Potravinarstvo, vol. 8, 2014, no. 1, p. 124-129
doi:10.5219/364
Received: 13 March 2014. Accepted: 23 March 2014.
Available online: 7 May 2014 at www.potravinarstvo.com

ISSN 1337-0960 (online)

SPECIES IDENTIFICATION OF ENTEROCOCCI BY BIOCHEMICAL TEST AND MOLECULAR-GENETIC METHODS

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ABSTRACT

The aim of this study was comparison different methods of species identification of enterococci. One hundred and fifty three suspected colonies were isolated from milk and dairy products (cheeses from cow´s, ewe´s and goat´s milk). On the bases of their growth on BEA agar, microscopic characteristic, results of Gram staining, catalase test and PYRAtest was thirty four isolates assigned to the genus *Enterococcus*. These isolates were identified by commercial biochemical test EN-COCCUS. 52.9% of them were included in species *E. faecalis*, 29.4% in *E. faecium*, 14.7% in *E. durans* and 2.9% in *E. group III*. This group includes 3 species: *E. durans, E. hirae, E. faecalis asaccharolytic var*. Then 16S rRNA sequencing nucleotide of all isolates was realized. Results of sequencing were compared with NCBI database. Only 14.7% of isolates were in 100% accordance. One from them was species *E. durans* and others were designated as *E. faecium.* For 20.6% of detected isolates was in accordance with more reference strains. Other isolates were identical with reference strain on 99%. For verification of all results species-specific PCR was used and 52.9% isolates were identified as species *E. faecalis*, 32.4% as *E. faecium* and 14.7% as *E. durans*. Strains belonging to the species *E. faecalis* were identified the most reliable by all used methods.

Keywords: enterococci; identification; EN-COCCUS test; PCR method

INTRODUCTION

Enterococci are frequently associated with many foods from animal (dairy and meat products) and vegetable origins **(Krebs-Artimová et al., 2013)**. The reason for the prevalence of enterococci in dairy products has long been considered as a result of unhygienic conditions during the production and processing of milk **(Fabianová et al., 2010)**. However, their presence in foods has often been shown to be unrelated with direct faecal contamination **(Krebs-Artimová et al., 2013)**. Enterococci are a bacterial group that is commonly found in a high population in a large number of traditional cheeses produced with raw or pasteurized milk **(Nieto-Arribas et al., 2011)**.

One possibly negative aspect of enterococci in cheeses is their ability to produce biogenic amines **(Valenzuela et al., 2008)**. On another side, enterococci belong to the probiotic microorganisms that are able to produce bacteriocins **(Čanigová et al., 2012)** and are component of starter culture **(Ducková et al., 2012)**. Several strains share interesting biotechnological traits and they have a positive effect on cheese flavour development, by means of citrate metabolism, proteolytic and lipolytic activity **(Serio et al., 2010)**. In spite of all this, the clinical research underlines that the safety of dairy products containing enterococci is an issue and the industry must carefully address before proceeding to their application for production of products **(Jamaly et al., 2010)**.

A lot of ways isolation, identification and confirmation of enterococci can be used. There are over 100 modifications of selective media for the isolation of enterococci from various specimens. Especially the BEA medium seems to be the best suited for selective enumeration since it still demonstrates sufficient selective properties, even in combination with other LAB bacteria (lactobacilli and pediococci) and bifidobacteria **(Domig et al., 2003)**. For genus identification phenotypic and biologic methods have conventionally been used. Biochemical methods and genotyping techniques have been recommended for taxonomical characterization.

The objective of the present study was to evaluate accuracy of identification molecular and biochemical methods for enterococci isolated from raw cow milk and dairy products.

MATERIAL AND METHODOLOGY

Isolation of enterococci.

Presence of enterococci in samples of raw cow milk from milk machines and dairy products (cheeses from cow´s, goat´s and ewe´s milk) was determined by cultivation at 37 ±1 °C on medium Slanetz-Bartley (HiMedia Laboratories, India) during 48 ±2 hours (**STN 56 0100, 1970**). Suspected colonies (n = 153) were isolated on selective medium containing bile, aesculin and azide - BEA agar (HiMedia Laboratories, India) during 24 ±2 hours at 37 ±1 °C. Bacteria of genus *Enterococcus* created creamy, pale gray or dark gray colonies with strong hydrolysis of aesculin on this medium.

Genus identification

The genus *Enterococcus* was confirmed by microscopic characteristic of colonies, Gram staining, production of catalase and pyrolidonylarylamidase enzyme. These microorganisms were Gram-positive, catalasa-negative

and PYRAtest positive (Lachema, Czech Republic) cocci that often occur in pairs or short chains.

Species identification

For species identification of enterococci isolates (n = 34) by means of EN-COCCUS test (Lachema, Czech Republic) the bacteria suspension from overnight culture was adjusted to equal the 2 McFarland standard with Densi-La-Meter (Pliva, Lachema Brno). This commercial method is based on biochemical reaction of arginine, sorbose, arabinose, mannitol, sorbitol, melibiose, raffinose and melezitose. Colored reactions were evaluated after 24 ±2 hours cultivation at 37 ±1 °C according to EN-COCCUS key (Analytic Profile Index).

Results of EN-COCCUS test were confirmed by 16S rRNA sequencing and species-specific PCR methods. DNA of enterococci was isolated by peqGOLD Bacterial DNA Kit (Peqlab, Germany) and concentration and purity was detected on spectrophotometer NanoDrop 2 000c spectrophotometer (Thermo Scientific, Germany). DNA sequencing was performed by primer amplification according to **di Cello et al. (1997)** and following purification of PCR product by Extra Mini Kit (5 prime, Germany). The nucleotide sequences were accomplished by commercial firm Eurofins (Austria). Results were evaluated by database NCBI (URL 1).

The PCR method for the species identification of enterococci isolates were performed using specific primers (Table 1). Mixture (25 µL) for *E. durans* and *E. hirae* identification was composed: 2.5 µl of PCR buffer 10 x concentrated (Finnzymes, Finland), 0.5 µl of dNTP with concentration 10 mM (Carl Roth, Austria), 0.5 µL of DynaZyme II (Finnzymes, Finland) with concentration 2 $U.\mu L^{-1}$, 18.5 µL of sterile water, 0.5 µL of each primer (25 $pmol.\mu L^{-1}$) and 1 µL of DNA template. Same mixture was used for identification of *E. faecalis* and *E. faecium*.

Optimal PCR program for each primer was described by **Arias et al. (2006)** and **Dutka-Malen et al. (1995)**.

Analysis of PCR products was performed on agarosa gel (2%) at 80 V and 200 mA during 50 minutes, staining by ethidium bromide and visualisation in UV light (ChemiDocTM XRS + System with Image Lab™, Software, Bio-rad Laboratories, USA).

RESULTS AND DISCUSSION

Of the 153 isolates from milk and dairy products were 34 included in *Enterococcus* genus.

Results of enterococci identification by three methods are summarized in Table 2.

On the basis on EN-COCCUS test 52.9% (n = 18) were identified as *E. faecalis*, 29.4% (n = 10) in *E. faecium*, 17.7% (n = 5) in *E. durans* and 2.9% (n = 1) in *E. group III* (*E. durans, E. hirae, E. faecalis asaccharolytic var.*). In samples from raw cow milk (n = 46) **Fabianová et al. (2010)** identified 56.5% *E. faecalis*, 19.5% *E. group III*, 15% *E. faecium*, 7% *E. mundtii* and 2% *E. casseliflavus* by same method. Also **Kročko et al. (2011)** determined *E. faecalis* (51.5%) as prevalence species followed *E. durans / E. hirae* (12%), *E. faecium* (11%), *E. mundtii* (2%), *E. casseliflavus* (1%) and *Enterococcus* spp. (22.5%) from total amount of enterococci (n = 101) isolated from cow milk. According to **Račková (2012)** is this method less accurate for identification of species *E. faecium*. **Brtkova et al. (2010)** stated that EN-COCCUS test are not able to recognize some enterococci, especially unusual species. EN-COCCUS test is able to identify only 19 enterococci species. For example lactose-negative strain of *E. faecalis* can be misidentified as *E. solitarius*. The problem is with ability of some strain of *E. faecium* to utilize sorbitol and identification is prolonged for next 1-2 days. Other problem with EN-COCCUS test is associated with individual personal experiences and manual reading of the results.

From this reason some authors use combination of biochemical and PCR method for enterococci identification. **Jurkovič et al. (2006)** found some discrepancies between results of enterococci identification from Bryndza cheese samples, obtained by commercial biochemical test and PCR method. Seven enterococci strains identified by commercial biochemical test were identified as *E. faecium* and by PCR method as *E. faecalis*. Three strains of *E. casseliflavus* were determined by PCR method as *E. faecium* (two strains) and *E. faecalis* (one strain).

Table 1 Primers used for species identification of enterococcal isolates

Species	Primer	Sequence (5´→ 3´)	Size of PCR product (bp)	References
E. durans	Mur-2ed/F	AAC AGC TTA CTT GAC TGG ACG C	177	**Arias et al., 2006**
	Mur-2ed/R	GTA TTG GCG CTA CTA CCC GTA TC		
E. hirae	MurG-F	GGC ATA TTT ATC CAG CAC TAG	521	**Arias et al., 2006**
	MurG-R	CTC TGG ATC AAG TCC ATA AGT GG		
E. faecium	Dut-F1	GCA AGG CTT CTT AGA GA	550	**Dutka-Malen et al., 1995**
	Dut-F2	CAT CGT GTA AGC TAA CTT C		
E. faecalis	Dut-E1	ATC AAG TAC AGT TAG TCT	941	**Dutka-Malen et al., 1995**
	Dut-E2	ACG ATT CAA AFC TAA CTG		

Table 2 Comparison biochemical and molecular genetic methods used for identification of enterococci

Isolate number	EN-COCCUS test	PCR methods	16S rRNA sequenation	
			Reference strain	Similarity
29	*E. durans*	*E. durans*	*E. durans (98D)*	99 %
			E. hirae (R)	99 %
			E. thailandicus (FP48-3)	99 %
96	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
98	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
99	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
100	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
101	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
108	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
110	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
114	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
118	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
125	*E. durans*	*E. durans*	*E. durans (98D)*	99 %
126	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
127	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
128	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
129	*E. durans*	*E. durans*	*E. durans (98D)*	99 %
131	*E. faecium*	*E. faecium*	*E. faecium (LMG 11423)*	100 %
132	*E. faecium*	*E. faecium*	*E. durans (98D)*	99 %
			E. hirae (R)	99 %
			E. ratti (ATCC 700914)	99 %
			E. thailandicus (FP48-3)	99 %
			E. faecium (LMG 11423)	99 %
			E. mundtii (ATCC 43186)	99 %
			E. villorum (88-5474)	99 %
133	*E. faecium*	*E. faecium*	*E. faecium (LMG 11423)*	100 %
134	*E. faecium*	*E. faecium*	*E. durans (98D)*	99 %
			E. hirae (R)	99 %
			E. ratti (ATCC 700914)	99 %
			E. thailandicus (FP48-3)	99 %
			E. faecium (LMG 11423)	99 %
			E. mundtii (ATCC 43186)	99 %
135	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
137	*E. group III*	*E. durans*	*E. durans (98D)*	100 %
138	*E. faecium*	*E. faecium*	*E. durans (98D)*	99 %
			E. hirae (R)	99 %
			E. ratti (ATCC 700914)	99 %
			E. faecium (LMG 11423)	99 %
			E. thailandicus (FP48-3)	99 %
			E. mundtii (ATCC 43186)	99 %
139	*E. faecium*	*E. faecium*	*E. faecium (LMG 11423)*	100 %
140	*E. faecium*	*E. faecium*	*E. durans (98D)*	99 %
			E. hirae (R)	99 %
			E. ratti (ATCC 700914)	99 %
			E. faecium (LMG 11423)	99 %
141	*E. faecium*	*E. faecium*	*E. faecium (LMG 11423)*	100 %

Continuation of the Table 2.

Isolate number	EN-COCCUS test	PCR methods	16S rRNA sequenation	
			Reference strain	Similarity
142	*E. faecium*	*E. faecium*	*E. durans (98D)*	99 %
			E. hirae (R)	99 %
			E. ratti (ATCC 700914)	99 %
			E. thailandicus (FP48-3)	99 %
			E. faecium (LMG 11423)	99 %
			E. mundtii (ATCC 43186)	99 %
143	*E. faecium*	*E. faecium*	*E. durans (98D)*	99 %
			E. hirae (R)	99 %
			E. ratti (ATCC 700914)	99 %
			E. faecium (LMG 11423)	99 %
			E. thailandicus (FP48-3)	99 %
			E. mundtii (ATCC 43186)	99 %
146	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
147	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
148	*E. durans*	*E. durans*	*E. durans (98D)*	99 %
149	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
150	*E. durans*	*E. faecium*	*E. faecium (LMG 11423)*	99 %
152	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %
153	*E. faecalis*	*E. faecalis*	*E. faecalis (JCM5803)*	99 %

Using of species-specific PCR was 52.9% isolates identified as *E. faecalis*, 32.4% as *E. faecium* and 14.7% as *E. durans*. One isolate previously identified by EN-COCCUS test as *E. durans* was identified by PCR method as *E. faecium*. Isolate no. 137 determined by EN-COCCUS test as *E. group III* was specified by PCR method as *E. durans*.

Also another authors **(Citak et al., 2006, Nieto-Arribas et al., 2011)** the most frequently identified *E. faecalis* (54.2%, 81.8%, respectively) from cow milk and cheeses by PCR method. However, from fresh cheeses **Pesavento et al. (2014)** identified mostly *E. faecium* (63.1%) followed *E. faecalis* (23.7%), *E. avium* (10.5%) and *E. durans* (2.63%).

According our results we can conclude that method of identification by 16S rRNA sequencing is not exact. Only 14.7% of isolates (no. 131, no. 133, no. 137, no. 139, no. 141) were in 100% accordance with reference strain. One of them was *E. durans* and four isolates were *E. faecium*. 20.6% of detected isolates was in accordance with more reference strains occurred in NCBI database. For example isolate no. 132 had similar nucleotide sequences with 7 reference strains and isolates no. 134, no. 138, no. 142, no. 143 with 6 reference strains and is not possible to exactly chosen only one species. On another side, strains that were detected by PCR methods were everytime confirmed. It may be explained by the 16S rRNA sequencing was performed in only one direction. If it was used two-sided sequencing, it would be possible to detect anomalies that may arise in the one direction sequencing and it would be more reliable in comparing sequences studied strains with reference strains in the database NCBI. In study of **Fei et al. (2006)** was found that one tested strain was phylogenetically closely related to *E. mundtii* (100% sequence similarity), *E. hirae* (99%) and *E. durans*, *E. faecium*, *E. azikeevi*, *E.villorum* (98%). They also stated that presently the acceptable standard is that if the similarity of strain under investigation and a reference strain sequences is 99-100%, they are regarded as belonging to the same species while if similarity is 97-98%, they are regarded as belonging to the same genus. According to this standard tested strain can belong to the species *E. mundtii*. **Nikolic et al. (2008)** used this method as a supplement rep-PCR, because two isolates of enterococci had same profile. By nucleotide sequence was shown that these isolates belong to the species *E. faecalis* with 99% similar to a reference strain. Results of other authors confirm that method 16S rRNA sequencing is suitable only as supplementary method for identification of enterococci.

The species E. faecalis was identified the most reliable by all three used methods. Suitable method for the identification of this species can be EN-COCCUS test. Problem was with identification of E. faecium and E. durans by commercial biochemical method and 16S rRNA sequencing. Therefore for the thorough identification of another species of enterococci (except E. faecalis) we recommend to use not only EN-COCCUS test but also PCR method.

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Acknowledgments:

This work was supported by grant VEGA grant no. 1/0679/13 from the Ministry of Education, Science, Research and Sport of the Slovak Republic.

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