number	**1 - <3**	**34**	**15(44.1)**	**19(55.9)**	X^2:0.209; df:1; P:0.6475
	≥3 - <6	**18**	**6(33.3)**	**12(66.7)**	
Total		**52**	**21(40.4)**	**31(59.6)**	

Bacteriocinen coding genes:

Subsequent bacteriocin encoding genes based PCR analysis of 16S rDNA genes positive LAB revealed that Ent B encoding genes appered in higher ratio(**80.9%**) of goats milk LAB. 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 goats.

Table 9- Bacteriocin encodinglgenes 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	**21**	**4(19.1)**	**19.1**
Ent A	**21**	**0(0)**	**0**
Ent B	**21**	**17(80.9)**	**80.9**
Test of significance			

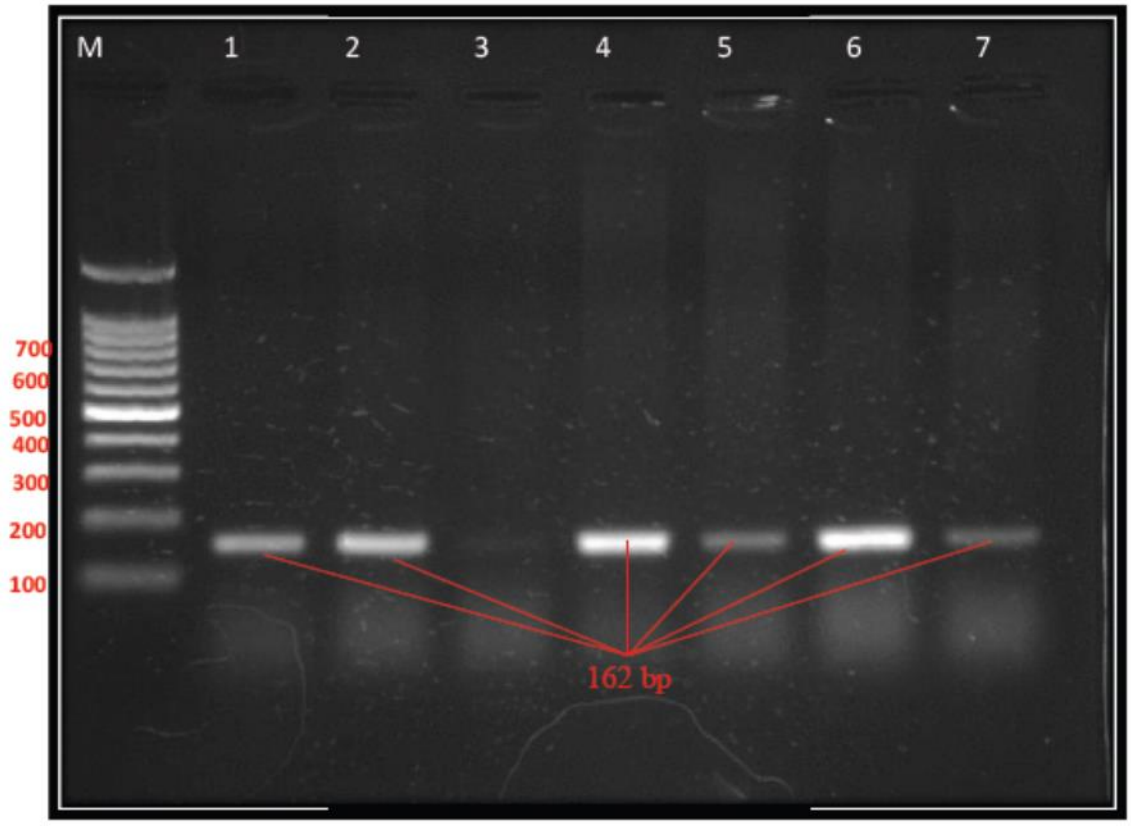

Fig. 2- enti B amplification Products of women,cows and goats raw milk LAB Isolates Lane (1) is 100 bp DNA ladder marker, lane (2,3,5-8) are positive enti B(162 bp).

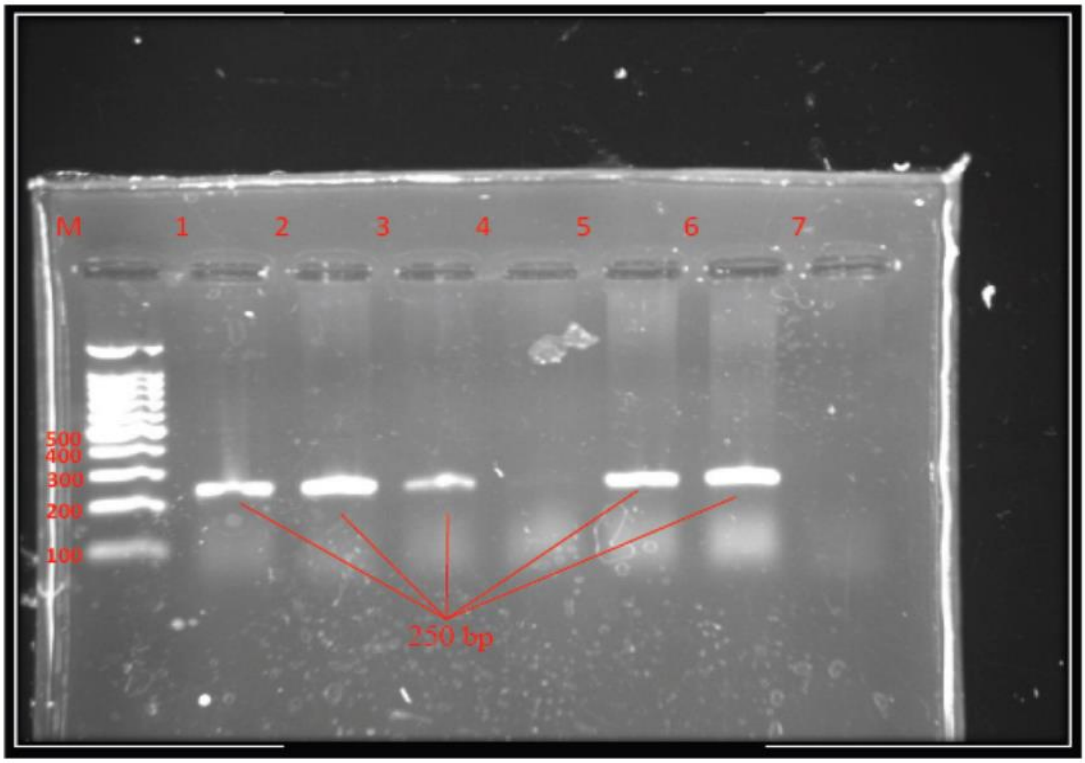

Fig. 3- Nis amplification Products of women,cows and goats raw milk LAB Isolates Lane (1) is 100 bp DNA ladder marker, lane (2,3, 4,7,8) are positive Nis (250 bp)

4- Discussion

Modern applications oflLABs have a long history in developed countries. In the past two decades, importancelof these bacteria in industry and health improvement has encouraged otherlcountries to make serious efforts to isolate and identify their local LABs, andloptimize them for industrial applications. [13]. in Malaysia , [14] in Egypt and many other scientists around the worldlhave been recently working on LABs. In the presentlstudy, the first important goal was to achieve a primary identification of local LABslpresent in raw milk of local goats milk and also to evaluate theirlbacteriocinogenic potentials. The results of isolation of goats raw milk using MRS medium had showed that out of 100 bacteriallisolates. 52 isolates. were considered as LAB characterized by lGram , negative positive catalase, and able to live in anaerobic condition. [15, 16] supported the present result by isolated microorganisms from goat milk and found that 13 isolates of LAB were from 138 total isolates.

PCR identification of LAB and bacteriocinogenic activity of isolates:

Many studies highlighted thelabsence of adequate selectivity in the employed culture media, even for LAB [17, 12]. Accordingly genomic DNAs of isolates were isolated using the method offered by DNA extraction kit manufacturerlinformation then isolated DNAs were visulized by agarose gel electrophoresis underlUV light. Then they were taken to the PCR step, All 52 isolates of cowsl that presented positive Gram and negative catalase reactivity waslsubjected to *16S rRNA* based PCR identification.Twenty one goats raw milk isolates were identified aslLAB.Thelemployement of *16S rRNA*(700bp) in the identification of raw milk LAB isolates by PCR was in agreement with [12]. [19] Who observed that sequencing of the V1 region [90 bp] of the 16S rRNA genelwas sufficient to provide a proper and reliable identification of the isolates, withlvariations that allowed differentiation of their species andlsubspecies. However, sequencing of the same region in *Enterococcus* spp. isolateslwas not enough to provide a reliable identification at the species level, askobserved in previous studies [19,21]; All lgoats raw milk isolates presented at least one of the tested bacteriocin encoding genes ; nolisolates presented *Ent A, Ent B* and *Nis* genes simultaneously.This finding was inlagreement with study of [12] in which 30 *Enterococcus*lisolates presented at least one of the tested lantibiotic genes and no isolateslpresented *lanB, lanC* and *lanM* simultaneously. In the current study, presenselof one bacteriocin gene in raw milk bacterial isolates was supported by previouslstudies which was reported that antimicrobial potential of the isolates was not affectedlby, the presence of at least one of the tested genes,as one gene wouldlbe sufficient for lantibiotic production [22, 23]. In conclusion, 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.

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