# *Enterococcus canintestini* sp. nov., from faecal samples of healthy dogs

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The taxonomic position of strain LMG 13590[T], originally isolated from dog faeces and classified as *Enterococcus dispar* in the BCCM/LMG Bacteria Catalogue, was reinvestigated. This strain and 12 recent isolates from faecal samples of healthy dogs occupied a clearly separate position when investigated with multilocus sequence analysis (MLSA) of the genes encoding the alpha subunit of ATP synthase (*atpA*), RNA polymerase alpha subunit (*rpoA*) and phenylalanyl-tRNA synthase alpha subunit (*pheS*). The 16S rRNA gene sequence of one representative strain showed highest similarities of 98–99 % with *E. dispar* LMG 13521[T], *Enterococcus canis* LMG 12316[T] and *Enterococcus asini* LMG 18727[T]. A further polyphasic taxonomic study based on whole-cell protein fingerprinting, DNA–DNA hybridization and biochemical features demonstrated that the 13 enterococcal dog faecal strains represent a single, novel *Enterococcus* species for which the name *Enterococcus canintestini* sp. nov. is proposed. The type strain is LMG 13590[T] (=CCM 7285[T]).

The genus *Enterococcus* currently comprises 30 species. Enterococci are natural inhabitants of the mammalian gastrointestinal tract (Devriese *et al.*, 1992; Klein, 2003). However, enterococci are ubiquitous and can be found free-living in soil, on plants or in dairy products (Baele *et al.*, 2000; Devriese *et al.*, 1995; Franz *et al.*, 1999). *Enterococcus dispar* was originally described by Collins *et al.* (1991) on the basis of two strains from human clinical sources, one from synovial fluid (LMG 13521[T]) and one from a stool culture. In 1993, an additional strain, LMG 13590, isolated from the faeces of a dog, was assigned to the species based on similar characteristics. A recent biodiversity study of the intestinal flora of dogs revealed that one enterococcal taxon was commonly occurring in dog faeces. These isolates were biochemically most similar to *E. dispar* but differed in a number of tests which were considered typical for the species (De Graef *et al.*, 2005). In the present study, the taxonomic relationships between *E. dispar* strains and the novel dog faecal isolates were investigated.

Two culture collection strains, *E. dispar* LMG 13521[T] and LMG 13590, and the following 12 isolates from faecal samples of healthy dogs from different owners distributed over Flanders were included in the study: LMG 22752 (eve 16a), LMG 22753 (eve 21a), LMG 22754 (eve 117b), LMG 22755 (eve 115a), LMG 22976 (eve 2b), LMG 22977 (eve 3b), LMG 22978 (eve 10a), LMG 22979 (eve 15b), LMG 22980 (eve 19c), LMG 22981 (eve 23b), LMG 22982 (eve 109c) and LMG 22983 (eve 125a). Cultivation and maintenance conditions for the isolates and reference strains were MRS agar (Oxoid) and incubation at 30 °C for 24 h, unless indicated otherwise.

Initially the two culture collection strains, *E. dispar* LMG 13521[T] and LMG 13590, were included in a multilocus sequence analysis (MLSA) study applied for the reliable identification of all currently described enterococcal species. MLSA compares the primary DNA sequences from multiple

conserved protein-coding loci to assess the diversity and relationships of different isolates across related taxa, thereby using an appropriate phylogenetic or cladistic approach. The criteria used for selecting the multiple protein-coding loci (housekeeping genes) are: (i) widely distributed among lactic acid bacterial (LAB) whole genome sequences; (ii) present in only one copy per organism; and (iii) mutually unlinked in location. Partial sequences for the genes encoding the alpha subunit of ATP synthase (*atpA*), RNA polymerase alpha subunit (*rpoA*) and phenylalanyl-tRNA synthase alpha subunit (*pheS*) were determined. Amplification and sequencing reactions were performed as described by Naser *et al.* (2005a, b). The designed primers enabled the amplification and final comparison of *atpA* (1102 bp), *rpoA* (533 bp) and *pheS* (455 bp) fragments of all *Enterococcus* species. The simultaneous use of several housekeeping genes in bacterial taxonomy gives higher taxonomic resolution between *Enterococcus* species and allows the discrimination of all currently recognized species of *Enterococcus* to a greater degree than the use of 16S rRNA gene sequences. The MLSA scheme is best suited for analysis at the species and genus levels as it integrates the information from different molecular markers from the bacterial chromosome (Stackebrandt *et al.*, 2002; Zeigler, 2003). At the interspecies level, all enterococcal species were clearly differentiated on the basis of *atpA*, *rpoA* and *pheS* gene sequences, with a maximum of 92, 97 and 86 % similarity, respectively (Naser *et al.*, 2005a, b). Evaluation of the intraspecies variation showed that *atpA*, *rpoA* and *pheS* genes had a high degree of homogeneity among strains of the same species. The MLSA approach for the analysis of the genus *Enterococcus* provides a reliable, highly reproducible, low-cost and rapid identification tool for the currently known species.

Neighbour-joining trees of the housekeeping genes investigated showed that strain LMG 13590 represents a separate
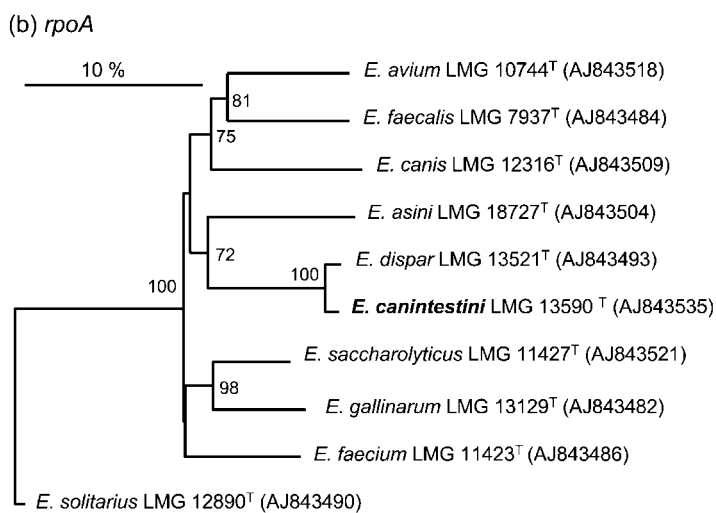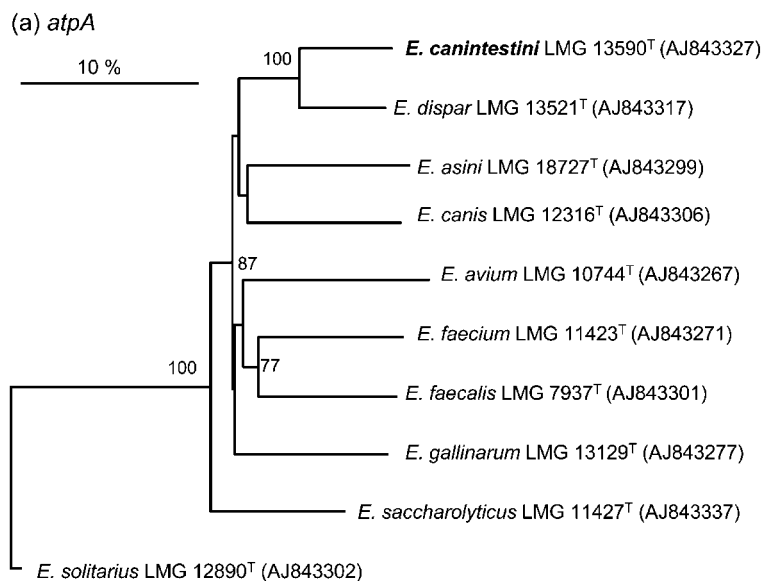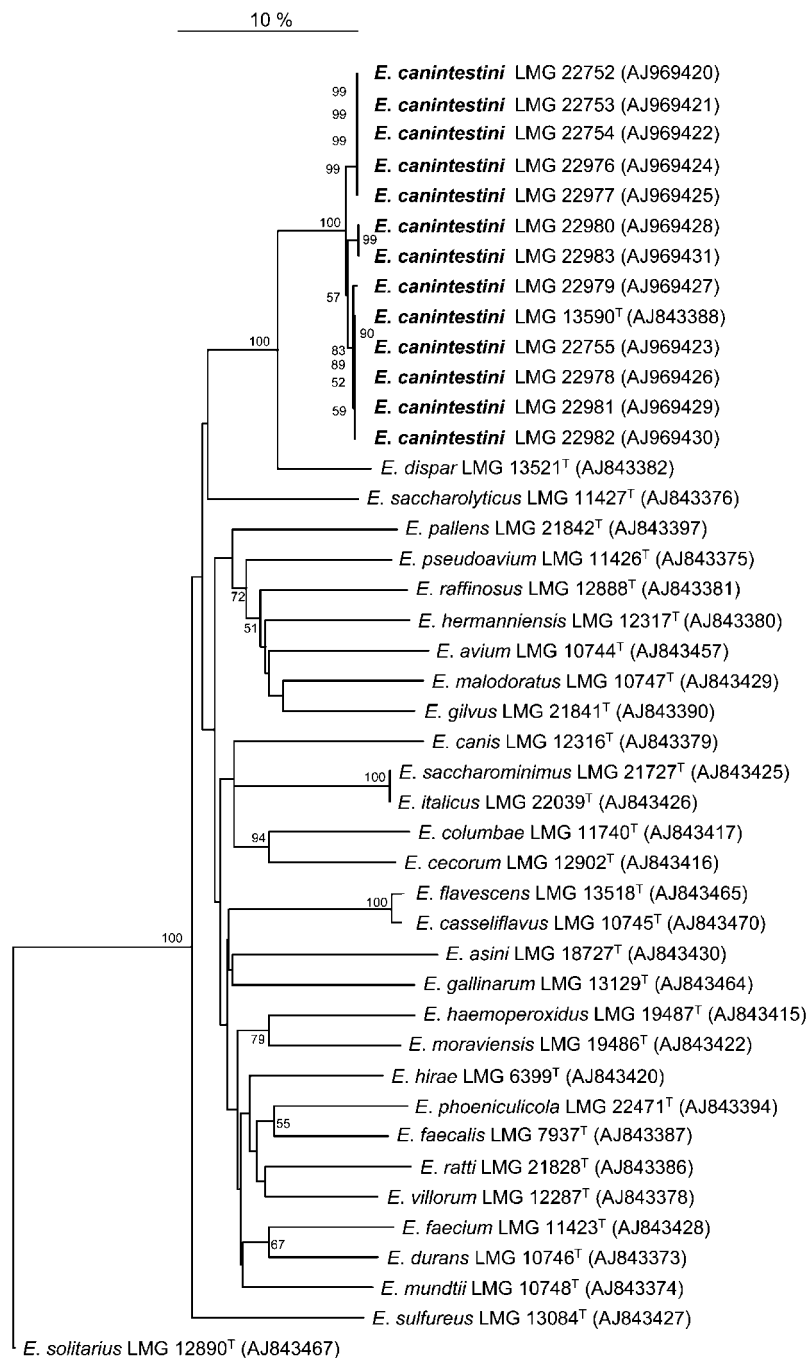


**Fig. 1.** Neighbour-joining trees showing the relationships of *E. canintestini* sp. nov. and other enterococcal reference species based on *atpA* (a) and *rpoA* (b) sequence comparisons. *E. solitarius* LMG 12890$^T$ was used as the outgroup. Bootstrap percentages ($\geqslant$50) after 500 simulations are shown. Bar, 10 % sequence divergence.

lineage distantly related to the other enterococcal species and to *E. dispar* LMG 13521$^T$ (90, 98 and 89 % *atpA*, *rpoA* and *pheS* gene sequence similarities, respectively). The other closest type strains were *Enterococcus asini* LMG 18727$^T$ (83 and 84 % *atpA* and *rpoA* gene sequence similarities, respectively), *Enterococcus canis* LMG 12316$^T$ (83 % *atpA* and *rpoA* gene sequence similarities, respectively) and *Enterococcus saccharolyticus* LMG 11427$^T$ (83, 84 and 84 % *atpA*, *rpoA* and *pheS* gene sequence similarities, respectively) (Figs 1 and 2 ). In a further study, the *pheS* gene sequences of the 12 isolates from dog faeces were determined and,

together with strain LMG 13590, they occupied a homogeneous and separate position in the *pheS* tree (98·6 % *pheS* gene sequence similarity) (Fig. 2).

The phylogenetic position of strain LMG 13590 was determined by complete 16S rRNA gene sequence analysis. Genomic DNA was prepared according to the protocol of Niemann *et al.* (1997). 16S rRNA gene amplification, purification and sequencing was performed as described by Vancanneyt *et al.* (2004) with the following modifications: PCR-amplified 16S rRNA genes were purified by using a



**Fig. 2.** Neighbour-joining tree showing the relationships of 13 *E. canintestini* sp. nov. strains and other enterococcal reference species based on *pheS* sequence comparisons. *E. solitarius* LMG 12890$^T$ was used as the outgroup. Bootstrap percentages (≥50) after 500 simulations are shown. Bar, 10 % sequence divergence.

NucleoFast 96 PCR clean-up kit (Macherey-Nagel). Sequencing reactions were performed with a BigDye terminator cycle sequencing kit (Applied Biosystems) and purified by using a Montage SEQ$_{96}$ sequencing reaction clean-up kit (Millipore). Sequencing was performed with an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Sequence assembly was done by using the GENEBUILDER program (Applied Maths). The 16S rRNA gene sequences of strain LMG 13590 and those of strains retrieved from GenBank/EMBL were aligned and a phylogenetic tree was constructed from the global alignment by using the neighbour-joining method using the BioNumerics software package, version 3.50 (Applied Maths). Bootstrap analysis was undertaken to test the statistical reliability of the topology of the neighbour-joining tree using 500 bootstrap resamplings of the data (Supplementary Fig. S1 available in IJSEM Online). The strain was classified in the genus *Enterococcus* with *E. dispar* LMG 13521[T], *E. asini* LMG 18727[T] and *E. canis* LMG 12316[T] as the nearest neighbours (99, 98 and 98 % 16S rRNA gene sequence similarities, respectively).

*E. dispar* LMG 13521[T], *E. canis* 12316[T], *E. asini* LMG 18727[T], strain LMG 13590 and the 12 dog faecal isolates were investigated using PAGE of whole-cell proteins. Whole-cell protein extracts were prepared and SDS-PAGE was performed as described by Pot *et al.* (1994). Densitometric analysis, normalization and interpolation of protein profiles and numerical analysis were performed by using the GelCompar software package, versions 3.1 and 4.0, respectively (Applied Maths). The 12 dog strains and LMG 13590 constituted a homogeneous and separate cluster (Fig. 3).

After extraction of genomic DNA as described by Baele *et al.* (2000), the 12 strains isolated from dog faecal samples, were subjected to tRNA intergenic length polymorphism analysis (tDNA-PCR). PCR was carried out using the tRNA-gene consensus primers T5A and fluorescently labelled T3B (Welsh & McClelland, 1991), which are directed outwardly against the conserved edges of the tRNA genes. PCR mixtures and cycle conditions were as described previously (Baele *et al.*, 2000). Capillary electrophoresis of the DNA fragments was performed with an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Electrophoretograms obtained with GENESCAN software were compared with a database developed at the Faculty of Veterinary Medicine, Ghent University, using a software program developed at our laboratory (Baele *et al.*, 2000). The pattern of the 12 dog isolates was composed of mean peak positions at 56·9, 62·6, 64·4, 79·4, 157, 230·1, 249·5 and 265·5 bp, which corresponded to the peak values of LMG 13590 included in our database as one of the *E. dispar* reference strains. A comparison of this pattern with the fragment lengths of all known LAB species in our database, including *E. dispar* type strain LMG 13521[T], revealed a separate grouping for the 12 dog faecal isolates and LMG 13590.

DNA G + C content was determined for *E. dispar* LMG 13521[T] and three dog faecal isolates, LMG 13590, LMG 22752 and LMG 22753. Cells were cultivated in MRS broth at 37 °C for 24 h. DNA was extracted from 0·5–0·75 g (wet
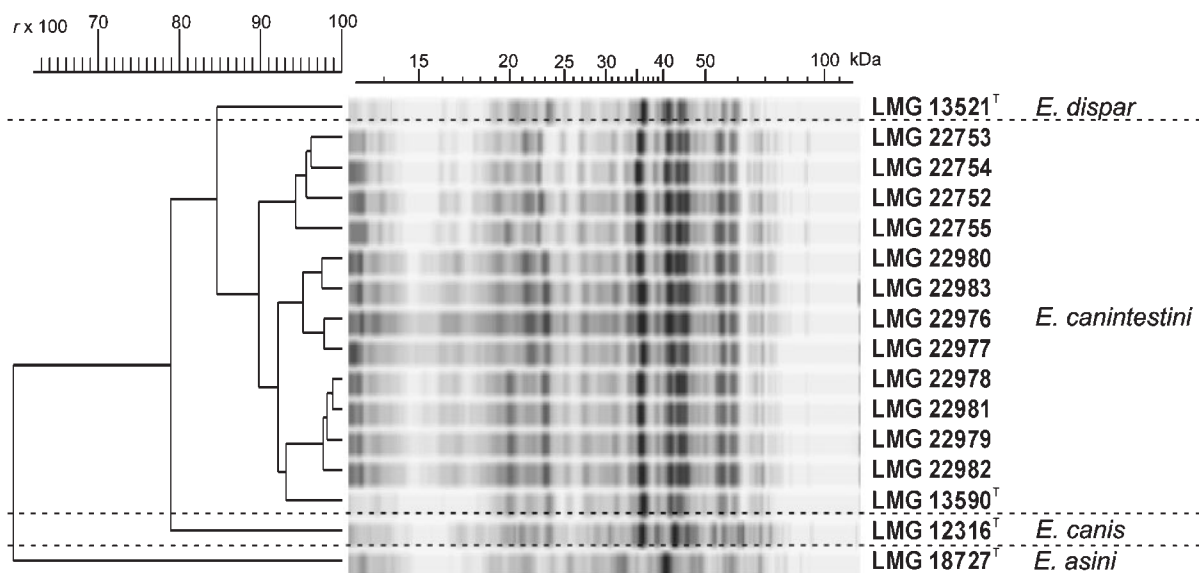


**Fig. 3.** Protein profiles and corresponding dendrogram derived from the unweighted pair group mean linkage of correlation coefficients *r* (expressed for convenience as a percentage value) of *E. canintestini* sp. nov. strains and some related reference species.

weight) using the protocol described by Marmur (1961) with the following modifications: (i) cells were suspended overnight in Tris/HCl buffer that contained lysozyme (8 mg ml$^{-1}$) before the addition of SDS and (ii) lysed cells were treated with proteinase K (360 mg l$^{-1}$; Merck) at 37 °C for 2 h. For determination of the DNA G+C content, DNA was degraded enzymically into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture was then separated by HPLC using a Waters SymmetryShield C8 column maintained at a temperature of 37 °C. The solvent was 0·02 M NH$_4$H$_2$PO$_4$ (pH 4·0) with 1·5 % acetonitrile. Non-methylated lambda-phage DNA (Sigma) was used as the calibration reference. The DNA G+C content of the dog faecal strains was in a range from 36 to 37 mol%. For *E. dispar* LMG 13521$^T$, a slightly higher value of 38 mol% was observed.

DNA–DNA hybridizations were performed between strains *E. dispar* LMG 13521$^T$ and LMG 13590, LMG 22752 and LMG 22753. Genomic DNA was prepared according to the protocol of Niemann *et al.* (1997). The microplate method was used as described by Ezaki *et al.* (1989) and Goris *et al.* (1998), using an HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. Biotinylated DNA was hybridized with unlabelled single-stranded DNA, which was bound non-covalently to microplate wells. Hybridizations were performed at 36 °C with the hybridization mixture (2× SSC, 5× Denhardt's solution, 2·5 % dextran sulfate, 50 % formamide, 100 μg denatured salmon sperm DNA ml$^{-1}$, 1250 ng biotinylated probe DNA ml$^{-1}$). The DNA–DNA relatedness percentages presented are means based on at least two independent hybridization experiments. Reciprocal reactions were performed and also considered as independent hybridization experiments. Hybridization values of 51·5 % were found with the type strain of *E. dispar*. Hybridization values of 93–96 % were found between LMG 13590, LMG 22752 and LMG 22753, indicating that these three dog faecal strains constitute a single novel species.

Growth tests were carried out as described by Švec *et al.* (2001). Lancefield antigens were detected by using a Streptococcal grouping kit (Oxoid). Biochemical reactions were determined in API 50 CH galleries under paraffin cover and in the API 20 STREP system (bioMérieux). The unknown dog faecal isolates present a number of characteristics in common with their closest phylogenetic relative, *E. dispar*, but, as outlined in Table 1, several phenotypic differences were noted compared with the *E. dispar* species description. These differences can easily be used to differentiate the unidentified dog faecal isolates from this related enterococcal species.

The overall results of the present study allowed us to assign 13 strains, LMG 13590$^T$, LMG 22752, LMG 22753, LMG 22754, LMG 22755, LMG 22976, LMG 22977, LMG 22978, LMG 22979, LMG 22980, LMG 22981, LMG 22982 and LMG 22983, to a novel species for which we propose the name *Enterococcus canintestini* sp. nov.

**Table 1.** Characteristics that differentiate *E. canintestini* sp. nov. and *E. dispar*

The data for *E. dispar* are as described by Collins *et al.* (1991) based on two strains, E18-1$^T$ and 174-85. For the *E. canintestini* strains, values shown are for the number of strains showing a positive reaction out of the number of strains tested. +, Positive; V, variable.

| Characteristic | *E. dispar* | *E. canintestini* sp. nov. |
|---|---|---|
| α-Galactosidase | + | 3/13 |
| β-Galactosidase | + | 0/13 |
| Glycerol | + | 1/13 |
| Hippurate hydrolysis | V | 0/13 |
| Leucine arylamidase | + | 4/13 |
| D-Melibiose | + | 0/13 |
| Pyrrolidonyl arylamidase | + | 1/13 |
| Raffinose | + | 0/13 |

## Description of *Enterococcus canintestini* sp. nov.

*Enterococcus canintestini* (can.in.tes′ti.ni. L. gen. n. *canis* of a dog; L. neut. n. *intestinum* gut; N.L. gen. n. *canintestini* of the gut of a dog).

Cells are non-motile, small, Gram-positive cocci. They are predominantly arranged in small groups. On Columbia sheep blood agar, colonies are circular, non-pigmented, smooth, shiny and milky, with diameters up to 3 mm. Strains show homogeneous growth in brain-heart infusion broth (Oxoid). Growth is not enhanced by the addition of 5 % CO$_2$. Growth is slow at 25 °C, better at 30 °C and optimal at 37 °C; growth at 42 °C is equal to or slower than at 37 °C. Strains grow in 6·5 % NaCl broth. On Edward's *Streptococcus* selective medium, aesculin-degrading colonies are formed. Blackening occurs on aesculin bile agar. Strains grow and tetrazolium chloride is reduced on Slanetz & Bartley agar. The strains do not react with Lancefield groups A, B, C, D, G or F antisera. Negative reactions in API tests for hippurate, β-galactosidase, β-glucuronidase and alkaline phosphatase. All strains are positive for arginine, aesculin and the Voges–Proskauer test. Few strains are positive in tests for leucine arylamidase, pyrrolidonyl arylamidase or α-galactosidase (4/13, 1/13 and 3/13 positive, respectively). Acid is produced in the API 20 STREP and/or API 50 CH kits in tests with ribose, D-galactose, D-glucose, D-fructose, D-mannose, methyl α-D-glucopyranoside, N-acetylglucosamine, amygdalin (sometimes weak), arbutin, salicin, D-cellobiose, D-maltose, D-sucrose, trehalose, gentiobiose and D-tagatose. Most strains react positively in tests with D-turanose (11/13 positive with two weak reactions), 2-ketogluconate (8/13 strains with often weak reactions) and lactose (12/13 positive in API 50 CH and 10/13 positive in API 20 STREP). Few strains produce acid from methyl α-D-mannopyranoside (2/13 strains) and only one of 13 strains

produces acid from glycerol. Reactions are also strain-dependent for acid production from D-lyxose (5/13 positive, 7/13 negative and one weak reaction). No acid is produced from erythritol, D- or L-arabinose, D- or L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, mannitol, sorbitol, D-melibiose, inulin, D-melezitose, raffinose, starch, glycogen, xylitol, D- or L-fucose, D- or L-arabitol, gluconate or 5-ketogluconate. DNA G+C content is 36–37 mol%.

The type strain, LMG 13590$^T$(=CCM 7285$^T$), was isolated from dog faeces.

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