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INCIDENCE OF *Enterococcus* SPECIES IN RAW MILK AND SOME CHEESES

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ABSTRACT

The incidence of enterococci in Raw milk, Karish cheese, Ras cheese and Domiati cheese was investigated by using three different selective media. All isolated enterococci were identified on genus and species levels.

Higher, minimum, and maximum log counts were obtained by using KF medium being 10^6 - 10^7 (min) and 10^8 (max). These figures were 10^3 - 10^5 (min) and 10^6 (max) by using BEA and CATC media. Accordingly, KF medium could be considered the best for detection of enterococci incidence.

The obtained results showed that the isolated *Enterococcus* from milk and cheeses studied were identified as *E. faecalis* 26 (32.5%), *E. faecium* 25 (31.25%) and *E. durans* 29 (36.25%), respectively.

Most identified *E. durans* was observed in Karish cheese (15 out of 20) , followed by Raw milk (9 out of 20) and only 3 and 5 *E. durans* were isolated from Ras cheese and Domiati cheese. On the other hand, most isolated *Enterococcus spp.* from Ras cheese (14 out of 20) were *E. faecalis*. However, approximately half (9 and 8 out of 20) of identified *Enterococcus spp.* from Domiati cheese and Raw milk were *E. faecium*.

All isolated strains were identified by using the biochemical characteristics. The results showed that all tested isolates belonged to genus *Enterococcus*.

INTRODUCTION

The most controversial species of lactic acid bacteria found in food products are the enterococci. They are present as a component of the natural microflora of certain foods such as dairy products. Enterococci are normal inhabitants of gastrointestinal tracts of both human and animals (Bhardwaj *et al*, 2011) . *E. faecium* and *E. faecalis* are the two predominant species in human intestine (Giraffa. 2003).

Although enterococci are considered to be important in foods, some strains have detrimental activities that include spoilage of foods and dairy products. Both *E. faecalis* and *E. faecium* species are relatively heat resistant as well.

The perception of the presence of enterococci in milk is due to contact with cows feces has now changed due to the fact that it is very common to find enterococci in other sources as well contradicting what was formerly a sole fecal contamination belief. Giraffa. (2003) stated that enterococci can enter into the cheese milk either primarily from human or animal feces, but also secondarily from contaminated water sources, the exterior of the animal or other contaminated milking equipment or bulk storage tanks handled in the processing plant. The objective of this work was to study the incidence of *Enterococcus spp.* in raw milk and some cheeses by

using different selective media , and to identify these isolates on genus and species levels.

MATERIALS AND METHODS

Milk and some dairy products samples (cheeses) were collected from Cairo and El Gammalia city markets at Dakahlia governorate in sterile glass containers or sterilized polyethylene bags. Samples were transferred to laboratory in ice box and analyzed at the same day.

Enterococci counts were determined by using different selective media, namely bile aesculin azide agar (BEA), citrate azide tween carbonate agar (CATC) and KF Streptococcus Agar (KF). Pouring plate method was used, and plates were incubated for 2-3 days at 30 ºC under aerobic condition.

Typical colonies isolated from different sources on BEA agar were transferred to TSA agar slants , which were incubated for 24 hr at37 ºC under aerobic condition. A maximum of four typical colonies from each sample were retained for complementary tests. After four successive purification on the same medium, a stock culture of each isolated culture was maintained through biomonthly transfers on trypticase soya agar (TSA) and stored at 4ºC. All isolates were transferred before use from stock culture into trypticase soya broth (TSB) and incubated at 37 ºC for 24 hr under aerobic condition. Subsequently, to insure an active culture, two transfers of each culture to new tubes of TSB were carried out , followed by incubation as described.

For the identification on the genus level , Isolated typical colonies were primary identified on genus level according to American Public Health Association (1992). Experiment was designed by using 18h culture grown in TSB broth at 37 ºC. One ml of each 18h culture was added to 9ml TSB broth and incubated at 10 ºC, 45 ºC and 60 ºC for 24hr, 24hr and 30min, respectively.

One ml of each 18h culture was added to 9ml of TSB broth adjusted to pH 9.6 by using sodium hydroxide, adjusted to Nacl 6.5% by using sodium chloride and adjusted to bile 40% by using bile salts and to sodium azide 0.04% by using sodium azide. Cultures were incubated at 37 ºC under aerobic condition for 24hr.

Regaring the Identification on the species level , peptone water medium was used for the carbohydrate fermentation for all purified *Enterococci* isolates. To 900 ml peptone water, 10 ml indicator solution were added (bromocrysol purple 0.2% ethyl alcohol). 10 g of the appropriate sugar were disolved in 90 ml distilled water and steamed for 30 min, and was added to sterile base. Peptone water medium was inoculated with *Enterococci* isolates , and then examined after 7 days for acid production. A positive reaction was recorded when the broth medium (purple color) turns yellow. The carbohydrate broth was inoculated with a drop or loopful of an overnight broth culture or by several colonies taken from nutrient agar plate's i.e, the inoculum was taken from a fresh culture. D(-) mannitol, L(-) sorbose, L(-) arginine , D(-) arabinose, D(-) sorbitol, D(-)raffinose, potassium tellurite, D(-)

sucrose and sodium pyruvate were used for such purpose. This assay was performed according to (Tahoun. 2010).

RESULTS AND DISCUSSION

Incidence of *Enteococci* in studied Raw milk and some cheeses:

In this study, the incidences of enterococci in Raw milk, Karish cheese, Ras cheese and Domiati cheese samples was investigated. Besides, comparative study of different selective media was also performed. All isolated enterococci were identified on genus and species levels.

Enterococci counts in Domiati cheese samples on BEA agar medium were of similar minimum and maximum log counts, being 10^4 and 10^6, expect one sample (Dc1 10^3). Table (3) show that the minimum and maximum log counts of enterococci grown on CATC medium were mostly similar and ranged from 10^5 to 10^6 expect five samples, being Rm8, Rm10, Rc4, Dc2 and Kc3, which were of maximum log count of 10^7. Higher minimum and maximum log count was observed with KF medium , which were 10^7 and 10^8 in Raw milk and Ras cheese samples, expect one sample, being Rm2, (10^6). In addition, enterococci counts on KF media also showed high, minimum and maximum log counts being 10^6 and 10^7 in all Domiati cheese and Karish cheese samples expect Dc2 and Kc3 samples (10^8). It seems to be true that imputably similar contamination level with enterococci in raw milk and different cheeses was observed.

KF medium could be considered as the best medium for enterococci count in the studied milk and cheeses. KF medium showed higher minimum and maximum count (Tables 1 and 2) for all tested Raw milk (10) and cheese samples (30) being 7×10^6 - 3.9×10^8 (Raw milk samples), 1.3×10^7 - 2.3×10^8 (Ras cheese samples), 1.5×10^6 - 1.4×10^8 (Domiati cheese samples) and 3.7×10^7 **-** 1×10^8 **(**Karish cheese samples). Followed by CATC medium, being 3.7×10^5 **-** 1.1×10^7 (Raw milk samples), 1.5×10^5 **-** 1.5×10^7 (Ras cheese samples), 1.4×10^5 - 2.8×10^7 (Domiati cheese samples) and 3.6×10^6 **-** 1.3×10^7 **(**Karish cheese samples), respectively.

Table (1): Enterococci count in studied Raw milk and Ras cheese samples by using different selective media.

Samples	Selective media			Samples	Selective media		
	●BEA	♦CATC	■KF		●BEA	♦CATC	■KF
*Rm1	6.5×10^5	2.9×10^6	2.9×10^7	*Rc1	6.2×10^4	1.5×10^5	1.3×10^7
Rm2	1×10^6	1.9×10^6	2×10^6	Rc2	1.3×10^5	1.7×10^5	1.3×10^8
Rm3	1×10^4	3.7×10^5	7×10^7	Rc3	7.8×10^5	4.8×10^6	2.03×10^7
Rm4	1.2×10^5	1.3×10^6	1.5×10^7	Rc4	1.9×10^6	1.5×10^7	2.3×10^8
Rm5	1.1×10^6	1.6×10^6	4.3×10^7	Rc5	5.9×10^5	3.08×10^6	5×10^7
Rm6	1.3×10^6	2.2×10^6	9×10^7	Rc6	3.8×10^5	1.29×10^6	1.36×10^7
Rm7	4.5×10^5	1.2×10^6	4.5×10^7	Rc7	1.33×10^6	3.8×10^6	3.54×10^7
Rm8	9.6×10^5	1×10^7	3.9×10^8	Rc8	2.06×10^6	3.26×10^6	2.5×10^7
Rm9	2.3×10^5	3.5×10^6	1.6×10^7	Rc9	1.13×10^6	1.42×10^6	1.7×10^7
Rm10	8.2×10^5	1.1×10^7	2.7×10^8	Rc10	1.5×10^6	4.22×10^6	3.44×10^7

***Rm= Raw Milk *Rc= Ras cheese ●BEA= Bile Aesculin Azide Agar**
♦CATC= Citrate Azide Tween Carbonate Agar ■KF= Streptococcus Agar

Table (2): Enterococci count in studied Domiati cheese and Karish cheese samples by using different selective media.

Samples	Selective media			Samples	Selective media		
	●BEA	♦CATC	■KF		●BEA	♦CATC	■KF
*Dc1	3×10^3	1.4×10^5	1.5×10^6	*Kc1	3.4×10^5	7×10^6	4.9×10^7
Dc2	2.9×10^6	2.8×10^7	1.4×10^8	Kc2	6.4×10^5	9.4×10^6	8.6×10^7
Dc3	1.4×10^4	2.8×10^5	2.7×10^6	Kc3	3.8×10^6	1.3×10^7	1×10^8
Dc4	8×10^4	4.4×10^5	1.9×10^7	Kc4	5.4×10^5	5.3×10^6	3.7×10^7
Dc5	8.9×10^4	4.2×10^5	9.8×10^6	Kc5	2×10^5	3.6×10^6	4.3×10^7
Dc6	4.3×10^5	8.13×10^5	4.2×10^7	Kc6	9.8×10^5	7.3×10^6	6.7×10^7
Dc7	1.8×10^5	6.6×10^5	2.3×10^7	Kc7	6×10^5	4.3×10^6	6×10^7
Dc8	3.9×10^5	2.1×10^6	7.3×10^7	Kc8	3.6×10^5	6.1×10^6	4.6×10^7
Dc9	6.2×10^5	1.8×10^6	3.9×10^7	Kc9	1.8×10^6	8.6×10^6	7.8×10^7
Dc10	3.4×10^5	1.8×10^6	3.7×10^7	Kc10	3×10^6	9.2×10^6	7.5×10^7

***Dc= Domiati cheese *Kc = Karish cheese ●BEA= Bile Aesculin Azide Agar**
♦CATC= Citrate Azide Tween Carbonate Agar ■KF Streptococcus Agar

Table (3): Minimum and maximum counts of Enterococci in studied dairy samples by using different selective media.

Samples	●BEA		♦CATC		■KF	
	Min.	Max.	Min.	Max.	Min.	Max.
Raw milk	1×10^4	1.3×10^6	3.7×10^5	1.1×10^7	7×10^6	3.9×10^8
Ras cheese	6.2×10^4	2.06×10^6	1.5×10^5	1.5×10^7	1.3×10^7	2.3×10^8
Domiati cheese	3×10^3	2.9×10^6	1.4×10^5	2.8×10^7	1.5×10^6	1.4×10^8
Karish cheese	2×10^5	3.8×10^6	3.6×10^6	1.3×10^7	3.7×10^7	1×10^8

●BEA= Bile Aesculin Azide Agar ■KF Streptococcus Agar
♦CATC= Citrate Azide Tween Carbonate Agar

Finally the lowest enterococci count was observed by using BEA being 1×10^4 - 1.3×10^6 (Raw milk), 6.2×10^4 - 2.06×10^6 (Ras cheese), 3×10^3 - 2.9×10^6 (Domiati cheese) and 2×10^5 - 3.8×10^6 **(**Karish cheese).

Approximately similar contamination levels were observed in literature. Enterococci are associated with traditional European cheeses manufactured in Mediterranean countries from raw or pasteurized goats, ewes, water – buffaloes or bovine milk (Xanthopoulos *et al*. 2000, Sarantinopoulos *et al*. 2002 and Manolopoulou *et al*. 2003). Levels of enterococci in different cheese curds ranged from 10^4 to 10^6 cfu /g , and in fully ripened cheese from 10^5 to 10^7 cfu /g (Sarantinopoulos *et al*. 2001, Franz *et al*. 2003, and Giraffa. 2002 and Manolopoulou *et al*. 2003). Similarly, the number of enterococci in different cheeses ranged from 4 to 6 cfu /g and from 4 to 7 cfu /g in full – matured cheese (Saaverdra *et al*, 2003). In certain cheeses enterococci can reach counts of up to 10^6 – 10^8 cfu /g and may play an important role in the organoleptic characteristics during ripening (Psoni *et al*, 2003). Giraffa. (2003) approved *E. faecium* K77D as a starter culture for ripening of cheese in Denmark.

On the other hand, high contamination levels in raw milk samples tested were observed comparing with that observed by Hill and Smyth. (1997), who found that minimum and maximum levels of enterococci in raw cows milk were < 10^1 cfu /g and 1.2×10^3. Other sources reported numbers in

European raw milk varying from 10^3 cells/ml to 10^5 cells/ml or more (Perez *et al*. 1982, Suzzi *et al*. 2000, Cogan *et al*. 1997 and Del Pozo *et al*. 1988).

The average, maximum and minimum counts of enterococci in Egyptian Ras cheese were 4.4×10^5 cfu /g, 8×10^6 cfu /g and 7.7×10^2 cfu /g respectively, (Sharaf *et al*. 1997). This figures were obtained by using thallous acetate tetrazolium glucose agar according to Barnes. (1956). In addition, Bahout *et al*. (2006) found that the average, maximum and minimum counts of enterococci in Karish cheese were 5.7×10^6 cfu /g, 3.5×10^7 cfu /g and 9.2×10^3 cfu /g , respectively. It showed be stated here that there is no any available informations concerning the were of the will known selective Enterococci media such as KF, BEA and CATC for studying the incidence of Egyptian dairy products or milk.

For identification of isolated bacteria on the genus level , grown on the Enterococci selective medium BEA, the classical phenotype – based (biotyping) method was followed. Domig *et al*. (2003) stated that in spite of the advantage of genotype methods, the biotyping methods are still important for dairy routine analysis. Jurkovic *et al*. (2006) identified Enterococci on the genus level by several conventional phenotypic methods similar to phenotypic method used in this study.

All isolated cultures (80) grown on BEA agar media were identified on the genus level by several conventional methods: Gram stain, Catalase production, growth at 10°C and 45°C and in 6.5% Nacl, pH 9.6 in combination with resistance to bile 40%, sodium azide 0.04% and 60°C /30 min. All tested cultures grown on *Enterococcus* selective medium BEA (untabulated results) showed similar behavior that all were able to grow at 10°C and 45°C, pH 9.6, Nacl 6.5% and resist 40% bile and 0.04% sodium azide, besides, survive 60°C/30 min.

Identification of isolated *Enterococcus* strains on the species level , was done by the identification program, according to (Tahoun. 2010).

In general all tested *Enterococcus spp* were belonged to *E. faecalis*, *E. faecium* and *E. durans*, being 26 (32.5%), 25 (31.25%) and 29 (36.25%), respectively. The study done by El - Shafei *et al*. (2002) showed that two – hundred strains of Lactic acid bacteria were isolated from traditional Egyptian fermented milk (Laben Rayeb) and identified according to their morphological and physiological characteristics. They found that 80 (40%) strains were identified as *Enterococcus*, and then carbohydrate fermentation were carried on 80 *Enterococcus* species and were identified as *E. faecium* (60) and *E. faecalis* (20). In addition, Dabiza *et al*. (2005) isolated two hundred strains of Lactic acid bacteria from samples of raw buffaloes and cows milk, traditional fermented milk (Laban Rayeb), and Karish, Ras and Cheddar cheeses collected from the local market. The strains were identified by using of API 20 and API 50 CH Strips and API CHL medium and the results showed that 46 strains were identified as *E. faecium* and 13 strains were identified as *E. faecalis*.

Ayad *et al*. (2006) isolated 1340 strains of lactic acid bacteria from Ras cheese during manufacture and ripening, and were identified by biochemical and physiological characteristics, 715 strains were identified as *Enterococcus spp* and these isolates were characterized to species by using

API system and identification of some strains was confirmed by using SDS – PAGE Technique. Results showed that 480 strains were identified as *E. faecium*, (63) *E. faecalis*, (120) *E. durans* and (52) *E. spp*.

Results of (Table 5) also show that most *E. faecalis* was isolated from Ras cheese samples followed by Domiati cheese, being 14, 6 and 3from each of Raw milk and Karish cheese. While *E. faecium* was mostly isolated from Domiati cheese samples (9) followed by Raw milk, Karish cheese and Ras cheese samples being 8, 5 and 3 isolates, respectively.

Finally, most *E. durans* were isolated from Karish cheese and Raw milk samples, being 21 out of the 29 isolates. These results are in agreement except with Karish cheese with that reported by Franz *et al*. (1999), Giraffa. (2003) and Sarantinopoulos *et al*. (2001). They stated that among enterococci, *E. faecium*, *E. faecalis* and to lesser extent *E. durans* are generally the most frequent and prevalent species in cheeses. As mentioned above, numbers of *E. durans* isolates were 3, 5 and 12 among the 20 *Enterococcus spp* of each cheese. In agreement with our results Ali. (2011) stated that *E. faecalis*, *E. faecium* and *E. durans* were only *Enterococcus spp* isolated from raw milk.

Table (4): Identification of isolated enterococci on species level.

No. of isolates	Phenotypic characteristics									Species
	Mannitol	Arabinose	Sorbose	Raffinose	Arginine	Sodium pyruvate	Sorbitol	0.04 % Tellurite	Sucrose	
*Rm1-1, Rm1-2, Rm2-2, Rm3-1, Rm3-2, Rm7-2, Rm8-1, Rm8-2, Rm9-1	-	-	-	-	+	+	-	-	-	***E.durans***
Rc3-2, Rc4-1, Rc5-1*	-	-	-	-	+	+	-	-	-	
Dc6-1, Dc6-2, Dc9-1, * Dc9-2, Dc10-1	-	-	-	-	+	+	-	-	-	
K1-1, K2-2, K4-1, K4-2, * K5-1, K5-2, K6-1, K7-2, K8-1, K9-1, K9-2, K10-1	-	-	-	-	+	+	-	-	-	
Rm2-1, Rm4-1, Rm4-2, Rm5-1, Rm5-2, Rm6-1, Rm6-2, Rm7-1	+	+	-	-	+	+	-	-	+	***E.faecium***
Rc2-1, Rc3-1, Rc7-1	+	+	-	-	+	+	-	-	+	
Dc1-1, Dc4-1, Dc4-2, Dc5-1,Dc7-1, Dc7-2, Dc8-1, Dc10-2	+	+	-	-	+	+	-	-	+	
K1-2, K3-1, K3-2, K6-2, K8-2	+	+	-	-	+	+	-	-	+	
Rm9-2, Rm10-1, Rm10-2	+	-	-	-	+	+	+	+	+	***E.faecalis***
Rc1-1, Rc1-2, Rc2-2, Rc4-2, Rc5-2, Rc6-1, Rc6-2, Rc7-2, Rc8-1, Rc8-2, Rc9-1,Rc9-2, Rc10-1, Rc10-2	+	-	-	-	+	+	+	+	+	
Dc1-2, Dc2-1, Dc2-2, Dc3-1, Dc3-2, Dc5-2	+	-	-	-	+	+	+	+	+	
K2-1, K7-1, K10-2	+	-	-	-	+	+	+	+	+	

***Rm= Raw Milk *Rc= Ras cheese *Dc= Domiati cheese *Kc = Karish cheese**

Table (5): Number and percentage of identified *Enterococcus spp*.

Species	No. of isolates								Total	
	Raw milk		Ras cheese		Domiati cheese		Karish cheese			
	No	%	No	%	No	%	No	%	No	%
E.faecalis	3	3.75	14	17.5	6	7.5	3	3.75	26	32.5
E.faecium	8	10	3	3.75	9	11.25	5	6.25	25	31.25
E.durans	9	11.25	3	3.75	5	6.25	12	15	29	36.25

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تواجد أنواع من الانتيروكوكس فى اللبن الخام وبعض أنواع الجبن

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تم دراسة ١٠ عينات من كلا من اللبن الخام والجبن الراس والجبن الدمياطى والجبن القريش من حيث العد الكلى باستخدام أوساط زراعية متخصصة لعزل الجنس *Enterococcus* . أظهرت النتائج أن الحد الأدنى للعد الكلى لجميع العينات فى بيئة BEA هو $١٠^{٤}$ والحد الأقصى $١٠^{٦}$ وفى بيئة CATA كان الحد الأدنى $١٠^{٥}$ والحد الأقصى $١٠^{٦}$ وفى بيئة KF كان الحد الأدنى $١٠^{٧}$ والحد الأقصى $١٠^{٨}$ وأوضحت الدراسة أن بيئة KF كانت أفضل البيئات المستخدمة.

من ناحية أخرى تم تعريف السلالات المعزولة على البيئات السابقة باستخدام الخصائص البيوكيمائية وأظهرت النتائج أن كل السلالات المعزولة كانت تابعة لجنس *Enterococcus* وعلى مستوى الأنواع أظهرت النتائج ان جنس *Enterococcus* المعزول من المنتجات تتبع ثلاث أنواع هى *E. faecalis* و *E. faecium* و *E. durans* وكان توزيعها ٢٦ (٣٢,٥%) , ٢٥ (٣١,٢٥%) , ٢٩ (٣٦,٢٥%) على التوالى.

قام بتحكيم البحث

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