

## ***Lactobacillus fornicalis* sp. nov., isolated from the posterior fornix of the human vagina**

L. M. T. Dicks,<sup>1</sup> M. Silvester,<sup>1</sup> P. A. Lawson<sup>2</sup> and M. D. Collins<sup>2</sup>

Author for correspondence: L. M. T. Dicks. Tel: +27 21 8084536. Fax: +27 21 8083611.  
e-mail: lmttd@maties.sun.ac.za

<sup>1</sup> Department of Microbiology, University of Stellenbosch, Stellenbosch 7600, South Africa

<sup>2</sup> Department of Food Science and Technology, University of Reading, Reading RG6 6AP, UK

**Twelve strains isolated from the posterior fornix fluid of the human vagina were identified as *Lactobacillus johnsonii*, *Lactobacillus acidophilus*, *Lactobacillus gallinarum* and *Lactobacillus crispatus* based on numerical analyses of total soluble cell protein profiles and randomly amplified polymorphic DNA (RAPD)-PCR banding patterns. Five strains grouped with the type strains of *Lactobacillus gasseri* (DSM 20077<sup>T</sup>) and *Lactobacillus jensenii* (DSM 20557<sup>T</sup>) at  $r \geq 0.83$  in one protein profile cluster, well separated from the other species included in this study. However, numerical analysis of the RAPD-PCR banding patterns of representative strains selected from the *L. gasseri*-*L. jensenii* protein cluster clearly indicated that they belong to two different species. Four strains (TV 1010, TG 1013, TV 1018<sup>T</sup> and TV 1045) grouped into another well separated protein profile cluster at  $r \geq 0.87$ . Strains selected from this cluster displayed very similar RAPD-PCR banding patterns and clustered at  $R^2 \geq 0.78$ , separate from the other strains examined. Sequencing of the 16S rRNA of two representative strains, TV 1018<sup>T</sup> and TG 1013, of this group indicated that it represents a new member of rRNA group I *Lactobacillus*, which includes *Lactobacillus delbrueckii*, the type of the genus, and close relatives *Lactobacillus acetotolerans*, *Lactobacillus kefiranofaciens*, *Lactobacillus iners*, *L. jensenii*, *L. crispatus*, *L. acidophilus*, *Lactobacillus helveticus*, *Lactobacillus amylovorus*, *Lactobacillus hamsteri*, *L. johnsonii*, *L. gasseri* and *Lactobacillus amyolyticus*. The name *Lactobacillus fornicalis* sp. nov. is proposed for strains TV 1010 (DSM 13172), TG 1013, TV 1018<sup>T</sup> and TV 1045, with strain TV 1018<sup>T</sup> (= DSM 13171<sup>T</sup> = ATCC 700934<sup>T</sup>) as the type.**

**Keywords:** *Lactobacillus fornicalis* sp. nov., human vagina, taxonomy, phylogeny, lactic acid bacteria

### **INTRODUCTION**

The genus *Lactobacillus* is divided into three groups based on the fermentative abilities of the species (Kandler & Weiss, 1986). Group I, the obligately homofermentative species, degrade hexoses almost completely to lactic acid and do not ferment pentoses or gluconate. Species of group II are facultatively heterofermentative and produce acetic acid, ethanol and formic acid under glucose limitation, in addition to lactic acid. Pentoses are usually fermented. Group III contains the obligately heterofermentative lacto-

bacilli which ferment hexoses to lactic acid, acetic acid, ethanol and carbon dioxide. Pentoses are fermented to lactic acid and acetic acid.

Group I contains 21 species, divided into two subgroups based on DNA-DNA hybridization (Kandler & Weiss, 1986). Subgroup 1 includes *Lactobacillus delbrueckii* (the type species of the genus) and *Lactobacillus jensenii* (Kandler & Weiss, 1986). Subgroup 2 is represented by *Lactobacillus acidophilus*, *Lactobacillus amylophilus*, *Lactobacillus farciminis*, *Lactobacillus kefiranofaciens*, *Lactobacillus vitulinus*, *Lactobacillus intestinalis*, *Lactobacillus uli* (Pot *et al.*, 1994) and the '*Lactobacillus casei*/*Pediococcus* group of lactobacilli', viz. *Lactobacillus aviarius*, *Lactobacillus animalis*, *Lactobacillus salivarius*, *Lactobacillus mali*, *Lactobacillus ruminis* and *Lactobacillus sharpeae* (Collins *et al.*, 1991). The *L. acidophilus* group was

**Abbreviations:** RAPD, randomly amplified polymorphic DNA; UPGMA, unweighted pair group method with arithmetic averages.

The GenBank accession number for the 16S rRNA gene sequence of strain TV 1018<sup>T</sup> is Y18654.

**Table 1.** Genotypic relatedness among *L. johnsonii*, *L. acidophilus*, *L. gallinarum*, *L. crispatus*, *L. gasseri*, *L. jensenii* and *L. fornicalis* sp. nov.

Strains printed in bold were selected for RAPD-PCR analysis.

Strain*	Protein cluster†	RAPD-PCR cluster‡
<i>L. johnsonii</i> TV 1016, TV 1035, TG 1038, <b>TG 1016, NCIMB 702241<sup>T</sup>,</b> <b>TV 1005, TV 1048, TG 1027</b>	I	V
<i>L. acidophilus</i> <b>TG 1025</b> TV 1001, ATCC 4356 <sup>T</sup> , <b>NCIMB 701748<sup>T</sup></b>	I II	IV IV
<i>L. gallinarum</i> <b>NCIMB 702235<sup>T</sup>, TV 1006</b>	III	III
<i>L. crispatus</i> TG 1010, NCIMB 45704, <b>NCIMB 702752<sup>T</sup>, NCIMB</b> 45705, TG 1006	IV	NC§
<i>L. gasseri</i> <b>DSM 20077<sup>T</sup>, TG 1034, TV</b> <b>1013</b>	V	VI
<i>L. jensenii</i> <b>DSM 20557<sup>T</sup>, TV 1036, TV</b> <b>1044, TG 1030</b>	V	II
<i>L. fornicalis</i> sp. nov. <b>TV 1010, TG 1013, TV 1018<sup>T</sup>,</b> <b>TV 1045</b>	VI	I

\* TV and TG, strains isolated from patients who attended pre- and post-natal clinics, respectively.

† From Fig. 1.

‡ From Fig. 2.

§ NC, Did not cluster with the other strains.

later divided into six genotypic groups based on DNA–DNA hybridization, with the species *L. acidophilus* in group A1 (Fujisawa *et al.*, 1992). Groups A2, A3 and A4 contain *Lactobacillus crispatus* (Cato *et al.*, 1983), *Lactobacillus amylovorus* and *Lactobacillus gallinarum* (Fujisawa *et al.*, 1992), respectively. Groups B1 and B2 include *Lactobacillus gasseri* (Lauer & Kandler, 1980) and *Lactobacillus johnsonii* (Fujisawa *et al.*, 1992), respectively.

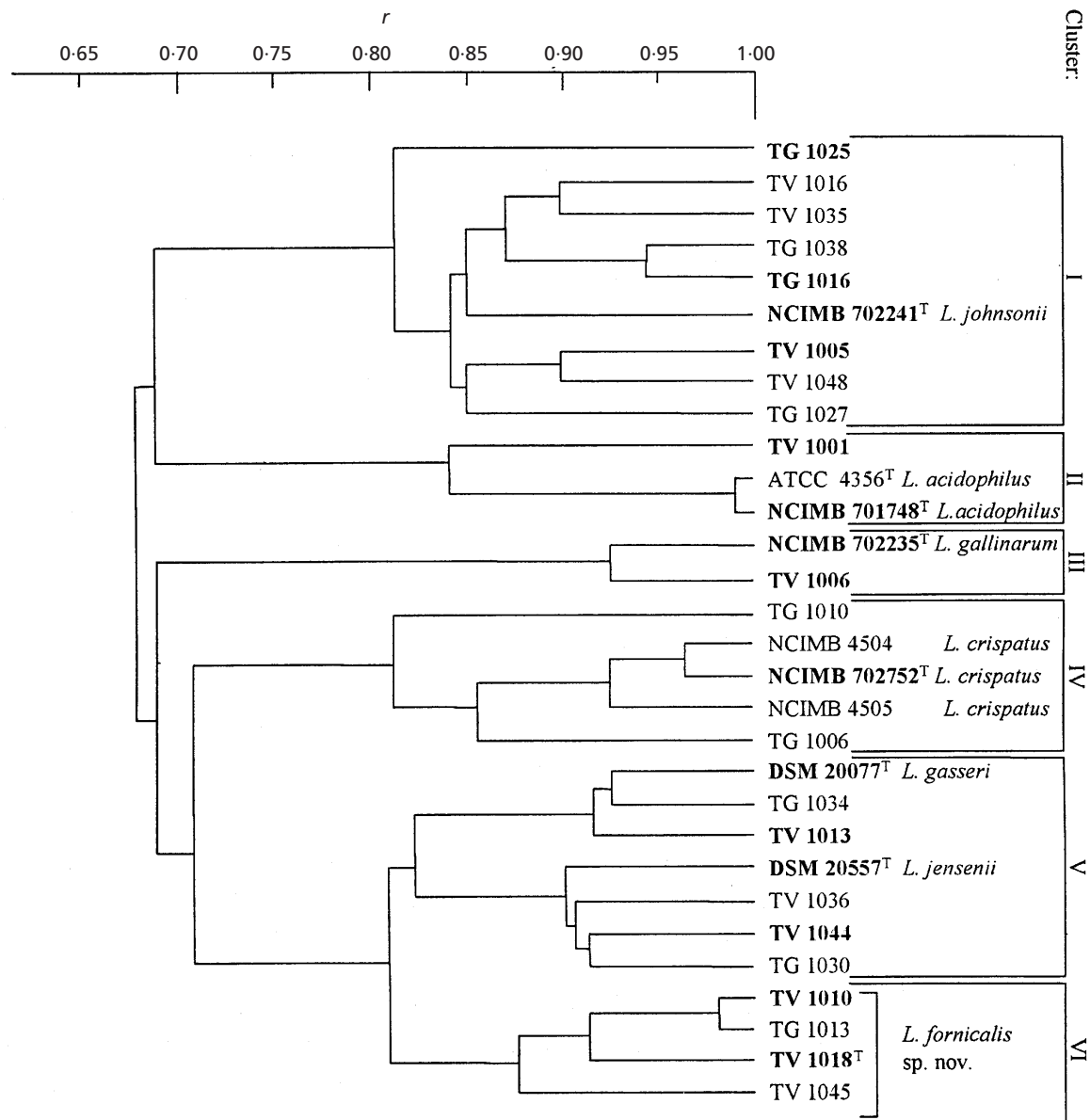
In humans, as many as  $10^7$ – $10^8$  *Lactobacillus* spp. ml<sup>-1</sup> have been reported in vaginal fluid (Redondo-Lopez *et al.*, 1990). The obligately homofermentative species thus far isolated include *L. acidophilus*, *L. crispatus* and *L. jensenii*; the facultatively heterofermentative species *Lactobacillus plantarum* and *L. casei*; and the obligately heterofermentative species *Lactobacillus brevis* and *Lactobacillus fermentum* (Fernandes *et al.*, 1987; Giorgi *et al.*, 1987).

In this study, we have investigated the relatedness among 21 strains of obligately homofermentative (group I) *Lactobacillus* spp. isolated from the posterior

fornix fluid of the vagina of healthy pre- and post-natal patients and representative strains of *L. acidophilus*, *L. crispatus*, *L. jensenii*, *L. gallinarum*, *L. gasseri* and *L. johnsonii* using both phenotypic and genotypic methods. Based on the results of this polyphasic taxonomic study we propose a new species, *Lactobacillus fornicalis*.

## METHODS

**Bacterial strains and growth conditions.** Twenty-one strains of lactic acid bacteria were isolated from the posterior fornix secretions of the vagina of 18 healthy patients who attended the pre- and post-natal clinics at the Tygerberg Hospital in Tygerberg, South Africa (Table 1). The swabs collected from the patients were immersed in 1 ml sterile physiological salt (Univar) and immediately spread-plated (100 µl) onto MRS agar (Biolab). Colonies were selected from the plates after 2 d incubation at 37 °C. Reference strains were obtained from the National Collection of Industrial and Marine Bacteria (NCIMB), American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and the Culture Collection, University



**Fig. 1.** Dendrogram showing the clustering of *L. johnsonii*, *L. acidophilus*, *L. gallinarum*, *L. crispatus*, *L. gasseri*, *L. jensenii* and *L. formicalis* sp. nov. obtained by numerical analysis of total soluble cell protein patterns. Clustering was by UPGMA.

of Göteborg, Sweden (CCUG). All strains were cultured in MRS broth (Biolab) at 37 °C.

**Numerical analysis of total soluble cell protein patterns.** Cultures were grown in MRS broth (Biolab) for 24 h at 37 °C. Protein isolation and gel electrophoresis were performed as described by Vauterin *et al.* (1993). The Gelcompar computer program (version 4.0) of Applied Maths was used to analyse the protein banding patterns. The program recorded the normalized electrophoretic patterns of the densitometric traces, grouped the isolates by the Pearson product moment correlation coefficient ( $r$ ) and performed UPGMA (unweighted pair group method with arithmetic averages) cluster analysis of the protein bands.

**Numerical analysis of randomly amplified polymorphic DNA (RAPD)-PCR profiles.** Strains representative of each subgroup within a cluster were selected (Table 1). The DNA of these

strains was isolated according to the method of Dellaglio *et al.* (1973). Three single primers [TGGGCGTCAA (OPL-02), ACGCAGGCAC (OPL-05) and ACGATGAGCC (OPL-11)] were used and the DNA amplification performed according to the methods described by Van Reenen & Dicks (1996). Lambda DNA, digested with *EcoRI* and *HindIII* (Boehringer Mannheim), was used as molecular mass marker. Numerical analysis of RAPD-PCR profiles was done using the CLUSTER program of SAS Institute, according to the methods described by Van Reenen & Dicks (1996).

**Determination of 16S rRNA gene sequences and phylogenetic analyses.** A large fragment of the 16S rRNA gene was amplified by PCR by using universal primers pA (5'-AGAGTTTGATCCTGGCTCAG; positions 8–27, *Escherichia coli* numbering) and pH\* (5'-AAGGAGGTG-ATCCAGCCGCA; positions 1541–1522). The PCR pro-

ducts were purified by using a Prep-A-Gene kit (Bio-Rad) according to the manufacturer's instructions and were sequenced by using a *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) and a model 373A automatic sequencer (Applied Biosystems). The closest known relatives of the new isolates were determined by performing sequence database searches and the sequences of closely related strains were retrieved from GenBank or Ribosomal Database Project libraries. Sequences were aligned by using the program PILEUP (Devereux *et al.*, 1984) and the alignment was corrected manually. Distance matrices were produced by using the programs PRETTY and DNADIST (using the Kimura-2 correction parameter) (Felsenstein, 1989). A phylogenetic tree was constructed according to the neighbour-joining method with the program NEIGHBOR (Felsenstein, 1989). The statistical significance of the groups obtained was assessed by bootstrapping (500 replicates) by using the programs DNABOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1989).

**DNA base composition.** DNA was isolated and purified by using the technique described by Dicks *et al.* (1990). The G+C content was calculated by using the method of Marmur & Doty (1962).

**Biochemical and physiological tests.** The biochemical and physiological characteristics of all strains were determined by using the methods described by Sharpe (1979). Carbohydrate fermentation characteristics of all strains were determined by using the API 50CHL system. Results were recorded after 48 h at 37 °C.

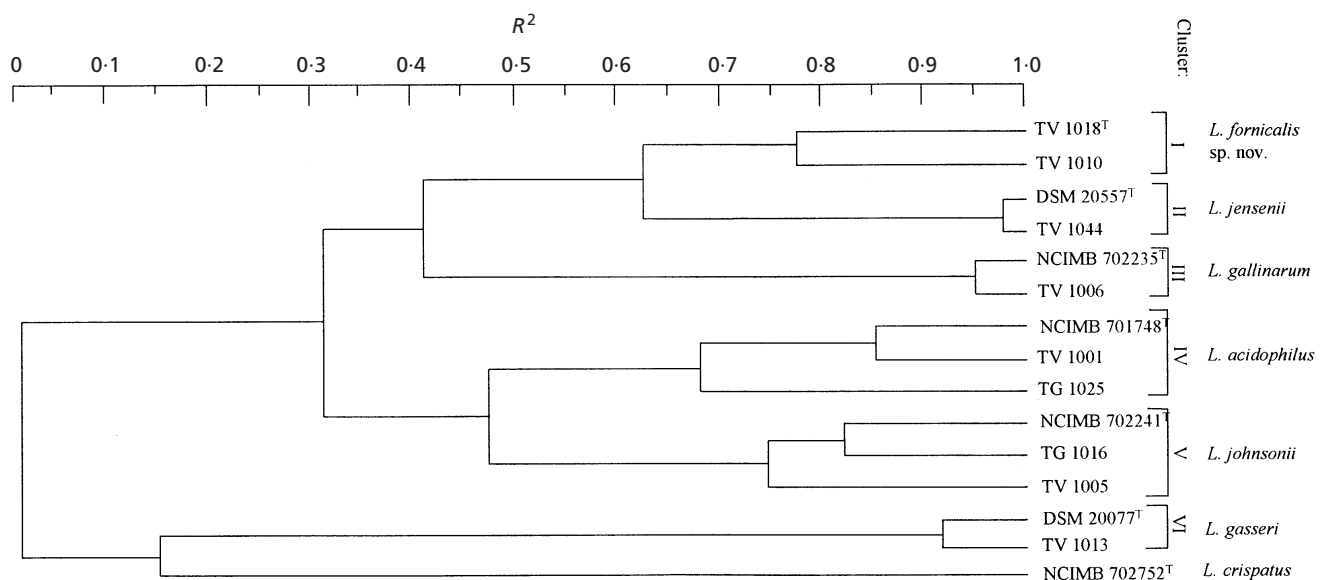
## RESULTS AND DISCUSSION

Twenty-one strains isolated from the posterior fornix fluid of the human vagina grouped into six protein profile clusters at  $r \geq 0.82$ , well separated from each other at  $r \geq 0.67$  (Fig. 1).

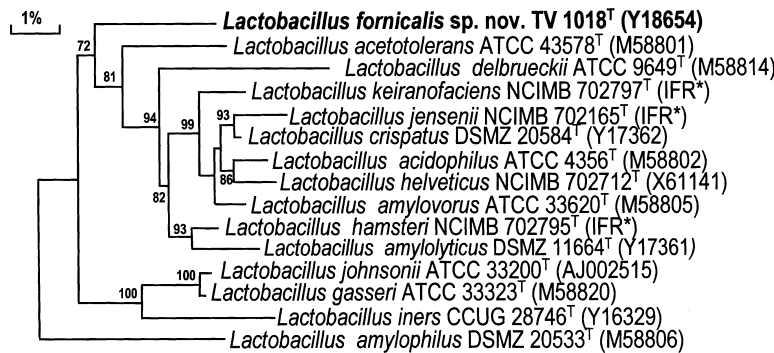
Eight strains of the vaginal isolates grouped with the

type strain of *L. johnsonii* (NCIMB 702241<sup>T</sup>) in cluster I at  $r \geq 0.82$ . The overall protein profile of strain TG 1025 was, however, different from the other strains in cluster I, as also indicated by its lower clustering at  $r \geq 0.82$ . Strain TG 1025 is thus considered an intruder in cluster I, a phenomenon which has been described by Kersters & De Ley (1975). One strain formed a tight cluster with the type strain of *L. acidophilus* (ATCC 4356<sup>T</sup>, NCIMB 701748<sup>T</sup>) in cluster II at  $r \geq 0.84$ , one strain linked with the type strain of *L. gallinarum* (NCIMB 702235<sup>T</sup>) in cluster III at  $r \geq 0.93$ , and two strains grouped with *L. crispatus* in cluster IV at  $r \geq 0.82$  (Fig. 1). These groupings were confirmed by numerical analysis of RAPD-PCR banding patterns (Fig. 2). Strains within each protein profile cluster displayed similar DNA profiles, whereas strains from different clusters displayed unique banding patterns (Table 1). Similar results were recorded in one of our previous studies on *L. acidophilus*, *L. crispatus*, *L. amylovorus*, *L. gallinarum*, *L. gasseri* and *L. johnsonii* (Du Plessis & Dicks, 1995). The RAPD-PCR profile of strain TG 1025 was, however, different from the other three strains of *L. johnsonii* in cluster I, but similar to the profiles recorded for *L. acidophilus* (Fig. 2). Based on these results, strains TV 1016, TV 1035, TG 1038, TG 1016, TV 1005, TV 1048 and TG 1027 (cluster I, Fig. 1) are classified as *L. johnsonii*, whereas strains TG 1025 (cluster I, Fig. 1) and TV 1001 (cluster II, Fig. 1) are considered members of *L. acidophilus* and strain TV 1006 (cluster III, Fig. 1) a strain of *L. gallinarum*. Strains TG 1010 and TG 1006 (cluster IV, Fig. 1) are classified as *L. crispatus*.

Five strains (TG 1034, TV 1013, TV 1036, TV 1044 and TG 1030) grouped with the type strains of *L. gasseri* (DSM 20077<sup>T</sup>) and *L. jensenii* (DSM 20557<sup>T</sup>) at



**Fig. 2.** Dendrogram showing the clustering of *L. fornicalis* sp. nov., *L. jensenii*, *L. gallinarum*, *L. acidophilus*, *L. johnsonii*, *L. gasseri* and *L. crispatus* obtained by numerical analysis of RAPD-PCR profiles. Clustering was by normalized average linkage analysis. Distances between clusters are expressed in  $R^2$  values.



**Fig. 3.** Unrooted tree showing the phylogenetic relationships of *L. formicalis* sp. nov. and closely related lactic acid bacteria. The tree constructed using the neighbour-joining method was based on a comparison of approximately 1320 nucleotides. Bootstrap values, expressed as a percentage of 500 replications, are given at branching points. Asterisks indicate sequences from the Institute of Food Research (Reading, UK) database.

**Table 2.** Differential carbohydrate fermentation reactions among strains of *L. johnsonii*, *L. acidophilus*, *L. gallinarum*, *L. crispatus*, *L. gasseri*, *L. jensenii* and *L. formicalis* sp. nov.

+, 90% or more of strains positive; -, 90% or more of strains negative; d, 11–98% of strains positive; ND, not determined. Data for *L. johnsonii*, *L. acidophilus*, *L. gallinarum*, *L. crispatus* and *L. gasseri* are from Fujisawa *et al.* (1992); data for *L. jensenii* are from Kandler & Weiss (1986). All strains fermented amygdalin, cellobiose, fructose, galactose, glucose, maltose, mannose, salicin and sucrose, and hydrolysed aesculin. None of the strains fermented arabinose, rhamnose and xylose. All strains produced DL-lactic acid from glucose, except *L. jensenii* which produced the D-isomer.

Carbohydrate	<i>L. johnsonii</i>	<i>L. acidophilus</i>	<i>L. gallinarum</i>	<i>L. crispatus</i>	<i>L. gasseri</i>	<i>L. jensenii</i>	<i>L. formicalis</i> sp. nov.
Dextrin	d	–	+	d	+	ND	ND
Lactose	d	+	d	d	+	–	–
Mannitol	–	–	–	d	–	d	+
Melibiose	d	–	+	d	d	–	–
Melezitose	–	–	–	–	–	–	+
Raffinose	d	d	+	+	–	–	–
Ribose	–	–	–	d	–	–	+
Sorbitol	–	–	–	–	–	–	+
Starch	d	–	d	d	d	ND	–
Trehalose	d	+	–	d	+	+	+

$r \geq 0.83$  in cluster V (Fig. 1). Two of the strains (TG 1034 and TV 1013) formed a tight grouping with *L. gasseri* DSM 20077<sup>T</sup>, whereas strains TV 1036, TV 1044 and TG 1030 linked closer to *L. jensenii* DSM 20557<sup>T</sup>. These groupings were confirmed by numerical analyses of the RAPD-PCR profiles. The RAPD-PCR profile of strain TV 1013 was almost identical to that of *L. gasseri* DSM 20077<sup>T</sup>, as shown by the high similarity value ( $R^2 \geq 0.92$ ) recorded between the two strains (Fig. 2). On the other hand, strain TV 1044 shared a high DNA similarity with *L. jensenii* DSM 20557<sup>T</sup>, as revealed by the high correlation ( $R^2 \geq 0.98$ ) recorded in their RAPD-PCR banding patterns (Fig. 2). These findings suggest that numerical analysis of RAPD-PCR banding patterns are more reliable than numerical analysis of total soluble cell protein patterns in differentiating strains of *L. gasseri* and *L. jensenii*. Additional strains will have to be studied to confirm this suggestion.

Four strains (TV 1010, TG 1013, TV 1018<sup>T</sup> and TV 1045) were phenotypically different from any of the species included in this study and formed a tight

phenotypic group at  $r \geq 0.87$  (cluster VI, Fig. 1). The RAPD-PCR profiles of two representative strains from this cluster (TV 1018<sup>T</sup> and TV 1010) were found to be very similar, as shown by the high correlation value ( $R^2 \geq 0.78$ ) recorded between the two strains (Fig. 2). To investigate the phylogenetic position of this group, the almost complete 16S rRNA gene sequence (>1400 nucleotides) of strain TV 1018<sup>T</sup> was determined, and subjected to a comparative analysis. Sequence database searches (data not shown) revealed the bacterium was closely related to the *L. delbrueckii* group of organisms (rRNA group I; see Collins *et al.*, 1991) with other lactobacilli more distantly related. A tree depicting the phylogenetic affinities of strain TV 1018<sup>T</sup> is shown in Fig. 3. It was evident from both sequence divergence values of >5% with other members of the *L. delbrueckii* rRNA cluster and the treeing analysis that the unidentified bacterium represents a hitherto unknown *Lactobacillus* species. The 16S rRNA gene sequence of a second isolate, TG 1013, of the novel vaginal bacterium was also sequenced (>1400 nucleotides) and showed 99.9% sequence similarity with strain TV 1018<sup>T</sup>, thereby confirming their genotypic

identity. Thus, based on phenotypic and phylogenetic findings, we propose the name *Lactobacillus fornicalis* sp. nov. for strains TV 1010, TG 1013, TV 1018<sup>T</sup> and TV 1045, with strain TV 1018<sup>T</sup> as the type. A full description of *L. fornicalis* is given below.

#### Description of *Lactobacillus fornicalis* sp. nov.

*Lactobacillus fornicalis* (for.nic.a'lis. L. gen. n. *fornicalis* of the posterior fornix).

Gram-positive rods. Non-motile, non-sporulating, catalase-negative and oxidase-negative. Colonies on MRS agar are round, smooth, white and approximately 1 mm in diameter. Growth occurs at temperatures between 20 and 40 °C on MRS agar, with the optimum temperature between 35 and 37 °C. Facultatively anaerobic, but grows well on the surface of MRS agar when not incubated under micro-aerophilic conditions. Obligately homofermentative, with no gas production from glucose or gluconate. D(-)- and L-lactic acid are produced from glucose. Aesculin is hydrolysed. Voges-Proskauer-negative. Indole is not formed and nitrates are not reduced. Polysaccharides are not produced from sucrose. *Lactobacillus fornicalis* is distinguished from *L. johnsonii*, *L. acidophilus*, *L. gallinarum*, *L. crispatus*, *L. gasseri* and *L. jensenii* on the basis of a few carbohydrate fermentation reactions (Table 2). Acid is produced from amygdalin, cellobiose, fructose, galactose, glucose, maltose, mannose, mannitol, melezitose, ribose, salicin, sorbitol, sucrose and trehalose. Arabinose, lactose, melibiose, raffinose, rhamnose, starch and xylose are not fermented. The G+C content of the type strain is 37 mol% (as determined by the thermal denaturation method). Isolated from the posterior fornix fluid of the human vagina. The type strain is TV 1018<sup>T</sup> (= DSM 13171<sup>T</sup> = ATCC 700934<sup>T</sup>).

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