IJSEM Papers in Press
Published online 7 June 2002 http://dx.doi.org/10.1099/ijs.0.02202-0

International Journal of Systematic and Evolutionary Microbiology (2002)

Molecular analysis of sourdough reveales *Lactobacillus mindensis* sp. nov.

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Genotypic fingerprints to analyze the bacterial flora of an industrial sourdough revealed a coherent group of strains, which could not be associated to a valid species. Comparative 16S rDNA sequence analysis showed that these strains form a homogeneous cluster distinct from their closest relatives *Lactobacillus farciminis*, *L. alimentarius* and *L. kimchii*.
For a further characterization, physiolgical (sugar fermentation, formation of DL-lactate, hydrolysis of arginine, growth temperature, CO_2 production), and chemotaxonomical properties have been determined. The G + C content of the DNA is 37.5 ± 0.2. The peptidoglycan is of the lysine-D-iso-asparagine (L-Lys-D-Asp) type. The strains are homofermentative, gram-positive, catalase-negative, non-spore-forming, non-motile rods. They were found as major stable component of a rye flour sourdough fermentation. Physiological, biochemical as well as genotypic data outed them as a new species of the genus *Lactobacillus*.
DNA-DNA hybridization of genomic DNA experiments confirmed that the isolates represent a new *Lactobacillus* species, for which we propose the name *Lactobacillus mindensis*. The type strain of this species is DSM 14500^T.

Key words: *Lactobacillus mindensis*, sourdough, taxonomy

INTRODUCTION

Within bacteria the group of lactic acid bacteria (LAB) plays the obviously most important role in human and animal nutrition and maintenance of health (Hammes & Vogel, 1995; Herrero *et al*., 1996). From an ecological point of view food fermentations represent special niches where communities of highly specialized organisms have been established. As in cereal fermentations educts cannot be subjected to heat-sterilization, the occurence of microorganisms as well as their numbers are strictly depending from substrates and technological parameters (Salovaara, 1998). With few exceptions, in sourdough fermentation for rye bread production mainly lactobacilli were shown to be responsible for acidification, inhibition of rye amylases, bread volume, texture and nutritional value or increased shelf life and flavour (Vogel *et al*., 1996; 1999). Depending on the tradition in production parameters of the sourdough, isolates were assigned to the obligately homofermentative species *Lactobacillus acidophilus*, *L. delbrueckii*, and *L. farciminis*, the facultatively heterofermentative *L. alimentarius*, *L. casei*, *L. paralimentarius*, *L. plantarum*, and the heterofermentative *L. brevis*, *L. buchneri*, *L. fermentum*, *L. fructivorans*, and *L. sanfranciscensis*. The consequent application of molecular techniques led to the identification of many new species.
Both, *L. pontis* and its phylogenetic relative *Lactobacillus panis* were described as endemic members to batters with an extended fermentation period and higher temperatures (Vogel *et al*., 1994; Wiese *et al*., 1996). The most recently described species were *Lactobacillus frumenti* (Müller *et al*., 2001) and *L. paralimentarius* (Cai *et al*., 2001).

Recently, we isolated an organism that occurred in small numbers beside dominating strains of *L. sanfranciscensis* in a commercial sourdough starter preparation that was not assignable to an hitherto known species. As this organism was also shown to persist after multiple consecutive propagations over six month in a bakeries sourdough, we considered it for a relevant member of the sourdough flora. According to phenotypic and genotypic results the purpose of the present study was to describe this *Lactobacillus* as a new species and for which we propose the name *Lactobacillus mindensis.*

METHODS

Strains, medium and culture conditions. Strain TMW 1.80 was isolated from an industrial rye sourdough starter preparation (BRS). Strains TMW 1.1201, TMW 1.1199 and TMW 1.1206 originate from a bakery sourdough initiated with BRS and propagated by back-slopping for 6 month without additional inoculation with BRS. Samples were subjected to serial dilutions and plated on modified MRS medium as described by Vogel *et al.* (1994). Strains of *L. mindensis* were incubated at 30 °C. Other lactobacilli were grown on regular MRS and incubated at the temperature recommended by the respective strain collection. Solid media were incubated under a modified atmosphere (N_2:CO_2 90 %:10 %, v/v). Cultures were maintained at –80 °C in glycerol stocks. The purity of cultures was checked microscopically. Strains have been deposited at the Deutsche Stammsammlung für Mikroorgansimen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) as DSM 14500^T.

Physiological characterization. Sugar fermentation patterns were determined by using a microtiter plate assay as described by Müller *et al.* (2001). The reproducibility was verified by repeated analyses. An pH dependent change of the indicator was documented after 24 h, 48 h and 168 h. Only definitive turnovers were rated as positive. Additional experiments were carried out using the API 50 CH kit (bioMérieux, France).

The formation of the lactate isomers in the fermented broth was determined enzymatically using the D/L-lactate test kit (Boehringer, Germany). Arginine hydrolysis was determined according the methods described by Sharpe (1979).

Morphological characteristics. Cell morphology was studied with a light microscope by phase-contrast modus. Gram determination was performed using the KOH method of Gregersen (1978).

DNA base composition. The G + C content in mol % of the strains TMW 1.80 was determined by a HPLC analytical method. The experiments were performed by the DSMZ (Germany). They were carried out using the protocol previously described by Tamaoka & Komagata (1984). The G + C content (mol %) was determined after Meshbah *et al.* (1989). Wild-type lambda phage DNA was used as standard.

Cell wall. The peptidoglycan structure of the cell wall was determined by the DSMZ (Germany). The absence of teichoic acids was determined as described by Baddiley and Davison, (1961).

Determined as described in : Baddiley, J. & Davison, A. L. (1961). The occurence and location of teichoic acid in lactobacilli. J Gen Microbiol. 24, 295-299

DNA isolation. DNA was isolated according to Marmur (1961) with some modifications. One hour before cells were harvested, penicillin G (Sigma, USA) was added to inhibit the synthesis of crosslinking of the cell wall, and therefore to facilitate the lysis. A wet weight of 70 mg of cells was utilized for the DNA isolation. Following the protocol, lysis was completed within 45-90 min after the addition of lysozyme and mutanolysine. For some strains a more effective lysis was obtained by an overnight lysis at 4°C with a subsequent proteinase K treatment at 60 °C for 1 h, and then continuing the protocol. The purified and vacuum dried DNA was dissolved in 2 x SSC (0.3 M NaCl, 0.03 M Na_3Citrate $2H_2O$, [pH 7.0]). This DNA preparation served for DNA-DNA hybridization experiments as well as for 16S rDNA amplification. DNA used for RAPD analyses were prepared in smale scale preparations as described by Lewington *et al.* (1997).

RAPD-PCR. The colonies subjected to RAPD-PCR were picked randomly. PCR was carried out with the oligonucleotide primer M13V (5‘-GTT TTC CCA GTC ACG AC-3‘). All reactions have been performed in TopYield™ Strips (Nunc, Denmark) with oil overlay (50µl) and TECAN sealing (Tecan, Kreilsheim, Germany). The conditions for PCR amplification were as follows: One µl of genomic DNA, 5 µl of 10x reaction buffer, 5 mM $MgCl_2$, 200 nM each of the four deoxynucleotides, 1.5 U Taq polymerase (all components from Amersham Pharmacia Biotech, Freiburg, Germany) and 20 pmol of primer M13V. The PCR reactions were carried out on a Hybaid Omni Gene thermocycler equipped with heated lid (MWG-Biotech, Germany). The cycling program was: 3 cycles, 96 °C for 3 min, 35 °C for 5 min, 75 °C for 5 min; 32 cycles, 96 °C for 1 min, 55 °C for 2 min, 75 °C for 3 min. Amplicons were electrophoretically separated on a 1.5 % TBE agarose gels.

DNA-DNA hybridization. The determination of DNA homology values were carried out using a modified procedure as described by Cardinali *et al.*, (2000). Hydroxyapatite (100mg; BioRad) was suspended in 1 ml 100 mM sodium phosphate buffer (NPB) pH 6.7, heated for 10 min at 65 °C and centrifuged (14000 x g) for 30 s at 4 °C. HTP pellet was resuspended with the DNA solution already equilibrated at 65 °C, incubated at 65 °C for 15 min and then centrifuged (14000 g) for 30 s at 4 °C. HTP-bound DNA was washed twice with 600 µl 120 mM NPB and once with 600 µl 180 mM NPB. Finally DNA was resuspended in

400 µl of 300 mM potassium/sodium phosphate buffer (NPPB, pH 7.2) incubated for 15 min at 65 °C and then centrifuged for 30 s at 14000 g.

Desalination was carried out with NAP-5 columns (Amersham-Pharmacia Biotech, Sweden).

DNA was diluted in water to reach a final concentration of 10 ng μl^{-1} (optical density at 260 nm of 0.200 ± 5 %). DNA was stored at –18 °C.

For dot-blotting of DNA samples were diluted in 0.4 M NaOH to a final concentration of 1 ng μl^{-1} and incubated for 30 min at room temperature. DNA samples (10 ng) were transferred by using a dot blot apparatus (Stratagene, USA) on nylon Hybaid-N+ membrane (Amersham Pharmacia, Biotech, Germany). Fixation of DNA on the membrane was achieved by incubation at 80 °C for 1 h.

For quantification a serial dilution (10, 8, 6, 4, 2 ng) of unlabelled DNA was doted.

DNAs used for probes were labelled using the non radioactive ECL random prime labeling and detection system (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Prehybridization, hybridization and stringency washings (0.5 NaCl) were carried out at 42 °C.

Membranes were exposed to a Kodak X-Omat film (Sigma-Aldrich, Germany). The spot intensity was calculated with the Image Master 2D Elite software (Amersham Pharmacia Biotech, Germany).

16S rDNA amplification and sequencing. PCR-mediated amplification of the complete 16S rDNA was carried out in a Gradient Master Thermocycler (Eppendorff, Germany). All reagents if not indicated were ordered from Amersham Pharmacia Biotech (Germany). The amplification conditions were as follows: 1 µl of genomic DNA, 10 µl 10x reaction buffer, 200 nM each of the four deoxynucleotides, 1.5 U Taq polymerase, 20 pmol primer (Interactiva, Germany) each (616V: 5'-AGAGTTTGATYMTGGCTCAG-3'; 630R: 5'-CAKAAAGGAGGTGATCC-3'), dH_2O to a final volume of 100 µl. The PCR conditions were: (94 °C/2 min)1x, (94 °C/45 sec, 52 °C/1 min, 72 °C/30 sec) 30x, (94 °C/1 min, 72 °C /4 min) 1x. PCR products were purified by the QIAquick PCR purification kit (Quiagen, Germany) and were eluted with 60 µl elution buffer. DNA sequences were determined by the chain-termination method (Sanger *et al*., 1977) using the ABI Prism™ Dye Terminator Cycle Sequencing Kit (Perkin Elmer, UK) on an ABI 373 stretch sequencing system by a commercial service (SequiServe, Germany). For sequencing, the amplification primer 616V together with the internal primers 609R (5'-ACT AC(CT) (AGC)GG GTA TCT AA(GT) CC-3'), 612R (5'-GTA AGG TT(CT) T(AGCT)C GCG T-3'), 607R (5'-ACG TGT GTA GCC C-3'), 606R (5'-T(AG)A CGG (GC)C(AG) GTG TGT ACA-3') and 607V (5'-GGG CTA CAC ACG TGC-3') were used.

Phylogenetic analysis. The complete 16S rDNA sequence of *L. mindensis* DSM 14500^T was fitted into alignments of almost full primary structures available in public databases (Ludwig, 1995). Additional sequences were obtained from the ribosomal database project (Maidak et al., 2001). Distance matrix, maximum parsimony and maximum likelihood methods were applied for tree reconstructions as implemented in the ARB software package (Ludwig & Strunk, 1997). Different data sets varying with respect to included outgroup reference sequences as well as alignment positions were analyzed. To exclude highly variable regions a filter with 50% invariance were applied.

Nucleotide sequence accession number. The 16S rDNA sequence of strain *L. mindensis* DSM 14500^T has been submitted to the EMBL nucleotide sequence database under the accession number AJ313530.

Species specific detection with PCR. The specific primer PmindR was checked for its specificity against other bacterial 16S rRNA sequences by using the probe checking software provided in the Ribosomal Database Project (Maidak *et al*., 2001). PmindR was applied in combination with primer 616V in the PCR assay. The amplification conditions were as follows: 1 µl of genomic DNA, 5µl 10x reaction buffer, 1.5 µl DMSO, 200 nM each of the four deoxynucleotides, 1.5 U Taq polymerase, 20 pmol primer each (616V, PminR), deionized H_2O to a final volume of 50 µl. The used PCR program was: (94 °C/2 min)1x, (94 °C/45sec, 62.5 °C/30sec., 72 °C/30sec) 30x. Primer sequence PminR: 5´- AAC AGT GAT CAT GTG AAG AC-3´. A control PCR to check the accessibility of DNA with universal primers 616V and 609R was performed as described previously (Garriga *et al*., 1994).

RESULTS AND DISCUSSION

Typical constituents found in a traditional 3-stage processed doughs were obligately heterofermentative species like *L. sanfranciscensisn*, *L. brevis*, *L. fermentum*, *L. fructivorans* (Stolz, 1999) among which *L. sanfranciscensis* were the numerically dominant organisms (Böcker , 1990; 1995; Spicher and Schröder, 1978).

The composition of the bacterial flora of the sourdough described in the Material and Methods section was analyzed by the use of the random amplified polymorphic DNA technique (RAPD). A database of RAPD reference patterns of lactobacilli all isolated from food fermentations were generated and shown to allow differentiation at species level (Fig. 2). The total bacterial cell count in the sourdough under investigation was 10.3 x 10^8 cfu/ g, whereas concentration of yeasts was 6 x 10^7 cfu/g.

DNAs isolated from 117 randomly selected bacterial colonies were compared to the RAPD database. According to pattern similarity we found a

composition consisting of 61 % of *L. sanfranciscensis*, 36 % of strains sharing identical patterns with *L. spec.* TMW 1.80. The latter strain was previously isolated from an industrial sourdough starter preparation and characterized as *Lactobacillus* species only.
A remainder of 3 % consisted of other lactic acid bacteria whose RAPD patterns could not be assorted to any *Lactobacillus* species available in our RAPD database (data not shown). The strains of the '36 %' group TMW 1.1201, TMW 1.119 and TMW 1.1206 and the previous isolate TMW 1.80, were subjected to a further taxonomic characterization.

Phylogenetic analysis. The complete sequence (1544 bp) of the 16S rRNA gene of the strain TMW 1.80 was determined. It was aligned to all available sequences of members of the gram positive organisms with low G + C content. The analysis placed the representative strain TMW 1.80 within the *L. plantarum* group of the heterogeneous *Lactobacillus casei* group as defined by Schleifer *et al.* (1995). It represents a cluster of related species consisting of *L. alimentarius*, *L. farciminis*, the recently described *L. paralimentarius* (Cai *et al.*, 1999) and *L. kimchii* (Yoon *et al.* 2000). Except of *L. kimchii* all above mentioned species have already been isolated from sourdoughs. The closest relatives were *L. kimchii* (98. 7%), *L. alimentarius (97.5 %)*, and *L. paralimentarius* (97.2 %). The phylogenetic position is shown in Fig. 3. Positions determined by the parsimony algorithm were identical with those obtained with the maximum likelihood approach. Minor differences in branching points were found by application of the neighbour joining method (data not shown).
The G + C content of the 16S rDNA was 51.2 mol %.
The DNA-DNA hybridization studies were performed according to the method of Cardinali *et al.* (2000). For each hybridization experiment a calibration by serial dilutions of homologous DNA was applied on the same membrane. The relation between spot intensity and amount of blotted DNA showed high linearity with correlation values (R^2) equal or over 95 %. (Fig. 4).
Whereas DNA-DNA relatedness between strains TMW 1.80, TMW 1201, TMW 1.1199 and TMW 1206 of *L. mindensis* revealed by DNA-DNA hybridization experiments was greater than 85 %, hybridizations of these strains against DNA from all relevant type strains showed values below 30 % (Fig. 5).
As these data revealed a phylogenetic homogeneous group of strains that is separated from other known species we propose a new species *L. mindensis*. The type strain is TMW 1.80 deposited at the DSMZ as *L. mindensis* DSM 14500^T.
Colony and cell morphology. Colonies of *L. mindensis* DSM 14500^T appeared white with a regular sharp edge and after 3 days of growth they had 1 mm in diameter. Colonies older than three days appeared frayed at the edges.
Cells were non-spore-forming, non-motile rods, that occurred singly or in pairs, seldom in chains. In the phase-contrast microscope cells grown in liquid culture appeared straight, rod-shaped measuring 0.3-0.7 x 3-5 µm (in mid- to late exponential growth phase). Cells on solid media were observed to elongate filamentously with a length between 5 to 20µm (Fig. 1). The KOH test indicated a Gram-positive behavior.
Physiological and biochemical properties. The sugar fermentation patterns and further physiological and biochemical characteristics of *L. mindensis* strains and the reference organisms are listed in Table 1. Lactate is produced in a ratio of 96 % L-lactate to 4 % of D-lactate. Gas was not produced from glucose. Acid was produced from glucose, fructose, mannose, esculin, salicine and cellobiose by all strains tested. Only weak fermentation of maltose was detected. Strain TMW 1.1206 did not ferment amygdaline.
The G + C content of genomic DNA is 37.5 mol % (T_M). Analysis of cell wall in strain TMW 1.80 revealed the presence of lysine and aspartic acid, which indicates the Lys-D-Asp peptidoglycan type.
Design of a species specific PCR detection assay. A diagnostic sequence was identified within the 16S rRNA gene (see table 2), that allows the identification of strains of *L. mindensis* and differentiation thereof from other relevant lactobacilli when used as target site in a PCR assay (Fig. 6). Primer PmindR generated in combination with the 16S rDNA universal primer 616V a 226 bp fragment. No cross reaction was detected for DNA of other lactobacilli. Accessibility of DNA preparations for amplification was successfully controlled by a simultaneous amplification with 16S rDNA specific universal primers.
The identity of RAPD fingerprints of all four strains (TMW 1.80, 1.1199, 1.1201, 1.1206) and their DNA-DNA homology values above 80 % with each other and values less than 30 % to other species provided enough phylogenetic data to propose a separate species status.
The G + C content of 37.5 % is within the range of the *L. plantarum* group (34 to 46 %) and the peptidoglycan type (L-Lys-D-Asp) felt into line of the majority of lactobacilli.
Taxonomic significance is provided by the lack of the fermentation of galactose, arbutine, lactose and trehalose, which allows differentiation from its closest relatives. A difference to *L. paralimentarius* is the lack of the ability to ferment ribose, arbutin, sucrose and trehalose. The limited fermentation spectrum is a typical trait as it can also be observed in other sourdough lactobacilli. The moderate fermentation of maltose also observed in some strains of *L. paralimentarius* (Cai *et al.*, 1999) seems to be at first unfavorable for this environment, but

may explain the observed coexistence with *L. sanfranciscensis*, who was shown to possess a highly optimized maltose metabolism resulting in deliberation of glucose (Stolz *et al*. 1995, Ehrmann & Vogel, 1998).

The increased occurrence of *L. mindensis* in the investigated sourdough may be caused by specific process parameters used in the bakery. Its effect on dough quality and aroma was not investigated in this study.

Description of *Lactobacillus mindensis* sp. nov. (Ehrmann, Müller, Vogel)

L. mindensis (min.den´sis. M. L. adj. *mindensis*, pertaining to the city of Minden, Germany, from where the first strain of this species was isolated).

Cells are Gram-positive, non-motile, non spore-forming rods (0.9 to 5 µm), occurring singly, in pairs or in chains. Colonies are usually small (2mm), smooth, low convex and flat with white colour on MRS-agar. Cells are catalase negative and homofermentative. Growth occurs at 15 °C to 30 °C but not above; growth optimum is at pH 4.6 to 5.2; no growth at or above pH 6.5. Acid is produced from glucose, maltose, fructose, mannose, N-acetyl-glucosamine, cellobiose and salicine. Some strains produce acid from amygdaline.

Neither acid nor gas are produced from, arabinose, dextrin, galactose, lactose, mannitol, melezitose, melibiose, raffinose, rhamnose, ribose, sucrose, sorbitol, trehalose and xylose. Arginine decarboxylase was not detected. Urease and H_2S are not produced. Nitrate is not reduced to nitrite. All strains produce L-lactate (4 % D-lactate and 96 % L-lactate) The peptidoglycan is of the lysine-D-iso-asparagine (L-Lys-DAsp) type and cell wall do not contain teichoic acid. The mol % G + C content of DNA is 37.5 mol % (T_M). Strains were isolated from commercial sourdough starter preparations and from bakeries sourdough after continuous propagations for long periods. The type strain is DSM 14500^T (LMG 21508^T).

ACKNOWLEDGEMENTS

This work was supported by a grant of the European Union (FAIR project: CT 96 1126). We thank Dr. Wolfgang Ludwig for the phylogenetic calculations and the reconstruction of the phylogenetic tree. We also wish to thank Monika Hadek for technical assistance.

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Table 1. Physiological and biochemical characteristics of *L. mindensis* and selected reference organisms

	species						
characteristic(s)	*L. mindensis* DSM 14500^{T} TMW 1.80	*L. frumenti* DSM 13145^{T†}	*L. kimchii* JCM 10707^{T*}	*L. farciminis* LMG 9200^{T§}	*L. alimentarius* JCM 1095^{T‡}	*L. paralimentarius* JCM 10415^{T‡}	*L. sanfrancisco-censis* ATCC27651$^{T\$}$
D,L-Lactate	D,L	D,L	D,L	D,L	D,L	n.d.	D,L
NH_3 from arginine	-	+	-	n.d.	-	n.d.	-
Growth at 15°C/45°C	+/-	-/+	+/-	+/-	+/-	n.d./+	+/-
Peptidoglycan type	L-Lys-D-Asp	L-Lys-D-Asp	L-Lys-D-Asp	L-Lys-D-Asp	L-Lys-D-Asp	n.d.	L-Lys-Ala
G + C content of DNA (mol %)	37.5±0.2	43.3±0.2	35.0	34-36	36-37	37.2-38	36-38
acid production from:							
D,L-Arabinose	-	+/-	+	-	-	-	-
Ribose	-	+	+	-	+	+	+
D,L-Xylose	-	-	+	-	+	-	-
Galactose	-	+	w	+	+	-	+-
Glucose	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	-
Mannose	+	+	+	+	+	+	-
Mannitol	-	+	n.d.	-	-	-	-
Sorbitol	-	+	n.d.	-	-	-	-
N-Acetylglucosamine	+	+	+	n.d.	+	+	-
Amygdalin	+	+	+	+	+	+	-
Arbutine	-	+	+	n.d.	+	+	-
Esculine	+	+	+	+	+	+	-
Salicin	+	+	+	+	+	+	-
Cellobiose	+	+	+	+	+	+	+
Maltose	w	+	+	+	+	+/-	+
Lactose	-	+	-	+	-	-	-
Melibiose	-	+	n.d.	-	-	-	-
Sucrose	-	+	+	+	+	+	-
Trehalose	-	+	+	+	+	+	-
Melezitose	-	-	+	-	-	-	-
Raffinose	-	+	n.d.	-	-	-	-
Gluconate	-	+	+	n.d.	-	-	-

[*] data from Yoon *et al.*, 2000;
[†] data from Müller *et al.*, 2001;
[‡] data from Cai etz al., 1999;
[$] data from Vogel *et al.*, 1994; Stolz 1994
[§] data from Kandler and Weiss, Bergeys
n.d., not determined; -, negative reaction; +, positive reaction; w, weak reaction;

Table 2. Diagnostic sequence signatures in the 16S rRNA to differentiate *L. mindensis* from its closest relatives. Sequence used for specific PCR (primer PmindR) is underlined.

Species	sequence (5´-3´)
L. mindensis	C A A C A G T C T T C A C A T G A T C A C T G T T T A A
L. kimchii	C A A C T T A G A T C A C A T G A T C T T T G T T T A A
L. farciminis	C A A C T A C T T T C A C A T G A T C G T A G C T T G A
L. alimentarius	C A A C A T T A A A C A C A T N T T N T T T G T T T A A
L. paralimentarius	C A A C T T A G A T C A C A T G A T C T T T G T T T A A

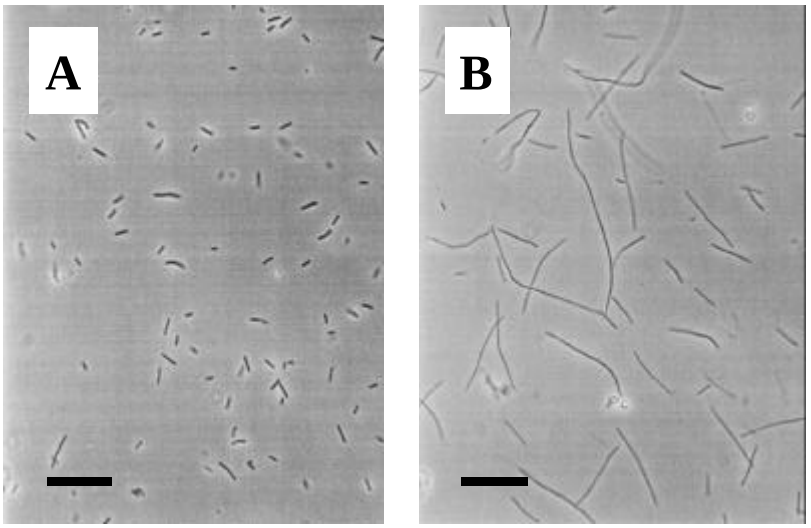

Fig. 1. Phase-contrast micrograph of cells of *Lactobacillus mindensis* DSM 14500^{T}.

Cells were grown on MRS. A, cells grown in liquid culture; B, cells grown on solid medium. Magnification, 800x. The bar represents 10µm.

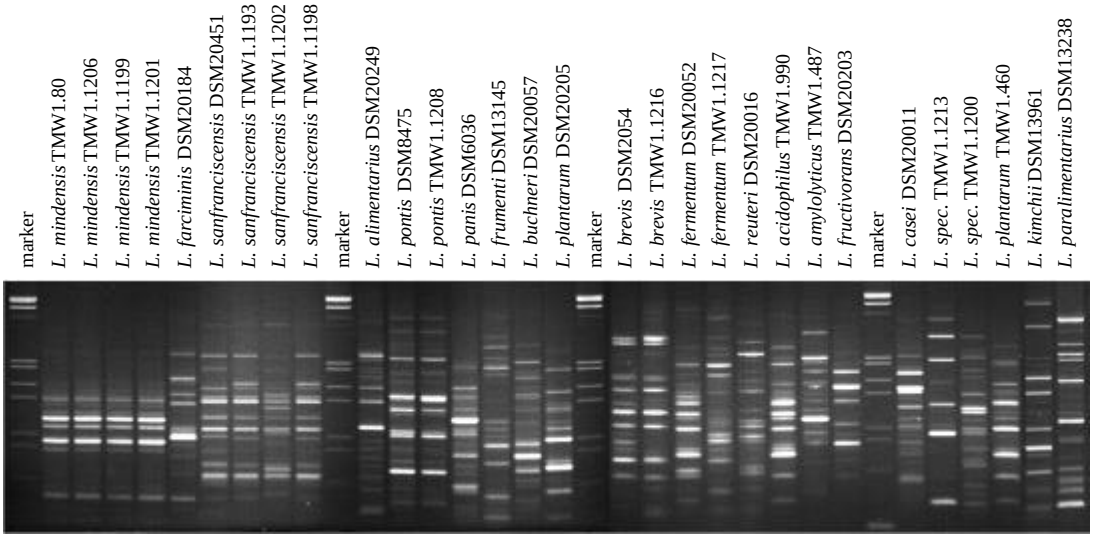

Fig. 2. RAPD patterns of various lactobacilli often described as typical organisms in sourdough fermentations.

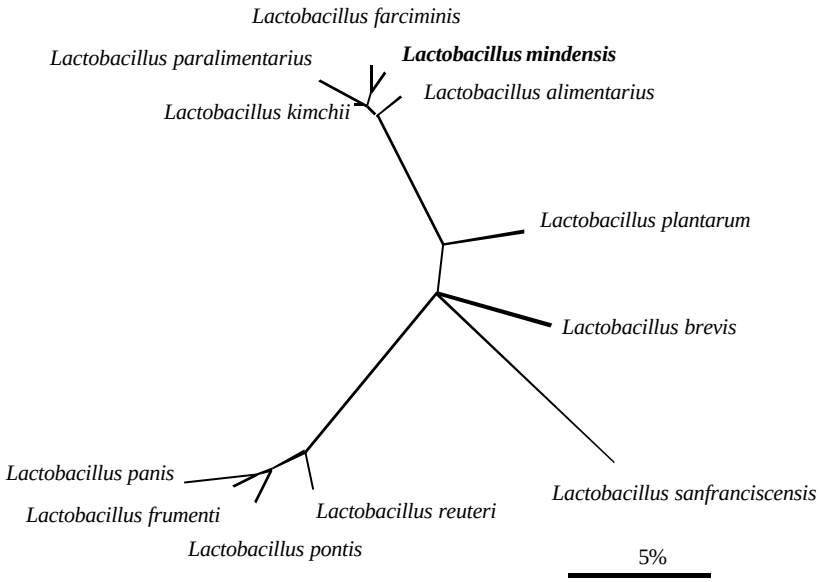

Fig. 3. Phylogenetic tree demonstrating the relationship of *L. mindensis* to the closest related lactobacilli. The tree was constructed by using the parsimony approach. The tree was based on a data set that included only positions which are present in at least 50 % of all available *Lactobacillus* 16S rRNA sequences. The bar indicates 5 % estimated sequence divergence.

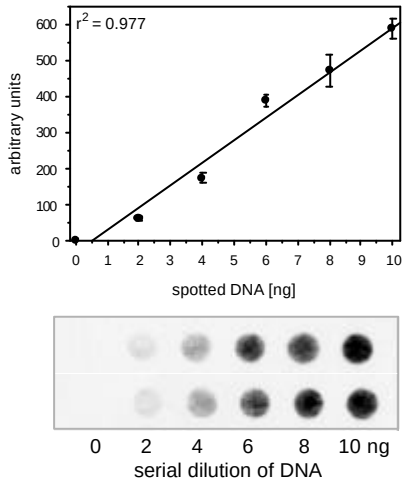

Fig 4. Calibration of the DNA-DNA hybridization assay. Upper part: Regression curve of densitometric intensities of spots (arbitrary units) and amount of DNA spotted to the membrane. Lower part: Accompanying hybridization of serial dilutions (double experiment) of DNA. DNA of *L. mindensis* TMW 1.1201 was used as probe and target.

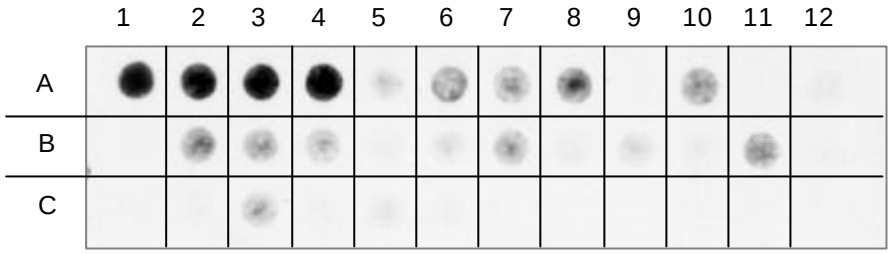

Fig. 5. DNA-DNA hybridization experiment between DNA of *L. mindensis* and other lactobacilli. Genomic DNA of *L. mindensis* TMW 1.1201 was used as a probe and 10 ng of DNA of the following strains were blotted: Lane A: 1, *L. mindensis* TMW 1.80; 2, *L. mindensis* TMW 1.1201; 3, *L. mindensis* TMW 1.1199; 4, *L. mindensis* TMW 1.1206; 5, *L. sanfranciscensis* TMW 1.1203; 6, *L. farciminis* DSM 20184^{T}; 7, *L. alimentarius* DSM 20249^{T}; 8, *L. sanfranciscensis* DSM20451^{T}; 9, *L. pontis* DSM 8475^{T}; 10, *L. oris* DSM 4864; 11, *L. panis* DSM 6035; 12, *L. panis* DSM 6036; lane B: 1; *L. frumenti* DSM 13143; 2, *L. frumenti* DSM 13145^{T}; 3, *L. buchneri* DSM 20057^{T} ; 4, *L. kefiri* DSM 20587^{T}; 5, *L. malefermentans* DSM 2017; 6, *L. plantarum* DSM 20174^{T}; 7, *L. brevis* DSM 20451^{T}; 8, *L. fermentum* DSM 20052^{T}; 9, *L. lindneri* DSM 20690^{T}; 10, *L. vaginalis* LMG 1289; 11, *L. hilgardii* DSM 20176; 12, *L. parabuchneri* DSM 5707^{T}; lane C: 1; *L. amylolyticus* DSM 11664^{T}; 2, *L. pontis* TMW 1.675; 3, *L. plantarum* TMW 1.535.

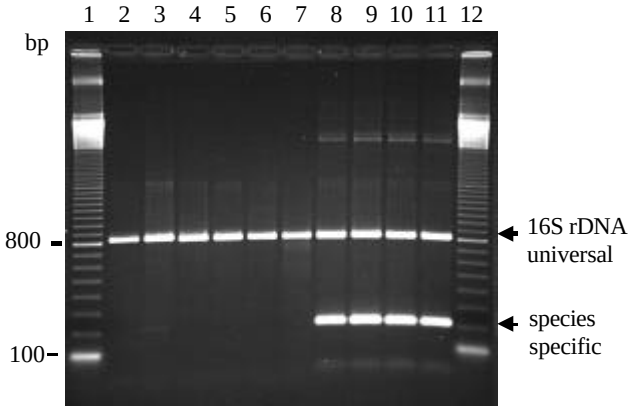

Fig. 6. Identification of *L. mindensis* with a specific PCR approach. For the species-specific PCR primer PmindR and 616V and for control PCR primer 616V and 609R were used. Specific and control PCR were performed separately, but run in a single lane. Lanes: 1 and 12, 100pb ladder (molecular weight standard); 2, *L. frumenti*; 3, *L. sanfranciscensis*; 4, *L. farciminis*; 5, *L. alimentarius*; 6, *L. kimchii*; 7, *L. paralimentarius*; 8, *L. mindensis* TMW 1.1201; 9, *L. mindensis* TMW 1.1199; 10, *L. mindensis* TMW 1.1206; 11, *L. mindensis* DSM 14500^{T}.