Identification and Virulence Factors of Enterococcus Species Isolated from Raw Milk

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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 harbor 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 (Flouque – 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; Kayaoğlu 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 ascinulin agar (BEA), and transferred to TSA agar slants, which were incubated for 24hr. at37°C 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 bi-monthly transfers on trypticase soya agar (TSA), and stored at 4°C. All isolates were transferred before use from stock culture into trypticase soya broth (TSB), and incubated at 37°C 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 18th culture grown in TSB broth at 37°C. One ml of each 18th culture was added to 9ml TSB broth and incubated at 10°C, and 45°C. and 60°C for 24hr., 24hr and 30min, respectively.

One ml of each 18th 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°C 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-l’Etoile, France.

Sensitivity to antibiotics:

Four strains of enterococci were tested for their sensitivity to ampicillin (10µg), erythromycin (15µg), penicillin (2µg), and vancomycin (30µg). 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 harbor 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.
The aggregation assay and cell surface hydrophobicity of Enterococci were performed according to Cariolato 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 growth of 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 species were characterized on account of their enzymatic activity and fermentation patterns using API 20 Strept.

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. (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).
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 ≥ 3 cm) to ampicillin and penicillin. In general, the most effective antibiotics against Enterococcus species were ampicillin and penicillin (Cariolato et al., 2008).

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

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Enterococcus species</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. faecium</td>
<td>Edurans</td>
</tr>
<tr>
<td></td>
<td>Rm1</td>
<td>Rm2</td>
</tr>
<tr>
<td>Inhibition zone/cm</td>
<td>R</td>
<td>M</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>2.5</td>
<td>3.2</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>2.1</td>
<td>2.4</td>
</tr>
<tr>
<td>Penicillin</td>
<td>2.3</td>
<td>3.0</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>2.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>1.8</td>
<td>2.5</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>1.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>1.2</td>
<td>1.5</td>
</tr>
</tbody>
</table>

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 enterococcci 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 Utely 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 required 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.

<table>
<thead>
<tr>
<th>Vancomycin concentrations</th>
<th>Enterococcus species</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. faecium</td>
<td>Edurans</td>
</tr>
<tr>
<td></td>
<td>Rm1</td>
<td>Rm2</td>
</tr>
<tr>
<td>Inhibition zone/cm</td>
<td>R</td>
<td>M</td>
</tr>
<tr>
<td>(10µg)</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td>(20µg)</td>
<td>1.4</td>
<td>1.6</td>
</tr>
<tr>
<td>(30µg)</td>
<td>1.8</td>
<td>2.0</td>
</tr>
</tbody>
</table>

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)

Haemolysin/cytolytic production is also considered a risk factor (Thacker et al., 1992 and Jett et al., 1994). Haemolysin plays an important role in enterococcal virulence as it may increase the chance of infection (Morandi et al., 2006).

Haemolysin 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 haemolysin activity. E. durans Rm2 showed α haemolysin activity while, E. faecalis Rm4 showed β haemolysin activity. However, Yoon et al. (2008) stated that no haemolysin 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 able 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 tested 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 of hydrophobicity (xylene adhesion) ranged from 57.30% to 64.15%. In general, four isolated Enterococcus spp. showed a strong affinity for xylene demonstrating 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.**

<table>
<thead>
<tr>
<th>Enterococcus species</th>
<th>Haemolysis</th>
<th>Gelatinase</th>
<th>Aggregation after 30 min (%)</th>
<th>Hydrophobicity after 30 min (%)</th>
<th>Biofilm formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. faecium Rm1</td>
<td>γ</td>
<td>-</td>
<td>22.54</td>
<td>58.20</td>
<td></td>
</tr>
<tr>
<td>E. durans Rm2</td>
<td>α</td>
<td>-</td>
<td>18.35</td>
<td>60.45</td>
<td></td>
</tr>
<tr>
<td>E. durans Rm3</td>
<td>γ</td>
<td>-</td>
<td>17.62</td>
<td>57.30</td>
<td></td>
</tr>
<tr>
<td>E. faecalis Rm4</td>
<td>β</td>
<td>-</td>
<td>24.15</td>
<td>64.15</td>
<td>+</td>
</tr>
</tbody>
</table>

α- Haemolysis = a partial hydrolysis and greening zone.  
β- Haemolysis = a clear zone of hydrolysis around the colonies.  
γ- 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 harbor 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 Yousef et al., (2005), who stated that most of the E. faecium strains in their study did not produce any of confirmed enteroococcal 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.**

<table>
<thead>
<tr>
<th>Enterococcus species</th>
<th>Haemolysis</th>
<th>Gelatinase</th>
<th>Vancocin resistant</th>
<th>Multi antibiotic resistant</th>
<th>Aggregation</th>
<th>Hydrophobicity (%)</th>
<th>Biofilm formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. faecium Rm1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>58.20</td>
<td>-</td>
</tr>
<tr>
<td>E. durans Rm2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>60.45</td>
<td>-</td>
</tr>
<tr>
<td>E. durans Rm3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>57.30</td>
<td>-</td>
</tr>
<tr>
<td>E. faecalis Rm4</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>64.15</td>
<td>+</td>
</tr>
</tbody>
</table>

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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