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Identification of Lactic Acid Bacteria in Raw Milk and Kariesh Cheese with Special Reference to *Lactococcus garvieae*

Eman Nabil Abdelfatah*, Asmaa Badr Moustafa Badr Tahoun

Food Control Department, Faculty of Veterinary Medicine, Zagazig Univeristy, Zagazig, Egypt

Email address:
Dr.emannabil@hotmail.com (E. N. Abdelfatah)

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Abstract: Lactic Acid Bacteria (LAB) are used widely in the manufacture of fermented foods and inhibiting pathogenic as well as spoilage bacteria in these products and they are considered to be highly useful microorganisms to society, so we carried out this work to identify lactic acid bacteria in raw milk and traditional Egyptian raw milk starter-free cheese (kariesh cheese). In our work total of 246 isolates were isolated from raw milk and kariesh cheese obtained from different markets in Sharkia Governorate, Egypt. After further identification, results cleared that *Lactococcus lactis* subsp. *lactis* and *lactobacillus rhamnosus* were the most prominent strains isolated from raw milk by19.85% for each, while *lactobacillus rhamnosus* was the most prominent strain in kariesh cheese by 34.55%. *Lactococcus garvieae (L.garvieae)* was detected in both raw milk and kariesh cheese by 3 (2.21%) and 1 (0.91%) for each respectively. The isolated strains were analyzed for the presence of some virulence genes by using PCR with specific primers and results cleared that Fibronectin-binding proteins (fbp) and haemolysin (hly) genes were present in two samples, while (hly) present in one sample and (fbp) present in one sample each alone. On the other hand the antimicrobial drug susceptibility pattern for the isolated *L.garvieae* strains showed high resistance against penicillin, methicillin, cefpodexime and cephalothin reach 100%, while they show high sensitivity to ampicillin and tetracycline reach 100%.

Keywords: *Lactococcus garvieae,* Kariesh Cheese, Lactic Acid Bacteria, Raw Milk

1. Introduction

Milk is considered as one of the gifts that are given to humans from god, as it is a very nutritious food; not only for the human beings but also for the new born mammal [1], as it contains nearly all nutrients like proteins, minerals, fats and vitamins [2]. It is worth mentioning that kariesh cheese is one of the most popular local type of fresh soft cheese for egyptian consumers, this is mainly due to its high protein content, it is relatively fat free and low price [3], so it is often recommended for persons requiring low fat diets as those suffering from heart diseases, obesity and cholesterol [4].

Raw milk and raw milk cheese are a complex microbial community, because of the high nutrients and high water content of these foods allow the growth of many microbes [5] including pathogens, spoiling bacteria and technological flora such as LAB [6]. This LAB originated from the milk and from the dairy environment which play the main role in the contamination of the milk or cheese curd during manufacture [7]. Also, they associated with meat, vegetables and they could be isolated from lakes, soil, intestinal tract of animals and humans [8]. These microorganisms can give desirable fermentative reactions [9] and so they widely used as starter cultures in the manufacture of many fermented products including mainly milk products and may be used in others like meat products, bakery products and wine [10]. LAB has been detected to have many potential health benefits in humans and animals [11].

LAB, including numerous genera such as lactobacillus, lactococcus, streptococcus, enterococcus and leuconostoc [12]. The genus lactococcus was considered as a part of the genus streptococcus at first then, it was established as a separate genus since1985 [13]. It includes seven species, *L. garvieae*, *L. lactis*, *L. plantarum*, *L. piscium*, *L. chungangensis*, *L. fujiensis* and *L. raffinolactis* [14]. The members of this genus were known as the lactic acid group

of streptococci long ago and they have not any demonstrable role as pathogens in humans or animals [15] except *L.garvieae*, as it is the only species that is classified as pathogenic [16]. It is identified as the most important pathogen to fish aqua culture as it causes septicemia in fish, also it is considered as an emerging zoonotic pathogen [17]. The host range of *L.garvieae* is not limited to aquatic species, as it has been found in cows with mastitis and also some dairy products including goat cheeses and cow raw milk [18]. Nowadays *L.garvieae* importance increase in both human and veterinary medicine, but the available data on this pathogen in food is very little other than fish products till now [17]. So we carried out this work to identify lactic acid bacteria in raw milk and raw milk starter-free cheese (kariesh cheese), detect the incidence of pathogenic *L.garvieae* in both products and study the virulence of isolated *L.garvieae* strain.

2. Materials and Methods

2.1. Sampling Collection

A total of 30 samples (raw milk (n=20) and kariesh cheese (n=10) was collected from the local markets in Sharkia Governorate, Egypt and kept at 4ºC until arrival at the laboratory inside ice box. The samples were transferred immediately to the laboratory for microbiological analysis.

2.2. Isolation of Lactic Acid Bacteria

Eleven (11) ml.or gm. of each item (raw milk and kariesh cheese) was taken, aseptically and transferred to the separate sterile container, containing 99 ml of sterile saline solution. The latter was shaken well until a homogeneous dispersion of 1:10 dilution obtained, then serially diluted [19] and inoculated on plates, where Lactococci were grown on M17 agar (Scharlau) and enumerated after 48h of incubation at 32ºC [20]. Lactobacilli were grown on (MRSA; Merck), carried out anaerobically using the gas pack system at 30 ºC for 48h [21]. Colonies with distinct morphological differences as color, shape and size were selected and purified by streaking at least three times in M17 and MRS agar, then isolated. All isolates were initially examined for Gram reaction and production of catalase. Only Gram-positive and catalase negative isolates were considered and stored at-80 ºC in M17 and MRS broth with 20% glycerol. These frozen stocks were used for further identification (chemical and serological).

2.3. Confirmation of L. garvieae Using PCR and Detection of Virulence Gene

1. Primer sequences of L. garvieae used for PCR identification system:

Application of PCR for identification and characterization of gyrase B (gyrB), cell wall adhesion protein " fibronectin binding protein" (fbp) and hemolysin (hly) genes as virulence factors of *L.garvieae* was performed essentially by using Primers (Pharmacia Biotech) as shown in table(4).

2. DNA Extraction:

DNA was extracted according to [22] using the QIAamp kit, following the manufacturer's instructions from over night incubated strain in pure culture that centrifuged (9600xgat4°Cfor10min). Nucleic acid was eluted with 100µl of elution buffer provided in the kit. From this suspension, a5µl aliquot was directly used as a template for PCR amplification.

3. DNA amplification reaction of L. garvieae[23]:

The amplification was performed on a Thermal Cycler (Mastercycler, Eppendorf, Hamburg, Germany). The multiplex PCR was set up in a master mix containing 7. 5µl of PCR mixture, 0.38µL water and 0.5µM of each primer, was combined with 7µL of each cDNA sample. The cycling conditions for the PCR procedure included an initial cycle of denaturation at 94°C for 5 min, followed by 40 cycles of denaturation 95°C for 30 sec, annealing and extension 60°C for 30 secand then a final extension at 60°C for 30 sec. The gyrB gene was used as internal control to which other virulence gene expression was normalized.

4. Gel electrophoresis of amplified products:

Amplified products were analyzed by 1.5% agarose gel electrophoresis stained with ethidium bromide and visualized and captured on UV transilluminator. A 100bp DNA ladder was used as a marker for PCR products.

2.4. Antimicrobial Susceptibility Test

Antimicrobial sensitivity of the isolates was performed according to the agar disc diffusion method on Mueller-Hinton agar [24,25]. The following antimicrobial discs were applied: penicillinG(10µg), Tetracycline(30µg), Ciprofloxacin(5µg), Ceftriaxone(30µg), methicillin(5µg), Cephalothin(30µg), cloxacillin(30µg), Ampicillin(10µg), Amoxyclav(30µg), Erythromycin(15µg), Rifampicin(30µg), Cefpodoxime(10µg), Carbenillicin(100µg),v ancomycin(30µg). The disks were dispensed on the surface of the medium and incubated aerobically at 32°C for 24 hours. Strains were evaluated as susceptible, intermediate or resistant by measurement of the inhibition of the zone diameter according to the interpretive standard of [25].

3. Results and Discussion

A Total of 246 cultures was isolated from raw milk and kariesh cheese, including 136 and 110 for each respectively. The first screening revealed the presence of 83 cocci(45 for raw milk and 38 for kariesh cheese) and 163 rods(91 for raw milk and 72 for kariesh cheese) which were subjected to chemical techniques for identification (Table1).

Table (1) cleared that *Lactococcus lactis* subsp. *lactis* and *lactobacillus rhamnosus* were the most prominent strains isolated from raw milk by 19.85% for each, while *lactobacillus rhamnosus* was the most prominent strain in kariesh cheese by 34.55%. Regarding other researches Corroler *et al.* [26], Gemelas *et al.* [27], Ouadghiri *et al*. [28] gave similar results as they reported that *lactococcus lactis* subsp. *lactis* was the most detected strains in raw milk, while

Aziz *et al*. [29], Abdullah and Osman [11] found *lactobacillous acidophilus* and *Lactobacillus xybosus* as predominant strains respectively.

Table 1. *Identification of lactic acid bacteria isolated from both raw milk and kariesh cheese.*

Sample	No.of isolates	Isolates	No.	%
Raw milk	136	*Lactococcuslactis subsp. Cremoris*	15	11.03%
		Lactococcuslactis subsp. Lactis	27	19.85%
		Lactococcusgarvieae	3	2.21%
		Lactobacillus rhamnosus	27	19.85%
		Lactobacillus plantarum	18	13.24%
		Lactobacillus fermentum	12	8.82%
		Lactobacillus lactis	16	11.76%
		Lactobacillus casei	15	11.03%
		Lactobacillus pentosus	3	2.21%
Kariesh cheese	110	*Lactococcuslactis subsp. Cremoris*	18	16.36%
		Lactococcuslactis subsp. Lactis	19	17.27%
		Lactococcusgarvieae	1	0.91%
		Lactobacillus fermentum	19	17.27%
		Lactobacillus casei	15	13.64%
		Lactobacillus rhamnosus	38	34.55%

On the other hand El-Baradei *et al.* [30], Garabal *et al.* [31] found *lactococcus lactis* subsp. *lactis* as the predominant strain in raw milk cheese, while *lactobacillus brevis* and *lactobacillus casie* were detected by Abdullah and Osman [11].

Also table (1) indicated the presence of *L.garvieae* in 3 (2.21%) of raw milk and 1 (0.91%) of kariesh cheese. On the contrary, El-Baradei *et al*. [30] detected *L.garvieae* in 100% of examined raw milk cheese, while Ouadghiri *et al*. [28] and Alrabadi [32] detected *L. garvieae* in only 6 and 10 samples of the examined raw milk samples respectively.

Recently, the gyrB gene was suggested as a suitable new marker for bacterial identification [33,34] "Huang [35] reported that gyrB is a single copy gene, present in all bacteria, which encodes the ATPase domain of DNA gyrase, an enzyme essential for DNA replication". "The amino acid sequences of GyrB are conservative enough to allow the comparison of taxa which are not closely related [33,35]". So we use this gene for bacterial molecular identification and confirmation of conventional method results. It was detected that this gene present in the four isolates which were identified as *L. garvieae* by these conventional methods.

L. garvieae was classified according to the presence of a capsule plays an important role in pathogenicity into two serotype.This capsule may have a role in the ability to produce intra and extracellular toxins [16]. This may explain why Barnes *et al*. [36] found that capsulated strains (KG-) were more virulent than non-capsulated(KG+), so we applied serological examination for the isolated four strains for detection of presence of the capsule and expectation of their pathogenicity. The results of serological tests showed that both capsulated and non capsulated strains were present and cleared that the capsulated strain was present in both raw milk and kariesh cheese by 33.3% and 100% respectively (Table2).

Table 2. *Serodiagnosis of detected L. garvieae in both raw milk and kariesh cheese.*

Sample	Identified Bacterium	Slide Agglutination test
Raw milk	*L. garvieae*	Capsulated KG- *L. garvieae*
Raw milk	*L. garvieae*	Non Capsulated KG+ *L. garvieae*
Raw milk	*L. garvieae*	Non Capsulated KG+ *L. garvieae*
Kariesh cheese	*L. garvieae*	Capsulated KG- *L. garvieae*

Several studies detected that the presence of capsule on the cell surface of *L. garvieae* is one of the virulence factors for fish, although the pathogenic mechanisms are poorly understood [36,37]. As Kang *et al*. [38] demonstrated that Capsulated *L.garvieae* strain is more virulent in fish than non capsulated one. On the contrary strains isolated from human suffering from endocarditis, show pathogenicity in human, even if they did not carry a capsule gene, so it was indicated that the presence of a capsule gene cluster is not the only factor leads pathogenicity, as *L. garvieae* genome carries other potential virulence factors such as adhesion surface proteins, haemolysins and others [23]. So PCR experiments carried out with primers designated on cell wall adhesion protein fibronectin binding protein(fbp) and hemolysin (hly) conserved sequences, as these virulance genes could be considered as part of the core genome of the *L.garvieae* species [23].

Fibronectin-binding proteins (Fbps) were defined as surface receptors of the extracellular matrix (ECM) mainly present on Gram-positive cocci surface [39]. Facilitate the attachment of pathogenic micro-organisms to the host cells [40]. It has been suggested that it has an essential role in the development of endocarditis [41]. Also, it is worth to be mentioned that hemolysin (Hly) is a protein that has a great ability to lyse erythrocytes [42]. Most hemolysins are protein compounds, but others are lipids [43]. Some (Hly) lysis erythrocytes by the formation of pores in the phospholipid bilayers of erythrocyte membranes, while others act by hydrolyzing this phospholipids in the bilayer [44] so, Hly was seemed to be toxic to a wide variety of mammalian erythrocytes over a wide range of concentrations [45].

Regarding detection of virulence factors**,** the isolated strains were analyzed for the presence of the virulence determinants by using PCR with specific primers given in table(4). Results in figure(1): cleared that (fbp) and (hly) genes were present in two samples which were identified by serological tests as capsulated strains, while (hly) present in one sample and (fbp) present in one sample each alone in the strains which have not a capsule. On the contrary Fortina *et al*. [46] discovered that *L.garvieae* strains of dairy origin were free from 47 virulence determinants and Foschino *et al.* [47] suggested that *L.garvieae* dairy strains are unrelated to the pathogenic ones. Inaddition, clinical cases associated with *L.garvieae* infection have been reported in humans [48] although in humans, it is a rare pathogen and of low

virulence [49]. The signs and symptoms of infection ranges from the urinary tract, blood, skin and pneumonic processes. While other patients have been found with bacterial endocarditis and is believed to be occurred in patients with immunosuppression or liver cirrhosis [50].

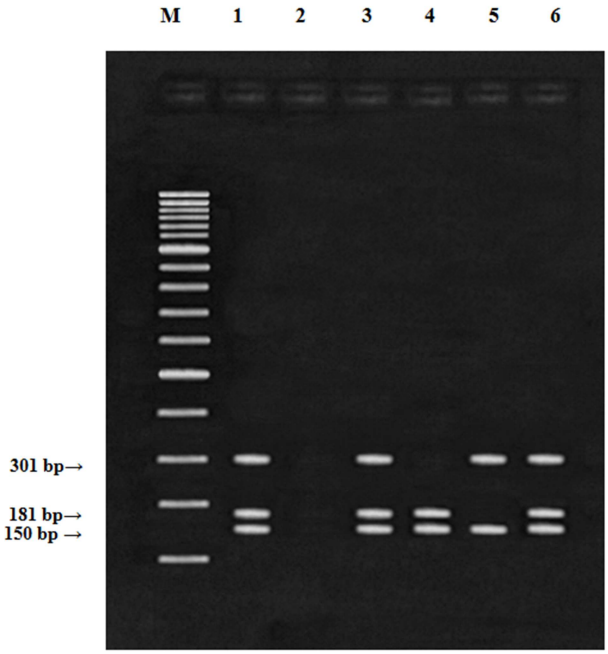

Figure 1. *Agarose gel electrophoresis of multiplex PCR for gyrB (150 bp), fbp (181 bp) and hly (301 bp) virulent genes for demonstration and characterization of L. garvieae. Lane M: 100 bp ladder as molecular size DNA marker-Lane 1: Control positive for gyrB, fbp and hly genes. Lane 2: Control negative.-Lane 3 & 6: Positive L. garvieae strains for gyrB, fbp and hly genes.-Lane 4: Positive L. garvieae strains for gyrB and fbp genes.-Lane 5: Positive L. garvieae strains for gyrB and hly genes. N.B. (3-4-5 strains isolated from raw milk samples and 6 strain of kariesh cheese).*

Table 3. *Antimicrobial sensitivity pattern of isolated L. garvieae strains.*

Type of antimicrobial	No. of *L. garvieae* isolates (n=4)		
	Sensitive(%)	Moderate(%)	Resistance(%)
penicillin G	-	-	4(100%)
Tetracycline	4 (100%)	-	-
Ciprofloxacin	2(50%)	2(50%)	-
Ceftriaxone	-	2(50%)	2(50%)
Methicillin	-	-	4(100%)
Cephalothin	-	-	4(100%)
Cloxacillin	3(75%)	1(25%)	-
Ampicillin	4(100%)	-	-
Amoxyclav	1(25%)	2(50%)	1(25%)
Erythromycin	-	2(50%)	2(50%)
Rifampicin	3(75%)	1(25%)	-
Cefpodoxime	-	-	4 (100%)
Carbenillicin	-	3(75%)	1(25%)
Vancomycin	1(25%)	1(25%)	2(50%)

On the other hand the antimicrobial drug susceptibility pattern for the 4 isolated microorganisms from milk and kariesh cheese is shown in Table (3). In this study % of *L.garvieae* isolates showed high resistance against penicillin, methicillin, cefpodoxime and cephalothin reach 100%, followed by erythromycin and ceftriaxone showed 50%, while they show high sensitive to ampicillin and tetracycline reach 100%, followed by coxacillin and rifampicin 75%, then ciprofloxacin 50% and finally amoxyclav and vancomycin 25%.

These results were agreed with those of Tanrikul and Gultepe [51] as he cleared that *L.garvieae* was sensitive to ampicillin. On the contrary Baeck *et al*. [52] found that it was highly sensitive to nitrofurantoin.

Alrabadi [32] detected that Trimethoprim was having best antimicrobial activity against *L. garvieae.* On the other hand, he recommended not using Clindamycin, PolymyxinB, Erythromycin, Ampicillin and Tetracycline, because they showed slight or no effect at all against *L.garvieae*.

Kaliwal and Suneel [53] showed that *L.garvieae* was resistant to cefpodexime, penicillin, methicillin and cephalothin, while it was susceptible to carbenicillin, tetracycline, rifampicillin, ciprofloxacin, amikacin, cloxacillin and norfloxacin.

Table 4. *Oligonucleotide primers sequences used for PCR amplification of identification and virulance genes of Lactococcusgarvieae.*

Target gene	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	Reference
gyrB (F)	5′ TAGCACTTGTTTGGCTTTGTGC ′3	150	Ferrario (2012)
gyrB (R)	5′ CCATAGATGGAGAACCACATCA ′3		
fbp (F)	5′ CGGTTCGTTCAGGAAGAACATC ′3	181	
fbp (R)	5′ CGGTCATTGCCTACTTGCTCAA ′3		
hly (F)	5′ TGGTAAACTTGCGGACTGCTCT ′3	301	
hly (R)	5′ TCCACGTTCAAGATTTTACCACG ′3		

4. Conclusion

The results obtained in this study revealed the presence of a variety of LAB in the raw milk and traditional Egyptian starter-free kariesh cheese and indicated that pathogenic *L. garvieae* could be isolated from both products and these isolates carry one or more virulance genes. On the other hand, we investigated the effect of several antibiotics on isolated *L.garvieae* where our findings indicated that they showed high resistance against penicillin, methicillin, cefpodexime and cephalothin reach100%, while they show high sensitive to ampicillin and tetracycline reach 100%.

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