Lactobacillus apis sp. nov., from the stomach of honeybees (Apis mellifera), having an in vitro inhibitory effect on the causative agents of American and European foulbrood

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A taxonomic study was performed on Gram-stain-positive, catalase-negative and regular rod-shaped bacterial strains R4B\textsuperscript{T} and R4C, isolated from the stomachs of honeybees. 16S rRNA gene sequence analysis revealed that the phylogenetic position of the novel strains was within the genus Lactobacillus; the highest sequence similarity to R4B\textsuperscript{T} was shown by Lactobacillus acidophilus BCRC 10695\textsuperscript{T} (93.6\%). Lower sequence similarities were found to other obligately homofermentative lactobacilli. A PCR–DGGE method could detect the sequence of the 16S rRNA gene of strain R4B\textsuperscript{T} at different developmental stages of honeybees occurring in two different locations in the Czech Republic. The distinctiveness of the strains from other lactobacilli was also confirmed by analysis of sequences of other phylogenetic markers applicable to the taxonomy of the genus Lactobacillus, ribotyping and rep-PCR analysis. The DNA G+C content of strain R4B\textsuperscript{T} was 41.3 mol%. The predominant cellular fatty acids of strain R4B\textsuperscript{T} were C\textsubscript{18} : 1\textit{v}9\textit{c}, summed C\textsubscript{19} : 1\textit{v}6\textit{c}/C\textsubscript{19} : 0, cyclo ω10c, C\textsubscript{16} : 0, summed C\textsubscript{18} : 1\textit{v}7\textit{c}/C\textsubscript{18} : 1\textit{v}6\textit{c} and summed C\textsubscript{16} : 1\textit{v}7\textit{c}/C\textsubscript{16} : 1\textit{v}6\textit{c}. The major polar lipids of strain R4B\textsuperscript{T} were glycolipids, lipids and phospholipids. Phenotypic and phylogenetic characteristics also confirmed the independent status of the strains at the species level. Interestingly, strain R4B\textsuperscript{T} was able to inhibit growth in vitro of Paenibacillus larvae subsp. larvae (causal agent of American foulbrood in honeybees) and Melissococcus plutonius (causal agent of European foulbrood). The name Lactobacillus apis sp. nov. is proposed for this novel taxon; the type strain is R4B\textsuperscript{T} (=CCM 8403\textsuperscript{T}=LMG 26964\textsuperscript{4}).

At the present time, the bacterial genus Lactobacillus comprises more than 100 species and subspecies (Hammes & Hertel, 2009) and represents a large group of Gram-positive bacteria within the phylum Firmicutes. Lactobacilli are well known as the main representatives of the lactic acid bacteria that occur in various carbohydrate-rich environments such as plant-derived materials, products of the dairy industry, the reproductive tract of women and the digestive tracts of mammals and insects (Hammes & Hertel, 2009; Forsgren et al., 2010). Lactobacilli are important members of healthy gastrointestinal tracts of animals, and some of them are used frequently as probiotics because of their beneficial influences on mammalian and human health (Zubillaga et al., 2001).

It is supposed that lactobacilli, other lactic acid bacteria and bifidobacteria have a beneficial effect on honeybee health. For this reason, attempts to describe the occurrence of these bacteria in the digestive tract of these important pollinators have increased in recent years (Olofsson & Vásquez, 2008). Novel species of lactobacilli have been detected from the digestive tract of Apis mellifera and Apis...
species of the genus *Lactobacillus* having in vitro inhibitory effects on both *P. larvae* subsp. *larvae* and *Paenibacillus alvei*.

The bacterial strains R4BT and R4C were isolated during the screening of the bacterial microflora of honeybees (*Apis mellifera* L.) performed in the private laboratory of one of the authors (S.D.). The strains were retrieved from the stomachs of two honeybees that originated from central Bohemia (locality Sluhy). Captured honeybees from a hive were transported to the laboratory and then decapitated and the stomach contents were serially diluted in tubes containing sterile anaerobic MRS broth (Oxoid). Aliquots of 0.1 ml were placed on fermented wheatgerm medium (FWGM) (Dubná et al., 2010) which contained free amino acids and peptides that improve the growth of lactobacilli. Samples were cultivated at 37 °C under anaerobic conditions (Oxoid anaerobic jars) for 48 h. Bacterial colonies were picked, transferred to tubes containing anaerobic FWGM and cultivated at 37 °C for 16 h.

The region carrying the 16S rRNA gene (1490 bp) of strains R4BT and R4C was amplified by PCR according to Killer et al. (2010). Strains R4BT and R4C were then characterized by multilocus sequence analysis. Partial sequences of atpA (ATP synthase alpha subunit), pheS (phenylalanine tRNA synthase alpha subunit), hsp60 (60 kDa heat-shock protein), rpoA (RNA polymerase alpha subunit) and tuf genes were amplified by the methods of Naser et al. (2005a, b), Dobson et al. (2002) and Ventura et al. (2003), respectively. All purified fragments were sequenced, checked, edited and compared with published sequences of related bacteria according to Killer et al. (2010). The 16S rRNA gene and the other gene sequences obtained in the present study and those of type strains of species of the genus *Lactobacillus* retrieved from GenBank (http://www.ncbi.nlm.nih.gov/nuccore) were aligned using CLUSTAL_X. These alignments were used for phylogenetic analyses. The MrBayes program (http://mrbayes.sourceforge.net/) using the maximum-likelihood algorithm recommended for reconstruction of phylogenetic trees (Tindall et al., 2010) was used for phylogenetic analyses. Strain R4BT is most closely related to *Lactobacillus acidophilus* BCRC 10695T (93.6 %) based on 16S rRNA gene sequence similarity. Lower sequence similarities were found in the group of obligately homofermentative lactobacilli. Sequences of the 16S rRNA gene revealed high similarity (99.86 %) between strains R4BT and R4C. The two tested strains had the same sequences for the other phylogenetic markers. For this reason, strain R4BT was selected for phylogenetic analyses. However, the small difference between these strains was confirmed by other genotypic and phenotypic characteristics. The highest atpA, hsp60, pheS, rpoA and tuf gene sequence similarities of strain R4BT were respectively found to *L. acidophilus* NCFM (84.3 %), *L. acidophilus* CIP 76.13T (86.4 %), *L. crispatus* CIP 102990T (84.4 %), *L. acidophilus* CIP 76.13T (84.4 %) and *L. acidophilus* NCFM (90.5 %). These similarities are much lower than those found between strains of the same species of the genus *Lactobacillus* (Ventura et al., 2003; Naser et al., 2005b).

The novel strain R4CT is clearly separated from related lactobacilli based on phylogenetic analyses of the 16S rRNA (Fig. 1), hsp60, pheS, rpoA and tuf genes (Figs S1–S4, available in IJSEM Online). atpA gene sequences are not available for many species of *Lactobacillus*; for this reason, a phylogenetic tree based on atpA gene sequences was not reconstructed. Phylogenetic analyses revealed that strain R4BT represents a separate lineage, most closely related to the *L. acidophilus* group.

The PCR–DGGE method has been used as a suitable tool to demonstrate the presence of novel species of the genus *Lactobacillus* in the digestive tracts of different developmental stages of honeybees. Samples of developmental stages of honeybees (1-, 3- and 7-day-old larvae, white and black pupae, 3-day-old honeybees, foraging workers and honeybee drones) were taken directly from hives from two localities within the Czech Republic (localities Postřížín, Central Bohemia, and Ústrašice, South Bohemia). Samples were then frozen and transported to the microbiology laboratory. Digestive tracts were removed aseptically and weighed. Approximately 100 mg samples of mixed digestive tracts of different developmental stages of honeybees were used for isolation of total bacterial DNA using the ZR Faecal DNA MiniPrep kit (Zymo Research). One-day-old larvae were first rinsed with 60 % ethanol and then used for isolation of total bacterial DNA. Amplification of total bacterial community DNA was performed by targeting 200 bp partial 16S rRNA gene sequences with universal bacterial primers FP338c1 and RP534 under conditions described previously (Mrázek et al., 2008). DNA isolated from strain R4BT and *Bifidobacterium asteroides* DSM 20089T was used for amplification of a 200 bp 16S rRNA gene region using the same primers. Amplicons were then used as a standard sample. PCR products were analysed on a DGGE gel (gradient from 35 to 65 %) according to the protocol of Muyzer et al. (1993). DNA bands of interest were sequenced and compared with the GenBank database using the BLAST algorithm (Mrázek et al., 2008). DNA bands belonging to strain R4BT (99–100 % sequence similarity) were detected mainly in samples of 3-day-old honeybees, foraging workers and honeybee drones from two different localities in the Czech Republic (Fig. S5).

Automated ribotyping with EcoRI was performed on strains R4BT and R4C using the RiboPrinter microbial characterization system (DuPont Qualicon) in accordance with the standard protocol provided by the manufacturer. The dendrogram was calculated with Pearson’s correlation coefficients with UPGMA using the BioNumerics software version 6.6. Ribotype patterns obtained from strains R4BT and R4C were compared with all entries included in the DuPont Qualicon reference database DUP 2011, containing more than 400 entries representing 74 species of the...
Fig. 1. Phylogenetic tree of species of the genus Lactobacillus that occur in the digestive tracts of humans and animals, showing the position of strain R4B T isolated from the stomach of a honeybee. The tree was reconstructed based on 16S rRNA gene sequences (1354 nt) using MrBayes software version 3.1.2. GenBank accession numbers are given in parentheses. The tree was rooted by Streptococcus bovis ATCC 33317 T. Posterior probabilities of Bayesian inference analysis are given at nodes. Bar, 0.05 substitutions per nucleotide position.
species description. Metabolic differences between strains R4B\textsuperscript{T} and R4C obtained from the Biolog GP2 MicroPlate (ThermoFisher Scientific) are listed in Table S1.

Anaerobic FWGM and TPY (tryptone phytone yeast extract) broth (pH 6.5) were found to be best for growth of strains R4B\textsuperscript{T} and R4C. However, these strains grew well in media intended for the cultivation of lactococci (MRS broth and agar, Rogosa agar). Differences in OD\textsubscript{620} in comparison with controls were used for evaluation of growth characteristics. The ability of strains R4B\textsuperscript{T} and R4C and L. acidophilus CCM 4833\textsuperscript{T} to grow at low and high pH was determined at 37°C in anaerobic TYP broth at pH 3.0–9.5 for 24–48 h. The pH was regulated using concentrated solutions of HCl and NaOH. The temperature range for growth was tested in the same medium at 5–46°C for 24–72 h (Killer et al., 2010). Strains R4B\textsuperscript{T} and R4C grew over a relatively narrow pH range (pH 5–9) in comparison with L. acidophilus CCM 4833\textsuperscript{T} (pH 3.5–8.5). Strains R4B\textsuperscript{T} and R4C grew over the same temperature range of 20–40°C; in contrast, L. acidophilus CCM 4833\textsuperscript{T} was able to grow at 45°C (Table 1).

The end products of hexose catabolism for the representative strain R4B\textsuperscript{T} were assessed using the capillary isotachophoresis method (Killer et al., 2011). Strain R4B\textsuperscript{T} was also tested for production of D- and L-lactic acid by using a d/l-lactic acid kit (Megazyme). Gas production from glucose was assayed using a Durham tube in MRS broth. We determined that lactic acid was the main end product of fermentation (89%), with very small amounts of propionic (4%), valeric (4%) and acetic (3%) acids also being produced. These results, along with the inability to utilize pentoses and gluconates, suggest that the novel species belongs to the obligately homofermentative lactobacilli (Hammes & Hertel, 2009). Cells of R4B\textsuperscript{T} produced only l-lactic acid from D-glucose.

Analyses of cellular fatty acids and polar lipids were carried out by the Identification Service of the DSMZ according to the methods of Miller (1982), Kämpfer & Kroppenstedt (1996), Bligh & Dyer (1959) and Tindall et al. (2007). Strain R4B\textsuperscript{T} was cultivated under anaerobic conditions in MRS medium for 24 h at 37°C for these analyses. Bacterial cells were collected by centrifugation and lyophilized. C\textsubscript{18}:1\textit{ω}9\textit{c}, summed C\textsubscript{19}:0\textit{ω6c}, C\textsubscript{19}:0\textit{ω6c} cyclo ω10c, C\textsubscript{16}:0 summed C\textsubscript{18}:1\textit{ω7c}L. acidophilus C; in anaerobic TYP broth. We determined that lactic acid was the main end product of fermentation (89%), with very small amounts of propionic (4%), valeric (4%) and acetic (3%) acids also being produced. These results, along with the inability to utilize pentoses and gluconates, suggest that the novel species belongs to the obligately homofermentative lactobacilli (Hammes & Hertel, 2009). Cells of R4B\textsuperscript{T} produced only l-lactic acid from D-glucose.

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Table 1. Differentiation of strains of Lactobacillus apis sp. nov. from phylogenetically related obligately homofermentative lactobacilli

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*Data from Hammes & Hertel (2009).*
In summary, the results obtained in the present study demonstrated that strains R4B and R4C isolated from a honey bee stomach represent a novel obligately homofermentative species of the genus *Lactobacillus*, for which the name *Lactobacillus apis* sp. nov. is proposed.

**Description of Lactobacillus apis sp. nov.**

*Lactobacillus apis* (a’pis. L. gen. fem. n. apis of/from a honey bee, the genus name of the true honey bee *Apis mellifera* L., referring to the insect host of the first strains).

Cells growing in liquid anaerobic media (FWGM, TPY) are Gram-stain-positive, non-spore-forming, non-motile, regular-shaped rods, 0.5–1.0 μm long. They are arranged mainly in long chains or singly and do not grow under aerobic conditions on solid media. Colonies on TPY and MRS agars under anaerobic conditions after 72 h of incubation at 37 °C are circular, white and smooth with entire edges and reach only 0.25–0.29 mm in diameter (measured using a light microscope with an extended scale). Cells are obligately homofermentative and produce L-lactic acid from d-glucose. Growth is found under strictly anaerobic and microaerophilic conditions (using the CampyGen gas-generating system; Oxoid). Results of Rapid ID 32A and API ZYM tests reveal production of β-galactosidase-6-phosphate, β-glucosidase, N-acetyl-β-glucosaminidase, glutamic acid decarboxylase, arginine arylamidase, phenylalanine arylamidase, leucine arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, serine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Weak activities of β-arylarnidase, glycine arylamidase, histidine arylamidase, serine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Weak activities of β-galactosidase and esterase (C4) are found. Activities of α-galactosidase, α-glucosidase, α-arabinosidase, α-fucosidase, β-glucoronidase, alkaline phosphatase, proline arylamidase, leucyl glycine arylamidase, pyroglutamyl acid arylamidase, glutamyl glutamic acid arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, urose, arginine dihydrolase, esterase lipase (C8), lipase (C14) and catalase are not detected. Able to use the following carbon sources by respiration: dextrin, N-acetyl-D-glucosamine, methyl β-D-glucoside, d-lactic acid methyl ester, l-lactic acid and pyruvic acid methyl ester. Negative for utilization of α- and β-cyclodextrin, mannann, Tween 40 and 80, N-acetyl-β-D-mannosamine, D-galacturonic acid, D-gluconic acid, myo-inositol, lactulose, methyl α-D-galactoside, methyl α-D-mannoside, patatinose, D-psicose, β- and γ-hydroxybutyric acid, p-hydroxyphenylacetic acid, α- and β-ketoglutaric acid, lactamid, D-and l-malic acid, succinic acid monomethyl ester, propionic acid, succinamic acid, succinic acid, N-acetyl-l-glutamic acid, l-alaninamide, D- and L-alanine, l-asparagine, l-glutamic acid, glycl l-glutamic acid, l-serine, putrescine, 2,3-butanediol, adenosine, 2′-deoxyadenosine, thymidine, uridine, adenosine 5′-monophosphate, thymidine 5′-monophosphate, uridine 5′-monophosphate, d-fructose 6-phosphate, 2-D-glucose 1-phosphate, D-glucose 6-phosphate and D,L-α-glycerophosphate. Other biochemical and phenotypic characteristics are shown in Table 1. Cells exhibit antagonistic effects in vitro against the agents of American foulbrood (*P. larvae* sp. *larvae*) and European foulbrood (*M. plutonius*). C18:1ω9c, summed C19:1ω6c/C19:0 cyclo ω10c, C16:0 summed C18:1ω7c/C18:1ω6c and summed C16:1ω7c/C16:1ω6c are major fatty acids. The major polar lipid groups are glycolipids, lipids and phospholipids.

The type strain, R4B (=CCM 8403T =LMG 26964T), was isolated from the stomach contents of honeybees (*Apis mellifera* L.) sampled from central Bohemia (locality Sluhy), Czech Republic, in 2009. The strain was deposited as a patent strain. The DNA G+C content of the type strain is 41.3 mol%.

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