**Lactobacillus kefir sp. nov., a Component of the Microflora of Kefir**

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**Summary**

About 100 strains of heterofermentative rod-shaped lactic acid bacteria have been isolated from kefir grains and drink kefir, respectively. These isolates differ from *Lactobacillus brevis* and the other species of heterofermentative lactobacilli so far validly published with respect to biochemical characteristics and DNA/DNA homology. They are thus considered as representatives of the new species *Lactobacillus kefir* sp. nov., with strain DSM 20587 (= A/K) being the type strain.

Key words: *Lactobacillus kefir* – Kefir – Heterofermentative lactobacilli – Taxonomy

**Introduction**

Although the microflora of kefir has been studied by many authors, the taxonomic position of the lactic acid rods dominating in kefir grains is still unclear. Most of the early investigators used the epithet "caucasicus" for their kefir isolates ("Dispora caucasica", Kern 1882; "Lactobacillus caucasicus", Beijerinck, 1889; Beijerinck 1901; "Bacillus caucasicus" Freudreich 1897; "Bacterium caucasicum" Nikolajewa 1907; "Betabacterium caucasicum" Orla-Jensen 1919). The homofermentative "Lactobacillus caucasicus" (Beijerinck, 1889) Beijerinck 1901 became the type species of the genus *Lactobacillus*. However, this name was rejected by the Judicial Commission (Opinion 38, Lapage et al., 1975) since no type strain was available and the various organisms isolated from kefir by different authors and called "caucasicus" were probably not identical. Thus, the organism isolated by Beijerinck was homofermentative and grew at temperatures up to 44 °C, while that isolated by Orla-Jensen (1919) was heterofermentative and did not grow at tempera-

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1 Names not contained in the Approved Lists of the Bacterial Names (Skerman et al., 1980) are placed between quotation marks.
tatures above 40 °C. Heterofermentative kefir isolates were also called "Lactobacillus desidiosus" by Vaughn et al. (1949). A close relationship between the rod isolated from kefir and Lactobacillus brevis was suggested by Pederson (1938) and, more recently, authors have usually considered the heterofermentative rod-shaped kefir isolates as strains of L. brevis (La Rivière et al. 1967; Rosi and Rossi, 1978). However, Vescovo et al. (1979) showed that lactic acid bacteria isolated from kefir exhibited a high DNA/DNA homology among each other, but not with strains of L. brevis. Thus, the taxonomic position of the most frequent lactic acid bacteria in kefir was reinvestigated (Kunath and Kandler, 1983) and the main heterofermentative rod is described as Lactobacillus kefir sp. nov. in this paper.

Materials and Methods

The type strains of Lactobacillus brevis DSM 20054 and Lactobacillus buchneri DSM 20057 were obtained from the Deutsche Sammlung von Mikroorganismen (DSM). Kefir grains were obtained from eight different sources:

Private homes (A, B, E, G), producers of starter cultures (C), Bakteriologisches Institut der Süddeutschen Versuchs- und Forschungsanstalt für Milchwirtschaft, Freising (D), Institute of Dairy Science, University of Helsinki (F), Institute for Milk Technology, Warsaw Agricultural University (H).

Growth Conditions

The strains were isolated by plating diluted samples of drink kefir or of a homogenate of kefir grains on MRS-agar (De Man et al., 1960) and incubation at 30 °C in an atmosphere of 90% N₂ + 10% CO₂. Stock cultures were transferred into MRS-agar stabs and kept at 4 °C.

Determination of various characteristics

The physiological characters were tested according to Sharpe (1962) and Rogosa (1970). Sugars were added to the basal medium as filter-sterilized solutions to a final concentration of 10 g/l.

Disc – Electrophoresis

The electrophoretic mobility of the lactate-dehydrogenases (LDH) was tested in polyacrylamide gels according to Maurer (1968), modified by Stetter (1973). Rabbit-L-lactate-dehydrogenase iso I (Boehringer, Mannheim) served as a reference.

Fermentation products from various carbohydrates

The configuration and amount of lactic acid was determined enzymatically (Hohorst, 1970) using L-lactate- and D-lactate-dehydrogenases (Boehringer, Mannheim).

Acetic acid was determined with acetate-kinase (Boehringer, Mannheim), according to Holz and Bergmeyer (1970) and ethanol with aldehyde-dehydrogenase and alcohol-dehydrogenase (Boehringer, Mannheim) as described by Beutler and Michal (1977).

Cell wall analysis

Cell walls were prepared and the murin components determined according to Schleifer and Kandler (1967, 1972). Teichoic acids were extracted from the cell walls using the hydrofluoric acid (HF) method described by Fiedler et al. (1981).

After hydrolysis of the HF-extract (2N HCl, 3 h, 100 °C) sugar alcohols were separated as the corresponding alditol-acetate derivatives (Albersheim et al., 1967) by gas liquid
chromatography using a nickel 200 column 6 ft. × 1/8 in. packed with 3% SP 2340 on 100/120 Supelcoport (Supelco, Inc.).

**Determination of the G + C content of DNA and DNA/DNA homology**

DNA was isolated and purified according to a modified method of Marmur (1961). The melting point (Tm) of the purified DNA was determined according to Marmur and Doty (1962) and the resulting G + C mol% was calculated by the equation of De Ley (1971). DNA extracted from *E. coli* B cells (Sigma) was used as a reference showing a G + C mol% value of 50.7.

Hybridization and calculation of the % homology values was carried out as described by De Ley et al. (1970), Gillis et al. (1970) and Huss et al. (1983).

**Results and Discussion**

As shown by direct microscopic count kefir grains from 8 different sources contained $4 \times 10^8$ yeasts and $3 \times 10^9$ lactobacilli per g grain. About 15% of the yeasts and 30% of the lactobacilli observed under the microscope could also be detected by plate count. Cells of leuconostocs and streptococci could not be seen under the microscope, but leuconostocs were found to be about two orders of ten less than lactobacilli by plate count and streptococci were seen in titer tubes of dilutions lower than $10^{-4}$ (Table 1, for details see Kunath and Kandler 1983). Sterile milk freshly inoculated with about 5 g grains/100 ml, after slight shaking, contained $10^5$ yeasts and $10^8$ lactobacilli washed off from the grains. They increased during 48 h of incubation to about $10^7$ yeasts and $10^8$ lactobacilli. In addition, $10^9$ streptococci and $10^8$ leuconostocs were found after 48 h when the kefir was ready to drink (Table 1).

Table 1. Composition of the microflora of kefir grains and drink kefir

<table>
<thead>
<tr>
<th>Source</th>
<th>Yeasts</th>
<th>Streptococcus</th>
<th>Leuconostoc</th>
<th>Total</th>
<th>Lactobacillus</th>
<th>Heterofermentative (%)</th>
<th>Homofermentative (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grain</td>
<td>$4 \cdot 10^8$</td>
<td>$&lt;10^9$</td>
<td>$2.5 \cdot 10^7$</td>
<td>$3 \cdot 10^9$</td>
<td>10</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Inoculated milk before incubation</td>
<td>$1 \cdot 10^6$</td>
<td>$&lt;10^9$</td>
<td>$10^6$</td>
<td>$1 \cdot 10^8$</td>
<td>20</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Drink kefir</td>
<td>$10^7$</td>
<td>$10^9$</td>
<td>$10^8$</td>
<td>$10^8$</td>
<td>80</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

1 Mixture of grains from 8 different sources (microscopic count).
2 Plate count.
3 Incubation: 24 h with grains at 20 °C; 24 h after decanting from the grains at 10 °C.

More than 100 strains of lactobacilli have been isolated and tested for their fermentation patterns. While the homofermentative strains dominated in the grains, about 80% of the lactobacilli present in the drink kefir after 48 h incubation were
Table 2. Characteristics of kefir isolates

<table>
<thead>
<tr>
<th>Strains</th>
<th>L-Arabinose</th>
<th>D-Ribose</th>
<th>D-Xylose</th>
<th>Galactose</th>
<th>Sorbitol</th>
<th>Mannose</th>
<th>Mannitol</th>
<th>Fructose</th>
<th>Raffinose</th>
<th>Maltose</th>
<th>Melizitose</th>
<th>Rhamnose</th>
<th>Sucrose</th>
<th>Malto</th>
<th>Trehalose</th>
<th>Lactose</th>
<th>Inulin</th>
<th>Cellulobiose</th>
<th>Gluconate</th>
<th>Salicin</th>
<th>Electrophoretic mobility of LDH (R buffer = 1.0)</th>
<th>Cell wall</th>
<th>Mannol type</th>
<th>Glycerol</th>
<th>teatoic acid</th>
<th>G + C mol%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kefir A/K; E40; 10a</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>1.23</td>
<td>1.07</td>
<td>Lys-D-Asp</td>
<td>+</td>
<td>41.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>isolates ATCC 8007</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>1.23</td>
<td>1.07</td>
<td>Lys-D-Asp</td>
<td>+</td>
<td>41.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. brevis DSM 20054 a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>1.75</td>
<td>1.40</td>
<td>Lys-D-Asp</td>
<td>+</td>
<td>45.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. buchneri DSM 20057 a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>1.33</td>
<td>1.22</td>
<td>Lys-D-Asp</td>
<td>+</td>
<td>44.7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All strains form CO₂ from hexoses and gluconate, NH₃ from arginine and grow at 15 °C but not at 45 °C.

A/K was isolated from kefir grains, E40 and 10a from drink kefir prepared from grains in the laboratory or by a dairy, respectively.

100 additional kefir isolates subjected to fermentation tests exhibited the same pattern of fermented carbohydrates, but L-arabinose was fermented by only 50% of the strains.

Type strain.
heterofermentative strains (Table 1). As shown in Table 2, 100 heterofermentative isolates from either grains or drink kefir, either prepared in the laboratory or manufactured by dairies, produced DL-lactic acid and fermented the same set of carbohydrates as did strain *L. brevis* ATCC 8007 (DSM 20 485) isolated from kefir and deposited at the ATCC originally under the name "*Lactobacillus caucasicus*" by C.S. Pederson in 1944. The only variation observed in the 100 isolates was the fermentation of L-arabinose which was positive in about 50% of strains. The fermentation balances, determined for several carbohydrates using strain A/K (Table 3), indicate the presence of the well-known hexose monophosphate pathway including the action of phosphoketolase.

Table 3. Fermentation balance of *L. kefir* A/K grown in MRS-medium containing different carbohydrates (mmol/l)

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>110 mmol/l</th>
<th>D-</th>
<th>Lactate</th>
<th>mmol/l</th>
<th>Acetate</th>
<th>Ethanol</th>
<th>C-2 compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>26</td>
<td>26</td>
<td>29</td>
<td>55</td>
<td>13</td>
<td>38</td>
<td>51</td>
</tr>
<tr>
<td>Lactose</td>
<td>22</td>
<td>22</td>
<td>30</td>
<td>52</td>
<td>10</td>
<td>39</td>
<td>49</td>
</tr>
<tr>
<td>Maltose</td>
<td>22</td>
<td>22</td>
<td>28</td>
<td>50</td>
<td>10</td>
<td>36</td>
<td>46</td>
</tr>
<tr>
<td>Sucrose</td>
<td>23</td>
<td>23</td>
<td>27</td>
<td>50</td>
<td>6</td>
<td>40</td>
<td>46</td>
</tr>
<tr>
<td>Arabinose</td>
<td>22</td>
<td>22</td>
<td>36</td>
<td>58</td>
<td>50</td>
<td>7</td>
<td>57</td>
</tr>
<tr>
<td>Gluconate</td>
<td>49</td>
<td>49</td>
<td>53</td>
<td>102</td>
<td>64</td>
<td>33</td>
<td>97</td>
</tr>
</tbody>
</table>

1 About 1 mol of CO₂ per mol of 6-carbon compound was formed in addition to the acids.

Optimal growth temperature, cell wall composition, electrophoretic mobility of LDH and G + C content of DNA were only determined in three isolates. They proved to be also the same in the three isolates and in strain ATCC 8007.

With respect to these criteria the heterofermentative kefir isolates are very similar to *L. brevis* and *L. buchneri*, with the exception of the electrophoretic mobility of D- and L-LDH. However, DNA/DNA hybridization (Table 4) showed high DNA/DNA homology among only the kefir isolates, but yielded very low values of about 20% and 40% between the kefir isolates and the type strains of *L. brevis* and *L. buchneri*, respectively. Since the applied hybridization technique (Gillis et al., 1970; De Ley et al., 1970) exhibits a relatively high background at an order of 30% (Huss et al., 1983) even a value of 40%, observed in the hybridization of kefir isolates with *L. buchneri*, may not indicate a significant relationship, while values of 80 to 90% among the kefir isolates, including strain ATCC 8007, indicate that these strains belong to one and the same species.

This result agrees with the data of Vescovo et al., (1979) who hybridized ATCC 8007 with strains of all heterofermentative species listed in Bergey's Manual 8th edition. There was no significant relationship to any of these species but some unnamed isolates from beer fell within the same homology group as ATCC 8007.

The kefir isolates also differ from all the other heterofermentative species in regard to either their G + C content of DNA, their cell wall composition or the
electrophoretic mobility of their D- and L-LDH. Therefore, they have to be considered as representatives of a distinct species different from any other species validly published as yet. Although, most likely, this species is the same as the one described by Orla-Jensen (1919) as “Betabacterium caucasicum”, we name it *L. kefir*, in order to avoid confusion. The epithet “caucasicus” was pre-empted for the homofermentative lactobacillus isolated from kefir by Beijerinck (1889) and this “Lactobacillus caucasicus” (Beijerinck, 1889) Beijerinck 1901 served as type species for the genus *Lactobacillus* until it was rejected by the Judicial Commission (Opinion 38, Lapage et al., 1975).

“*Lidesidiosus*” Vaughn et al. 1949, also isolated from kefir, was considered by its authors to be identical with “Betabacterium caucasicum” Orla-Jensen, 1919. However, the description states that “*Lidesidiosus*” ferments only arabinose and several monomeric hexoses but no oligosaccharides which is not the case in Orla-Jensen’s and our kefir isolates. Strain Co 23 of “*Lidesidiosus*” does not hybridize with the kefir isolate ATCC 8007 but with *L. brevis* ATCC 11577, isolated from saliva, and with other strains of non-kefir-origin (Vescovo et al., 1979). No type strain of “*Lidesidiosus*” has yet been designated and the name is not included in the “Approved Lists of Bacterial Names” (Skerman et al., 1980). It is doubtful if “*Lidesidiosus*” Vaughn et al. 1949 is the same organism as that isolated from kefir by Orla-Jensen, C.S. Pederson (ATCC 8007) and by us. Therefore, the specific epithet “desidiosus” is not revived.

While Rosi and Rossi (1978) suggested that the polysaccharides forming the kefir grains are produced by homofermentative lactic acid bacteria which they called “atypical streptobacteria”, La Rivière et al. (1967) found heterofermentative rods forming capsules of “kefran”, a polysaccharide constituting about 25% of the dry weight of the grains. They ascribed this organism to the species *L. brevis* and reported that capsule formation was already lost with the first transfer after the strains had been isolated. Although we applied the same techniques to detect capsule

Table 4. DNA/DNA homology % of heterofermentative lactobacilli from kefir

<table>
<thead>
<tr>
<th>Strains</th>
<th>A/K</th>
<th>E40</th>
<th>10a</th>
<th>ATCC 8007</th>
<th>L. buchneri DSM 20057</th>
<th>L. brevis DSM 20054</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kefir-isolates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(grain)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E40</td>
<td>91</td>
<td>80</td>
<td>80</td>
<td>85°</td>
<td>42°</td>
<td>5</td>
</tr>
<tr>
<td>10a</td>
<td>90</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>41</td>
<td>19</td>
</tr>
<tr>
<td>ATCC 8007</td>
<td>91</td>
<td>85°</td>
<td>80</td>
<td>-</td>
<td>48°</td>
<td>16°</td>
</tr>
<tr>
<td><em>L. buchneri</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSM 20057</td>
<td>40</td>
<td>42°</td>
<td>41</td>
<td>48°</td>
<td>-</td>
<td>20°</td>
</tr>
<tr>
<td>Type strain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. brevis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSM 20054</td>
<td>17</td>
<td>5</td>
<td>19</td>
<td>16°</td>
<td>20°</td>
<td>-</td>
</tr>
<tr>
<td>Type strain</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

formation of kefir isolates we were not successful. Thus it remains undecided which organism is responsible for grain formation. *L. kefir* may not be the kefiran producer but it is certainly the main lactic acid bacterium in drink kefir, whereas it is only a minor component in the grain.

![Fig. 1. L. kefir grown in MRS-bouillon. a) Phase contrast micrograph. b) Thin section electron micrograph, showing polyphosphate (?) granules.](image)

**Description of Lactobacillus kefir sp.nov.**

*L. kefir* (ke'fir, Turkish noun kefir, a Caucasian sour milk). Gram-positive, non spore-forming rods with rounded ends (Fig. 1a), often containing polyphosphate granules usually terminal (Fig. 1b); generally 0.6 to 0.8 by 3–15 μm, with a tendency to form chains of short rods or long filaments. Non motile. Colonies on MRS agar greyish, smooth and flat, 2–4 mm in diameter. Microaerophilic, surface growth is greatly enhanced by reduced O₂ tension or anaerobiosis; growth in agar stab occurs throughout the stab until shortly beneath the surface. Growth occurs between 10 and 40 °C with an optimum at 30 °C. Obligately saccharoclastic. Heterofermentative (Table 3). The fermented carbohydrates are listed in Table 2. Ammonia is formed from arginine; nitrate is not reduced to nitrite; indole and H₂S are not formed; catalase and benzidine test negative. Cell wall contains murine of the Lys-D-Asp type and glycerol teichoic acid. The data for electrophoretic mobility of D- and L-LDH are shown in Table 2.

G + C content of DNA of the type strain is 41.5 mol % (Tm).

Habitat: Kefir grains and drink kefir.

Type strain: DSM 20 587 = (A/K).
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