**Bifidobacterium aquikefiri** sp. nov., isolated from water kefir

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A novel **Bifidobacterium**, strain LMG 28769ᵀ, was isolated from a household water kefir fermentation process. Cells were Gram-stain-positive, non-motile, non-spore-forming, catalase-negative, oxidase-negative and facultatively anaerobic short rods. Analysis of its 16S rRNA gene sequence revealed **Bifidobacterium crudilactis** and **Bifidobacterium psychraerophilum** (97.4 and 97.1 % similarity towards the respective type strain sequences) as nearest phylogenetic neighbours. Its assignment to the genus **Bifidobacterium** was confirmed by the presence of fructose 6-phosphate phosphoketolase activity. Analysis of the hsp60 gene sequence revealed very low similarity with nucleotide sequences in the NCBI nucleotide database. The genotypic and phenotypic analyses allowed the differentiation of strain LMG 28769ᵀ from all recognized **Bifidobacterium** species. Strain LMG 28769ᵀ (=CCUG 67145ᵀ=R 54638ᵀ) therefore represents a novel species, for which the name **Bifidobacterium aquikefiri** sp. nov. is proposed.

Bifidobacteria are Gram-stain-positive, non-motile, non-spore-forming bacteria that are usually associated with the gut microbiota of humans and animals (Simpson et al., 2004; Biavati & Mattarelli, 2006). They are generally obligately anaerobic but some species can also grow aerobically (Simpson et al., 2004; Delcenserie et al., 2007; Watanabe et al., 2009). Bifidobacteria are considered to be non-pathogenic (Borriello et al., 2003) and some species occur in fermented foods and beverages (Delcenserie et al., 2007; Watanabe et al., 2009; Hsieh et al., 2012; Gulitz et al., 2013; Laureys & De Vuyst, 2014). Sometimes, bifidobacteria are added to foods and beverages because their consumption is associated with positive health effects (Tojo et al., 2014). Recently, an unknown **Bifidobacterium** species was detected in several water kefers from different origins via PCR denaturing gradient gel electrophoresis community fingerprinting with both the universal prokaryotic primer pair 357f-GC/518r and the **Bifidobacterium**-specific primer pair bif164f/bif662r-GC (Laureys & De Vuyst, 2014; D. Laureys & L. De Vuyst, unpublished results). The 0.5 kb partial 16S rRNA gene sequences, obtained with the latter primer pair, were 100 % identical to the 16S rRNA gene sequence of an unknown **Bifidobacterium** species detected in a water kefir fermentation process in Germany (Gulitz et al., 2013), but were only 98 % identical to the 16S rRNA gene sequence of the closest relative **Bifidobacterium psychraerophilum**. These data suggested that a novel **Bifidobacterium** species was present in water kefirs, which may even be a water kefir-specific micro-organism. Its present characterization was based on an isolate from a water kefir fermentation process carried out at household level.

A water kefir fermentation process was carried out as described by Laureys & De Vuyst (2014). Strain R 54638ᵀ (=LMG 28769ᵀ) was isolated from the water kefir liquor by plating on mTY agar medium (Gulitz et al., 2013), supplemented with cycloheximide (final concentration of 0.1 g l⁻¹; Sigma-Aldrich), kanamycin sulfate (final concentration of 0.05 g l⁻¹; Sigma-Aldrich), mupirocin (final concentration of 0.05 g l⁻¹; AppliChem) and amphotericin B (final concentration of 0.005 g l⁻¹; Sigma-Aldrich). The agar media were incubated anaerobically (AnaeroGen; Thermo Fisher Scientific) at 30 °C for 6 days.

For its genotypic characterization, strain LMG 28769ᵀ was grown on M144 agar medium (per litre: 23.0 g special peptone (Oxoid), 1.0 g soluble starch (Merck), 5.0 g

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**Abbreviation:** F6PPK, fructose 6-phosphate phosphoketolase.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and hsp60 gene sequences of strain LMG 28769ᵀ are LN849254 and LN849255, respectively. That for the hsp60 gene sequence of **Bifidobacterium crudilactis** LMG 23609ᵀ is LN849256.

Three supplementary figures are available with the online Supplementary Material.
NaCl (Merck), 0.3 g cysteine hydrochloride (Sigma-Aldrich), 5.0 g glucose (Merck) and 15.0 g agar (Oxoid) and DNA was obtained via alkaline lysis of the cells, as described previously (Niemann et al., 1997). The nearly complete 16S rRNA gene was amplified and sequenced according to both Coenye et al. (1999) and Kim et al. (2010). The results obtained were identical and the consensus sequence (1445bp) was deposited in the NCBI nucleotide database (accession no. LN849254). Comparison of the 16S rRNA gene sequence of strain LMG 28769T with sequences in the EzTaxon database (Kim et al., 2012) revealed *Bifidobacterium crudulactis* FR62/b/3T (97.42% pairwise similarity; accession no. AY952449) and *B. psychraerophilum* T16T (97.06% pairwise similarity; accession no. AY952449) as nearest phylogenetic neighbours. Comparison of the 16S rRNA gene sequence of strain LMG 28769T with sequences in the EzTaxon database (Kim et al., 2012) revealed *Bifidobacterium adolescentis* ATCC 15703T (D86197) (100% identical to the 16S rRNA gene sequence obtained from *Bifidobacterium* strain LMG 28769T (Laureys & De Vuyst, 2014; D. Laureys & L. De Vuyst, unpublished results). For the construction of a phylogenetic tree, the 16S rRNA gene sequences of the type strains of all *Bifidobacterium* species were retrieved from the NCBI nucleotide database. The MEGAS software package (Tamura et al., 2013) was used to align the sequences with the MUSCLE algorithm (Edgar, 2004) and to reconstruct the phylogenetic trees with both the maximum-likelihood method based on the Tamura–Nei model (Tamura & Nei, 1993) and the neighbour-joining method (Saitou & Nei, 1987). The statistical reliability of the tree topology was evaluated via a bootstrapping analysis based on 1000 replicates. The topologies as reconstructed with the two methods were similar and only the phylogenetic tree reconstructed with the neighbour-joining method is shown (Fig. 1 and Fig. S1, available with the online Supplementary Material).

The hsp60 gene of strain LMG 28769T was amplified and sequenced with the primer pairs HspF3/HspR4 and HspBF3/HspBR4, as described by Kim et al. (2010). The hsp60 gene sequence results were identical for both primer pairs and the consensus sequence (605 bp) was deposited in the NCBI nucleotide database (accession no. LN849255). Comparison of this hsp60 gene sequence with those of the *Bifidobacterium* type strains retrieved from the NCBI nucleotide database (Johnson et al., 2008) revealed an unexpectedly low level of similarity with its...
nearest neighbours, B. crudilactis LMG 23609<sup>T</sup> (84.27 % pairwise similarity; accession no. LN849256) and Bifidobacterium adolescentis JCM 1275<sup>T</sup> (84.16 % pairwise similarity; accession no. AF210319). As a consequence, strain LMG 28769<sup>T</sup> appeared only remotely related to other members of the genus Bifidobacterium in the phylogenetic tree based on hsp60 gene sequences (Fig. S2). All hsp60 nucleotide sequences were translated into protein sequences and compared using the tblastx tool (Altschul et al., 1997) in the NCBI nucleotide database. This comparison revealed that B. adolescentis JCM 1275<sup>T</sup> (95.02 % pairwise similarity; 98.10 % positives; accession no. AF210319) and Bifidobacterium kashiwahone DSM 21854<sup>T</sup> (94.03 % pairwise similarity; 98.01 % positives; accession no. AB491759) had the most similar amino acid sequences to the amino acid sequence of the Hsp60 protein of strain LMG 28769<sup>T</sup> (data not shown).

The G + C content of the DNA from strain LMG 28769<sup>T</sup> was determined by HPLC, using a high-performance liquid chromatograph equipped with an XBridge BEH Shield RP18 column and an UV detector (all from Waters). Genomic DNA was hydrolysed enzymatically, as described by Mesbah & Whitman (1989), and the hydrolysate was injected into the column and isocratically eluted with a mixture of 0.02 M NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> at pH 4.0 (98.5 %, v/v) and acetonitrile (1.5 %, v/v). Non-methylated lambda phage DNA (Sigma-Aldrich) was used as a calibration reference and genomic DNA from Escherichia coli LMG 2093 was included as a control. The mean G + C content of the DNA of strain LMG 28769<sup>T</sup> was 52.6 ± 0.5 mol%, which is within the range of 50–67 mol% G + C reported previously for the genus Bifidobacterium (Mattarelli et al., 2014).

The colony and cell morphologies of strain LMG 28769<sup>T</sup> were assessed after 6 days of anaerobic growth (AnaeroGen) at 28 °C on M144 agar medium (Fig. S3). Growth of strain LMG 28769<sup>T</sup>, B. crudilactis LMG 23609<sup>T</sup> and B. psychraerophilum DSM 21775<sup>T</sup> was assessed at 28 °C in M144 broth. After 24, 48 and 72 h, the cultures were centrifuged (5 min, 2700 g, 4 °C) and the cell-free culture supernatants were used for metabolite analyses. The presence of formic acid and the concentrations of lactic acid and acetic acid were determined by HPLC, using a high-performance liquid chromatograph equipped with an ICSep ICE-ORH-801 column (Interchim) and coupled to a refractive index detector (Waters), as described by Makras et al. (2005). The concentrations of d- and l-lactic acid were measured with HPLC, using a high-performance liquid chromatograph (Waters) equipped with a Shodex ORpak CRX-853 column (Showa Denko) and a UV detector (Waters). For the above, 250 µl of cell-free culture supernatant was added to a mixture of 500 µl acetonitrile and 250 µl ultrapure water. The samples were vortexed, centrifuged (21 000 g, 20 min, 4 °C) and filtered (0.2 µm pore size Whatman filters; GE Healthcare Life Sciences) before they were injected into the columns. Quantifications were performed with an external standard curve with standards prepared in the same way as the samples.

To assess the presence of fructose 6-phosphate phosphoketolase (F6PPK), strain LMG 28769<sup>T</sup>, B. crudilactis LMG 23609<sup>T</sup> and B. psychraerophilum LMG 21775<sup>T</sup> were incubated anaerobically (AnaeroGen) at 28 °C in M144 broth. After 72 h, the cultures were centrifuged (5 min, 2700 g, 4 °C) and the cell pellets were used for an F6PPK assay, as described by Orban & Patterson (2000). Briefly, the cell pellets were washed twice with 0.05 M phosphate buffer supplemented with 0.5 mg cysteine-HCl 1<sup>−1</sup> (adjusted to pH 6.5) and lysed with cetyl-trimethylammonium bromide. After the addition of NaF, sodium iodacetate and dipotassium fructose 6-phosphate, the samples were incubated at 37 °C for 30 min. This was followed by addition of hydroxylamine-HCl, trichloroacetic acid and alkali and the growth was visually assessed after 6 and 13 days of incubation. The production of gas during growth was visually assessed with inverted Durham tubes.

Enzyme activities and acid production from different substrates by strain LMG 28769<sup>T</sup>, B. crudilactis LMG 23609<sup>T</sup> and B. psychraerophilum LMG 21775<sup>T</sup> were assessed after 4 days of anaerobic growth (AnaeroGen) at 28 °C on M144 agar medium. Cells were suspended in 0.85 % (w/v) NaCl to prepare a suspension with turbidity similar to a McFarland No. 5 standard, from which 65 µl was added to each enzyme test of the API ZYM kit (bioMérieux). The results were read after 5 h of anaerobic incubation (AnaeroGen) at 28 °C. Cells were also suspended in 0.85 % (w/v) NaCl to prepare a suspension with turbidity similar to a McFarland No. 2 standard, from which eight drops were added to the API 50CHL medium, which was used to inoculate each substrate test of the API 50CHL kit (bioMérieux). The results were read after 6 and 13 days of anaerobic incubation (AnaeroGen) at 28 °C.

To assess the production of metabolites from glucose, strain LMG 28769<sup>T</sup>, B. crudilactis LMG 23609<sup>T</sup> and B. psychraerophilum LMG 21775<sup>T</sup> were incubated anaerobically (AnaeroGen) at 28 °C in M144 broth. After 24, 48 and 72 h, the cultures were centrifuged (5 min, 2700 g, 4 °C) and the cell-free culture supernatants were used for metabolite analyses. The presence of formic acid and the concentrations of lactic acid and acetic acid were determined by HPLC, using a high-performance liquid chromatograph equipped with an ICSep ICE-ORH-801 column (Interchim) and coupled to a refractive index detector (Waters), as described by Makras et al. (2005). The concentrations of d- and l-lactic acid were measured with HPLC, using a high-performance liquid chromatograph (Waters) equipped with a Shodex ORpak CRX-853 column (Showa Denko) and a UV detector (Waters). For the above, 250 µl of cell-free culture supernatant was added to a mixture of 500 µl acetonitrile and 250 µl ultrapure water. The samples were vortexed, centrifuged (21 000 g, 20 min, 4 °C) and filtered (0.2 µm pore size Whatman filters; GE Healthcare Life Sciences) before they were injected into the columns. Quantifications were performed with an external standard curve with standards prepared in the same way as the samples.
acid, HCl and FeCl, after which the colour reaction to red-
dish violet was visually assessed. A pellet of non-inoculated
M144 broth and a cell pellet of strain LMG 28769T without
fructose 6-phosphate were used as negative controls.
B. crudilactis LMG 23609T and B. psychraerophilum LMG
21775T were used as positive controls.

To assess gelatin degradation, strain LMG 28769T, B. crudi-
lactis LMG 23609 and B. psychraerophilum LMG 21775T
were inoculated into test tubes containing 5 ml of M144
broth supplemented with 12 % (w/v) gelatin. After
6 days of anaerobic incubation (AnaeroGen) at 28 °C, the
test tubes were cooled to 7 °C and gelatin degradation
was visually assessed by liquefaction. Non-inoculated test
tubes were used as negative controls and test tubes inocu-
lated with Serratia marcescens LMG 2792T were used as a
positive control. To assess casein degradation, the same
strains were grown on M144 agar medium supplemented
with skimmed milk powder (13 g l–1; Oxoid) and incu-
bated as described above. Casein degradation was visually
assessed by a clear zone around the colonies. Non-inocu-
lated agar media were used as negative controls and agar
media inoculated with Bacillus subtilis LMG 7135T were
used as a positive control.

Strain LMG 28769T, B. crudilactis LMG 23609T and
B. psychraerophilum LMG 21775T grew at 4–37 °C, and
under aerobic and anaerobic conditions. The three strains

**Table 1.** Differential characteristics between strain LMG 28769T and its closest relatives

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
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</thead>
<tbody>
<tr>
<td>Growth*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>4–37</td>
<td>4–37</td>
<td>4–37</td>
</tr>
<tr>
<td>Optimal temperature (°C)</td>
<td>28</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>pH</td>
<td>4.0 to 8.0</td>
<td>4.0 to 8.0</td>
<td>4.5 to 8.0</td>
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<tr>
<td>Enzyme activity‡</td>
<td></td>
<td></td>
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<tr>
<td>Alkaline phosphatase</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Cystine arylamidase</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Naphthol-AS-BI-phosphohydrolase</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Production of acid from*:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Methyl α-D-glucopyranoside</td>
<td>+</td>
<td>+ (−†)</td>
<td>+</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>±</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Amygdalin</td>
<td>±</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Salicin</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>–</td>
<td>+</td>
<td>– (±)</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>+</td>
<td>+ (−§)</td>
</tr>
<tr>
<td>Lactose</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Melezitose</td>
<td>–</td>
<td>+ (−†)</td>
<td>+</td>
</tr>
<tr>
<td>Gentiofuran</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Turanose</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Potassium gluconate</td>
<td>+</td>
<td>+ (−†)</td>
<td>+</td>
</tr>
<tr>
<td>Metabolites in M144 broth§</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molar ratio of acetic acid/lactic acid</td>
<td>4.83 ± 0.35</td>
<td>1.49 ± 0.01</td>
<td>2.77 ± 0.16</td>
</tr>
<tr>
<td>Production of formic acid</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>52.6</td>
<td>56.4</td>
<td>59.2</td>
</tr>
</tbody>
</table>

*Assessed after 13 days of anaerobic incubation at 28 °C.
†Data obtained from Delcenserie et al. (2007).
‡Assessed after 5 h of anaerobic incubation at 28 °C.
§Data obtained from Simpson et al. (2004).
†Assessed after 72 h of anaerobic incubation at 28 °C.
showed activity of F6PPK, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, z-galactosidase, b-galactosidase, z-glucosidase and b-glucosidase, but did not show activity of esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, trypsin, z-chymotrypsin, b-gluconuridase, N-acetyl-b-glucosaminidase, z-mannosidase or z-fucosidase. None of the three strains degraded gelatin or casein. Strain LMG 28769T, B. crudilactis LMG 23609T and B. psychraerophilum LMG 21775T produced acid from D-ribose, D-galactose, D-glucose, D-fructose, methyl z-D-glucopyranoside, maltose, melibiose, sucrose, raffinose and potassium gluconate, but not from glycerol, erythritol, D-ribonose, L-xylitol, D-adonitol, methyl b-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl z-D-mannopyranoside, arbutin, aesculin ferric citrate, salicin, cellobiose, lactose, trehalose, inulin, melezitose, starch, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabinose, D-arabitol, L-arabitol, potassium 2-ketogluconate or potassium 5-ketogluconate. Activity of F6PPK, leucine arylamidase, acid phosphatase (weak), naphthol-AS-BI-phosphohydrolase (weak), z-galactosidase, b-galactosidase, z-glucosidase and b-glucosidase is present. Activity of alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, trypsin, z-chymotrypsin, b-glucuronidase, N-acetyl-b-glucosaminidase, z-mannosidase and z-fucosidase is not present, and gelatin and casein are not degraded.

The type strain, LMG 28769T (=CCUG 67145T=RM 54638T), was isolated from a household water kefir fermentation process carried out in Brussels, Belgium, in 2014. The DNA G+C content of the type strain is 52.6 mol%.

Acknowledgements

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References


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