Six Gram-positive-staining, microaerophilic, non-spore-forming, fructose-6-phosphate phosphoketolase-positive bacterial strains with a peculiar morphology were isolated from faecal samples of baby common marmosets (*Callithrix jacchus*). Cells of these strains showed a morphology not reported previously for a bifidobacterial species, which resembled a coiled snake, always coiled or ring shaped or forming a ‘Y’ shape. Strains MRM 3/1T and MRM 4/2 were chosen as representative strains and characterized further. The bacteria utilized a wide range of carbohydrates and produced urease. Glucose was fermented to acetate and lactate. Strain MRM 3/1T showed a peptidoglycan type unique among members of the genus *Bifidobacterium*. The DNA base composition was 64.7 mol% G+C. Almost-complete 16S rRNA, hsp60, clpC and rpoB gene sequences were obtained and phylogenetic relationships were determined. Comparative analysis of 16S rRNA gene sequences showed that strains MRM 3/1T and MRM 4/2 had the highest similarities to *Bifidobacterium scardovii* DSM 13734T (94.6%) and *Bifidobacterium stellenboschense* DSM 23968T (94.5%). Analysis of hsp60 showed that both strains were closely related to *B. stellenboschense* DSM 23968T (97.5% similarity); however, despite this high degree of similarity, our isolates could be distinguished from *B. stellenboschense* DSM 23968T by low levels of DNA–DNA relatedness (30.4% with MRM 3/1T). Strains MRM 3/1T and MRM 4/2 were located in an actinobacterial cluster and were more closely related to the genus *Bifidobacterium* than to other genera in the family *Bifidobacteriaceae*. On the basis of these results, strains MRM 3/1T and MRM 4/2 represent a novel species within the genus *Bifidobacterium*, for which the name *Bifidobacterium aesculapii* sp. nov. is proposed; the type strain is MRM 3/1T (=DSM 26737T=JCM 18761T).

Bifidobacteria are known to exert beneficial effects and to play an important role in maintaining the health of their host (Turroni *et al.*, 2011). Hence, it is important to understand the diversity of bifidobacteria in the gastrointestinal tract and faeces.

During the characterization of bifidobacterial distribution in primates, six bifidobacterial strains with similar morphology were isolated from fresh faecal samples of baby subjects of the common marmoset (*Callithrix jacchus*), which were individually collected from five animals kept in animal houses at Aptuit s.r.l. Verona, in northern Italy. The common marmoset is a small exudivore monkey from the New World that has developed a large specialized caecum for the digestion of the complex carbohydrates found in tree exudates (Caton *et al.*, 1996; Bailey & Coe, 2002). As microbiota growth and composition are affected by gastrointestinal tract function, such as motility and nutrient availability in the intestinal lumen, it is likely that this evolutionary adaptation may influence the concentrations and types of Bifidobacteria.
bacteria that form part of the normal intestinal microbiota (Bailey & Coe, 2002).

Samples of fresh rectal swabs from common marmosets were serially diluted with peptone water (Merck) supplemented with cysteine hydrochloride (0.5 g L⁻¹); aliquots of each dilution were inoculated onto TPY agar supplemented with mupirocin (100 mg L⁻¹; Applichem), which is a selective agent for bifidobacteria (Rada & Petr, 2000). In each subject, we observed cells of a bacterium with a novel and unusual morphology, resembling a coiled snake. A total of six isolates with this morphology were obtained from the five baby marmosets. They were namely MRM 3/1², MRM 4/2, MRM 5/13, MRM 8/7, MRM 4/6 and MRM 4/7. The isolates were subcultured on TPY agar and cells were suspended in a 10 % (w/v) sterile skimmed milk solution supplemented with lactose (3 %) and yeast extract (0.3 %) and kept both freeze-dried and frozen at −120 °C. For all experiments, the strains were cultivated under anaerobic conditions and maintained in TPY broth, pH 6.9, at 37 °C, unless indicated otherwise.

In the present study, the morphological, biochemical and molecular characterization of the isolates was carried out. Chromosomal DNA was obtained from the isolates according to the procedure of Rossi et al. (2000), with slight modifications. Briefly, cells of overnight cultures were pelleted and resuspended in 1 ml TE buffer (pH 7.6) containing 50 mg lysozyme ml⁻¹ and then incubated overnight at 37 °C.

For discrimination of the isolates, molecular typing was performed using enterobacterial repetitive intergenic consensus sequences (ERIC) PCR with the primers ERIC1 (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC2 (5'-AGTAAGTCGTTGGGTAGG-3') (Ventura et al., 2003). Each 20 μl reaction mixture contained 3.5 mM MgCl₂, 20 mM Tris/HC1, 50 mM KCl, 200 μM each dNTP (HotStarTaq plus DNA polymerase MasterMix kit; Qiagen), 30 ng DNA template and 2 μM each primer. Amplifications were performed using an Applied Biosystems Veriti Thermal Cycler with the following temperature profile: 1 cycle at 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 48 °C for 30 s and 72 °C for 4 min; and 1 cycle at 72 °C for 6 min. Aliquots of each amplification reaction mixture (15 μl each) were separated by electrophoresis in 2 % (w/v) agarose gels at a voltage of 7 V cm⁻¹. Gels were stained with ethidium bromide (0.5 μg ml⁻¹) and photographed under 260 nm UV light. Given that the isolates revealed two different ERIC-PCR profiles (Fig. S1, available in the online Supplementary Material), strains MRM 3/1² and MRM 4/2 were selected as representatives and further characterized.

The partial 16S rRNA genes of strains MRM 3/1² and MRM 4/2 were amplified by PCR using the primers Bif285 (5'-GGAGGTTGCAATTGCTGCA-3') and Bif261 (5'-AAAGGAGGTATCTCCAGGCA-3') (Kim et al., 2010). Partial hsp60, rpoB and clpC gene sequences were also obtained using the primer pairs HspF3 (5'-ATCGCAAGAGGAGATCGAGCT-3') and HspR4 (5'-AAGGTTGCGCGGATCGTGGGCTTTGTT-3'), BifF (5'-TCGATGCGGCCACATACGG-3') and BifR2 (5'-CGACCATTGGCAACCCG-3') (Kim et al., 2010) and BClpC-F (5'-ATCGCSGARACBATYGAGA-3') and BClpC-R (5'-ATRATGGGTTGTGCARYT-3') (Watanabe et al., 2009), respectively. Each PCR mixture (20 μl) contained 1.5 mM MgCl₂, 20 mM Tris/HCl, 50 mM KCl, 200 μM each dNTP (HotStarTaq plus DNA polymerase MasterMix kit; Qiagen), 0.1 μM each primer and 30 or 200 ng DNA template for the 16S rRNA gene and for each housekeeping gene, respectively. Amplifications were performed using a TGradient thermal cycler (Biomera). A touchdown PCR was used to amplify the 16S rRNA gene and the other phylogenetic markers as follows: initial denaturation (95 °C, 5 min) for HotStarTaq plus activation; four cycles of denaturation at 94 °C for 60 s, annealing at 62 °C for 60 s and extension at 72 °C for 90 s; 21 cycles of denaturation at 94 °C for 60 s, annealing at 60 °C for 60 s and extension at 72 °C for 90 s; and 15 cycles of denaturation at 94 °C for 60 s, annealing at 58 °C for 60 s and extension at 72 °C for 90 s. The PCR was completed with a single elongation step (10 min at 72 °C). The resulting amplicons were separated on 2 % agarose gels, followed by ethidium bromide staining. PCR fragments were purified using the NucleoSpin extract II kit (Macherey-Nagel) following the manufacturer's instructions.

16S rRNA genes were directly sequenced whereas hsp60, clpC and rpoB gene sequences were cloned using an InsTaClone PCR Cloning kit (Fermentas). All sequencing reactions were performed by Eurofins MWG Operon. Almost-complete 16S rRNA gene sequence assembly was performed using CAP (contig assembly program; Huang, 1992) in BioEdit (Hall, 1999). After editing, the closest known relatives of the novel strains were determined by comparison with database entries and the sequences of closely related strains were retrieved from the EMBL and GenBank nucleotide databases. Pairwise nucleotide sequence similarity values were calculated using the EzTaxon server (http://www.eztaxon.org/), which provides a web-based tool (Kim et al., 2012).

The 16S rRNA gene sequences (about 1421 bp) of strains MRM 3/1² and MRM 4/2 and of those of their closest relatives retrieved from the DDBJ/GenBank/EMBL databases were aligned by using the CLUSTAL_X2 program (version 1.82) (Thompson et al., 1997). A phylogenetic tree based on a total of 43 partial 16S rRNA gene sequences, including those of members of the genus Bifidobacterium and of related genera, was reconstructed with the neighbour-joining method (Saitou & Nei, 1987) and evolutionary distances were computed using Kimura’s two-parameter method (Kimura, 1980) by using the MEGA 5.05 program (Tamura et al., 2011). The tree was rooted using Micrococcus luteus DSM 20030² (Fig. 1). The statistical reliability of the tree was evaluated by bootstrap analysis of 1000 replicates (Felsenstein, 1985) and the tree topology was also confirmed with the maximum-likelihood (Cavalli-Sforza & Edwards, 1967), maximum-parsimony (Fitch, 1971) and least-squares (Fitch & Margoliash, 1967) methods, by using MEGA 5.05
The 16S rRNA gene sequence similarity between strains MRM 3/1T and MRM 4/2 was about 99.6%. They showed low sequence similarity to known bifidobacteria; the highest similarities were found to the type strains of *Bifidobacterium scardovii* and *Bifidobacterium stellenboschense* (94.6 and 94.5%, respectively), a recently described species from a red-handed tamarin (*Saguinus midas*) (Endo et al., 2012). Based on the neighbour-joining analysis, the novel strains are related phylogenetically to *B. scardovii* (Fig. 1). Similar tree topologies were obtained by using the maximum-likelihood (Fig. S2), maximum-parsimony and least-squares methods (not shown).

Multilocus sequence analysis is a reliable and robust technique for the identification and classification of bacterial isolates to the species level as an alternative to 16S rRNA gene sequence analysis (Martens et al., 2008). For this reason, the phylogenetic location of the novel strains was verified by analysis of three additional phylogenetic markers, hsp60, clpC and rpoB, which have proven to be discriminative for classification of the genus *Bifidobacterium* (Jian et al., 2001; Ventura et al., 2006; Kim et al., 2010).

For hsp60, clpC and rpoB genes, the sequences of strains MRM 3/1T and MRM 4/2 and of those of their closest relatives retrieved from the DDBJ/GenBank/EMBL databases were aligned by using the MAFFT program, at CBRC (http://mafft.cbrc.jp/alignment/software/) (Katoh & Standley, 2013). The Gblocks program (version 0.91b) as server tool at the Castresana Lab (http://molevol.cmima.csic.es/castresana/Gblocks.html) was then used to eliminate poorly aligned positions and divergent regions of DNA alignments, so that they became more suitable for phylogenetic analysis (Talavera & Castresana, 2007).

To complete our phylogenetic determination, the partial hsp60 gene was amplified, purified and directly sequenced from *B. scardovii* DSM 13734T as described above, whereas, for *B. stellenboschense* DSM 23968T, we used the partial gene sequence obtained by Stenico et al. (2014) and retrieved from GenBank.

Three phylogenetic trees were then reconstructed using the neighbour-joining method. Approximately 645 bp of the hsp60 gene, 500 bp of the clpC gene and 524 bp of the rpoB gene sequence of the isolates and related strains were used in the analyses.

The level of similarity for the partial hsp60 gene sequences of strains MRM 3/1T and MRM 4/2 was 99.5% and, in relation to the type strains of their closest relatives, the levels of similarity were about 97.5% with *B. stellenboschense*, 96.2% with *Bifidobacterium sacculare*, 96% with *Bifidobacterium pullorum* and *Bifidobacterium gallinarum*, 94.4% with *Bifidobacterium biavatii*, 94% with *Bifidobacterium callitrichos* and 90.8% with *B. scardovii*. Strains MRM 3/1T and MRM 4/2 formed a subcluster in the *B. pullorum* group (Fig. 2).

The sequence similarity between the clpC genes of strains MRM 3/1T and MRM 4/2 was 99.2%. The highest sequence similarities were found to the type strains of *B. scardovii* and *Bifidobacterium bifidum* (about 86.7 and 86.5%, respectively). Strains MRM 3/1T and MRM 4/2 produced a subcluster in the *B. scardovii* group.

The clpC phylogenetic tree is shown in Fig. S3.

The level of similarity for the partial rpoB gene sequences of strains MRM 3/1T and MRM 4/2 was 99.8%, and the levels of similarity in relation to their closest relatives were about 95.2, 95 and 94% to the type strains of *Bifidobacterium cuniculi*, *Bifidobacterium choerinum* and *B. pullorum*, respectively. Based on the partial rpoB sequences, MRM 3/1T and MRM 4/2 are placed in a distinct cluster and were related to *B. cuniculi*. The rpoB phylogenetic tree is shown in Fig. S4.

These findings correlated with the results of Ventura et al. (2006) and Endo et al. (2012) and indicated that the phylogenetic positions of species of the genus *Bifidobacterium* are highly influenced by the genes used for the analysis.

The 16S rRNA gene sequence similarity of strains MRM 3/1T and MRM 4/2 to known species was less than 97% and it was lower than the recommended value for species differentiation (98.7–99%; Tindall et al., 2010). However, analysis of hsp60 showed that both strains were closely related to *B. stellenboschense* DSM 23968T (97.5% similarity). Due to this high level of similarity (the cut-off value for bifidobacterial species differentiation of hsp60 is 96%; Zhu et al., 2003), DNA–DNA hybridization between strain MRM 3/1T and *B. stellenboschense* DSM 23968T was also performed. Estimation of the level of relatedness between *B. stellenboschense* DSM 23968T and strain MRM 3/1T was determined by the DSMZ, Braunschweig, Germany. Cells were disrupted by using a Constant Systems TS 0.75 kW (IUL Instruments). DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashion et al. (1977). DNA–DNA hybridization was carried out as described by De Ley et al. (1970) under consideration of the modifications described by Huss et al. (1983) using a model Cary 100 Bio UV/Vis spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multichannel changer and a temperature controller with in situ temperature probe (Varian). Strain MRM 3/1T shared 30.4% DNA–DNA relatedness with *B. stellenboschense* DSM 23968T, unequivocally supporting the assignment of strain MRM 3/1T to a novel species.

Estimation of the G+C content in bacterial chromosomal DNA of strain MRM 3/1T was done by the DSMZ. DNA was purified on hydroxyapatite according to the procedure of Cashion et al. (1977) and enzymically hydrolysed by the method of Mesbah et al. (1989). The resulting deoxyribonucleosides were analysed by HPLC as described by Tamaoka & Komagata (1984). Strain MRM 3/1T had a DNA G+C content of 64.7 mol%. This value was within the range of DNA G+C content reported for the genus *Bifidobacterium*, 52–67 mol% (Biavati & Mattarelli, 2012; Killer et al., 2010), and in particular was very similar to that
obtained for *B. callitrichos*, described recently from a marmoset by Endo et al. (2012).

Morphological, cultural and biochemical characterization of the isolates according to standard techniques was performed at 37 °C unless otherwise stated. Morphology as examined by phase-contrast microscopy is shown in Fig. 3(a, b). Morphological characteristics determined using a scanning electron microscope (SEM) are shown in Fig. 1.

**Fig. 1.** Phylogenetic relationships of the novel bifidobacteria to related species based on 16S rRNA gene sequences. The tree was reconstructed by the neighbour-joining method and rooted with *Micrococcus luteus* DSM 20030T. Percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to branches. Bootstrap values above 70% are given at branching points. Bar, 0.02 substitutions per nucleotide position.

**Fig. 2.** Phylogenetic tree based on *hsp60* gene sequences showing the relationships of the novel strains isolated from baby marmosets to closely related species. The tree was reconstructed by the neighbour-joining method on the basis of a comparison of 559 positions, and the sequence of *Mycobacterium tuberculosis* H37RvT was used as an outgroup. Percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to branches. Bootstrap values above 70% are given at branching points. Bar, 0.05 substitutions per nucleotide position.
3(c). For SEM observation, strains were cultured on TPY agar at 37°C for 48 h under anaerobic conditions. After culturing, a slice of agar was excised and dehydrated with a series of increasing ethanol concentrations (50, 70, 80, 90, 95 and 100% for 15 min each). The prepared cells were subsequently critical-point-dried in a critical point dryer apparatus (CPD Emitech K850) using liquid CO₂ as a transitional fluid. Dried samples were mounted on aluminium stubs with silver glue, coated with gold palladium film using an ion-sputtering unit (Emitech K500) and observed in a Philips 515 SEM at 7–10.0 kV.

The temperature range for growth of the strains was tested using an anaerobic TPY broth at 20, 25, 30, 35, 37, 40, 42, 45 and 47 °C for 48 h. The sensitivity of the strains to low pH was determined at 37°C in anaerobic TPY broth (pH 3.5, 4.0, 4.5, 5.0 and 5.5) for 48 h. The ability of the strains to grow under aerobic and microaerophilic conditions (CampyGen; Oxoid) was tested using TPY agar, TPY soft agar (0.6 %), TPY broth, skimmed milk and UHT whole milk at 37°C for 48 h.

Haemolytic activity was determined in Columbia blood agar (Bioline) at 37°C under anaerobic conditions for 48 h (Pineiro & Stanton, 2007).

Spore staining was performed using malachite green dye. Phase-contrast microscopy (Zeiss) was used to observe the morphology of individual cells as well as spore staining.

Gram staining and catalase and oxidase activities were respectively determined from cells grown on TPY agar at 37°C for 48 h under anaerobic conditions using Gram staining individual reagents (Merck Millipore), a 3% (v/v) hydrogen peroxide solution and cotton swabs impregnated with N,N,N',N'-tetramethyl p-phenylenediamine dihydrochloride and dried (Oxibioswab; Bioline). The motility of strains was determined by stabbing into TPY medium containing 0.4% agar, knowing that motile strains show diffuse growth spreading from the line of inoculation. Fermentation products (short-chain fatty acids) were extracted with diethyl ether. A Carlo Erba 5300 gas chromatograph, with a Nukol capillary column (30 cm) at 170°C, flame-ionization detector and hydrogen carrier gas, was used for the analysis. All strains tested fermented glucose to acetate and lactate in a variable ratio ranging from 2:1 to 1.5:1.

Biochemical characterization was carried out by using the API 20A, API 20E and API 50CHL systems (bioMérieux) following the manufacturer’s instructions. The results are summarized in Table 1.

Bifidobacteria and members of related genera degrade hexoses via the fructose-6-phosphate phosphoketolase (F6PPK) pathway. F6PPK is the key enzyme in this pathway and is considered a taxonomic marker for identification of species of Bifidobacterium and related genera (Biavati & Mattarelli, 2012). F6PPK activity was determined according to the method described by Scardovi (1986) and modified by Orban & Patterson (2000). All the isolates possessed F6PPK activity.

The cell-wall murein composition of strain MRM 3/1T was examined by the DSMZ. Analysis of partial acid hydrolysates revealed the presence of A4-7 type, L-Lys–D-Ser–D-Asp. This murein type is unique among members of the genus Bifidobacterium and related genera, confirming the novelty of this species.

According to our phylogenetic analyses based on 16S rRNA gene and partial hsp60, clpC and rpoB sequences and the other data obtained, strains MRM 3/1T, MRM 4/2, MRM 5/13, MRM 4/6, MRM 4/7 and MRM 8/7 are genetically and phenotypically distinguishable from currently recognized species of bifidobacteria and thus represent a novel species, for which we suggest the name Bifidobacterium aesculapii sp. nov.

**Description of Bifidobacterium aesculapii sp. nov.**

*Bifidobacterium aesculapii* (aes.cu.la’pi.i. L. gen. masc. n. aesculapii of Aesculapius, from the snake-like appearance of the bacterium, resembling the serpent-entwined rod wielded by the Roman god Aesculapius).

Cells grown in TPY broth are rods of various shapes, occasionally swollen, always coiled or ring shaped or...
forming a ‘Y’ shape at both ends. They are Gram-positive-staining, non-motile, asporogenous, non-haemolytic, F6PPK-positive, catalase- and oxidase-negative, indole-negative and microaerophilic. There is no difference in growth under either anaerobic or microaerophilic conditions. Negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization and H₂S production. Does not reduce nitrate or nitrite. Well-isolated colonies on the surface of TPY agar under anaerobic conditions are white, opaque, smooth and circular with entire edges, while imbedded colonies are lens-shaped or elliptical. Colonies reach 1.7–2.5 mm in diameter after 3 days of incubation. The temperature range for growth is 25–42 °C; no growth occurs at 20 or 47 °C. The optimum temperature for growth is 35–37 °C. Grows at pH 4.5–7.0 with an optimum at pH 6.5–7.0. Can grow in milk, under aerobic, microaerophilic and anaerobic conditions. Acid is produced from D-glucose, lactose, maltose, salicin, D-xylose, L-arabinose, melezitose, D-sorbitol, D-ribose, D-galactose, gentiobiose, D-turanose, arbutin, melibiose and potassium gluconate. Acid production from D-mannitol, sucrose, glycerol, cellobiose, D-mannose, raffinose, L-rhamnose, trehalose, D-fructose, starch, inulin and glycogen is strain dependent. Acid is not produced from xylitol, amygdalin, methyl α-D-glucopyranoside, N-acetylglucosamine or potassium gluconate. Lactic and acetic acids are produced as end products of glucose fermentation in a variable ratio ranging from 1:2 to 1:1.5. Aesculin is hydrolysed and urease is produced. The peptidoglycan type is A4α L-Lys–D-Ser–D-Asp. Phylogenetic analysis of the 16S rRNA gene sequence places the species in the B. scardovii subgroup of the genus Bifidobacterium.

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<td>Arbutin</td>
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<tr>
<td><strong>DNA G+C content (mol%)</strong></td>
<td>64.7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>66.3*</td>
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<tr>
<td><strong>Urease activity</strong></td>
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*Data from Endo et al., 2012.
The type strain, MRM 3/1T (=JCM 18761T=DSM 26737T), and the reference strain MRM 4/2 (=JCM 18762=DSM 26738) were isolated from fresh faecal samples of infant common marmosets (Callithrix jacchus) that were individually collected from animals kept in animal houses in Aptuit s.r.l. Verona, northern Italy, in 2012. The DNA G+C content of the type strain is 64.7 mol%.

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References


