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INDUSTRIAL AND SAFETY CHARACTERIZATION OF *Lactococcus garvieae* ISOLATED FROM TRADITIONAL EGYPTIAN DAIRY PRODUCTS

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ABSTRACT

A total of 90 samples of traditional Egyptian dairy products including 25 samples of each of Kariesh cheese, Domiati cheese, and Ras cheese and 15 samples of Laban Rayeb were collected and exmained for the presence of *Lactococcus garvieae.* Potential isolates were identified using physiological and PCR examinations. PCR analysis confirmed 66 out of 84 physiologically identified *Lc. garvieae* isolates. This indicated the importance of employing a relevant PCR assay to confirm the belonging of isolates to *Lc. garvieae* rather than relying only on phsiological tests. *Lc. garvieae* was found to be associated with all the examined products at incidence rates ranging from 20% to 32%. *Lc. garvieae* isolates recovered from these products were found to be able to develop acidity to levels that caused milk coagulation after 24 h. Acidity levels ranged from 0.52% to 0.78% (T.A.%). *Lc. garvieae* isolates also showed proteolytic activities ranging from 0.00 to 0.04 mg tyrosine/ mL. Isolates from Domiati and Ras cheese generally showed higher proteolytic activities than those associated with Kariesh cheese and Laban Rayeb. None of the examined isolates were able to express a lipolytic activity. All isolates were sensitive to vancomycin, whereas 3%, and 9% of them showed resistance to kanamycin and tetracycline, respectively. A limited number of the *Lc. garvieae* isolates (7.6%) could produce the biogenic amine tyramine, but all isolates were unable to produce histamine. These results suggest a potential role of *Lc. garvieae* in the processing and ripening of dairy products, but also highlight the necessity of applying safety assessments before any potential industrial use of its strains.

Keywords: *Lactococcus garvieae,* cheese, traditional dairy products, proteolytic activity, antibiotic resistance, biogenic amines, PCR

INTRODUCTION

Lactococcus garvieae is a fish-borne bacterium that has been reported as a causative agent of lactococcosis, infecting different fish species (Eyngor *et al*. 2004). It was also detected in milk from mastitic cows and was shown to be involved in human infections (Chan *et al*. 2011). Nevertheless, *Lc. garvieae* has been very frequently isolated from artisanal cheese varieties prepared from raw milk, with no reports suggesting human infection due to the consumption of such cheeses (Florez *et al*. 2012). This was explained by the observation that dairy-borne *Lc. garvieae* strains did not harbor virulence factors detected in *Lc. garvieae* strains isolated from fish (Fortina *et al*. 2007). Furthermore, *Lc. garvieae* was suggested to contribute to the development of typical sensory traits of traditional dairy products (Fernandez *et al*. 2010).

Lc. garvieae could be detected in different Greek, Italian, and Spanish cheese varieties (Hatzikamari *et al*. 1999; Prodromou *et al*. 2001; Foschino *et al*. 2006; Algeria *et al*. 2009). It was also isolated as a dominant bacterial

component of the natural microflora of traditional Egyptian cheeses (El-Baradei *et al*. 2005; El-Baradei *et al*. 2007) and Zabady (El-Baradei *et al*. 2008). For instance, *Lc. garvieae* could be recovered from 94%, 93%, and 78% of Domiati cheese, Ras cheese, and Kariesh cheese samples, respectively (El-Baradie *et al*. 2005). El-Baradie *et al*. (2007) could also detect *Lc. garvieae* in 100% of 11 samples of Domiati cheese. However, no further studies have characterized *Lc. garvieae* isolates associated with traditional Egyptian dairy products as to demonstrate their potential role in the processing and ripening of these products. The present study was thus designed to characterize the industrial and safety traits of *Lc. garvieae* isolates recovered from traditional Egyptian dairy products.

MATERIALS AND METHODS

Sample collection

A total of 90 samples of traditional Egyptian dairy products including Kariesh cheese (*n* = 25), Laban Rayeb (*n* = 15), Domiati cheese (*n* = 25), and Ras cheese (*n* = 25) were aseptically collected from local markets in Aga, Mansoura, and Domiatta cities during 2013 - 2014.

Isolation of *Lc. garvieae* from dairy samples

Twenty-five grams of each sample were homogenized with 225 ml of sterile saline solution (0.85% NaCl). Serial dilutions of the resultant suspensions were made in Maximum Recovery Diluent (Oxoid, Basingstoke, UK) and plated onto M17 agar (Oxoid) followed by incubation at 30 °C for 48 h (Alegria *et al*. 2009). Suspect colonies were picked up and examined for Gram-staining reaction and catalase expression. Gram-positive, catalase-negative cocci isolates were maintained for further physiological identifications. These involved growth at 10 °C and 45 °C (Sharpe 1979), growth in 4% and 6.5% NaCl (Abd-El-Malek and Gibson 1948), hydrolysis of esculin in the presence of bile, arginine hydrolysis (Facklam and Collins 1989), gas production from glucose (De Man *et al.* 1960), and acid production from the fermentation of lactose, maltose, ribose, and mannitol (Facklam 1972).

PCR identification of *Lc. garvieae* isolates

Potential isolates of *Lc. garvieae* that showed typical physiological reactions were further confirmed by using a PCR assay described by Pu *et al*. (2002). DNA was extracted from 24 h cultures grown in M17 broth (Oxoid) using the GenElute^{TM} Bacterial Genomic DNA kit (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. A PCR reaction mixture of a final volume of 50 µL was formulated to contain DNA template (7.5 µL), forward primer (1RL) (600 nM) and reverse primer (LgR) (600 nM), PCR master mix (one*Taq*® quick-load 2x, New England Biolabs, Hitchin, Hertfordshire, UK) (25 µL), and completed with H_2O. The 1RL forward primer (5´-TTTGAGAGTTTGATCCTGG-3´) and LgR reverse primer (5´-AAGTAATTTTCCACTCTACTT-3´) were designed to target a DNA sequence specific to *Lc. garvieae* within a 16S rRNA gene (Pu *et al*. 2002). PCR reactions were conducted at 95 °C for 10 min, and 35 cycles of 94 ºC for 30 s, 45 ºC for 30 s and 72ºC for 2 min using the Primus 25 Advanced® PCR

thermal cycler (PEQLAB Biotechnology GmbH, Erlangen, Germany). PCR products were analyzed by gel electrophoresis using 1.2% agarose in TBE buffer and visualized using the BioDocAnalyzer gel documentation system (Biometra, Goettinge, Germany).

Assessment of proteolytic activity of *Lc. garvieae* isolates

Proteolytic activity of *Lc. garvieae* isolates were quantified using the colorimetric method of Hull (1947), modified by Citti *et al*. (1963) as follows. *Lc. garvieae* isolates were grown for 24 h at 30 °C in M17 broth. Five mL of the resultant culture were washed and re-suspended in 0.32 mM Na phosphate, pH 7.2. Cell suspension was inoculated (1%) into 100 mL reconstituted skim milk (RSM) (10% TS) pasteurized at 63 °C for 30 min (autoclaved milk was reported by Citti *et al*. (1963) not to be suitable for this assessment). Cultures were grown at 22 °C, and samples were taken after 24 h, mixed with trichloroacetic acid (TCA) (0.72 N) and left for 10 min. The solution was filtered and the TCA filtrate containing protein lysates were mixed with the Folin-Ciocalteu's phenol reagent and left for 5 min. Absorbance of the resultant blue color was measured at 650 nm and results were expressed as mg tyrosine/mL using a standard curve relating different tyrosine concentrations with light absorbance.

Assessment of acidity development in milk by *Lc. garvieae* isolates

Lc. garvieae isolates were grown for 24 h at 30 °C in M17 broth and inoculated in sterilized RSM (10% TS) to provide an initial viable numbers of 10^5 cfu mL^{-1}. Cultures were incubated at 30 °C for 24 h and samples were taken to assess titratable acidity.

Assessment of lipolytic activity of *Lc. garvieae* isolates

Lc. garvieae isolates were grown for 24 h at 30 °C in the M17 broth followed by streaking on the M17 agar supplemented with 1% (w/v) neutral tributyrin (Sigma). Lipolysis was indicated by the formation of clear zones around the colonies (Katz *et al*. 2002).

Assessment of antibiotic resistance

Lc. garvieae isolates were assessed for their resistance to vancomycin, kanamycin and tetracycline employing the minimum inhibitory concentration (MIC) method as described by Fortina *et al*. (2007). Isolates were grown for 24 h at 30 °C in M17 broth and inoculated into the same broth containing different concentrations of the examined antibiotics to provide an initial inoculum of 10^5 cfu mL^{-1}, followed by incubation at 37 °C for 24 h. Results were interpreted according to the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) (FEEDAP Panel 2005). Resistance to vancomycin, kanamycin, and tetracycline was considered at MIC greater than 8 µg mL^{-1}, 1024 µg mL^{-1}, and, 16 µg mL^{-1}, respectively.

Screening of biogenic amines production

Lc. garvieae isolates were screened for their ability to produce the biogenic amines tyramine and histamine using the method described by Bover-Cid and Holzapfel (1999). To induce the expression of the decarboxylase enzymes, *Lc. garvieae* isolates were successively sub-

cultured in M17 containing 0.1% of tyrosine or histidine (Merck, Darmstadt, Germany) for 5 – 10 time at 30 °C for 24 h. Isolates were then streaked on the improved decarboxylase medium with and without amino acids (control) and incubated at 37 °C for 4 days.

Statistical analysis

Data were analyzed using analysis of variance (ANOVA) and Duncan multiple range test ($p < 0.05$).

RESULTS and DISCUSSION

Incidence of *Lc. garvieae* in traditional Egyptian dairy products

The occurrence of *Lc. garvieae* in traditonal Egyptina dairy products including Kariesh cheese, Laban Rayeb, Domiati cheese, and Ras cheese was examined. A total of 90 samples of these products were collected, serially diluted and plated on the M17 agar, followed by physiological and PCR indentification testing. *Lc. garvieae* was found to be associated with all the examined products (Table 1). The incidence rate ranged from 20% in the Labany Rayeb samples to 32% in the Kariesh cheese samples (Table 1). This confirmed previous studies reporting the association of *Lc. garvieae* with different Italian, Greek, and Spanish artisanal cheese varieties (Hatzikamari *et al*. 1999; Prodromou *et al*. 2001; Foschino *et al*. 2006; Alegria *et al*. 2009). The organism was also isolated before from traditional Egyptian cheese (El-Baradei *et al*. 2005; El-Baradei *et al*. 2007) and Zabady (El-Baradei *et al*. 2008). However, *Lc. garvieae* was detected at higher incidence rates than those reported in the present study. For example, El-Baradie *et al*. (2005) detected *Lc. garvieae* in 94%, 93%, and 78% of Domiati cheese, Ras cheese, and Kariesh cheese samples, respectively. These differences in the rates of occurrence of *Lc. garvieae* could be due to variations in the samples' numbers, and locations, and the season of the year, where they were collected. Variations in atmospheric temperatures between seasons could affect the growth and survival of the organism in the examined products.

As shown in Table 1, the PCR assay did not confirm all the physiologically identified *Lc. garvieae* isolates, but 66 out of 84 isolates could be confirmed by this analysis. This could be due to the fact that the physiological characteristics do not differentiate between *Lc. garvieae* and *Lc. lactis* subsp. *lactis* (Schleifer 1987). It was reported that these two organisms can be differentiated from each other based on the pyrrolidonylarylamidase (PYRase) enzyme test (Collins *et al*. 1983). However, Facklam *et al*. (1990) showed that both *Lc. garvieae* and *Lc. lactis* subsp. *lactis* could produce positive results in this test. Taken together with the present results, this highlights the importance of employing a relevant molecular-based method such as PCR in the identification of *Lc. garvieae.*

The physiological characteristics of PCR confirmed *Lc. garvieae* isolates recovered from different traditional dairy products are shown in Table 2. The isolates showed typical physiological traits of *Lc. garvieae,* with the exception of mannitol fermenation. Isolates recovered from Kariesh cheese and Laban Rayeb were variable in their ability to ferment mannitol (Table 2).

This agrees with a previous study reporting the isolation of atypical *Lc. garvieae* strains from water buffalos with subclinical mastitis that could not ferment mannitol (Teixeira *et al*. 1996). This may indicate that the Kariesh cheese, and Laban Rayeb samples containing mannitol non-fermenting *Lc. garvieae* cultures were probably made from raw mastitic milk.

Table 1: Detection of *Lactococcus garvieae* in traditional Egyptian dairy products using physiological and PCR identification tests

Samples	No. of Samples	No. of Physiologically Identified Isolates	No. of PCR Confirmed Isolates	No. of Positive Samples (%)
Kariesh cheese	25	23	20	8 (32%)
Laban Rayeb	15	15	10	3 (20%)
Domiati cheese	25	20	15	6 (24%)
Ras cheese	25	26	21	7 (28%)
Total	90	84	66	24 (26.7%)

Table 2: Physiological characteristics of PCR confirmed *Lc. garvieae* isolates recovered from traditional Egyptian dairy products

Dairy Product	Physiological Characteristics										
	Growth at 10 °C	Growth at 45 °C	Growth in 4% NaCl	Growth in 6.5% NaCl	Hydrolysis of esculin	Hydrolysis of arginine	Gas from glucose	Acid from lactose	Acid from maltose	Acid from ribose	Acid from mannitol
Kariesh cheese	+	-	+	-	+	+	-	+	+	+	v*
Laban Rayeb	+	-	+	-	+	+	-	+	+	+	v
Domiati cheese	+	-	+	-	+	+	-	+	+	+	+
Ras cheese	+	-	+	-	+	+	-	+	+	+	+

***Variable reactions**

Industrial traits of *Lc. garvieae* isolated from traditional Egyptian dairy products

A total of 66 physiologically and PCR confirmed isolates of *Lc. garvieae* were characterized for traits relevant to their potential industrial role in dairy products. These involved the ability of the isolates to develop acidity in milk, and their proteolytic and lipolytic activities. As shown in Table 3, *Lc. garvieae* isolates recovered from all products were able to develop acidity to levels that

caused milk coagulation after 24 h. Acidity levels ranged from 0.52% for isolates from Ras cheese to 0.78% for isolates from Kariesh cheese (Table 3). Significant variations in acidity development were also noticed in isolates recovered from the same product ($p < 0.05$). *Lc. garvieae* isolates recovered from different Egyptian dairy products showed proteolytic activity ranging from 0.00 to 0.04 mg tyrosine/ mL (Table 3). Isolates from Domiati and Ras cheese generally showed higher proteolytic activities than those associated with Kariseh cheese and Laban Rayeb ($p < 0.05$). None of the examined isolates could however show a lipolytic activity (Table 3). These results agree with those of Fortina *et al*. (2007) who reported the ability of *Lc. garvieae* cultures isolated from Italian cheese to develop acidity, coagulate milk and show proteolytic activities. This suggests that *Lc. garvieae* could contribute to the processing and ripening of dairy products by their ability to develop acidity and hydrolysis milk proteins.

Table 3: Industrial and safety characterization of *Lc. garvieae* isolated from traditional Egyptian dairy products

Dairy product	Industrial Traits			Safety Traits				
	Acidity Development (T.A.%)	Proteolytic Activity (mg tyrosine/ mL)	Lipolytic Activity	Antibiotic Resistance** (Number of resistant isolates)			Biogenic Amine Production (Number of isolates)	
				Van	Kan	Tet	Tyramine	Histamine
Kariesh cheese	0.66 – 0.78*	0.00-0.02*	-	0	1	2	0	0
Laban Rayeb	0.55 – 0.75	0.00-0.01	-	0	1	1	0	0
Domiati cheese	0.55 – 0.77	0.02-0.04	-	0	0	3	3	0
Ras cheese	0.52 – 0.77	0.02-0.03	-	0	0	0	2	0
Total (% of total isolates, *n*= 66)				0 (0%)	2 (3%)	6 (9%)	5 (7.6%)	0 (0)%

*** Minimum – Maximum**

****Examined antibiotics involved vancomycin (Van), kanamycin (Kan), and tetracycline (Tet).**

Safety traits of *Lc. garvieae* isolated from traditional Egyptian dairy products

Lc. garvieae isolates were also subjected to a safety assessment of their response to 3 relevant antibiotics and ability to produce the biogenic amines tyramine and histamine. None of the examined isolates could resist vancomycin, whereas 2 isolates (3%) from Kariesh cheese and Laban Rayeb showed resistance to kanamycin (Table 3). A higher number of 6 isolates (9%) recovered from all products but Ras cheese were found to be resistant to tetracycline (Table 3).

A limited number of 5 *Lc. garvieae* isolates (7.6%) recovered from Domiati cheese and Ras cheese could produce the biogenic amine tyramine (Table 3). However, none of the examined isolates could develop histamine (Table 3). These results were consistent with those of a previous study reporting the sensitivity of *Lc. garvieae* isolated from cheese to vancomycin, compared with intermediate resistance to kanamycin, and relatively higher resistance to tetracycline (Fortina *et al*. 2007). The same authors also

showed 18% of the examined *Lc. garvieae* isolates produced tyramine, but none of the isolates expressed histamine. These results highlight the importance of conducting safety assessments of *Lc. garvieae* strains before any potential incorporation into dairy products.

Taken together, the above findings show that *Lc. garvieae* is an important component of the microflora of traditional Egyptian dairy products. The ability of the *Lc. garvieae* isolates described in this study to develop acidity and express proteolytic activities suggests a potential role of this organism in the preparation and ripening of dairy products. However, some isolates showed resistance to antibiotics and were able to produce the biogenic amine tyramine. This highlights the importance of examining the safety of *Lc. garvieae* strains before including them in any potential application in dairy industries.

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تقييم الصفات الصناعية والأمان الصحي لميكروب لاكتوكوكس جارفيا المعزول من منتجات الألبان المصرية التقليدية.

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تم تجميع ٩٠ عينة من المنتجات اللبنية المصرية التقليدية متضمنةً ٢٥ عينة من كل من الجبن القريش والجبن الدمياطي والجبن الرأس و ١٥ عينة من اللبن الرائب، وتم فحص هذه العينات من حيث وجود بكتريا لاكتوكوكس جارفيا. تم تعريف عزلات لاكتوكوكس جارفيا بإستخدام الأختبارات الفسيولوجية وتحليل PCR. ووُجد أنه بإستخدام تحليل PCR فإنه تم التأكد من ٦٦ عزلة من مجموع ٨٤ عزلة تم التعرف عليها بواسطة الأختبارات الفسيولوجية. ولقد دل ذلك علي أهمية إستخدام إختبار PCR مناسب للتأكد من عزلات لاكتوكوكس جارفيا وعدم الاعتماد علي الأختبارات الفسيولوجية بمفردها. وقد أمكن عزل الميكروب من كل المنتجات التي تم إختبارها وذلك بنسبة تواجد ٢٠% - ٣٢% من مجموع العينات. إستطاعت عزلات لاكتوكوكس جارفيا أن تعمل علي تطور الحموضة وتجبن اللبن خلال ٢٤ ساعة. وتراوحت مستويات الحموضة الناشئة ما بين ٠,٥٢% إلي ٠,٧٨%. أظهرت عزلات لاكتوكوكس جارفيا القدرة علي تحليل البروتين بمستويات تراوحت ما بين ٠,٠٠ إلي ٠,٠٤ ملليجرام تيروسين/ المللي، وكانت العزلات الناتجة من عينات البجن الدمياطي والجبن الرأس أكثر قدرة بصفة عامة علي تحليل البروتين بالمقارنة بعزلات الجبن القريش واللبن الرائب. لم تظهر أي من العزلات المختبرة القدرة علي تحليل الدهون. كما أظهرت كل العزلات المختبرة حساسية للمضاد الحيوي فانكوميسن، ولكن بعضها كان مقاوماً للكاناميسن والتتراسيكلن. إستطاع عدد محدود من عزلات لاكتوكوكس جارفيا (٧,٦%) إنتاج الأمين الحيوي تيرامين، ولكن كل العزلات لم تنتج الهستامين. توضح هذه النتائج أن ميكروب لاكتوكوكس جارفيا يمكنه أن يلعب دوراً في تصنيع وتسوية المنتجات اللبنية، ولكنها تظهر أيضاً أهمية القيام بإختبارت لتقييم الأمان الصحي لسلالات هذا الميكروب قبل أي إستخدام محتمل لها في الصناعات اللبنية.