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Occurrence of *Enterococcus* spp. in Waters

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ABSTRACT. We studied 630 bacterial strains isolated from surface waters and determined as enterococci on the basis of their growth on Slanetz–Bartley agar in typical colonies. The strains were tested and characterized by several key conventional tests for basic differentiation of enterococci and by commercial test kits. We identified 135 strains of *E. fæcium* (21 %), 115 *E. fæcalis* (18 %), 30 *E. mundtii* (5 %), 27 *E. hirae* (4 %), 22 *E. casseliflavus* (3 %), 21 *E. gallinarum* (3 %), 17 *E. durans–E. hirae* complex (3 %), 5 *E. durans* (1 %), and 1 strain of *E. avium*. 150 strains were classified only as *Enterococcus* sp. (25 %) and 107 strains (17 %) isolated from Slanetz–Bartley agar were not enterococci. We found that the non-enterococcal group consisted of other Gram-positive cocci and Gram-positive and Gram-negative rods. Based on the identification we tried to find a relation between taxonomic position of isolated strains and their colony morphology on Slanetz–Bartley agar. Out of the total of 523 identified enterococci, 345 strains (66 %) formed purple colonies, 136 red colonies (26 %), 37 pink colonies (7 %) and 5 cream colored colonies (1 %). There was no correlation among the color, size or colony morphology and the taxonomic characterization of enterococcal strains.

Although enterococci form part of the common microflora of men, they have been found as a frequent cause of a wide variety of infections in humans (Jett *et al.* 1994; Facklam and Sahm 1995; Devriese *et al.* 1992; Aguirre and Collins 1993). The recent interest in enterococci has been spurred on by the increasing antibiotic resistance which may soon render conventional chemotherapy inadequate for serious enterococcal infections (Facklam and Sahm 1995; Devriese *et al.* 1992; Dever *et al.* 1995). They have been also isolated as nosocomial pathogens (Jett *et al.* 1994; Herwaldt and Wenzel 1995). Enterococci naturally occur in large numbers in intestines of mammals, birds, reptiles or insects, and we can also find them in the environment. Besides, they can be associated with plants, some kinds of food, soil and can be isolated from water (Devriese *et al.* 1992; Leclerc *et al.* 1996).

Enterococci are important indicators of fæcal pollution of waters and their presence is commonly monitored during the microbiological testing of water. The detection of enterococci (and other "fæcal streptococci") in drinking water is carried out by membrane filtration (this method is most suitable for the examination of drinking and bottled water) or by enrichment in a liquid medium (Leclerc *et al.* 1996).

The aim of this work was the study of composition of enterococcal flora and species distribution in waters. We focused on species distribution of enterococci in waters and did not perform physical or chemical characterizations of the waters or the characterizations of sampling sites. The colony morphology of isolates on Slanetz–Bartley agar was compared with the identification results.

MATERIALS AND METHODS

Bacterial strains. A total of 630 bacterial strains were isolated during microbial analysis of surface waters in the *District Public Health Station*, Dobrá, Frýdek-Místek (Czech Republic). We studied 422 samples of various kinds of surface waters. The samples were collected weekly from various sampling sites over two years (1995–1996). The basic isolation of bacteria was done by membrane filtration of 10 mL water samples through Millipore filters (max. pore size 0.45 μm). Slanetz–Bartley agar (*HiMedia*, cat. no. M612), which contains NaN_3 as a selective agent for enterococci (Devriese *et al.* 1992; *Anonymous* 1991) was used for the cultivation of the filters. The incubation of Slanetz–Bartley agar plates was performed at 37 °C for 1 d. From all Petri plates 1–4 strains with different-but "enterococcal"-colony morphology (= red-maroon colonies) were picked up (*Anonymous* 1991). We tried to intercept all strains of enterococci. The purity of the isolated strains was checked by the streak plate technique on blood agar. The work was done during 1995–1996 and the strains were maintained on YGLP agar slant tubes (Jackman *et al.* 1990) or in Litmus Milk with Chalk (Jackman *et al.* 1990) at 4 °C until use.

Identification. From slant agars each isolate was subcultured onto horse or sheep blood agar and after checking the purity all isolates were tested by several conventional tests and by commercial

test kits. At first we checked if the isolated strains belong to genus *Enterococcus* by the following tests: Gram stain, appearance of colonies on blood agar, catalase production, growth at 10 °C, 45 °C and in 6.5 % NaCl broth (Facklam and Wilkinson 1981). Then we used the commercial test kits STREPTOtest, STREPTOtest 16 and STAPHYtest (*Lachema Co.*, Brno, Czech Republic) for basic differentiation of our isolates. Furthermore we used other key conventional tests for more accurate identification: acid production from ribitol (*formerly 'adonit'*), L-arabinose, galactitol (*'dulcit'*), maltose, melezitose, melibiose, rhamnose, ribose, glucitol (*'sorbit'*), L-sorbose, sucrose, D-turanose, trehalose, xylitol and D-xylose (Facklam and Wilkinson 1981); hydrolysis of tyrosine (Gordon *et al.* 1973); production of pyrrolidonyl arylamidase (PYRAtest, *Lachema*, cat. no. 1306711) and α-galactosidase (modified microtiter plate method, *Lachema*); motility (Barrow and Feltham 1993) and production of yellow pigment (Facklam and Collins 1989). Group D antigen (Streptococcal grouping kit, *Oxoid*, cat. no. DR 585A) was determined only for non *E. fæcalis* and non-*E. fæcium* strains. The spectrum of the conventional tests was chosen on the basis of identification results obtained by the commercial tests kits. Identification of isolated strains was done by the identification programme TNW (distributed by *Czech Collection of Microorganisms*, Brno) as well as *via* identification schemes described previously for enterococci (Facklam and Sahm 1995; Devriese *et al.* 1993; Facklam and Collins 1989; Ruoff 1995).

Cluster analysis. The cluster analysis was performed by the statistical program SPSS Professional Statistics 7.5 (*SPSS, Inc.*, Chicago, Illinois). The dendograms were created using average linkage (between groups) with the coefficient of Jaccard.

RESULTS AND DISCUSSION

The results of identification of the strains isolated from waters and belonging to the enterococci are summarized in Table I.

We determined 135 strains as *E. fæcium* (21 %), 115 as *E. fæcalis* (18 %), 30 as *E. mundtii* (5 %), 27 as *E. hirae* (4 %), 22 as *E. casseliflavus* (3 %), 21 as *E. gallinarum* (3 %), 17 as *E. durans–E. hirae* complex (3 %), 5 as *E. durans* (1 %), and 1 strain as *E. avium*. One hundred and fifty intermediate strains (25 %) were classified only to the genus *Enterococcus*. The remaining 107 strains (17 %) isolated from Slanetz–Bartley agar were not enterococci. We found Gram-positive cocci and Gram-positive and Gram-negative rods in this non-enterococcal group, but we did not identify those strains in detail.

Table I. Identification results of bacterial strains isolated from waters

Results of identification	Number of isolated strains
E. fæcium	135
E. fæcalis	115
E. mundtii	30
E. hirae	27
E. casseliflavus	22
E. gallinarum	21
E. durans–E. hirae complex	17
E. durans	5
E. avium	1
Enterococcus sp.	150
Non-enterococcal strains	107
Number of studied strains	630

Key tube tests used for checking isolated Gram-positive and catalase negative cocci belonging to genus *Enterococcus* were the following: growth at 10 °C, 45 °C and growth in 6.5 % NaCl broth. Growth on bile–esculin, on NaCl–esculin and esculin hydrolysis was tested by the STREPTOtest kit. All isolated enterococcal strains grew in 6.5 % NaCl broth tubes and on bile–esculin wells in STREPTOtest, hydrolyzed esculin and grew (except for 3 strains) at 10 °C. Those results agree with species descriptions of isolated species. Unfortunately 17 strains (2.7 %) of *Enterococcus* spp. did not grow at 45 °C. Negative growth at this temperature was only described for *E. dispar* (Collins *et al.* 1990), *E. sulfureus* (Martinez-Murcia and Collins 1991), *E. malodoratus* (Collins *et al.* 1984) and some strains of *E. flavescens* (Pompei *et al.* 1992). However, we found the test negative for 2 strains of *E. mundtii*, 1 strain of *E. gallinarum*, 1 strain of *E. fæcium* and 13 strains of *Enterococcus* sp. Although it was only a small number of isolates in proportion to the number of analyzed strains, which did not influence the differentiation of isolates, we wanted to note these results.

The phenotypic characteristics of some our isolates are thoroughly described in the following three tables. Table II shows the results of tests for the *E. gallinarum* strains, Table III describes non-

pigmented and non-motile strains, and Table IV shows various phenotypes of isolated yellow-pigmented strains. The dendograms under the tables show phenotypical comparison of the isolated strains.

Table II. Biochemical characterization of *E. gallinarum* strains[a]

	GLY	VPT	ARG	SOR	RAF	INU	45 °C
			E. gallinarum				
g1(1)[b]	−	+	−	−	+	−	+
g2(1)	−	+	−	−	+	+	−
g3(1)	−	+	+	−	−	−	+
g4(3)	−	+	+	−	+	−	+
g5(5)	+	−	−	−	+	+	+
g6(1)	+	−	−	+	+	+	+
g7(1)	+	+	−	−	+	−	+
g8(3)	+	+	−	−	+	+	+
g9(1)	+	+	−	+	+	−	+
g10(2)	+	+	−	+	+	+	+
g11(2)	+	+	+	−	+	+	+

[a]For test abbreviations see Table IV. All strains were positive for: 10 °C, BIE, ESL, LAC, MAN, MOT, NaCl, NAE, SUC, TRE; negative for: MLZ, TYR, YEP.
[b]Numbers in parentheses indicate the number of isolated strains.

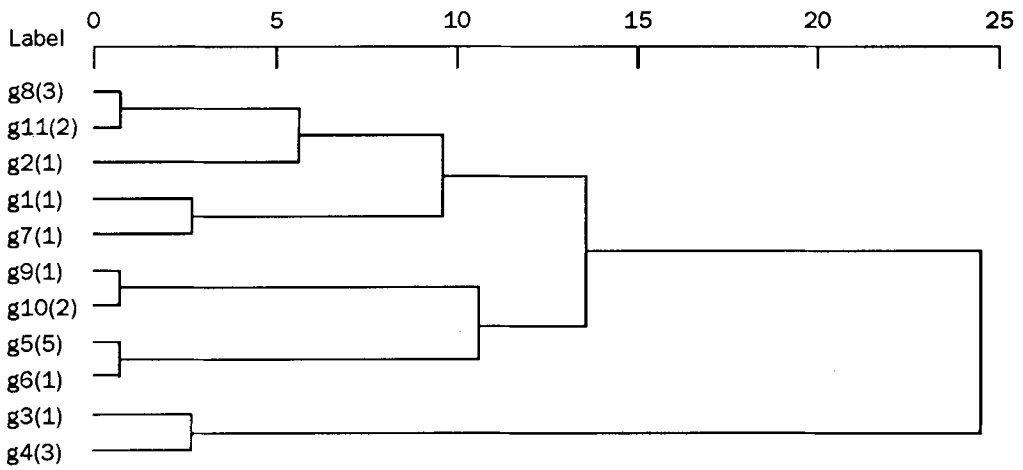

Fig. 1. Cluster analysis of *E. gallinarum* strains.

We had no problems with identification of motile and non-pigmented species (*E. gallinarum*). It was not difficult to classify clearly individual strains to the species level by biochemical tests. Cluster analysis of *E. gallinarum* (see Fig. 1) separated isolated strains into two groups. The strains labelled g3 and g4 differ from the others strains, but their biochemical characteristics allow us to identify the strains as *E. gallinarum*.

We found many intermediate phenotypes in the *E. fœcium–E. durans–E. hirae–E. avium* group. Fig. 2 shows the similarity of *E. durans–E. hirae–E. avium* strains. Isolated *E. avium* is clearly differentiated from the others strains, but isolated strains of *E. durans* and *E. hirae* show high similarity. There are many phenotypical similarities among the mentioned species (Devriese *et al.* 1993; Merquior *et al.* 1994). We also isolated many atypical non-motile and non-pigmented strains which were determined only as *Enterococcus* sp. For example, we isolated 17 strains which formed an intermediate group between *E. durans* and *E. hirae* species (*E. durans–E. hirae* complex). However, we did not find any biochemical test suitable for clear differentiation of these two species.

We did not find any misidentification among pigmented and/or motile species (*E. mundtii*, *E. casseliflavus*). We had no problem in classifying individual strains to the species level. Fig. 3 shows

Table III. Biochemical characterization of *E. avium* – *E. durans* – *E. hirae* strains[a]

	GLY	VPT	ARG	MAN	SOR	MLZ	RAF	SUC	ARA	MLB	aGA	ADO	DUL	TUR	RHA	XYL	XLT
E. avium																	
a1(1)[b]	–	+	–	+	+	+	–	+	+	–	–	+	+	+	+	–	+
E. durans																	
d1(1)	–	+	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–
d2(3)	–	+	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–
E. durans – atypical																	
da1(1)	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
E. hirae																	
h1(14)	–	+	+	–	–	–	–	+	–	+	+	–	–	–	–	–	–
h2(6)	–	+	+	–	–	–	+	+	–	+	+	–	–	–	–	–	–
h3(1)	–	+	+	–	–	–	+	+	–	+	+	–	–	+	–	+	–
h4(1)	+	+	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
h5(2)	+	+	+	–	–	–	–	+	–	+	+	–	–	–	–	–	–
h6(1)	+	+	+	–	–	–	+	+	–	+	+	–	–	–	–	–	–
E. hirae – atypical																	
ha1(1)	+	+	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–
ha2(1)	+	+	–	–	–	+	–	+	–	–	+	–	–	–	–	–	–
E. durans – *E. hirae* complex																	
d/h1(9)	–	+	+	–	–	–	–	–	–	+	+	–	–	–	–	–	–
d/h2(8)	+	+	+	–	–	–	–	–	–	+	+	–	–	–	–	–	–

[a]For test abbreviations see Table IV. All strains were positive for: 10 °C, BIE, Datg, ESL, LAC, MLT, NaCl, NAE, PYR, RIB, TRE, TYR; negative for: INU, MOT, SOE, YEP.
[b]Numbers in parentheses indicate the number of isolated strains.

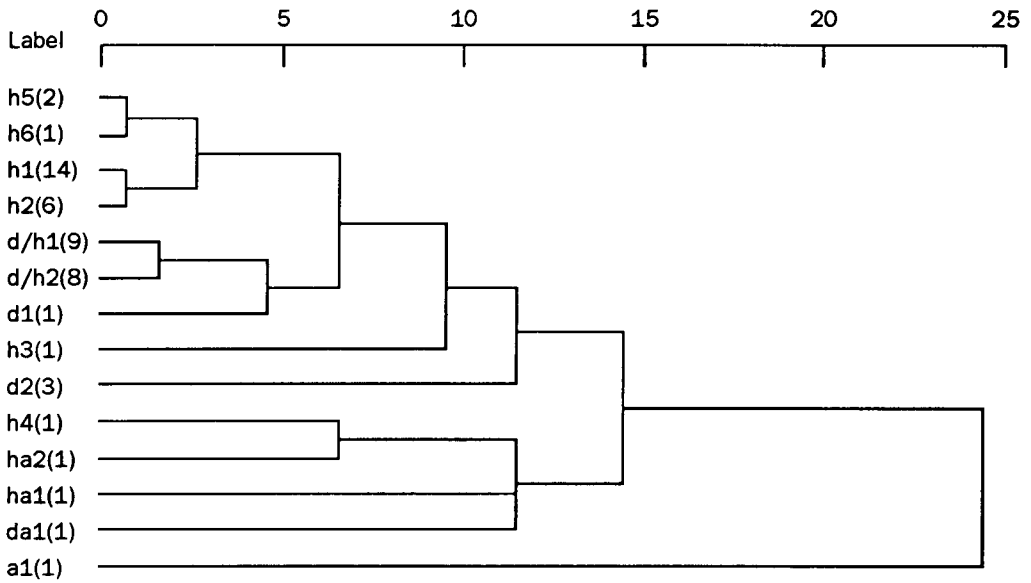

Fig. 2. Cluster analysis of *E. avium–E. durans–E. hirae* strains.

cluster analysis of yellow pigmented species. The two isolated species are clearly distinguished. The ability of isolates to produce yellow pigment could not be observed directly on the blood agars. It was discovered on the loop immediately after picking up the culture from the plating medium.

We found a relatively wide spectrum of atypical enterococcal strains which had different characteristics on comparison with species descriptions and it was difficult to classify these strains (*results not shown*). The high occurrence of the atypical strains probably depends on the source of isolation, because we did not find so many atypical strains among isolates from human infections in our previous study (Švec *et al.* 1996). Also, the genus *Enterococcus* is still being studied and reclassified and new groups of enterococci with uncertain taxonomic position which differ from the species descriptions have been isolated (Leclerc *et al.* 1996; Devriese *et al.* 1993).

As mentioned above we isolated a relatively wide spectrum of *Enterococcus* spp. Except for the common and the best known species — *E. fæcalis* and *E. fæcium*, which are commonly isolated from clinical materials as well as from environmental samples or food, we identified many less frequently common species, which are rarely isolated and identified. We suppose that these *E. mundtii*, *E. casseliflavus*, *E. gallinarum*, *E. hirae* and *E. durans* species form an important part in water environment and that the "rare" species usually occur in waters. Similar results were obtained in other studies, although the results show distribution of enterococci only in sewage or waste waters. Valdivia *et al.* (1996) isolated 45 strains of enterococci from municipal waste water; 32 of them were *E. fæcalis*, 10 isolates belonged to *E. fæcium* and 3 to *E. hirae*. Pourcher *et al.* (1991) analyzed 37 enterococcal strains isolated from urban raw sewage. They identified 20 strains of *E. fæcalis*, 13 strains of *E. fæcium* and 4 strains of the *E. durans–E. hirae* complex. Finally, Lauková and Jurič (1997) analyzed 2000 strains isolated from municipal sewages. Fifty % of these were *E. fæcium* strains, 15.5 % *E. gallinarum* strains, 10.1 % *E. casseliflavus* and 14.4 % *Enterococcus* sp. In addition, they found that the location of the source of isolation and the sewage composition did not affect the enterococcal species distribution. The results listed above show that *E. fæcalis* and *E. fæcium* form predominant enterococcal species in water environments, although the other enterococci can be isolated as well.

After identification we tried to find some correlation among the taxonomic position of enterococcal strains and the color, size or form of their colonies on Slanetz–Bartley agar. We did not find any relation. From a total of 523 identified enterococci, 345 strains (66 %) formed purple colored colonies, 136 red colonies (26 %), 37 pink colonies (7 %) and 5 cream colored colonies (1 %) if they grew on Slanetz–Bartley agar at 37 °C. Very similar results were obtained for separate species (*results not shown*) with the exception of *E. fæcium*. Colony morphology of *E. fæcium* was somewhat different from the colonies formed by the other isolated species, because we found a very wide spectrum of various combinations of colors, sizes or forms of colonies that were formed by those species on Slanetz–Bartley agar. Identification of isolates as *E. fæcium* may not be as reliable because of phenotypic similarities among *E. fæcium* and other species (Leclerc *et al.* 1996; Devriese *et al.* 1993; Merquior *et al.* 1994). We think that the taxonomic position and description of *E. fæcium* is not strict and that our

isolates which have been determined as *E. fæcium* could include other enterococci. Although colony morphology is not such an important taxonomic trait, our atypical results may support this idea.

Table IV. Biochemical characterization of yellow-pigmented strains[a]

	MOT	YEP	ARG	ARA	SOR	MLB	RAF	MLZ	VPT	TYR	INU	RIB	XYL	GLY	45 °C
E. casseliflavus															
c1(1)[b]	+	+	−	+	−	+	+	−	−	−	+	−	+	+	+
c2(1)	+	+	−	+	−	+	+	−	+	−	+	−	+	+	+
c3(1)	+	+	−	+	−	+	+	−	+	−	+	+	−	+	+
c4(2)	+	+	−	+	−	+	+	−	+	−	+	+	+	+	+
c5(1)	+	+	−	+	+	+	+	−	−	−	+	+	+	+	+
c6(1)	+	+	−	+	+	+	+	−	+	−	+	+	−	+	+
c7(3)	+	+	−	+	+	+	+	−	+	−	+	+	+	+	+
c8(1)	+	+	+	+	−	+	+	−	+	−	+	−	+	−	+
c9(1)	+	+	+	+	−	+	+	−	+	−	+	−	+	+	+
c10(1)	+	+	+	+	−	+	+	−	+	−	+	+	−	−	+
c11(1)	+	+	+	+	−	+	+	−	+	−	+	+	+	−	+
c12(4)	+	+	+	+	−	+	+	−	+	−	+	+	+	+	+
c13(1)	+	+	+	+	+	+	+	−	+	−	+	+	+	−	+
c14(2)	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+
c15(1)	+	+	+	+	+	+	+	+	+	−	+	+	−	+	+
E. mundtii															
m1(1)	−	+	+	−	−	+	−	−	+	−	−	+	+	+	+
m2(1)	−	+	+	+	−	−	−	−	+	−	−	+	+	+	−
m3(1)	−	+	+	+	−	−	−	−	+	+	−	+	+	+	+
m4(1)	−	+	+	+	−	+	−	−	+	−	−	+	−	+	+
m5(1)	−	+	+	+	−	+	−	−	+	−	−	+	+	−	+
m6(3)	−	+	+	+	−	+	−	−	+	−	−	+	+	+	+
m7(1)	−	+	+	+	−	+	−	−	+	−	+	+	+	+	+
m8(4)	−	+	+	+	−	+	−	−	+	+	−	+	+	−	+
m9(3)	−	+	+	+	−	+	−	−	+	+	−	+	+	+	+
m10(1)	−	+	+	+	−	+	+	−	+	−	−	+	+	−	+
m11(1)	−	+	+	+	−	+	+	−	+	−	−	+	+	+	+
m12(1)	−	+	+	+	−	+	+	−	+	+	−	+	+	−	+
m13(3)	−	+	+	+	−	+	+	−	+	+	−	+	+	+	+
m14(1)	−	+	+	+	+	+	−	−	+	−	−	+	+	+	+
m15(2)	−	+	+	+	+	+	−	−	+	+	−	+	+	+	+
m16(1)	−	+	+	+	+	+	−	+	+	−	−	+	+	−	−
m17(1)	−	+	+	+	+	+	+	−	+	−	−	+	−	+	+
m18(1)	−	+	+	+	+	+	+	−	+	−	−	+	+	+	+
m19(2)	−	+	+	+	+	+	+	−	+	+	−	+	+	+	+

[a]Acidification of: ADO − ribitol (*formerly 'adonit'*), ARA − L-arabinose, DUL − galactitol (*'dulcit'*), GLY − glycerol, INU − inulin, LAC − lactose, MAN − mannitol, MLB − melibiose, MLT − maltose, MLZ − melezitose, MNE − mannose, RAF − raffinose, RHA − rhamnose, RIB − ribose, SOE − sorbose, SOR − glucitol (*'sorbit'*), SUC − sucrose, TRE − trehalose, TUR − D-turanose, XLT − xylitol, XYL − xylose; growth: at 10 °C, at 45 °C, BIE − in bile-esculin, NaCl − in 6.5 % NaCl broth, NAE − in NaCl-esculin; production of: aGA − α-galactosidase, ARG − arginine dihydrolase, PYR − pyrrolidonyl arylamidase, YEP − yellow pigment; hydrolysis of: ESL − esculin, TYR − tyrosine; Datg − streptococcal D group antigen, MOT − motility, VPT − Voges-Proskauer test. All strains were positive for: 10 °C, Datg, NaCl, NAE, BIE, ESL, PYR, MAN, TRE, LAC, SUC, MLT, MNE; negative for: SOE.

[b]Numbers in parentheses indicate the number of isolated strains.

To maintain isolates over long periods we used at first YGLP agar slant tubes; the medium was not convenient for the maintenance of enterococci. During a short time we lost nearly all strains of *E. gallinarum* (that is why we did not characterize these strains by more tests) and some strains of the other species. We therefore used Litmus Milk with Chalk which is also recommended for enterococci. This medium was better suitable for the preservation of enterococci than YGLP agar. We detected

very low viability of *E. gallinarum* strains during the maintenance on YGLP slant agar but we have no explanation for this fact.

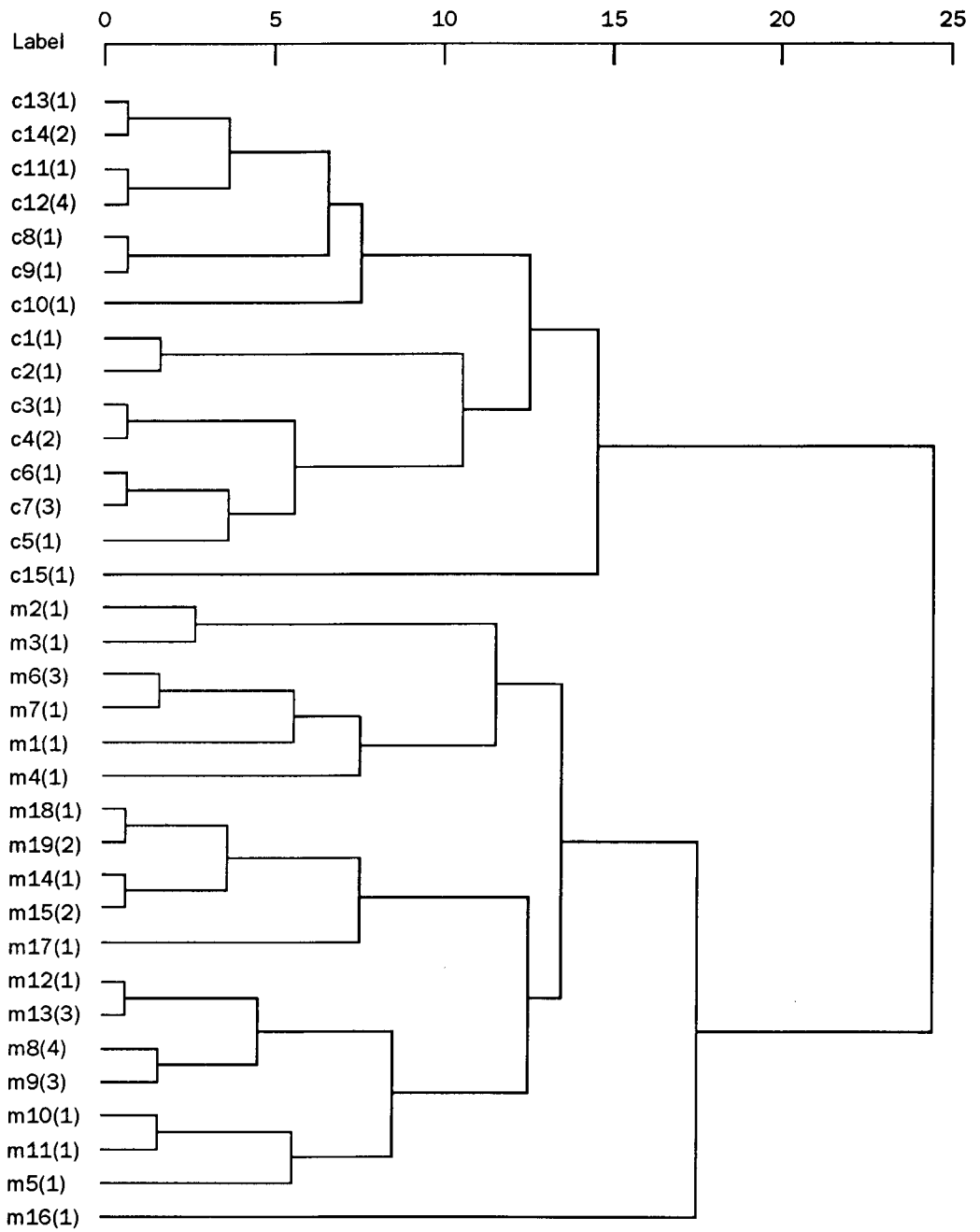

Fig. 3. Cluster analysis of *E. casseliflavus–E. mundtii* strains.

The results of our work can be summarized in three main points: (*i*) there is a wide spectrum of enterococcal species in surface waters; (*ii*) we isolated a lot of atypical strains; and finally (*iii*) we did not find any correlation between the taxonomic position of enterococcal strains and their colony morphology on Slanetz–Bartley agar.

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