J. Food and Dairy Sci., Mansoura Univ., Vol. 4 (10): 487 - 496, 2013

PREVALENCE OF *Enterococcus* IN EGYPTIAN DAIRY PRODUCTS

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ABSTRACT

This study examined the prevalence and diversity of *Enterococcus* species in traditional Egyptian dairy products. A total of 24 samples of traditional Egyptian dairy products including pickled Domiati cheese (7 samples), fresh Domiati cheese (5 samples), Kariesh cheese (6 samples), and Mish cheese (6 samples) were randomly collected from local markets in Mansoura city and examined for the presence of *Enterococcus* by plating on Kanamycin Asculin Azid agar. Out of 77 potential *Enterococcus* isolates recovered from those samples, 61 cultures were confirmed as belonging to the *Enterococuss* genus. These confirmed cultures were isolated from pickled Domiati cheese (19 isolates), fresh Domiati cheese (28 isolates), Kariesh cheese (7 isolates) and Mish cheese (7 isolates). These cultures were further identified to the species level by examining their ability to ferment glucose, sucrose, lactose, sorbitol, and mannitol, produce pigment and hydrogen sulphide (H_2S), show motility, and utilize pyruvate. Based on these physiological examinations, 80% of the isolates were identified as *E. faecium*. Other *Enterococcus* species including *E. faecalis*, *E. gallinarum*, and *E. malodoratus* also existed in the examined samples, but at minor incidence rates of 8%, 2%, and 10%, respectively. *E. faecium* isolates could be recovered from all examined traditional Egyptian dairy products. *E. faecalis*, and *E. gallinarum* were isolated from Domiati cheese, but could not be detected in Kariesh cheese or Mish cheese. Whereas, *E. malodoratus* could be isolated from both Kariesh cheese and Mish cheese, but could not be cultured from pickled or fresh Domiati cheese. These results highlighted *Enterococcus* as prevalent lactic acid bacteria that existed as diverse species in traditional Egyptian dairy products.

Keywords: *Enterococcus,* dairy products, *Enterococcus faecium*, diversity, Lactic acid bateria

INTRODUCTION

The *Enterococcus* genus involves Gram-positive, catalase-negative cocci that ferment lactose into lactic acid. Members of the *Enterococcus* genus are ubiquitous in diverse niches, but more frequently occur in vegetables, plant material, and foods of animal origin including dairy products (Giraffa *et al.* 1997; Franz *et al*. 1999). *Enterococcus* is considered the third-largest genus of lactic acid bacteria (LAB) after the genera *Lactobacillus* and *Streptococcus*. It currently involves 37 species, which fall into 7 species groups based on the 16S rRNA sequence similarities (Devriese *et al*. 2003; Franz and Holzapfel 2006). However, *E. faecium* and *E. faecalis* are the most important enterococcal species. Enterococci were found to play an important role in the ripening of various traditional cheese varieties (Giraffa 2003). They were shown to be involved in the proteolysis, lipolysis, and citrate breakdown occurring during ripening, and thus contributing to the development of the typical taste and flavor of ripened cheese. Certain *Enterococcus* strains have been also reported to produce bacteriocins and others have been employed

as probtiotics (Giraffa 2003). In contrast, *enterococci* have been reported among the most common nosocomial pathogens that have been incriminated in several cases of endocarditis, bacteremia, and infections of the urinary tract, central nervous system, intra-abdominal and pelvic infections (Endtz *et al*. 1999; Franz *et al*. 1999).

The association of *Enterococcus* with traditional European cheese varieties prepared in Mediterranean countries, such as Greece, Italy, Spain and Portugal, using raw or pasteurized goats', ewes', buffalos' or bovine milk has been extensively studied (Pe´rez Elortondo *et al*. 1999; Xanthopoulos *et al*. 2000; Manolopoulou *et al*. 2003). These studies showed *Enterococcus* as predominant LAB in the fully ripened product. However, little is known on the prevalence and diversity of *Enterococcus* in traditional Egyptian dairy products. The present study was therefore designed to assess the presence and diversity of *Enterococcus* in Egyptian dairy products including pickled Domiati cheese, fresh Domiati cheese, Kariesh cheese, and Mish cheese.

MATERIALS AND METHODS

Collection of dairy product samples

A total of 24 samples of traditional Egyptian dairy products were randomly collected from local markets in Mansoura city. These samples included 7 samples of pickled Domiati cheese, 5 samples of fresh Domiati cheese, 6 samples of Kariesh cheese and 6 samples of Mish cheese.

Isolation of *Enterococcus* from dairy product samples

Twenty-five grams of each examined dairy product sample were ground in 225 ml of sterile saline solution (0.85% NaCl), followed by preparing serial dilutions of the resultant sample suspension using the same sterile solution. Suitable dilutions were selected and plated onto kanamycin aesculin azide (KAA) agar (Oxoid, Basingstoke, UK), followed by incubation at 37°C for 24 h. Round, white or grey colonies surrounded by black zone were picked up and maintained for the identification tests.

Identification of *Enterococcus* isolates

Suspected isolates of *Enterococcus* were subjected to the following morphological and physiological examinations:

Gram staining

A 24 h culture of each suspected *Enterococcus* isolate grown on MRS agar (Oxoid) was Gram-stained and microscopically examined as described by Pollack *et al*. (2005).

Catalase activity

A loopful from one discrete colony of each suspected *Enterococcus* isolate grown for 24 h on MRS agar was transferred onto a clean glass slide to be mixed with a drop of H_2O_2 (30%). A positive result was indicated by immediate bubbling due to gas formation (Macfaddin 1977).

Growth at 45°C and 10°C

A 24 h culture of each suspected *Enterococcus* isolate grown in the MRS broth (Oxoid) was inoculated at 1% (v/v) into MRS broth, followed by

incubation at 45°C for 48 h and 10°C for 2 weeks. Bacterial growth was observed within these incubation times (Sharpe 1979).

Growth in 6.5% NaCl

A 24 h culture of each suspected *Enterococcus* isolate grown in MRS broth was inoculated at 1% (v/v) into MRS broth containing 6.5% NaCl followed by incubation at 37°C (Abd-El-Malek and Gibson 1948). Cell growth was observed after 24 h of incubation.

Growth at pH 9.6

A 24 h culture of each suspected *Enterococcus* isolate grown in MRS broth was inoculated at 1% (v/v) into the same broth adjusted to pH 9.6, followed by incubation at 37°C (Sharpe 1979). Cell growth was observed after 24 h of incubation.

Growth on bile *aesculin* agar (BAA) medium

One hundred microliters of a 24 h culture of each suspected *Enterococcus* isolate grown in MRS broth were spread onto bile aesculin agar (BAA) (Oxoid), followed by incubation at 37°C for 24 h. The development of brown colored growth on BAA indicated a positive result of the test.

Gas production from glucose

A 24 h culture of each suspected *Enterococcus* isolate grown in MRS broth was inoculated at 1% (v/v) into MRS broth, followed by incubation at 37°C for 24 h (De Man *et al.* 1960). Gas production was indicated by bubble formation in the inverted Durham tube in the MRS broth.

Biochemical speciation of *Enterococcus* isolates

Confirmed *Enterococcus* isolates were further identified to the species level. They were grown in MRS broth at 37°C for 24 h and subjected to the following examinations:

Carbohydrate fermentation

Enterococcus cultures were inoculated at 1% (v/v) into nutrient broth (Oxoid) supplemented with 1% (w/v%) glucose, sucrose, lactose, arabinose, sorbitol, glycerol, or mannitol and 0.0018% (w/v%) bromocresol purple (BCP) (1 ml from 1.6% BCP solution in 95% ethanol was added to 900 ml nutrient broth). A positive reaction was recorded when the indicator changed from purple to yellow, which indicated acid production. Tubes were observed on a daily basis for 3 days (Facklam 1972).

Pigment production

One hundred microliters of each *Enterococcus* culture were spread onto tryptone soya agar (TSA) (Oxoid), followed by incubation at 37°C for 24 h. The development of yellow colored growth on TSA indicated a positive result of the test (Facklam and Collins 1989).

Motility and hydrogen sulfide (H_2S) production

Enterococcus cultures were inoculated into test tubes containing the sulfide-indole-motility (SIM) medium (Oxoid), followed by incubation at 37°C for 24 h (Harrigan and McCance 1966). Non-motile isolates showed growth only along the line of inoculation, whereas motile cultures had either a diffuse

even growth spreading from the inoculum, turbidity of the whole medium, or more rarely, localised outgrowths which were usually fan-shaped or occasionally nodular. Hydrogen sulfide production was indicated by blackening of the line of inoculation.

Pyruvate utilization

Enterococcus isolates were inoculated at 1% (v/v) into nutrient broth, to which 1% sodium salt of pyruvic acid and 0.004% bromothymol blue were added. Cultures were incubated for 16 to 18 h at 35°C and observed for colour changes. Colour turned into bright yellow in pyruvate-utilizing cultures (Gross *et al*. 1975).

RESULTS AND DISCUSSION

Isolation of *Enterococcus* from traditional Egyptian dairy products

The prevalence of *Enterococcus* in traditional Egyptian dairy products was studied. Samples of dairy products were randomly collected from local markets in Mansoura city and examined for the presence of *Enterococcus* by plating on Kanamycin Asculin Azid (KAA) agar. Suspected colonies were subjected to biochemical and physiological identification tests. Isolates that were found to be Gram-positive cocci, and gave a negative reaction in the catalase test were considered as potential *Enterococcus* cultures. Table 1 shows the number of examined dairy samples and potential *Enterococcus* isolates recovered from these samples. It could be seen that potential *Entorococcus* isolates could be cultured from all examined traditional Egyptian dairy products. Higher percentages of positive samples containing potential isolates of the organism were found with pickled Domiati cheese (71%) and Mish cheese (83%), compared with fresh Domiati cheese (60%), and Kariesh cheese (67%) (Table 1).

Potential *Enterococcus* isolates were further examined for physiological traits shown in Table 2. Out of 77 potential *Enterococcus* isolates, 61 cultures could grow at 10°C, 45°C, and pH 9.6 and in the presence of 6.5% NaCl. (Table 2). They also gave positive results in the bile aesculin test, and fermented glucose without gas production (Table 2). Since these traits are typical phenotypic characteristics of the members of the *Enterococcus* genus (Sherman 1937; Stiles and Holzapfel 1997), those 61 cultures were confirmed as *Enterococuss* isolates.

Biochemical speciation of *Enterococcus* isolates cultured from traditional Egyptian dairy products

Confirmed *Enterococcus* isolates cultured from Egyptian dairy products were further identified to the species level based on their biochmical activities. This involved examining the ability of these isolates to ferment glucose, sucrose, lactose, sorbitol, and mannitol, produce pigment and hydrogen sulphide (H_2S), show motility, and utilize pyruvate (Table 3). The results of these biochemical examinations were interpretted according to Collins *et al*. (1986), Facklam and Collins (1989), and Day *et al*. (2001). It could be seen in Table 3 that *E. faecium* was the most frequently isolated *Enterococcus* species since 80% of the total isolates were found to belong to

it. *E. faecium* isolates could be recovered from all examined traditional Egyptian dairy products (Table 3). Higher incidence rates of *E. faecium* were observed with fresh Domiati cheese (25 isolates) and pickled Domiati cheese (16 isolates), compared with Kariesh cheese (5 isolates) and Mish cheese (3 isolates). Other *Enterococcus* species including *E. faecalis, E. gallinarum,* and *E. malodoratus* also existed in the examined traditional Egyptian dairy products, but at minor incidence rates of 8%, 2%, and 10%, respectively (Table 3). *E. faecalis,* and *E. gallinarum* were isolated from Domiati cheese, but could not be detected in Kariesh cheese or Mish cheese. Whereas, *E. malodoratus* could be isolated from both Kariesh cheese and Mish cheese, but could not be cultured from pickled or fresh Domiati cheese.

Table 1: Association of *Enterococcus* with traditional Egyptian dairy products

Samples	No. of Samples	No. of Positive Samples (%)	No. of potential *Enterococcus* isolates*
Pickled Domiati cheese	7	5 (71%)	35
Fresh Domiati cheese	5	3(60%)	28
Kariesh cheese	6	4(67%)	7
Mish cheese	6	5(83%)	7
Total	24	17(71%)	77

***Isolates that were Gram-positive cocci and gave negative results in the catalase test.**

Table 2: Physiological confirmation of potential *Enterococcus* isolates recovered from traditional Egyptian dairy products

Dairy product (Number of samples)	Number of confirmed/ potential *Enterococcus* isolates (%)	Physiological Characteristics					
		Growth at 10°C	Growth at 45°C	Growth in 6.5% NaCl	Growth at pH 9.6	Bile-aesculin reaction	Gas from glucose
Pickled Domiati Cheese	**19/35 (54%)**	**+**	**+**	**+**	**+**	**+**	**-**
Fresh Domiati Cheese	**28/28 (100%)**						
Kariesh Cheese	**7/7 (100%)**						
Mish cheese	**7/7 (100%)**						
Total	**61**						

Table 3: Biochemical speciation of *Enterococcus* cultures isolated from traditional Egyptian dairy products

Isolat umbrs (%)[a]	Source (Number of isolates)	Glucose	Sucrose	Lactose	Sorbitol	Mannitol	Pigment	H_2S	Motility	Pyruvate	Identification Result
49 (80%)	Fresh Domiati cheese (25) Pickled Domiati cheese (16) Kariesh cheese (5) Mish cheese (3)	+	+ (98%)[b] - (2%)[c]	+	+ (96%)[b] - (4%)[c]	+	-	-	-	-	*E. faecium*
5 (8%)	Fresh Domiati cheese (2) Pickled Domiati cheese (3)	+	+	+	+	+	-	-	-	+	*E. faecalis*
1 (2%)	Fresh Domiati cheese (1)	+	+	+	+	+	-	-	+	-	*E. gallinarum*
6 (10%)	Kariesh cheese (2) Mish cheese (4)	+	+	+	+	+	-	+	-	-	*E. malodoratus*

[a]Percent of total confirmed *Enterococcus* isolates.
[b]percent of isolates producing positive reaction.
[c]percent of isolates producing negative reaction.

The above results showed that diverse *Enterococcus* species including *E. faecium, E. faecalis, E. gallinarum,* and *E. malodoratus* could be isolated from traditional Egyptian dairy products, which was also reported in previous studies. For instance, El-Awady *et al*. (2011) could isolate 80 *Enterococcus* cultures from 100 samples of Kariesh cheese, Zabady, Laban Rayeb, and Ras cheese. Ayad *et al*. (2004) had also studied the technological characteristics of 333 *Enterococcus* isolates cultured from artisanal Egyptian dairy products including Domiati cheese, Ras cheese, Mish, Zabady and Laban Rayeb. Those isolates involved *E. faecium* (n= 255), *E. durans* (n= 48), *E. faecalis* (n= 23), *E. avium* (n= 3), and *E. cassseliflavus* (n= 4). However, the authors did not describe the prevalence of each *Enterococcus* species in each examined dairy product. Süßmuth (1995) could isolate 300 isolates of lactic acid bacteria from Domiati cheese and Ras cheese, most of which were identified as *Enterococcus* with only 3.5% of the strains belonging to *Lactococcus* and 1.5% belonging to *S*. *thermophilus* and *S. bovis*. The frequent occurrence of *Enterococcus* strains in traditional Egyptian dairy products as shown in those studies and the present work could reflect the ability of *Enterococcus* to tolerate relatively high salt levels of Domiati and Mish cheeses, and acidity of Kariesh cheese. It was reported that *Enterococcus* strains could persist during the preparation and storage of dairy

products due to their ability to withstand adverse environmental conditions of extreme pH, temperatures, and salinity (Giraffa 2003).

The diversity of *Enterococcus* species in Egyptian dairy products examined in this study was consistent with other reports of Teuber *et al*. (1996), Giraffa *et al.*, (1997), Franz *et al*. (1999), and Sarantinopoulos *et al*. (2001). In those studies, *E. faecium*, *E. faecalis*, and, to a lesser extent, *E. durans* were the most frequently *Enterococcus* species to be isolated from dairy products. In agreement with the present study, Jokovic *et al.* (2008) found that *E. faecium* was the most prevalent *Enterococcus* species in the Serbian fermented cream product, Kajmak.

In conclusion, this study presented *Enterococcus* as prevalent LAB in traditional Egyptian dairy products. It also showed that diverse *Enterococcus* species existed in these products, with *E. faecium* being the most frequently isolated enterococci.

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تواجد ميكروب الانتيروكوكس فى منتجات الالبان المصرية .

طه عبد الحليم نصيب ، وليد محمود الشارود وعلا محمد عادل كامل شلبى
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تم دراسة تواجد ميكروب الانتيروكوكس فى منتجات الألبان المصرية التقليدية والتعرف علي أنواعه المختلفة بها. حيث تم تجميع ٢٤ عينة من هذه المنتجات اللبنية إشتملت علي ٧ عينات من الجبن الدمياطى الخزين و٥ عينات من الجبن الدمياطى الطازج و٦ عينات من الجبن القريش و٦ عينات من الجبن المش وذلك من الأسواق المحلية بمدينة المنصورة، وبفحص هذه العينات عن طريق التخطيط على بيئة Kanamycin Asculin Azid agar أمكن عزل ٧٧ مزرعة محتملة من ميكروب الانتيروكوكس تم التأكد من أن ٦١ مزرعة منها تنتمي إلي هذا الجنس. وقد كانت مصادر هذه المزارع المؤكدة هى الجبن الدمياطى الخزين (١٩ عزلة)، والجبن الدمياطى الطازج (٢٨ عزلة)، والجبن القريش (٧ عزلات)، والجبن المش (٧ عزلات). وقد تم فحص هذه المزارع لتحديد أنواعها عن طريق إختبار قدرتها على تخمير السكريات (الجلوكوز والسكروز واللاكتوز والسوربيتول والمانيتول) وقدرتها أيضا على إنتاج الصبغات وكبريتيد الهيدروجين (H_2S) وقدرتها على الحركة وإستخدام البيروفات. ولقد أظهرت نتائج هذه الاختبارات الفيسيولوجية أن ٨٠ % من هذه المزارع ينتمي إلي النوع *E. faecium* .كما تم التعرف علي أنواع أخري وهي *E. faecalis* و *E. gallinarum* و *E. malodoratus* ولكنها تواجدت بنسب محدودة في العينات حيث مثلت أعدادها ٨ % و ٢ % و١٠ % من المجموع الكلي للعزلات على الترتيب. مزارع *E. faecium* تم عزلها من كل منتجات الالبان المصرية التقليدية التي تم فحصها في هذه الدراسة. أما مزارع *E. faecalis* و*E. gallinarum* فقد تم عزلها من الجبن الدمياطى ولكنها لم تتواجد فى الجبن القريش أوالجبن المش. بينما أمكن عزل *E. malodoratus* من الجبن القريش وجبن المش ولم يمكن عزلها من الجبن الدمياطى الطازج أو الخزين . أظهرت هذه النتائج إنتشار ميكروب الأنتيروكوكس وتنوعه كأحد ميكروبات بكتريا حامض اللاكتيك في منتجات الألبان المصرية التقليدية .

قام بتحكيم البحث

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