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Identification and Virulence Factors of *Enterococcus* Species Isolated from Raw Milk

Abdella, S. A. S.[1]; M. M. M. Refaey[2*] and M. T. Fouad[3]

[1] Dairy Department, Faculty of Agriculture, Al Azhar University, Cairo, Egypt
[2] Dairy Department, Faculty of agriculture Mansoura University, Egypt
[3] Dairy Department, Division of Food Industries and Nutrition, National Research Centre, 12311 Doki, Giza, Egypt

ABSTRACT

Four enterococci isolated from raw milk samples grown on *Enterococcus* selective medium were identified on genus level by several conventional methods, and species levels using API 20 Strep. The incidence of virulence factors (antibiotics susceptibility, vancomycin resistance, haemolytic activity, production of gelatinase, aggregation substances, hydrophobicity and biofilm formation) were also examined for the isolated *Enterococcus* species. Results showed that the tested cultures grown on *Enterococcus* selective medium BEA showed similar behavior that all were able to grow at 10°C and 45°C, pH 9.6, NaCl 6.5%, tolerate growth in the presence of 40% bile and 0.04% sodium azide. They also survived 60°C/30 min. Four tested *Enterococcus spp.* Were found belonging to *E. faecium*, *E. durans* and *E. faecalis*, being one strain, two strains and one strain, respectively. *E.faecium* Rm1, *E.durans* Rm2 and *E.durans* Rm3 strains were found to be safe as they did not harbored any of tested virulence trait or multi antibiotic resistant. Results also showed that *E.faecalis* Rm4 was not safe as it harbored some of virulence factors and resisted multi antibiotics. Finally, *E.faecium* Rm1, *E.durans* Rm2 and *E.durans* Rm3 could be considered ideal strains, which could be used as adjunct or starter cultures as they were free from virulence determinants and sensitive to all antibiotic examined.

***Keywords*:** enterococci, raw milk, identification, virulence factors.

INTRODUCTION

Some species of lactic acid bacteria present in food 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 and *E. faecium* and *E. faecalis* are the two predominant species in human intestine (Bhardwaj *et al*., 2011) and (Giraffa. 2003).

Although enterococci are important in certain foods, certain strains may deteriorate dairy products *E. faecalis* and *E. faecium* species, for example are relatively heat resistant.

Several virulence factors such as aggregation substance, gelatinase, extracellular superoxide and extracellular surface protein have been regretted for enterococci particularly those associated with *E. faecalis* (Flouquie – Moreno *et al*., 2006; Mannu *et al*., 2003). In addition biofilm formation, hydrophobicity, cytolycin formation, gelatinase production, aggregation was also found in enterococci bacteria as virulence factors (Galli *et al*., 1990; Gilmore *et al*., 1994; Su *et al*., 1991; Kayaoglu and Orstavik. 2004 and Pillar and Gilmore. 2004). In addition, enterococci are known as adjunct or starter cultures, where they play an important role, thanks to their fermenting activity. Enterococci are also acknowledged as contributors to humans digestibility and therefore are additionally known for their role as probiotics (Franz *et al*., 2003).

Therefore, this research aims to study identification and incidence of virulence factors among isolated *Enterococcus* species from some raw milk samples. This may allow evaluation of the probable safety of strains intended for use as adjuncts and probiotics or starter cultures.

MATERIALS AND METHODS

Samples:

Raw milk samples were collected from Nasr city markets, Cairo and kept under sanitary conditions.

Experimental Procedures:

Isolation of *Enterococci*:

Typical colonies isolated from raw milk samples on bile aesculin azide agar (BEA), and transferred to TSA agar slants, which were incubated for 24hr. at37ºC under aerobic condition. A maximum of four typical colonies from each sample were retained for the complementary tests. After four successive transfers for purification on the same medium, a stock culture of each isolates 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 were transferred to new tubes of TSB, followed by incubation as previously described.

General characteristics of isolated *Enterococci*:

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 37ºC. One ml of each 18h culture was added to 9ml TSB broth and incubated at 10ºC. and 45ºC. and 60ºC 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 concentration of 40% by using bile salts and sodium azide to concentration of 0.04% by using sodium azide. Cultures were incubated at 37 ºC under aerobic condition for 24hr.

Identification on the species level:

The isolates of enterococci were identified using Rapid API 20 as mentioned in bioMerieux, Marcy-1'Etoile, France.

Sensitivity to antibiotics:

Four strains of enterococci were tested for their sensitivity to ampicillin (10µg), erythromycin (15µg), penicillin

* **Corresponding author.**
E-mail address:
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(10µg), nitrofurantion (300µg), streptomycin (10µg), chloramphenicol (30 µg), norfloxacin (10µg) and gentamicin (10µg). The examined overnight cultures were then streaked onto TSA medium. Plates were left to dry about 20 to 30 min. Antibiotic disks were put with sterilized forceps on the surface of the medium, and gently touched with the tip of forceps to assure proper contact. The plates were then incubated for 16-24 hr, at 37°C , zones were then examined for the inhibitory effect by measuring the zone of diameters(mm)

Vancomycin Resistance:

Four strains of enterococci were tested for their vancomycin resistance (10 µg, 20 µg and 30 µg). Overnight cultures of the tested isolates were streaked onto TSA medium. Plates were left to dry about 20 to 30 min. Vancomycin solution was placed in holes on the surface of agar medium with sterilized pipette. The plates were incubation was then carried out at 16 – 24 hr, at 37°C and then examined for zones of inhibition. Inhibition zones diameters were measured and recorded.

Haemolytic activity of enterococci:

Haemolytic activity of enterococcal strains was detected according to (Jurkovic *et al*., 2006).

Assay of gelatinase activity:

Gelatinize was performed according to (Cariolato *et al*., 2008).

Aggregation assay and Hydrophobicity studies:

The aggregation assay and cell surface hydrophobicity of the examined isolates were detected due to Fortina *et al*., (2008).

Biofilm formation:

Capability of biofilm formation by the examined enterococci was determined according to (Necidova *et al*., 2009).

RESULTS AND DISCUSSION

Identification of isolated Enterococci (on the genus level):

For identification of isolated bacteria grown on the Enterococci selective medium BEA, the classical phenotype – based (biotyping) method was followed (Domig *et al*. (2003). Physiological properties of enterococci species were growth at 10 and 45°C, growth in the presence of pH 9.6, 6.5% Nacl, 40% bile and 0.04% sodium azide, esculin hydrolysis and grow D antigen (Domig *et al*., 2003 and Mirtha. 2005). Similar results were obtained by , Jurkovic *et al*. (2006).

All isolated cultures (4 isolates) 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 the presence of 6.5% NaCl, pH 9.6 in combination with resistance to bile 40%, sodium azide 0.04% and survival of 60°C./30 min. All tested cultures grown on *Enterococcus* selective medium (BEA) showed similar behavior that all were able to grow at pH 9.6, NaCl 6.5% and at 10°C - 45°C, and resist the presence of 40% bile and 0.04% sodium azide, and could survive 60°C/30 min.

Identification on the species level:

Identification of four isolates of *Enterococcus* strains were characterized on account of their enzymatic activity and fermentation patterns using API 20 Strep.

The four tested *Enterococcus spp*. Were, generally, found to be belonging to one strain of *E. faecium*, two strains of *E. durans* and one strain of *E. faecalis*. El- Shafei *et al*., (2002), Dabiza *et al*., (2005).and Ayad *et al*., (2006) could get and identified many isolates belonging to the genus from Ras cheese during manufacture and ripening,

Results in Table (1) revealed that the biochemical tests of *Enterococcus* species resulted in positive reactions with VP, HIP, ESC, PYRA and ADH tests except *E. durans* Rm2, which gave negative reaction with HIP test. Four strains also gave negative reactions with αGAL, βGUR, βGAL, PAL and LAP tests.

Results of Table (1) also show that *E. faecium* Rm1 resulted in positive reactions with RIB, ARA, MAN, LAC and TRE tests and gave negative reactions with SOR, INU, RAF, AMD and GLYG tests. However, two strains of *E. durans* (Rm2 and Rm3) resulted in positive reactions with RIB, LAC and TRE tests, and gave negative reactions with ARA, MAN, SOR, INU, RAF, AMD and GLYG tests. *E. faecalis* Rm4 also resulted in positive reactions with RIB, MAN, SOR, LAC, TRE and AMD tests and gave negative reactions with ARA, INU, RAF and GLYG tests. These results came in agreement with those mentioned by Ali. (2011).

Table 1. Identification of isolated enterococci on species level.

Test	Active ingredients	Reactions /Enzymes	*E.faecium* Rm1	*E.durans* Rm2	*E.durans* Rm3	*E.faecalis* Rm4
VP	Sodium pyruvate	Acetoin production	+	+	+	+
HIP	Hippuric acid	Hydrolysis	+	-	+	+
ESC	Esculin ferric citrate		+	+	+	+
PYRA	Pyroglutamic acid β-naphthlamide	Pyrroliddonyl arylamidase	+	+	+	+
αGAL	6-bromo-2-naphthyl-αD-galactopyranoside	α-galactosidase	-	-	-	-
βGUR	Naphthol ASBI-glucuronic acid	β-glucuronidase	-	-	-	-
βGAL	2-naphthyl-βD-galactopyranoside	β-galactosidase	-	-	-	-
PAL	2-naphthyl phosphate	Alkaline phosphatase	-	-	-	-
LAP	L-leucine-β-naphthylamide	Leucine aminopeptidase	-	-	-	-
ADH	L-arginine	Arginine dihydrolase	+	+	+	+
RIB	D-ribose	Acidification	+	+	+	+
ARA	L-arabinose		+	-	-	-
MAN	D-mannitol		+	-	-	+
SOR	D-sorbitol		-	-	-	+
LAC	D-lactose		+	+	+	+
TRE	D-trehalose		+	+	+	+
INU	Inulin		-	-	-	-
RAF	D-raffinose		-	-	-	-
AMD	Starch		-	-	-	+
GLYG	Glycogen		-	-	-	-

Rm= Raw Milk

Virulence factors of isolated *Enterococcus* species:

Antibiotics susceptibility:

Data presented in Table (2) show the antibiotics susceptibility of *Enterococcus* species against the eight antibiotics the (ampicillin, erythromycin, penicillin, nitrofurantion, streptomycin, chloramphenicol, norfloxacin and gentamicin. Antibiotic resistance of *Enterococcus* is well documented (Fisher and Philips. 2009). The results presented here indicate that the

presence of antibiotic –resistance strains in Egyptian milk could not be considered as a potential source for spreading.

Results in Table (2) illustrate that the four *Enterococcus* species were sensitive to all of the examined antibiotics, except *E. faecalis* Rm4, which was resistant to erythromycin and streptomycin. *E. durans* Rm2 and *E. durans* Rm3 possessed an intermediate sensitivity against streptomycin, and *E. faecium* Rm1 and *E. faecalis* Rm4 characterized with the same degree of sensitivity to gentamicin.

E. durans Rm3 and *E. durans* Rm2 is the most sensitive (IZ ≥ 3cm) to ampicillin and penicillin. In general, the most effective antibiotics against *Enterococcus* species were ampicillin and pencillin(Cariolato *et al*., 2008).

Table 2. Antibiotics susceptible(S), intermediate (M) and resistant(R) patterns of *Enterococcus* species isolated from Raw milk.

Antibiotics	*Enterococcus* species (4 strains)						
	E.faecium Rm1	*E.durans* Rm2	*E.durans* Rm3	*E.faecalis* Rm4	Reaction		
	Inhibition zone/cm				R	M	S
Ampicillin	2.5	3.2	3.8	2.4	0	0	4
Erythromycin	2.1	2.4	2.5	0.6	1	0	3
Penicillin	2.3	3.0	3.4	1.8	0	0	4
Nitrofurantion	2.0	1.8	2.0	1.8	0	0	4
Streptomycin	1.5	1.0	1.4	0.6	1	2	1
Chloramphenicol	1.8	2.5	2.6	2.2	0	0	4
Norfloxacin	1.5	2.0	2.0	1.5	0	0	4
Gentamicin	1.2	1.5	1.5	1.2	0	2	2

R= Resistant (inhibition zone = 0.6 cm)
M=intermediate sensitivity (inhibition zone >0.6 – < 1.5 cm)
S= susceptible (inhibition zone 1.5 - > 1.5 cm)

Vancomycin resistance:

Vancomycin was first used in clinical arena in 1972 and the first vancomycin – resistant enterococci were recognized only 15 years later (Metan *et al*., 2005) and VRE were first detected in the UK and France in 1986 (Leclercq *et al*., 1988 and Uttely *et al*., 1988) and are an important cause of nosocomial infections worldwide (Koluman *et al*., 2009).

Susceptibility of *Enterococcus* species against different vancomycin concentrations; 10µg, 20µg and 30µg are presented in Table (3). Results in this Table cleared that four *Enterococcus* species were found sensitive to vancomycin concentrations. *E. faecium* Rm1 and *E. faecalis* Rm4 resulted in an intermediate sensitivity (inhibition zone >0.6 – < 1.5 cm) against vancomycin concentrations 10µg and 20µg, and *E.durans* Rm2 was of the same degree of sensitivity to vancomycin concentration 10µg.

VRE is considered to be serious problem. Over a 15 year period there was a 20 – fold increase in VRE associated with nosocomial infections reported to National Nosocomial Infections Surveillance (NNIS) (National Nosocomial Infections Surveillance, 2004), (Katie and Carol. 2009).

Table 3. Susceptibility of *Enterococcus* species against different vancomycin concentrations.

Vancomycin concentrations	*Enterococcus* species (4 strains)						
	E.faecium Rm1	*E.durans* Rm2	*E.durans* Rm3	*E.faecalis* Rm4	Reaction		
	Inhibition zone/cm				R	M	S
(10µg)	1.0	1.2	1.8	1.0	0	3	1
(20µg)	1.4	1.6	2.0	1.2	0	2	2
(30µg)	1.8	2.0	2.3	1.5	0	0	4

R= Resistant (inhibition zone = 0.6 cm)
M=intermediate sensitivity (inhibition zone >0.6 – < 1.5 cm)
S= susceptible (inhibition zone 1.5 - > 1.5 cm)

Haemolytic activity:

Haemolycin production can increase severity of enterococcal injections and presence of genes involved in haemolysin/cytolycin production is also considered a risk factor (Thacker *et al*., 1992 and Jett *et al*., 1994). Haemolycin plays an important role in enterococcal virulence as it may increase the chance of infection (Morandi *et al*., 2006).

Haemolytic activity of isolated enterococci from raw milk was studied. Results in Table (4) show the haemolytic activity of four *Enterococcus* strains. *E. faecium* Rm1 and *E. durans* Rm3 resulted in γ haemolytic activity. *E. durans* Rm2 showed α haemolytic activity while, *E. faecalis* Rm4 showed β haemolytic activity), however ,Yoon *et al*. (2008)stated that no haemolytic activity was observed for the *E.faecium* strains.

Production of gelatinase:

Results of Table (4) showed that none of the studied *Enterococcus species*, *E. faecium*, *E. durans* and *E. faecalis* were found to be abile to produce gelatinase. Similar results were obtained when studying 7 strains of *E. faecium* by Yoon *et al*. (2008). On the other hand, the presence of gelatinase production among food *E. faecalis* strains is high (Eaton and Gasson. 2001 and Franz *et al*., 2001). In contrast to *E. faecalis*, where 48 out of the 80 strains showed gelatinase activity and none of studied *E. faecium* produced gelatinase (Gomes *et al*., 2008).

The relation between an enterococcal gelatinize and virulence was stated by many authors. Accordingly, all our studied *Enterococcus* strains might be considered as safe and should be further studied for other different virulence factors.

Production of an aggregation substance:

Aggregation substances increase the hydrophobicity of the enterococcal surface, might induce localization of cholesterol to phagosomes and prevent or delay fusion with lysosomal vesicles (Mundy *et al*., 2000).

Data presented in Table (4) show the production of aggregation (% increase) in *E. faecium* Rm1, *E. durans* Rm2, *E. durans* Rm3 and *E. faecalis* Rm4 being, 22.54, 18.35, 17.62 and 24.15, respectively. In general, four strains of *Enterococcus* show moderate content of aggregation, being 17 – 30%. similarly Fortina *et al*.,(2008) cleared that the examined strains exhibited a moderate autoaggregation phenotype with values ranging from 17 to 30%.

Cell surface hydrophobicity:

Four isolated *Enterococcus spp*. from milk were tasted for their cell surface hydrophobicity (CSH) towards one hydrocarbon i.e xylene. It has been proposed that the presence of ESP could increase cell surface hydrophobicity and facilitate hydrophobic interaction Shankar *et al*. (1999). Results in Tables (4 and 5) indicate that presence (%) hydrophobicity (xylene adhesion) ranged from 57.30% to 64.15%. In general, four isolated *Enterococcus spp*. showed a strong affinity for xylene demonstring hydrophobic cell surface of these isolates as well as cell surface hydrophobicities of the studied enterococci ranged from 57.30% to 64.15%. Similar conclusion was recorded by Fortina *et al*. (2008) with their strains, which exhibited xylene adhesion ranged from 57% to 99%.

Biofilm formation:

Formation of biofilm on abiotic surface is an important criterion of virulence of *Enterococcus* (Donlan. 2002). A biofilm is an assemblage of microbial cells associated with a surface and enclosed in matrix of primarily polysaccharide material (Esther *et al*., 2007. and Tendolkar *et al*., 2006).

Results in Tables (4 and 5) show the biofilm formation by 4 *Enterococcus spp*. *E. faecium* Rm1, *E. durans* Rm2 and *E. durans* Rm3 strains were biofilm- negative, and the capability of biofilm formation was proven in *E. faecalis* Rm4. These results indicated that most studied *Enterococcus spp*.

strains were biofilm negative. These results agree with those claimed by Necidova *et al*., (2009).

Table 4. Haemolytic activities, gelatinase production, aggregation, hydrophobicity and biofilm formation of *Enterococcus* species.

Enterococcus species	Haemolysis	Gelatinase	Aggregation after 5 hour (%)	Hydrophobicity After 30 min (%)	Biofilm formation
E.faecium Rm1	γ	-	22.54	58.20	-
E.durans Rm2	α	-	18.35	60.45	-
E.durans Rm3	γ	-	17.62	57.30	-
E.faecalis Rm4	β	-	24.15	64.15	+

α- Haemolysis = a partial hydrolysis and greening zone. γ- Haemolysis = no reaction.
β- Haemolysis = clear zone of hydrolysis around the colonies.
Moderate of aggregation= (17-30%)

In this study, The opportunity in the present study to elucidate the incidence of virulence factors among isolated *Enterococcus* species from some local raw milk samples. This may allow evaluation of the probable safety of strains intended for use as adjuncts and probiotics or starter cultures. It is well known that the risk of enterococci has to be interpreted as a sum of several factors rather than individual trait. So, numbers of factors were studied for each isolate.

Generally, *E. faecium* Rm1, *E. durans* Rm2 and *E. durans* Rm3 strains were found to be safe as they did not harbored any of the tested virulence trait or multi antibiotic resistant. Result also showed that *E. faecalis* Rm4 was not safe as it harbored some of virulence factors and resist to multi antibiotics (Table 5). Confirmatory results were obtained by Yousif *et al*., (2005), who stated that most of the *E. faecium* strains in their study did not produce any of confirmed enterococcal virulence factors (Esp, enterococcal surface protein; Ace, adhesion to collagen; Cyl, cytolysin; As, aggregation substance; Gel, gelatinase; EfaAfm *E. faecium* endocarditis antigen).

Table 5. Virulence factors and antibiotic resistant of *Enterococcus* species.

Enterococcus species	Haemolysis	Gelatinase	Vancomycin resistant	Multi antibiotic resistant	Aggregation	Hydrophobicity (%)	Biofilm formation
E.faecium Rm1	-	-	-	-	-	58.20	-
E.durans Rm2	-	-	-	-	-	60.45	-
E.durans Rm3	-	-	-	-	-	57.30	-
E.faecalis Rm4	+	-	-	+	-	64.15	+

Finally, *E. faecium* Rm1, *E. durans* Rm2 and *E. durans* Rm3 strains could be considered as ideal and could be used as adjunct or starter cultures as they were free from virulence determinants and sensitive to all antibiotic examined.

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التعرف على أنواع *Enterococcus* ودراسة العوامل المرضية لها فى اللبن الخام

شريف عادل سعد عبداللا[1] ، محمود مصطفي محمد رفاعي[2]* و محمد توفيق فؤاد توفيق[3]

[1] قسم الألبان ـ كلية الزراعة ــ جامعة الأزهر ـ القاهرة

[2] قسم الالبان ـ كلية الزراعة ــ جامعة المنصورة ــمصر

[3] قسم الالبان ـ قسم الصناعات الغذائية والتغذية ، المركز القومي للبحوث ، 12311 الدقي ، الجيزة ، مصر

أظهرت البحوث فى السنوات الأخيرة أهمية سلالات enterococci لأنها تمتلك تأثير ثنائى. لهذا تم عزل enterococci من عينات اللبن الخام التى نمت على وسط تخصصى (أربع عزلات) و تعريفها إلى مستوى الجنس بإستخدام العديد من الطرق التقليدية ثم إلى مستوى النوع بإستخدام API 20 Strep. كذلك تمت دراسة تواجد العوامل المرضية وهى الحساسية للمضادات الحيوية، المقاومة للمضاد الحيوى vancomycin، نشاط التحلل الدموى، إنتاج gelatinase، التجمع، hydrophobicity، تكوين biofilm. لقد أظهرت النتائج أن عزلات enterococci أوضحت سلوك مماثل حيث كان لديها القدرة على النمو فى 10، 45 درجة مئوية، والنمو فى 6.5 % Nacl، 9.6 pH، والمقاومة لأملاح الصفراء بتركيز 40%، sodium azide بتركيز 04.% وايضا النمو عند درجة حرارة 60 درجة مئوية لمدة 30 دقيقة وبذلك تنتمى العزلات الأربع إلى جنس *Enterococcus*. كما أظهرت النتائج أن هذه السلالات تنتمى إلى أنواع *E. faecium*(سلالة واحدة), *E. durans*(سلالتين)، *E. faecalis*(سلالة واحدة). من حيث دراسة تواجد العوامل المرضية أظهرت النتائج أن سلالات Rm1 *E. faecium*، Rm2 *E. durans* ، Rm3 *E. durans* كانت امنة لأنها لا تشارك فى العامل المرضى المختبر أو المقاومة للعديد من المضادات الحيوية، كما أوضحت النتائج أن سلالة Rm4 *E. faecalis* كانت غير امنة لأنها تمتلك بعض العوامل المرضية ومقاومة للعديد من المضادات الحيوية. فى النهاية، تعتبر *E. faecium* Rm1، Rm2 *E. durans* ، Rm3 *E. durans* سلالات مثالية وتستخدم adjunct أو كبادئ لأنها خالية من العوامل المرضية وحساسة لكل المضادات الحيوية المختبرة.