Enterococcus lactis sp. nov., from Italian raw milk cheeses

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Ten atypical *Enterococcus* strains were isolated from Italian raw milk cheeses. The 16S rRNA gene, phenylalanyl-tRNA synthase alpha subunit (*pheS*), RNA polymerase alpha subunit (*rpoA*) and the 16S–23S rRNA intergenic transcribed spacer (ITS) sequences, randomly amplified polymorphic DNA (RAPD) PCR and the phenotypic properties revealed that the isolates represent a novel enterococcal species. On the basis of 16S rRNA gene sequence analysis, the isolates were closely related to *Enterococcus hirae* ATCC 8043^T, *Enterococcus durans* CECT 411^T and *Enterococcus faecium* ATCC 19434^T, with 98.8, 98.9 and 99.4 % sequence similarity, respectively. On the basis of sequence analysis of the housekeeping gene *pheS*, the reference strain, BT159^T, occupied a position separate from *E. faecium* LMG 16198. The group of isolates could be easily differentiated from recognized species of the genus *Enterococcus* by 16S–23S rRNA ITS analysis, RAPD-PCR and phenotypic characteristics. The name *Enterococcus lactis* sp. nov. is proposed, with BT159^T (=DSM 23655^T=LMG 25958^T) as the type strain.

Enterococci belong to a group of organisms known as lactic acid bacteria and large numbers are usually present in vegetables, plant material and foodstuffs, especially those of animal origin such as dairy products (Giraffa, 2003; Fisher & Phillips, 2009). Studies on the microbiota of traditional cheeses of Mediterranean countries, produced mainly from raw milk from sheep, goats or cows, indicate that enterococci are a relevant component of the natural cultures involved in fermentation and that they play an important role in cheese ripening, hence contributing to the cheeses' typical taste and flavour (Foulquié Moreno *et al.*, 2006). In addition, enterococci are also used to extend the shelf life and improve the hygienic safety of foodstuffs because they produce antimicrobial substances such as lactic acid, hydrogen peroxide and bacteriocins (enterocins). Bacteriocins have become of great interest as they are frequently active against several Gram-positive, food-borne pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus* and *Clostridium botulinum* and it has been suggested that they should be used as biopreservatives in foods (Franz *et al.*, 2007). Enterococci have also been investigated for their potential as probiotics, a role that is still controversial as some strains

isolated from foods and human samples have been associated with human infections, several virulence factors and antibiotic resistance including resistance to glycopeptide antibiotics (vancomycin) (Foulquié Moreno *et al.*, 2006).

Since the revival of the genus *Enterococcus* by Schleifer & Kilpper-Bälz (1984), phylogenetic studies based on 16S rRNA gene sequences have established the presence of some species groups, namely '*faecium*', '*faecalis*', '*avium*', '*gallinarum*' and a fifth group comprising *Enterococcus columbae* and *Enterococcus cecorum*. Other described enterococci form individual lines of descent (Devriese *et al.*, 1993; Devriese & Pot, 1995; Stiles & Holzapfel, 1997; Fortina *et al.*, 2004; Fisher & Phillips, 2009). To date, more than 30 species have been added to the genus *Enterococcus* on the basis of phylogenetic evidence. Phenotypically, no definite characteristics distinguish enterococci unequivocally from other Gram-positive, catalase-negative, coccus-shaped bacteria or within the genus at the species level. Thus, it is desirable to use a polyphasic approach to correctly identify 'unusual' enterococcal strains (Fortina *et al.*, 2004).

During a study on the autochthonous microflora of an Italian raw milk cheese (Bitto), we isolated some atypical *Enterococcus* strains that appeared to represent a novel species of the genus *Enterococcus* (Morandi *et al.*, 2011). Similar strains had been observed a few years ago in sour milk products in Stavropol' Krai (Botina & Sukhodolets, 2006) but our present paper describes 10 strains isolated

Abbreviations: ITS, intergenic transcribed spacer; RAPD, randomly amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BT159^T is GU983697.

A supplementary figure is available with the online version of this paper.

from Bitto cheese; results of our polyphasic study indicate that these 10 strains represent a novel species.

Strains BT159^T, BT160, BT161, BT171, BT188, BT190, BT204, BT218, BT219 and BT220 were isolated from cheese samples using M17 agar, de Man Rogosa and Sharpe (MRS) agar and kanamycin aesculin azide (KAA) agar (Scharlau Microbiology). They were routinely maintained at 4 °C after growth at 37 °C for 18 h in M17 broth (Scharlau Microbiology). Cell morphology, Gram staining, catalase activity, hydrolysis of aesculin and production of gas from glucose were tested as reported by Morandi *et al.* (2011). The effects of temperature (10–45 °C), initial pH (pH 9.6) and NaCl concentration (2, 4 and 6.5 %) were determined in M17 broth. Moreover, growth on KAA agar, haemolysis on defibrinated sheep blood agar (Merck) and growth in litmus milk were determined. Lipolytic activity on tributyrin agar and hydrolysis of gelatin were tested as reported by Morandi *et al.* (2006) and Harrigan (1998), respectively. Biochemical tests were also performed using the API 20 STREP and API 50 CHL systems (bioMérieux), according to the manufacturer's instructions. Acid production was determined using bromocresol purple (0.002 %, w/v) in 1 % (w/v) peptone water as indicator. Susceptibility to vancomycin was evaluated by the disc-diffusion method on Mueller–Hinton agar (Biolife) with antibiotic discs containing 30 µg vancomycin (Oxoid), according to the Clinical and Laboratory Standards Institute (CLSI, 2007).

DNA was isolated and purified using the method of Cremonesi *et al.* (2006). PCR amplification of 16S rRNA was obtained as reported by Edwards *et al.* (1989). Multilocus sequence analysis based on partial sequences for genes encoding the phenylalanyl-tRNA synthase alpha subunit (*pheS*) and the RNA polymerase alpha subunit (*rpoA*) were detected as described by Naser *et al.* (2005). Sequencing of the 16S rRNA gene, *rpoA* and *pheS* was provided by the sequencing service of Primms (Milan, Italy) as previously described (Morandi *et al.*, 2011). Sequence similarity searches were performed using BLAST in the GenBank database. The sequence information was then imported into CLUSTAL W version 2 for assembly and alignment. The 16S rRNA gene, *rpoA* and *pheS* sequences of strain BT159^T were compared with those of the most closely related species retrieved from GenBank. Amplification of the 16S–23S rRNA intergenic transcribed spacer (ITS) was performed as reported by Jensen *et al.* (1993). The PCR products were quantified using a BioAnalyser 2100 applied to the DNA 1000 LabChip kit (Agilent Technologies). Randomly amplified polymorphic DNA (RAPD) analysis was carried out using primers M13, D11344 and D8635 and conditions as previously described (Andrighetto *et al.*, 2002; Morandi *et al.*, 2006). Grouping of the RAPD-PCR patterns was obtained with BioNumerics version 5.0 (Applied Maths) using cluster analysis with unweighted pair group method using arithmetic averages; the value for repeatability of the RAPD-PCR assay, DNA extraction and running conditions was 90 %.

Cellular fatty acid composition was determined according to the procedure of Miller & Berger (1985). The MIDI protocol for standardization of the physiological age of cells was applied (http://www.microbialid.com/PDF/TechNote_101.pdf). About 40 mg bacterial cells were saponified with 1 ml basic methanol (45 g NaOH dissolved in 300 ml deionized water:methanol, 1/1, v/v); tubes containing the saponified mixture were vortexed for 5–10 s and kept in a boiling water bath for 30 min. After cooling to room temperature, 2 ml 6 M HCl/methanol (325:275, v/v) was added and the sample was heated in an 80 °C water bath for 10 min. The sample was cooled rapidly, 1.25 ml methyl *tert*-butyl ether/hexane (1:1, v/v) was added and the tube was turned end over end for about 10 min. The lower aqueous phase was discarded by pipetting, 3 ml 0.3 M NaOH was added and the tube was turned end over end for 5 min. Then, the organic phase was transferred into a 2 ml glass vial. Fatty acid methyl ester analysis was performed using a HP 6890 series GC (Agilent) equipped with a Supelcowax 10 capillary column (30 m length, 0.32 mm i.d., 0.25 µm film thickness; Supelco), a split–splitless injector at 250 °C and a flame-ionization detector at 250 °C. One microlitre was injected in splitless mode. Hydrogen was used as the carrier gas at a constant flow of 1 ml min⁻¹. The oven temperature was held at 40 °C for 4 min, increased to 150 °C at a rate of 25 °C min⁻¹, held at 150 °C for 1 min, increased to 220 °C at a rate of 4 °C min⁻¹ and held at 220 °C for 5 min. Identification of individual fatty acid methyl esters was performed by analysing both the certified reference material CRM 164 (Commission of the European Communities, 1993) and the Bacterial Acid Methyl Esters standard mixture (Supelco). Values were expressed as relative percentages of the total fatty acid content.

The isolates were Gram-positive, spherical or ovoid cells occurring in pairs or short chains, catalase-negative, non-motile and non-endospore-forming. The isolates grew on KAA agar, showed β-haemolysis on defibrinated sheep blood and were able to grow in litmus milk, in which they caused reduction and clotting within 24 h of incubation. All of the isolates were susceptible to vancomycin. Growth occurred at 10 and 45 °C and pH 9.6. The isolates grew in M17 broth containing 6.5 % NaCl, which is in accordance with the genus *Enterococcus*. The species description gives a detailed description of other characteristics. Biochemical tests useful for differentiating the novel isolates from other enterococci are given in Table 1.

The almost-complete 16S rRNA gene sequence of strain BT159^T obtained in this study (1437 bp) indicated that the isolate belonged to the genus *Enterococcus* and was closely related to *Enterococcus hirae* ATCC 8043^T (98.8 %), *Enterococcus durans* CECT 411^T (98.9 %) and *Enterococcus faecium* ATCC 19434^T (99.4 %). Lower sequence similarities (<98.5 %) were found with other recognized species of the genus *Enterococcus*. Strain BT159^T belonged to the *E. faecium* species group, comprising *E. faecium*, *E. durans*, *E. hirae*, *Enterococcus mundtii* and *Enterococcus villorum*. The multi-locus sequence analysis based on *rpoA* and *pheS* for the

Table 1. Biochemical tests useful for the differentiation of *Enterococcus lactis* sp. nov. from other enterococci

Taxa: 1, *Enterococcus lactis* sp. nov.; 2, *E. durans*; 3, *E. faecalis*; 4, *E. faecium*; 5, *E. gilvus*; 6, *E. italicus*; 7, *E. hirae*; 8, *E. casseliflavus*; 9, *E. mundtii*. Data for columns 2–9 were taken from Collins *et al.* (1984, 1986), Farrow & Collins (1985), Tyrrell *et al.* (2002), Klein (2003), Fortina *et al.* (2004) and Tanasupawat *et al.* (2008). All taxa are positive for acid production from lactose. +, Positive with rare exceptions; w, weakly positive; v, variable; –, negative with rare exceptions; NR, not reported.

Characteristic	1	2	3	4	5	6	7	8	9
Growth at/with:									
10 °C	+	+	+	+	+	w	+	w	+
45 °C	+	+	+	+	+	v	+	+	+
6.5 % NaCl	+	+	+	+	+	–	+	+	+
Acid production from:									
L-Arabinose	+	–	–	+	+	–	–	+	+
Glycerol	–	–	+	+	+	–	–	–	v
Mannitol	+	–	+	+	+	v	–	+	+
Melibiose	+	–	–	v	+	–	+	+	+
Melezitose	–	–	+	–	NR	–	v	–	–
Raffinose	–	–	–	–	+	–	+	v	+
Ribose	+	+	+	+	+	–	+	+	+
Salicin	+	+	+	+	+	+	–	+	+
Sorbitol	–	–	+	v	+	v	–	–	v
Sucrose	–	–	+	v	+	+	+	+	+
Xylose	–	–	–	–	–	–	–	+	+

identification of *Enterococcus* species (Naser *et al.*, 2006) confirmed the separation of strain BT159^T from species of the genus *Enterococcus*. The sequences of *rpoA* (675 bp) and

pheS (455 bp) of strain BT159^T showed 98 and 95 % similarity, respectively, with *E. faecium* LMG 16198. The isolates produced a characteristic 16S–23S rRNA ITS pattern compared with other members of the genus *Enterococcus* (Fig. 1). The isolates demonstrated genetic homogeneity and were separated from the reference strains by the presence of six bands of 1288, 588, 555, 490, 315 and 250 bp. Moreover, the RAPD-PCR analysis indicated a distinct clustering of the 10 isolates (coefficient similarity 25 %; Fig. S1, available in IJSEM Online).

The fatty acid composition of the isolates was compared with those of the type strains of *E. faecium* and *E. durans*, their closest phylogenetic neighbours. The major fatty acids of the isolates were C_{16:0} (30.0 %), C_{19:0} cyclo 9c (20.3 %), C_{18:1} (17.5 %), C_{16:1} (15.4 %) and C_{14:0} (10.4 %). In addition, small amounts of C_{17:0} cyclo (2.2 %) and C_{18:0} (0.8 %) were found. *E. faecium* contained the same fatty acids but with some quantitative differences: smaller amounts of C_{16:0} (23.7 %) and C_{17:0} cyclo (1.4 %) and a larger amount of C_{19:0} cyclo 9c (26.6 %). *E. durans* contained smaller amounts of C_{16:1} (10.5 %), C_{14:0} (9.1 %) and C_{17:0} cyclo (0.6 %) and a larger amount of C_{18:0} (2.3 %); moreover, *E. durans* showed the presence of small amounts of odd-numbered and branched-chain fatty acids that were not found in the isolates.

All of the data from the present study suggest that the isolates should be assigned to a novel species of the genus *Enterococcus*, for which we propose the name *Enterococcus lactis* sp. nov.

Description of *Enterococcus lactis* sp. nov.

Enterococcus lactis (lac'tis. L. gen. n. *lactis* from milk, referring to dairy products, from which the species was first isolated).

Fig. 1. ITS analysis of *Enterococcus lactis* sp. nov. and other enterococcal species. Lanes: M, DNA ladder (DNA 500 LabChip Kit; Agilent Technologies); 1, *Enterococcus hirae* ATCC 8043^T; 2–4, *Enterococcus lactis* sp. nov. (BT504, BT159^T, BT161); 5, *E. durans* DSM 20633^T; 6, *E. italicus* DSM 15952^T; 7, *E. gilvus* VS 495 (CNR-ISPA collection); 8, *E. faecalis* ATCC 27332; 9, *E. faecium* DSM 20477^T; 10, negative control.

The description is based on 10 strains. Cells are Gram-positive, facultatively anaerobic, non-motile, non-spore-forming, spherical and arranged in pairs or short chains. On M17, MRS and blood agar, colonies are whitish, smooth and circular with entire margins, whereas on KAA agar, colonies are typical of presumptive enterococci (surrounded by a black halo). Grows with 2.0–6.5 % NaCl, at 10–45 °C and at pH 9.6. Positive for hydrolysis of aesculin and negative for catalase, hydrolysis of gelatin and tributyrin, and production of gas from glucose. In litmus milk, grows and causes acidification, reduction and clotting within 24 h. Shows haemolysis on blood agar [α -haemolysis ($n=8$) and γ -haemolysis ($n=2$)] and is susceptible to vancomycin. Positive for the Voges–Proskauer test and leucine aminopeptidase, pyrrolidonyl arylamidase and arginine dihydrolase, but negative for alkaline phosphatase, α -galactosidase, β -glucuronidase and hippurate. β -Galactosidase activity is strain-dependent. Produces acid from arabinose, arbutin, *N*-acetylglucosamine, cellobiose, D-fructose, galactose, β -gentiobiose, D-glucose, lactose, maltose, L-mannitol, D-mannose, methyl α -D-mannoside, melibiose, ribose, salicin, D-tagatose and trehalose, but not from adonitol, amygdalin, D-arabinose, D-arabitol, L-arabitol, dulcitol, erythritol, D-fucose, L-fucose, gluconate, methyl α -D-glucoside, glycerol, glycogen, inositol, inulin, 2-ketogluconate, 5-ketogluconate, D-lyxose, melezitose, raffinose, rhamnose, sorbitol, L-sorbose, sucrose, turanose, xylitol, methyl β -D-xyloside, D-xylose or L-xylose; acid production from starch is weak.

The type strain is BT159^T (=DSM 23655^T=LMG 25958^T), isolated from Bitto cheese, a Protected Designation of Origin (PDO) raw milk cheese produced in a restricted Italian alpine area.

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