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TAXONOMIC DESCRIPTION

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Lactobacillus mulieris sp. nov., a new species of *Lactobacillus delbrueckii* group

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Abstract

One Gram-stain-positive, non-motile, non-spore-forming, catalase-negative, and coccobacilli-shaped strain, designated c10Ua161M^T, was isolated from a urine sample from a reproductive-age healthy woman. Comparative 16S rRNA gene sequence analysis indicated that strain c10Ua161M^T belonged to the genus *Lactobacillus*. Phylogenetic analysis based on *pheS* and *rpoA* gene sequences strongly supported a clade encompassing strains c10Ua161M^T and eight other strains from public databases, distinct from currently recognized species of the genus *Lactobacillus*. *In silico* Average Nucleotide Identity (ANI) and Genome-to-Genome Distance Calculator (GGDC), showed 87.9 and 34.3% identity to the closest relative *Lactobacillus jensenii*, respectively. The major fatty acids of strain c10Ua161M^T were $C_{18:1}\omega9c$ (65.0%), $C_{16:0}$ (17.8%), and summed feature 8 (10.2%; comprising $C_{18:1}\omega7c$, and/or $C_{18:1}\omega6c$). The DNA G+C content of the strains is 34.2 mol%. On the basis of data presented here, strain c10Ua161M^T represents a novel species of the genus *Lactobacillus*, for which the name *Lactobacillus mulieris* sp. nov. is proposed. The type strain is c10Ua161M^T (=CECT 9755^T=DSM 108704^T).

The genus *Lactobacillus* is a paraphyletic group of lactic acid bacteria comprising more than 200 species [http://www.bacterio.net/lactobacillus.html], belonging to the phylum *Firmicutes*. *Lactobacillus* species are anaerobic, facultative anaerobic or microaerophilic, Gram-stain-positive, non-spore-forming rods, with a wide habitat range attributed to their metabolic versatility [1, 2].

Lactobacillus comprises a high number of species generally recognized as safe and/or included in the qualitative presumption of safety list, with several strains being widely used in food and human nutrition, due to their contribution to fermented food production or their probiotic usage [3, 4]. Members of this genus contribute to the health status of different body sites, namely the gastrointestinal tract and vagina [5].

Recently, *Lactobacillus* species (e.g. *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, *Lactobacillus iners*), amongst other bacterial genera, were also detected as members of the human urinary tract microbiota, changing the prevailing dogma that urine from healthy individuals is sterile [6, 7]. In the present study, we characterized a novel species of the genus *Lactobacillus* isolated from the urine of a healthy female individual based on a polyphasic approach.

Strain c10Ua161M^T was isolated from a urine sample in the course of a study on the urinary microbiota of healthy reproductive-age women in Porto, Portugal (2017). Urine was inoculated on blood agar, and colonies were selected after 48 h at 37 °C under microaerophilic conditions. Strain was preliminarily identified as *Lactobacillus jensenii* (99.9%) by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), analysis using VITEK MS (bioMérieux, France) system and *In Vitro* Diagnostic database version 3.0. The strain was maintained on de Man Rogosa Sharpe medium (MRS; Liofilchem) for short-term storage and in tryptic soy broth (TSB; Liofilchem) supplemented with 20 % (v/v) glycerol at −80 °C for long-term storage.

Complete nucleotide sequences of the housekeeping genes *rrs* (16S rRNA), *pheS* (phenylalanyl-tRNA synthase alpha

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Keywords: 16S rRNA; pheS; rpoA; genome; fatty acid.
Abbreviations: ANI, Average Nucleotide Identity; FA, fatty acid; GGDC, genome-to-genome distance calculator; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MRS, de Man Rogosa Sharpe medium; pheS, phenylalanyl-tRNA synthase alpha subunit gene; rpoA, RNA polymerase alpha subunit gene; TSB, Tryptic Soy Broth.
One supplementary figure is available with the online version of this article.

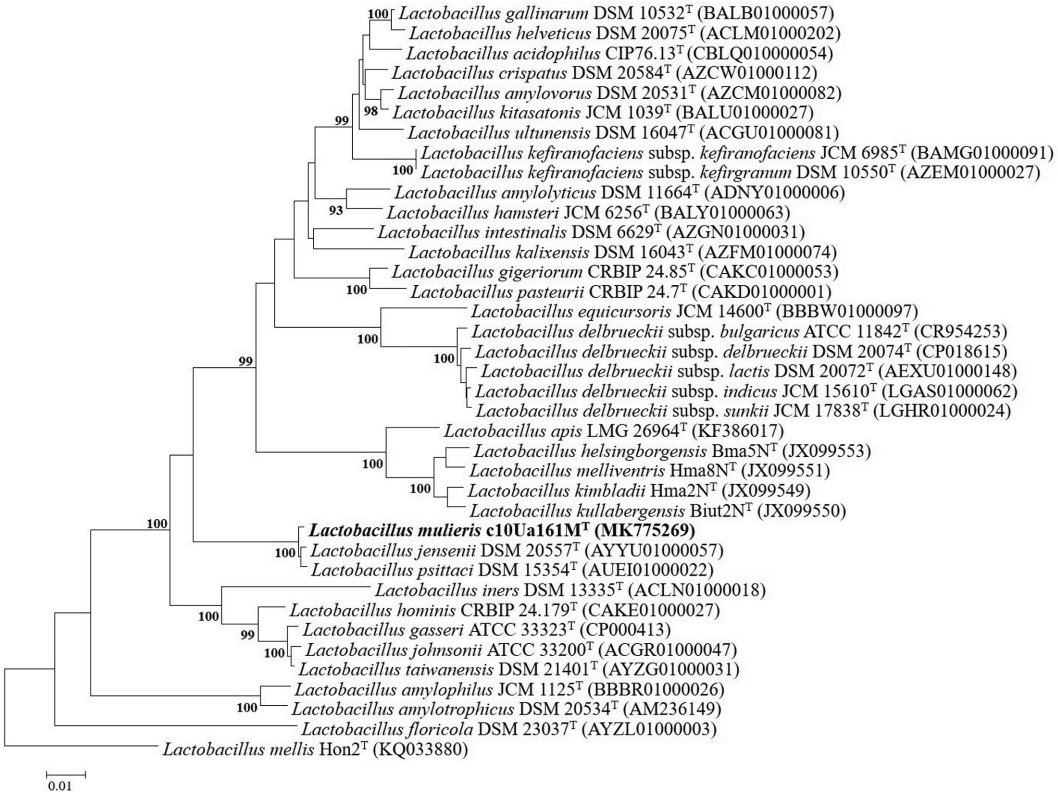

Fig. 1. Neighbour-joining tree (Kimura's two-parameter model and pairwise-deletion option) based on 16S rRNA gene sequences showing the phylogenetic relationships of Lactobacillus *mulieris* c10Ua161M^T (boldface type), and type strains of the genus Lactobacillus. Lactobacillus mellis Hon2 was used as the outgroup. Bootstrap percentages (based on 1000 replications) are shown at nodes. Only values above 90% are shown. Bar, 0.01 substitutions per nucleotide position.

subunit), and *rpoA* (RNA polymerase alpha subunit) of strain c10Ua161M^T and closely related *Lactobacillus* species were extracted, after genome annotation provided by the NCBI Prokaryotic Genome Annotation Pipeline [8], aligned and similarity scores were generated using MEGA version 7.0 (http://www.megasoftware.net/) [9]. Phylogenetic trees based on 16S rRNA gene sequences were constructed according to two different methods, neighbour-joining and maximum-likelihood [10], and genetic distances were estimated using Kimura's two-parameter model [11]. The reliability of internal branches was assessed from bootstrapping based on 1000 resamplings [12]. The 16S rRNA gene sequence variation provides limited resolution to discriminate among closely related species of the genus *Lactobacillus* [13, 14]. Indeed, phylogenetic analysis based on 16S rRNA gene sequences, obtained by the two methods, revealed that strain c10Ua161M^T clustered with type strains of *Lactobacillus jensenii* and *Lactobacillus psittaci* (Figs 1 and S1, available in the online version of this article). Phylogenetic analyses based on *pheS* or *rpoA* genes were constructed according to the neighbour-joining method [10], and genetic distances were estimated using Kimura's two-parameter model [11]. The reliability of internal branches was assessed from bootstrapping based on 1000 resamplings [12]. The phylogenetic trees based on *pheS* or *rpoA* genes showed that strain c10Ua161M^T and eight other strains with currently available genomes in public databases were grouped together and shared high similarity (100% sequence similarity), representing a well-separated lineage supported by bootstrap values of 100% (Figs 2 and 3). Furthermore, Figs 2 and 3 clearly delineate strain c10Ua161M^T and closely related strains in a separate and distinct cluster from the type strains of *Lactobacillus jensenii,* and *Lactobacillus psittaci* (91.6 and 80.3% of *pheS* sequence similarity and 97.7 and 88.7% of *rpoA* sequence similarity, respectively). These *pheS* and *rpoA* sequence divergence values observed indicate that strain c10Ua161M^T represents a novel species within the genus *Lactobacillus* [13, 15].

Whole-genome sequencing of strain c10Ua161M^T was performed by Illumina MiSeq 2×250 nt. The draft-genomes were obtained using INNUca-INNUENDO Reads Control and Assembly (https://github.com/INNUENDOCON/INNUca), which provides a pipeline to check reads quality using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), followed by *de novo* assembly with SPADes [16]. Annotation of the draft-genome was provided by the NCBI Prokaryotic Genome Annotation Pipeline [8]. *In silico* genome-to-genome comparison was assessed by ANI based on BLAST+ using PyANI v0.2.7 (https://github.com/widdowquinn/pyani) [17], and Genome-to-Genome

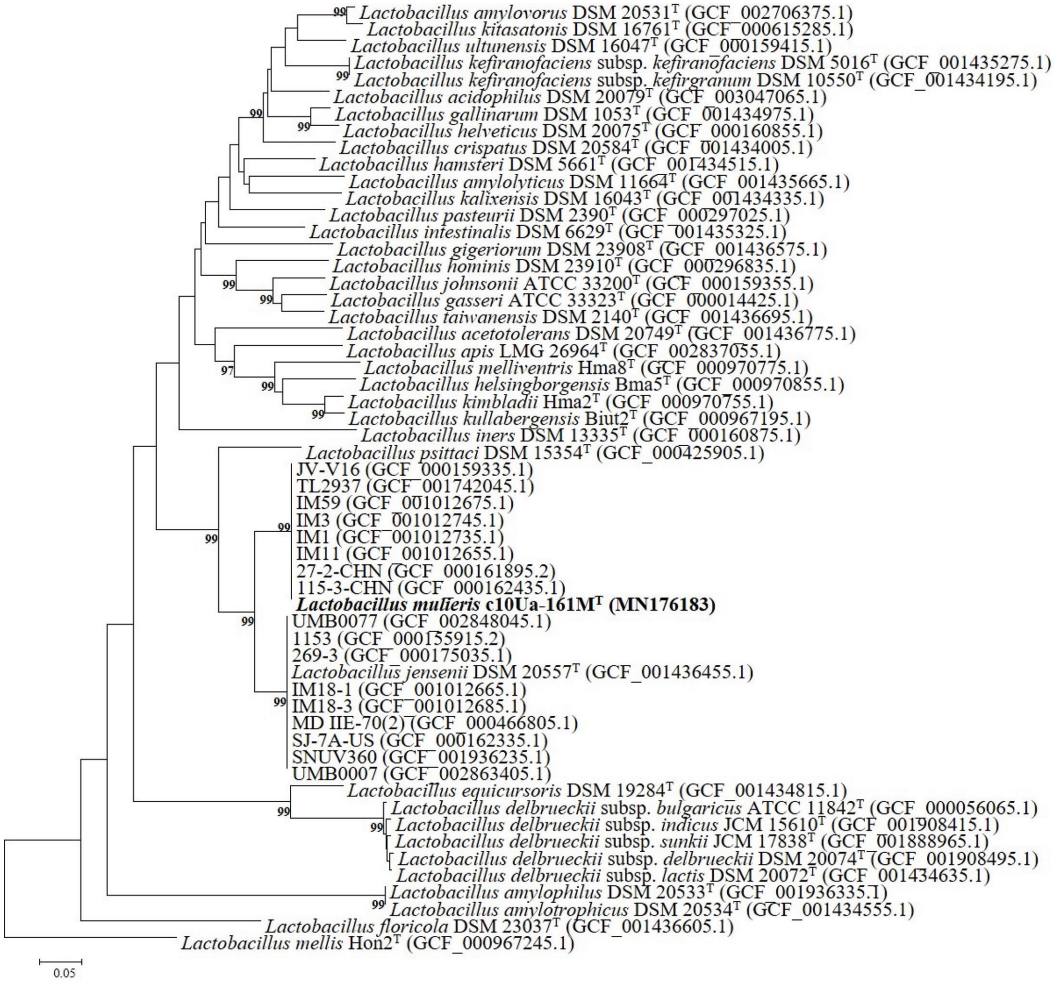

Fig. 2. Neighbour-joining tree (Kimura's two-parameter model and pairwise-deletion option) based on *pheS* gene sequences showing the phylogenetic relationships between Lactobacillus *mulieris* c10Ua161M^T (boldface type), closely related strains, and type strains of the genus Lactobacillus. Lactobacillus mellis Hon2 was used as the outgroup. Nucleotide sequences were extracted from draft/complete genomes obtained from the NCBI Assembly Database, for which the accession numbers are shown in the parenthesis. Bootstrap percentages (based on 1000 replications) are shown at nodes. Only values above 90% are shown. Bar, 0.05 substitutions per nucleotide position.

Distance Calculator (GGDC 2.1) under the recommended Formula 2 (http://ggdc.dsmz.de/distcalc2.php) [18]. The ANI value between strain c10Ua161M^T and type strain of *L. psittaci* (80.5%) and even *L. jensenii* (87.9%) were below the species cut-off level of 95% [19]. Remarkably, publicly available genomes of strains IM1, IM3, TL2937, 115–3-CHN, JV-V16, IM11, IM59, 27–2-CHN deposited as *L. jenseni* should be reclassified as *L. mulieris* based on whole-genome relatedness, since ANI values were all above 95% (99.7%, 99.5 %, 99.5 %, 99.6 %, 99.9 %, 99.6 %, 99.7 %, 99.6 %, respectively). Likewise, the intergenomic distance between strain c10Ua161M^T and the closest relative type strain of *L. jensenii* presented a GGDC value of 34.3%, which is clearly below the proposed criterion for bacterial species delineation (70 %) [20], and supports our findings of strain c10Ua161M^T as a new species.

Cell and colony morphology were observed with cells grown on MRS agar plates at 37 °C under anaerobic conditions for 72 h. Gram-staining was carried out using Gram-Staining kit (bioMérieux). Motility, catalase activity, spore formation, and gas production from glucose were determined using established procedures [21]. Growth at different temperatures [4, 10, 20, 30, 37, 40, 45 and 50 °C], pH [3.0, 3.5, 4.0, 5.0, 6.0, 7.0, 7.5, 8.0, 8.5 and 9.0] and NaCl concentrations [0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 4.5 and 5.0% (w/v)] were evaluated for cultures in MRS broth incubated for 7 days under aerobic conditions. Growth was estimated by monitoring the optical density at 600 nm. Biochemical characterization was performed using the standardized API 50 CH and API ZYM strips (incubation at 37 °C for 48 h) (bioMérieux) following the manufacturer's instructions. The novel isolates stained as Gram-positive. The coccobacilli to rod-shaped cells (0.9–3.0 µm) were non-motile.

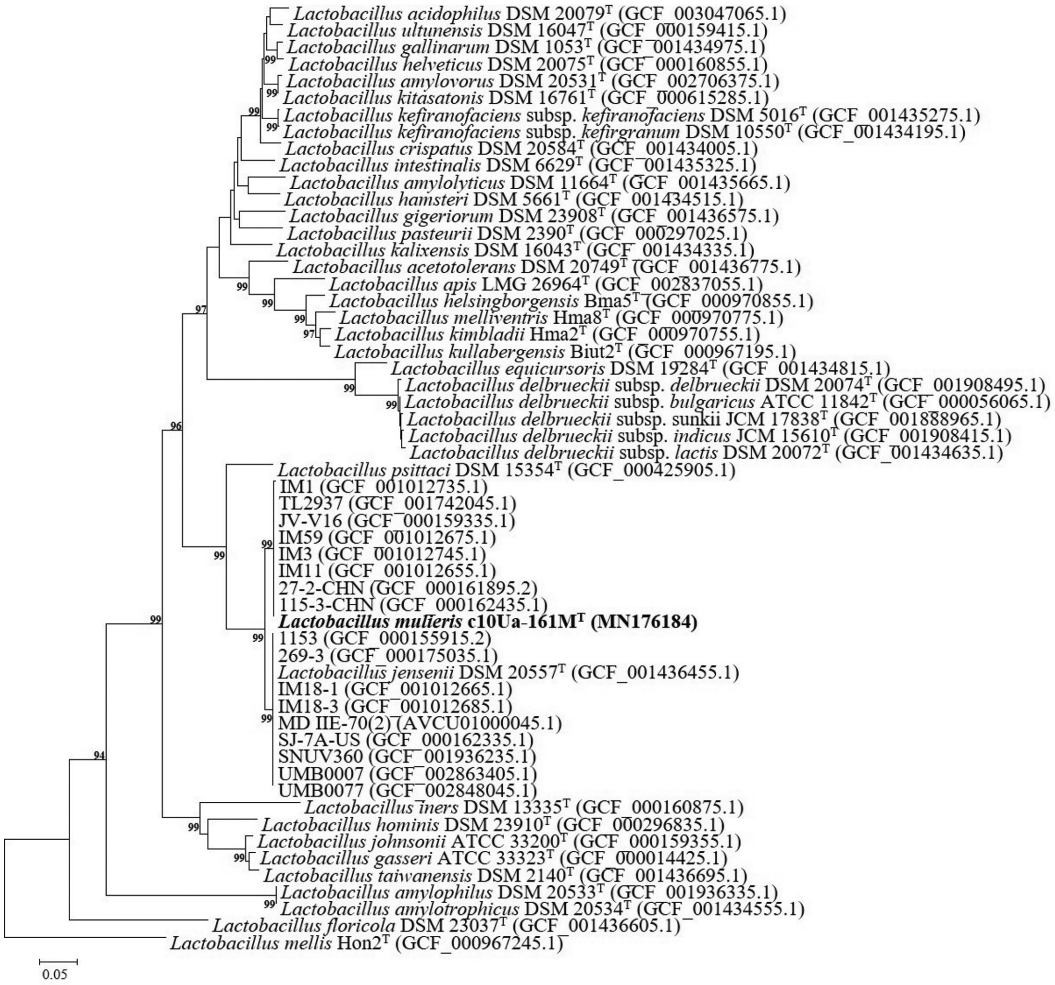

Fig. 3. Neighbour-joining tree (Kimura's two-parameter model and pairwise-deletion option) based on *rpoA* gene sequences showing the phylogenetic relationships between Lactobacillus *mulieris* c10Ua161M^T (boldface type), closely related strains and type strains of the genus Lactobacillus. Lactobacillus mellis Hon2 was used as the outgroup. Nucleotide sequences were extracted from draft/complete genomes obtained from the NCBI Assembly Database, for which the accession numbers are shown in the parenthesis. Bootstrap percentages (based on 1000 replications) are shown at nodes. Only values above 90% are shown. Bar, 0.05 substitutions per nucleotide position.

Growth occurred between 30–45 °C, with optimum temperature at 37 °C, until the maximum of 3.0% (w/v) NaCl, and in the range of pH 5.0–8.5. Different biochemical characteristics of strain c10Ua161M^T as compared to type strains of closely related species are summarized in Table 1. In particular, strain c10Ua161M^T was able to metabolize melibiose and starch, contrasting to *L. jensenii* and *L. psittaci*.

The fatty acid profile of strain c10Ua161M^T and closely related type strains of *L. jensenii* and *L. psittaci* were performed and analysed at the Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures GmbH) [22, 23, https://www.dsmz.de/services/microorganisms/microbiological-analysis/fatty-acid-fingerprint]. The major cellular fatty acids contained by c10Ua161M^T were $C_{18:1}\omega9c$ (65.0 %), $C_{16:0}$ (17.8 %), and summed feature 8 (10.2%; comprising $C_{18:1}\omega7c$, and/or $C_{18:1}\omega6c$) (Table 2). The fatty acid composition of c10Ua161M^T was similar to that of type strains of *L. jensenii* and *L. psittaci*, with small variations in the proportion. (Table 2).

Our data support the conclusion that strain c10Ua161M^T represents a novel species of the genus *Lactobacillus*, for which the name *Lactobacillus mulieris* sp. nov. is proposed. The type strain is c10Ua161M^T.

DESCRIPTION OF *LACTOBACILLUS MULIERIS* SP. NOV.

Lactobacillus mulieris (mu.li′.e.ris. L. gen. n. *mulieris* of a woman, from where the bacterium was isolated).

Gram-stain-positive, non-motile, non-spore-forming, catalase-negative, coccobacilli-shaped bacterium. Colonies

Table 1. Differential characteristics of strain c10Ua161M^T compared with those of closely related type strains of the genus *Lactobacillus*. Strains: 1 - c10Ua161M^T (this study); 2 L. *jensenii* DSM 20557^T [24], 3 L. *psittaci* CCUG 42378^T [25]. +, Positive; –, negative; w, weakly positive; ND, no data available

Characteristics	1	2	3
Isolation source	urine	vaginal discharge	bird lung
Morphology	coccobacilli	rod-shaped	coccobacilli to rod-shaped
Growth at/with:			
15 °C	–	ND	+
45 °C	+	–	+
G+C content (mol%)	34.2	34.3	35.65
API 50 CH:			
Ribose	–	+	–
N-Acetylglucosamine	+	–	ND
Arbutin	–	+	ND
Aesculin/ferric citrate	+	+	–
Melibiose	+	–	–
Trehalose	–	+	–
Raffinose	+	–	+
Amylum (starch)	+	w	ND
API ZYM:			
Alkaline phosphatase	–	ND	+
Leucine arylamidase	+	ND	+
Valine arylamidase	–	ND	w
Cystine arylamidase	–	ND	+
Naphthol-AS-BI-phosphohydrolase	+	ND	

Table 2. Comparative cellular fatty acid content (%) of strain c10Ua161M^T and related type strains of species of the genus *Lactobacillus*. Strains: 1, *L. mulieris* c10Ua161M^T; 2, *L. jensenii* DSM 20557^T; 3, *L. psittaci* DSM 15354^T. All data are from this study. Fatty acids present at >10% are indicated in bold. -, Not detected

Fatty acid	1	2	3
$C_{10:0}$	0.2	0.2	0.2
$C_{12:0}$	0.1	–	0.2
$C_{14:0}$	0.3	0.3	0.3
$C_{16:0}$	**17.8**	**15.6**	**15.9**
$C_{18:1}\omega9c$	**65.0**	**70.5**	**68.4**
$C_{18:0}$	2.9	3.1	2.6
$C_{19:1}$ iso	–	–	0.4
$C_{19:0}$ iso	2.5	–	2.1
Summed Features*			
3: $C_{16:1}\omega7c/_{C16:1}\omega6c/_{C15:0}$ iso 2-OH	1.0	0.9	1.0
8: $C_{18:1}\omega7c/_{C18:1}\omega6c$	**10.2**	9.4	8.9

*Summed features consist of one or more fatty acids that cannot be separated by the Sherlock Microbial Identification system.

are circular white, glossy, and 1.0–2.0 mm in diameter. They are facultative anaerobic and microaerophilic, homofermentative, and do not produce gas from glucose. Acids are produced from D-glucose, D-fructose, D-mannose, amygdalin, aesculin ferric citrate, salicin, cellobiose, maltose, melibiose, sucrose, raffinose, starch, *N*-acetyl-glucosamine but not from glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, lactose, trehalose, inulin, melezitose, glycogen, xylitol, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-keto-gluconate, 5-keto-gluconate, arbutin or gentiobiose. In the API ZYM test system, cells are positive for D-glucose, D-fructose, D-mannose, *N*-acetylglucosamine, amygdalin, aesculin, maltose, cellobiose, melibiose, Sucrose, raffinose, starch, leucine arylamidase and α-glucosidase. The major fatty acids are $C_{18:1}\omega9c$, $C_{16:0}$, and summed feature 8 (comprising $C_{18:1}\omega7c$, and/or $C_{18:1}\omega6c$).

The type strain is c10Ua161M^T (=CECT 9755^T=DSM 108704^T), isolated from human urine. The DNA G+C content is 34.2 mol%.

The annotated genome data was deposited in GenBank under the accession number SDGL00000000. The 16S rRNA, *pheS* and *rpoA* nucleotide sequences data from *Lactobacillus mulieris* c10Ua161M^T isolate are available in the GenBank database under accession numbers MK775269, MN176183 and MN176184, respectively.

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Author contributions
J.R. performed the phenotypic, biochemical and genomic methodologies, phylogenetic analysis, submission of genomes to public databases, and wrote the original draft preparation. JB contributed to ***in silico*** genomic comparisons and genome annotation. M.K. contributed to strains isolation, phenotypic and biochemical characterization. SUP performed MALDI-TOF MS analysis, and contributed to data curation

and reviewing the manuscript. MM and JAC contributed to genome annotation. LLP, SS, LMR-A, and MP performed the chemotaxonomic methodologies. TGR contributed to genomic and phylogenetic analysis, writing, reviewing and editing of the manuscript. LP contributed to reviewing the manuscript, supervision, project administration and funding.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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