Lactobacillus fabifermentans sp. nov. and Lactobacillus cacaonum sp. nov., isolated from Ghanaian cocoa fermentations

Katrien De Bruyne,1 Nicholas Camu,2 Luc De Vuyst2 and Peter Vandamme1

1Laboratory of Microbiology, Ghent University, Ledeganckstraat 35, B-9000 Ghent, Belgium
2Research Group of Industrial Microbiology and Food Biotechnology (IMDO), Department of Applied Biological Sciences and Engineering, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium

Two Gram-positive bacterial strains, LMG 24284T and LMG 24285T, were isolated from different spontaneous cocoa bean heap fermentations in Ghana. Analysis of their 16S rRNA gene sequences indicated that they were members of the Lactobacillus plantarum and Lactobacillus salivarius species groups, respectively. DNA–DNA hybridization experiments with their nearest phylogenetic neighbours demonstrated that both strains represented novel species that could be differentiated from their nearest neighbours by pheS sequence analysis, whole-cell protein electrophoresis, fluorescent amplified fragment length polymorphism analysis and biochemical characterization. Therefore, two novel Lactobacillus species are proposed, Lactobacillus fabifermentans sp. nov. (type strain LMG 24284T = DSM 21115T) and Lactobacillus cacaonum sp. nov. (type strain LMG 24285T = DSM 21116T).

Abbreviation: FAFLP, fluorescent amplified fragment length polymorphism.

In chocolate manufacture, the desired characteristic flavour is influenced by the cocoa cultivar, cocoa bean fermentation, drying and roasting of the beans and final processing (Camu et al., 2008; Hansen et al., 1998; Schwan & Wheals, 2004; Thompson et al., 2001). Natural cocoa bean fermentation is being studied intensively (Camu et al., 2007, 2008; De Vuyst et al., 2008; Lagunes Gálvez et al., 2007; Nielsen et al., 2007). In this process, lactic acid bacteria are responsible for the conversion of citric acid and sugars into mainly lactic acid. Biodiversity studies report lactobacilli as the dominant species in the fermentation (Camu et al., 2007, 2008); species of the genera Leuconostoc, Pediococcus, Weissella and Lactococcus have also been reported (Camu et al., 2008; Carr et al., 1979; Passos et al., 1984a, b; Thompson et al., 2001). The population dynamics of lactic acid bacteria during spontaneous heap fermentations of cocoa beans in Ghana have been studied by Camu et al. (2007). In the course of this biodiversity study, two lactobacillus strains that could not be identified at the species level as belonging to any existing species were isolated. In the present study, additional analyses allocated these strains to two novel Lactobacillus species.

Strains LMG 24284T and LMG 24285T were isolated during the main crop of 2005 (October–December 2005) in New Tafo, Ghana, from fermenting cocoa beans, which were sampled both for microbiological analysis and for plating, isolation and monitoring of lactic acid bacteria as described by Camu et al. (2007). The isolates were checked for purity by microscopy and catalase and oxidase tests were performed. Dereplication and preliminary identification of all isolates was obtained by rep-PCR using the (GTG)5 primer (Gevers et al., 2001). In the numerical analysis of (GTG)5 profiles, strains LMG 24284T and LMG 24285T occupied distinct positions in the dendrogram.

The phylogenetic position of both strains was investigated by 16S rRNA gene sequence analysis, performed as described by De Bruyne et al. (2008). FASTA analysis of both 16S rRNA gene sequences [continuous stretches of 1532 bp (strain LMG 24284T) and 1527 bp (strain LMG 24285T)] identified members of the genus Lactobacillus as the most closely related bacteria. 16S rRNA gene sequence analysis of strain LMG 24284T indicated that it belonged to the Lactobacillus plantarum species group. 16S rRNA gene sequence similarities between strain LMG 24284T and the type strains of all species in this group were at least 98.2 %, i.e. similarities of 98.8, 98.7, 98.6 and 98.2 % to the type strains of Lactobacillus pentosus, L. paraplantarum, L.
plantarum subsp. plantarum and L. plantarum subsp. argentoratensis, respectively. Strain LMG 24285\(^T\) belonged to the Lactobacillus salivarius group with Lactobacillus mali LMG 6899\(^T\) (97.5\% 16S rRNA gene sequence similarity) as the nearest neighbour; 16S rRNA gene sequence similarities with the type strains of all other species in this group were below 97\%. The obtained 16S rRNA gene sequences of strains LMG 24284\(^T\) and LMG 24285\(^T\) and sequences of the type strains of species of both groups (retrieved from GenBank/EMBL) were aligned using CLUSTAL_X. A phylogenetic neighbour-joining tree was constructed using the BioNumerics software package, version 4.61 (Applied Maths). The statistical reliability of the topology of the neighbour-joining tree was evaluated by bootstrap analysis of 500 replicates (Fig. 1). Maximum-parsimony and maximum-likelihood cluster analysis confirmed the tree topology obtained by the neighbour-joining method (data not shown). However, analysis of 16S rRNA gene sequences often does not enable closely related Lactobacillus species to be differentiated, e.g. members of the L. plantarum species group (Fig. 1), where a maximum interspecies variation of 1.3\% is observed. The usefulness and accuracy of any bacterial species identification system depends on the distinction between intraspecific variation and interspecific divergence in selected loci (Meyer & Paulay, 2005). In the search for an accurate identification system, a higher degree of resolution was obtained when using phenylalanyl-tRNA synthase alpha subunit (pheS) partial gene sequences to differentiate members of the genus Lactobacillus (Naser et al., 2007). The conditions for amplification and sequencing of the pheS genes from strains LMG 24284\(^T\) and LMG 24285\(^T\) were as described by Naser et al. (2007). The primer combination pheS-21-F/pheS-23-R was applied to amplify the target genes of both strains. Additional sequence data from type strains within the two species groups under discussion were retrieved from the study of Naser et al. (2007). The pheS phylogenetic trees are based on the neighbour-joining method and were obtained by importing the external sequence alignments from CLUSTAL_X into the BioNumerics software package. Within the L. plantarum species group, strain LMG 24284\(^T\) showed a maximum pheS gene sequence similarity value of 81.9\% to L. pentosus LMG 10755\(^T\). For strain LMG 24285\(^T\), the highest pheS gene sequence similarity value was 85.0\% with L. nagelii LMG 21593\(^T\). Fig. 2 clearly shows a higher resolution than the 16S rRNA gene sequence analysis and suggests that strains LMG 24284\(^T\) (Fig. 2a) and LMG 24285\(^T\) (Fig. 2b) both represent novel Lactobacillus species.

The relatedness of the two cocoa isolates with their nearest neighbours was further investigated by SDS-PAGE of whole-cell proteins and fluorescent amplified fragment length polymorphism (FAFLP) analysis. SDS-PAGE of cellular proteins was performed as described by Pot et al.

---

**Fig. 1.** Phylogenetic neighbour-joining tree based on 16S rRNA gene sequences showing the relationship between L. fabifermantans LMG 24284\(^T\), L. cacaonum LMG 24285\(^T\) and other strains belonging to the L. plantarum and L. salivarius species groups, respectively. Lactobacillus delbrueckii subsp. delbrueckii DSM 20074\(^T\) was used as the outgroup organism. Bootstrap values (%) based on 500 tree replications are indicated at branching points. Bar, 2\% sequence divergence.

**Fig. 2.** Neighbour-joining analysis of the pheS gene sequences of Lactobacillus type strains belonging to the L. plantarum species group (a) and the L. salivarius species group (b). Distance estimations were obtained by the Kimura-2 model. L. delbrueckii subsp. delbrueckii LMG 6412\(^T\) was included as the outgroup organism. Bars, 10\% sequence divergence.
Densitometric analysis, normalization and interpolation of protein profiles of strains LMG 24284<sup>T</sup> and LMG 24285<sup>T</sup> were performed and the resulting profiles were added to the in-house database. Numerical analysis was performed using the BioNUMERICS software package, version 4.61 (Applied Maths). The whole-cell protein profiles of strains LMG 24284<sup>T</sup> and LMG 24285<sup>T</sup> were clearly different from those of the type strains of their nearest phylogenetic neighbours (Fig. 3). Similarly, FAFLP analysis performed as described by Franz et al. (2006) confirmed the separate taxonomic status of both strains (Fig. 4).

Finally, genomic DNA was prepared as described by Stackebrandt & Kandler (1979). DNA–DNA hybridizations were performed according to a modification (Goris et al., 1998) of the microplate method described by Ezaki et al. (1989). Reciprocal hybridization experiments were performed for each pair of strains. DNA–DNA hybridization values between strain LMG 24285<sup>T</sup> and <i>L. mali</i> LMG 6899<sup>T</sup> were 20 ± 2%, confirming that strain LMG 24285<sup>T</sup> represents a novel species. For strain LMG 24284<sup>T</sup>, hybridizations were performed against representatives of all members of the <i>L. plantarum</i> group. Hybridization values between strain LMG 24284<sup>T</sup> and <i>L. pentosus</i> LMG 10755<sup>T</sup>, <i>L. paraplantarum</i> LMG 16673<sup>T</sup>, <i>L. plantarum</i> subsp. <i>plantarum</i> LMG 6907<sup>T</sup> and <i>L. plantarum</i> subsp. <i>argentoratensis</i> LMG 9205<sup>T</sup> were 16, 16, 26 and 26%, respectively, again confirming that strain LMG 24284<sup>T</sup> represents a novel species within the genus <i>Lactobacillus</i>.

The DNA G+C content was determined according to the enzymic DNA degradation method of Mesbah et al. (1989). The DNA nucleotide mixture was analysed chromatographically using a Waters Breeze HPLC system. A thermostable XBridge Shield RP18 column was used at 37 °C with 0.02 M NH₄H₂PO₄ (pH 4.0)/1.5% (v/v) acetonitrile as solvent. Non-methylated lambda phage

---

**Fig. 3.** Cluster analysis of whole-cell protein profiles and dendrogram derived from UPGMA linkage of correlation coefficients of <i>L. fabifermentans</i> sp. nov. LMG 24284<sup>T</sup> and the type strains of all species in the <i>L. plantarum</i> species group and those of <i>L. cacaonum</i> sp. nov. LMG 24285<sup>T</sup> and its closest phylogenetic neighbours.

**Fig. 4.** FAFLP patterns and dendrograms based on the UPGMA linkage of Dice coefficients of <i>L. cacaonum</i> sp. nov. LMG 24285<sup>T</sup> and its closest neighbours from the <i>L. salivarius</i> species group (a) and of <i>L. fabifermentans</i> sp. nov. LMG 24284<sup>T</sup> and representatives of the <i>L. plantarum</i> species group (b).
DNA (Sigma) was used as calibration reference and Escherichia coli LMG 2093 DNA was used as control. The G+C content of strain LMG 24285\textsuperscript{T} was 34.5 mol%, which was comparable to that of L. mali (32–34 mol%; Kato et al., 2000). The G+C content of strain LMG 24284\textsuperscript{T} was 44.9 mol%, which was consistent with the G+C contents determined for members of the L. plantarum group (44–47 mol%; Bringel et al., 2005; Curk et al., 1996; Zanoni et al., 1987).

Cell and colony morphology were investigated after growth on MRS agar (pH 5.4; Oxoid) for 48 h aerobic incubation at 37 °C, except where stated otherwise. Conventional biochemical tests and growth characteristics were determined as described by De Bruyne et al. (2008). Carbohydrate fermentation tests were performed in duplicate using the API 50 CHL system (bioMérieux) and enzyme activities were tested in duplicate using the API ZYM system (bioMérieux), both following the manufacturer’s instructions. For the detection of glucose metabolites and the proportion of D- and L-lactate, strains were grown for 24 h at 30 °C in MRS broth (pH 5.4; Oxoid). Detailed phenotypic descriptions are given in the species descriptions. Characteristics that differentiate LMG 24284\textsuperscript{T} and LMG 24285\textsuperscript{T} from their closest relatives are summarized in Table 1.

Based on the discussed polyphasic analysis, it is proposed that these strains should be classified as representatives of two novel Lactobacillus species: Lactobacillus fabifermentans sp. nov. (LMG 24284\textsuperscript{T}) and Lactobacillus cacaonum sp. nov. (LMG 24285\textsuperscript{T}).

**Description of Lactobacillus fabifermentans sp. nov.**

Lactobacillus fabifermentans (fa.bi.fer.men’tans. L. n. faba a bean; L. part. adj. fermentans fermenting; N.L. part. adj. fabifermentans of fermenting beans).

Cells are Gram-positive, catalase-negative, facultatively anaerobic and non-motile. Cells are long rods (1.0–3.0 μm wide and 10.0 μm long) that appear singly, in pairs or in short chains. Colonies are greyish white, opaque, smooth and circular with a convex elevation and an entire margin (diameter approx. 1.0 mm). Growth is observed at temperatures from 10 °C (from day 8 of incubation) up to 37 °C (immediate growth from day 1 of incubation). At 37 °C, growth is observed in MRS broth (pH 3.9). Growth occurs in MRS broth supplemented with 6 % NaCl. The type strain (LMG 24284\textsuperscript{T}) produces lactic acid only as a metabolite from glucose. The ratio of production of D- and L-lactic acid isomers is 80 : 20. No gas production is observed. Arginine is deaminated. Acid is produced from L-arabinose, ribose, D-xylene, galactose, glucose, fructose, mannose, mannotol, N-acetylglosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, and L-fabifermentans bean; N.L. part. adj. fermentans fermenting; N.L. part. adj. fabifermentans of fermenting beans).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid from:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellobose</td>
<td>+</td>
<td>d</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Galactose</td>
<td>−</td>
<td>d</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mannitol</td>
<td>−</td>
<td>d</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Melibiose</td>
<td>−</td>
<td>NA</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Methyl α-D-mannopyranoside</td>
<td>−</td>
<td>NA</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Melezitose</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>d</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Raffinose</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Ribose</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Trehalose</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Growth at 45 °C</td>
<td>−</td>
<td>NA</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Lactic acid configuration</td>
<td>DL</td>
<td>L</td>
<td>DL</td>
<td>DL</td>
<td>DL</td>
<td>DL</td>
<td>DL</td>
</tr>
<tr>
<td>Maximum NaCl concentration for growth (%)</td>
<td>−*</td>
<td>NA</td>
<td>6</td>
<td>NA</td>
<td>8</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>34.5</td>
<td>32–34</td>
<td>44.9</td>
<td>46–47</td>
<td>44–45</td>
<td>44–46</td>
<td>44–46</td>
</tr>
</tbody>
</table>

*No growth with added NaCl in MRS broth.
sucrose, trehalose and gentiobiose. Acid is not produced from glycerol, erythritol, D-xylose, adonitol, methyl β-D-xylopyranoside, sorbose, rhamnose, dulcitol, inositol, sorbitol, methyl α-D-glucopyranoside, lactose, melibiose, inulin, melezitose, raffinose, starch, glycogen, xylitol, turanose, D-lyxose, D-tagatose, D- or L-fucose, D- or L-arabitol, gluconate or 2- or 5-ketogluconate.

The type strain is LMG 24284T (=DSM 21115T), isolated from a cocoa bean heap fermentation in Ghana. The DNA G+C content of the type strain is 44.9 mol%.

**Description of Lactobacillus cacaonum sp. nov.**

*Lactobacillus cacaonum* (ca.ca.o.num. N.L. n. cacao -onis a cacao bean; N.L. gen. pl. n. cacaonum of cacao beans).

Cells are Gram-positive, catalase-negative and facultatively anaerobic. Motility is not observed. Cells are small rods (0.8–1.0 μm wide and 2.0–3.0 μm long) that appear singly, in pairs or short chains. Colonies are beige, opaque, smooth and circular with a convex elevation and an entire margin (diameter approx. 0.5 mm). Growth is observed at temperatures from 10 °C (from day 6 of incubation) up to 37 °C (immediate growth from day 1 of incubation). At 37 °C, growth is observed in MRS broth (pH 3.9). No growth is observed in MRS broth supplemented with NaCl. Besides the production of (mainly) lactic acid, acetic acid is also observed as an end product of glucose metabolism. The type strain (LMG 24285T) produces both D- and L-lactate in a 10:90 ratio. No gas production is observed. Arginine is deaminated. Acid is produced from glucose, fructose, mannose, N-acetylglicosamine, aesculin, salicin, cellobiose and maltose. Acid is not produced from glycerol, erythritol, D- or L-xylose, adonitol, methyl β-D-xylopyranoside, galactose, sorbose, rhamnose, dulcitol, inositol, mannotol, sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, amygdalin, arbutin, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D- or L-fucose, D- or L-arabitol, gluconate or 2- or 5-ketogluconate.

The type strain is LMG 24285T (=DSM 21116T), isolated from a cocoa bean heap fermentation in Ghana. The DNA G+C content of the type strain is 34.5 mol%.

**Acknowledgements**

This work was supported by the Federal Research Policy [Action for the promotion of and Cooperation with the Belgian Coordinated Collections of Microorganisms (C3/00/17)], the Research Council of the Vrije Universiteit Brussel (GOA project), the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Project 040043) and Barry Callebaut NV. The cooperation of the Ghanaian Cocoa Producers' Alliance (COCOBOD, Accra, Ghana) and the Cocoa Research Institute of Ghana is highly appreciated. Approval was obtained by COCOBOD to cooperate with local farmers.

**References**


