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ORIGINAL PAPER

Novel lactic acid bacteria isolated from the bumble bee gut: *Convivina intestini* gen. nov., sp. nov., *Lactobacillus bombicola* sp. nov., and *Weissella bombi* sp. nov.

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Abstract Twelve isolates of lactic acid bacteria (LAB) were obtained in the course of a bumble bee gut microbiota study and grouped into four matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry clusters. Comparative 16S rRNA gene sequence analysis revealed that cluster 1 isolates, represented by strain LMG 28288^T, are most closely related to *Lactobacillus apis* (97.0 % sequence similarity to that of *L. apis* LMG 26964^T). Cluster 2 isolates represented by strain LMG 28290^T are most closely related to *Weissella hellenica* (99.6 % sequence similarity to that of *W. hellenica* LMG 15125^T). The single cluster 3 and 4 isolates had identical 16S rRNA gene sequences which were 94.8 % similar to that of *Leuconostoc mesenteroides* subsp. *mesenteroides* LMG 6893^T, their nearest phylogenetic neighbour. A polyphasic taxonomic study additionally including comparative *pheS* sequence analysis, DNA–DNA hybridization experiments, DNA G+C content analysis, $(GTG)_5$-PCR fingerprinting and a biochemical characterization, demonstrated that cluster 1 isolates represent a novel *Lactobacillus* species for which we propose the name *Lactobacillus bombicola* sp. nov. with LMG 28288^T (= DSM28793^T) as the type strain; and that cluster 2 isolates represent a novel *Weissella* species for which we propose the name *Weissella bombi* sp. nov. with LMG 28290^T (= DSM28794^T) as the type strain. Cluster 3 and 4 isolates, in contrast, represented a very distinct, novel taxon that could be distinguished from members of the genera *Leuconostoc* and *Fructobacillus*, its nearest phylogenetic neighbours, by its cellular morphology, non-fructophilic metabolism and DNA G+C content. We therefore classify both isolates into a novel species representing a novel LAB genus for which the name *Convivina intestini* gen. nov., sp. nov. is proposed with LMG 28291^T (= DSM28795^T) as the type strain.

Keywords Lactic acid bacteria · *Lactobacillus* · *Weissella* · Leuconostocaceae · Bumble bee · Gut microbiota

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Introduction

Bumble bees are essential pollinators of wild plants and commercial crops, especially in the north

temperate zone of the world. Although most commercial crops do not depend on insect pollination, it greatly improves the yield and the quality of many crops, such as tomatoes and sweet pepper. Therefore, insect pollination of commercial crops to which bumble bees contribute considerably, is of major value to agriculture (Gallai et al 2009; Garibaldi et al. 2013). The maintenance of bumble bee biodiversity is also important for ecosystem integrity of wild plants and, consequently, there is considerable anxiety about worldwide declines of bumble bee populations (Cameron et al. 2011; Goulson et al. 2006; Grixti et al. 2009; Rasmont et al. 2005; Williams et al. 2009; Williams 1982). These declines are presumably caused by a combination of factors with pesticide use, changes in agricultural practices and pathogen emergence commonly regarded to be the most important ones (Goulson et al. 2008; Meeus et al. 2011).

Over the past 10 years, intestinal dysbiosis has gained attention to explain emergent diseases. A study of Cox-Foster et al. (2007) showed that the abundance of lactic acid bacteria (LAB) in diseased honey bees was significantly lower than in healthy bees. In addition, several studies indicated that LAB play a major role in the health of bumble bees and honey bees. They may prevent pathogens such as *Paenibacillus larvae*, *Melissococcus plutonius*, *Nosema bombi* and *Crithidia bombi* from causing diseases by either competing for the gut niche (Anderson et al. 2011) or by direct inhibition through the production of antimicrobial compounds such as hydrogen peroxide, organic acids and antimicrobial peptides and acidification of the gut environment (Audisio et al. 2011). Although the bumble bee gut microbiota is only partially characterized, LAB and bifidobacteria belong to a core set of bumble bee gut bacteria (Olofsson et al. 2014; Killer et al. 2009, 2014; Koch and Schmid-Hempel 2011; Martinson et al. 2011; Mohr and Tebbe 2006). Metagenomic studies have demonstrated the presence of several phylotypes of LAB in the guts of honey bees and bumble bees (Koch and Schmid-Hempel 2011; Mohr and Tebbe 2006; Olofsson and Vásquez 2008) and several cultured representatives have been isolated, including members of the genera *Lactobacillus*, *Fructobacillus* and *Enterococcus* (Audisio et al. 2011; Endo and Salminen 2013; Killer et al. 2013, 2014). In the course of a bumble bee gut microbiota study we isolated several LAB which could not be allocated to formally named species.

Materials and methods

Sampling of bumble bees and preparation of cell suspensions

Bumble bees of *Bombus pascuorum*, *Bombus lapidarius*, *Bombus hypnorum* and *Bombus terrestris* were caught in the field in the region of Ghent, Belgium, and identified by their colour pattern. In addition, the cytochrome oxidase I (COI) gene sequence was determined for bumble bees identified as *B. lapidarius* and *B. terrestris*, which can be confused with other *Bombus* species based on colour pattern. The COI gene was amplified as described by Carolan et al. (2012) after Chelex® 100 resin (Bio-Rad) DNA-extraction on two bumble bee legs which were ground with a micropestle (Walsh et al. 1991). The bumble bees were immobilized at −20 °C for 10 minutes and surface sterilized with 2.5 % Umonium38® Master (Laboratoire Huckert's International, Brussels, Belgium) before dissecting out their crop and gut. The crops and guts were homogenized in 125 µl saline solution (0.1 % peptone, 0.1 % Tween 80, 0.85 % NaCl) with a sterile micro-pestle. Afterwards, 125 µl of a 10 % DMSO solution was added to the cell suspensions which were stored at −80 °C until further use.

Isolation of bumble bee gut bacteria and dereplication

Cell suspensions were serially diluted to 10^{-4} in physiological saline (0.85 % NaCl), plated on MRS agar (Oxoid), modified trypticase phytone yeast extract (MTPY) agar (Rada and Petr 2000) and all culture (AC) agar (Sigma-Aldrich) and incubated aerobically (MRS), anaerobically (MRS and MTPY) and microaerobically (AC) at 37 °C. After 5 days, colonies were picked up from the agar plates and third generation axenic isolates were dereplicated by MALDI-TOF MS followed by curve-based data analysis (Ghyselinck et al. 2011) using the BioNumerics 5.1 software (Applied Maths, Sint-Martens-Latem, Belgium). Representative isolates of each MALDI-

TOF MS cluster were selected for further identification.

Phylogenetic analysis

The 16S rRNA gene sequences and *pheS* sequences were determined as previously described (De Bruyne et al. 2007; Snauwaert et al. 2013). EzTaxon-e (Kim et al. 2012) was used for the analysis of the 16S rRNA gene sequences and NCBI BLAST (www.ncbi.nlm.nih.gov/BLAST) for the analysis of the *pheS* sequences. The MEGA5 software package was used to align the sequences obtained with the corresponding sequences of their phylogenetic neighbour species by MUSCLE and to obtain phylogenetic trees by using the maximum-likelihood method and the general time-reversible model with invariant sites (Tamura et al. 2011). The robustness of the topology of the trees was estimated by bootstrap analysis with 100 replicates (Felsenstein, 1985). MEGA5 was also used to calculate the sequence similarity values.

DNA G+C content and DNA–DNA hybridization

The DNA G+C content of the type strains and the DNA–DNA hybridization between strain LMG 28290^T and the type strain of *Weissella hellenica* LMG 15125^T were determined as previously described (Cleenwerck et al. 2008).

$(GTG)_5$-PCR fingerprinting

Genotypic fingerprints of all isolates were obtained via $(GTG)_5$-PCR as described by Gevers (2001) and analysed with the BioNumerics 5.1 software (De Vuyst et al. 2008).

Phenotypic analysis

The isolates and type strains of their closest relatives (*Lactobacillus. apis* LMG 26964^T, *W. hellenica* LMG 15125^T and *Leuconostoc. mesenteroides* subsp. *mesenteroides* LMG 6893^T) were routinely grown anaerobically on MRS agar at 37 °C, except *W. hellenica* LMG 15125^T which was routinally grown aerobically at 25 °C. To test substrate utilization and enzyme activity, API 50CHL strips and APIZYM strips (bioMérieux) were inoculated with dense cell suspensions (McFarland 2 and 5, respectively) of the isolates. The API 50CHL strips were read after 5 days of incubation at 37 °C. Gram-stain behaviour, endospore staining and verification of oxidase and catalase activity were performed using standard microbiological procedures (MacFaddin 1980). Growth was determined in triplicate in MRS broth (Oxoid) at different temperatures (10, 15, 25, 37 and 45 °C), pH values (pH 3, 5, 7 and 9) and NaCl concentrations (5, 6, 7 and 8 %). Gas production from glucose was determined using inverted Durham tubes in MRS broth. Cell morphology and motility was checked with light microscopy and for LMG 28291^T also with electron microscopy as described in Houf et al. (2009). Verification of fructophilic growth characteristics of strains LMG 28291^T and LMG 28625 was performed by growth on MRS agar and MRS agar supplemented with 2 % fructose in aerobic and anaerobic atmospheres. Production of short chain fatty acids was determined after growth in MRS broth for 3 days as described by De Baere et al. (2013). The D-/L-Lactic Acid (D-/L-Lactate) (Rapid) Assay Kit (Megazyme) was used to determine D- or L-lactic acid production.

Results

MALDI-TOF MS dereplication

LAB isolates obtained from the bumble bee gut samples assembled into several MALDI-TOF MS clusters; isolates of most of these clusters were identified as *Fructobacillus tropeaoli*, *Leuconostoc mesenteroides*, *Lactobacillus kunkeei*, *Enterococcus faecalis* and *Lactococcus lactis* based on the profiles of reference strains present in our in-house database (data not shown). However, the profiles of the isolates of four MALDI-TOF MS clusters (Fig. 1a–c) were different from those of the LAB in the in-house reference database (Supplementary Fig. 1a–c). Cluster 1 and 2 profiles originated from isolates from multiple bumble bee species each; in contrast, cluster 3 and 4 profiles originated from biological and technical replicates of a single isolate each. An overview of all isolates, their hosts and sampling details is provided in Table 1.

Phylogenetic analyses

Strains LMG 28288^T, LMG 28290^T, LMG 28291^T and LMG 28625 were chosen as representatives of the four

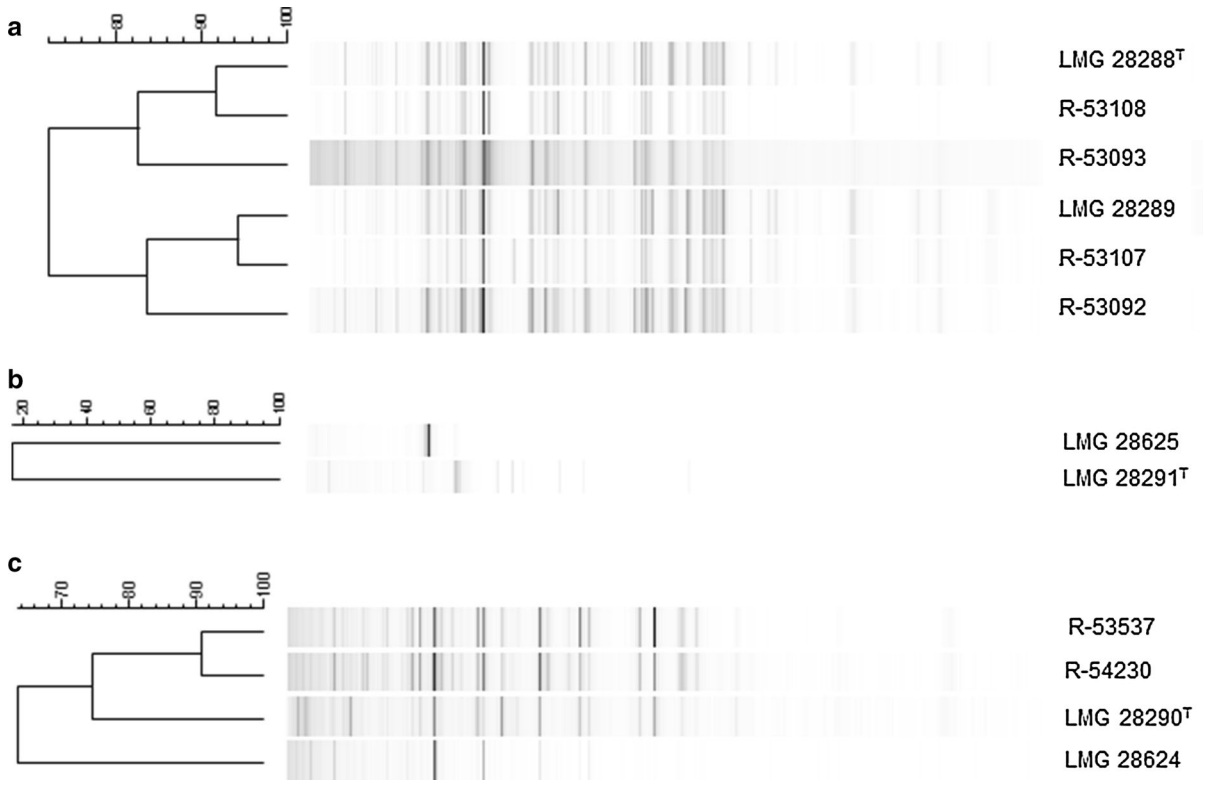

Fig. 1 MALDI-TOF MS profiles of the *Lactobacillus bombicola* (**a**), *Convivina intestini* (**b**), and *Weissella bombi* isolates (**c**). Cluster analysis was performed by Pearson correlation and the hierarchical clustering method UPGMA

clusters. Analysis of their 16S rRNA gene sequences demonstrated that LMG 28288^T and LMG 28290^T are most closely related to *Lactobacillus apis* LMG 26964^T (97.0 % sequence similarity) and *W. hellenica* LMG 15125^T (99.6 % sequence similarity), respectively (Fig. S2 and S3). The 16S rRNA gene sequence of strain LMG 28288^T was also 99.9 % similar to that of a *Lactobacillus* phylotype (clone Q05008Plasm3 1486 bp) detected in a bumble bee gut metagenomics study (Koch and Schmid-Hempel 2011). The 16S rRNA gene sequences of strains LMG 28291^T and LMG 28625 were identical and showed the highest sequence similarity (94.8 %) to that of *L. mesenteroides* subsp. *mesenteroides* LMG 6893^T; they were also 99 % similar to that of an uncultured Firmicutes phylotype (murgBL2to) detected by Vásquez et al. (2012) in honey bees (Fig. S4). A 16S rRNA gene sequence based phylogenetic tree comprising strains LMG 28288^T, LMG 28290^T and LMG 28291^T and their nearest phylogenetic neighbour species is shown in Fig. 2.

The *pheS* sequences of all six cluster 1 isolates were determined and revealed 98.9–100 % sequence similarity to that of strain LMG 28288^T; the MEGA5 analysis confirmed *L. apis* as the nearest neighbour species and revealed 81.5 % similarity to the *pheS* sequence of *L. apis* LMG 26964^T. The *pheS* sequences of all four cluster 2 isolates were identical; the MEGA5 analysis of the *phe*S sequence of strain LMG 28289^T confirmed *W. hellenica* as the nearest neighbour species and revealed 89.2 % similarity to the *pheS* sequence of *W. hellenica* LMG 15125^T. Finally, the *pheS* sequences of strains LMG 28291^T and LMG 28625 were identical and sequence analysis in MEGA5 showed the highest sequence similarity to the *pheS* sequence of *Fructobacillus ficulneus* LMG 21928^T (81.5 %). Figures S5, S6 and S7 present *pheS* sequence based phylogenetic trees of strains LMG 28288^T, LMG 28290^T and LMG 28291^T and some of their neighbouring taxa.

$(GTG)_5$-PCR-fingerprinting

The $(GTG)_5$-PCR profiles (Supplementary Fig. 8a–c) of two cluster 1 isolates (LMG 28289 and R-53092) were

Table 1 Isolates, their sources and MALDI-TOF MS cluster numbers

Isolates	Isolation source[a]	MALDI-TOF MS cluster
LMG 28288^T	Bumble bee H70 *B. lapidarius*	1
	Bourgoyen Ghent (51.06840°NL/3.685100°EL)	
LMG 28289	Bumble bee H53 *B. pascuorum*	
	Coupure Ghent (51.05130°NL/3.706°EL)	
R-53092	Bumble bee H66 *B. pascuorum*	
	Bourgoyen Ghent (51.06840°NL/3.685100°EL)	
R-53093	Bumble bee H113 *B. lapidarius*	
	Ledeganck Ghent (51.0368°NL/3.7221°EL)	
R-53107	Bumble bee H87 *B. vestalis*	
	Den Blakken Wetteren (51.0086°NL/3.899100°EL)	
R-53108	Bumble bee H18 *B. terrestris*	
	Ledeganck Ghent (51.0368°NL/3.7221°EL)	
LMG 28290^T	Bumble bee H24 *B. terrestris*	2
	Gentbrugge (51.0462°NL/3.7608°EL)	
R-53537	Bumble bee H1 *B. terrestris*	
	Ledeganck Ghent (51.0368°NL/3.7221°EL)	
R-54230	Bumble bee H15 *B. terrestris*	
	Ledeganck Ghent (51.0368°NL/3.7221°EL)	
LMG 28624	Bumble bee H69 *B. hypnorum*	
	Bourgoyen Ghent (51.06840°NL/3.685100°EL)	
LMG 28291^T	Bumble bee H79 *B. terrestris*	3
	Bourgoyen Ghent (51.06840°NL/3.685100°EL)	
LMG 28625	Bumble bee H79 *B. terrestris*	4

[a] Isolation source (bumble bee individual, bumble bee species and sampling location)

highly similar; all others were different (Fig. 3a). The $(GTG)_5$-PCR profile of one cluster 2 isolate (LMG 28290^T) differed slightly from those of the remaining three cluster 2 isolates (Fig. 3b). Finally, the $(GTG)_5$-PCR profiles of cluster 3 isolate LMG 28291^T and cluster 4 isolate LMG 28625 were highly similar (Fig. 3c).

DNA–DNA hybridization and DNA G+C content analysis

DNA–DNA hybridizations were performed between strain LMG 28290^T and *W. hellenica* LMG 15125^T. The DNA–DNA hybridization value was 37.2 ± 4.5 % (the reciprocal values were 34.1 and 40.3 %). The DNA G+C content of strains LMG 28288^T, LMG 28290^T and LMG 28291^T was 34.5, 37.2 and 31.9 mol%, respectively.

Phenotypic analyses

Phenotypic analyses were performed for all 12 isolates and the type strains of their closest neighbours (*L. apis* LMG 26964^T, *W. hellenica* LMG 15125^T and *L. mesenteroides* subsp. *mesenteroides* LMG 6893^T) as determined through 16S rRNA gene sequencing, unless stated otherwise. All isolates were found to be facultative anaerobes and Gram-stain positive, non-motile, oxidase- and catalase-negative. They did not form endospores.

Cluster 1 isolates and *L. apis* LMG 26964^T were found to produce acid from D-ribose, D-glucose, D-fructose, D-mannose, *N*-acetylglucosamine, amygdalin, arbutin, esculin ferric citrate, salicin, D-trehalose, gentiobiose and D-cellobiose. They did not produce acid from glycerol, erythritol, D-arabinose, D- and 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, D-maltose, D-lactose, D-melibiose, sucrose, inulin, D-melezitose, D-raffinose, starch, glycogen, xylitol, D-turanose, D- and L-fucose, D-arabitol, L-arabitol, potassium gluconate or potassium 2-gluconate. LMG 28288^T and *L. apis* LMG

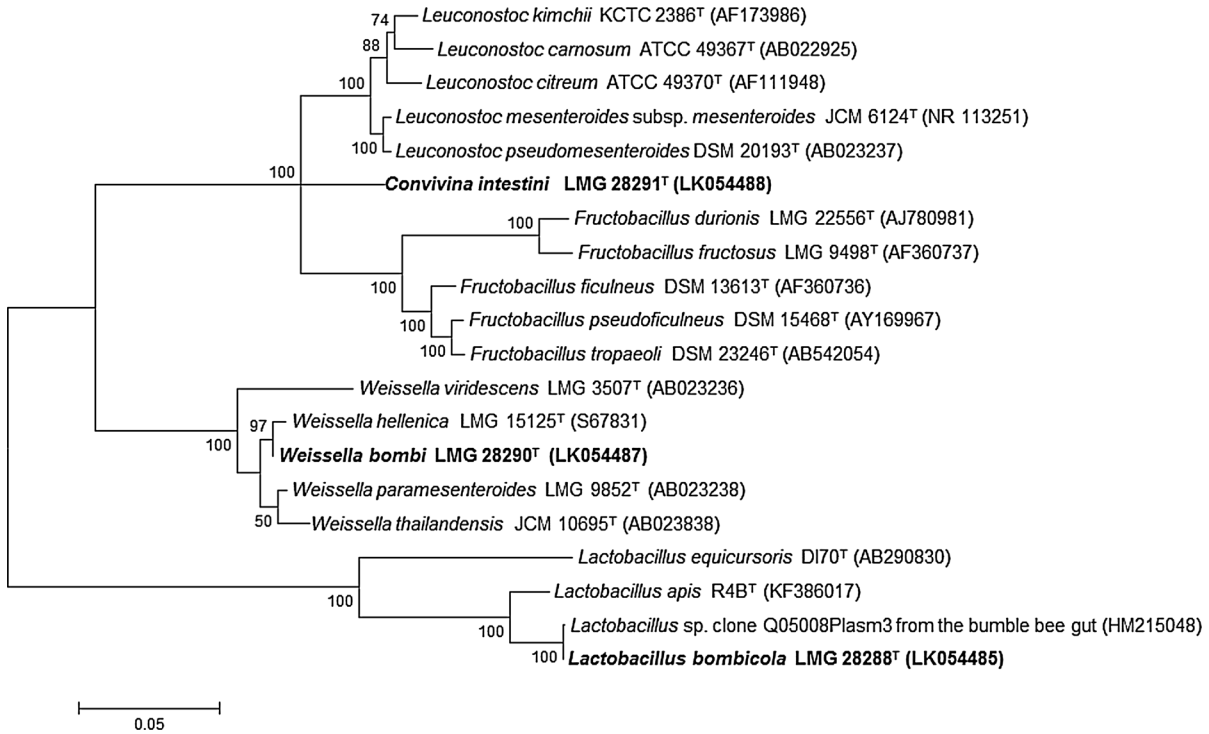

Fig. 2 Restricted phylogenetic tree based on 16S rRNA gene sequences of *Lactobacillus bombicola* LMG 28288^T, *Weissella bombi* LMG 28290^T and *Convivina intestini* LMG 28291^T and established *Lactobacillus*, *Weissella*, *Fructobacillus* and *Leuconostoc* species. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model (Nei and Kumar 2000). The bootstrap consensus tree inferred from 100 replicates is taken to represent the evolutionary history of the taxa analysed (Felsenstein 1985). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches if the percentage is 50 % or higher (Felsenstein 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites [five categories (+G, parameter = 0.2022)]. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 57.0917 % sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 20 nucleotide sequences. There were a total of 1575 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011)

26964^T exhibit activity of leucine arylamidase, valine arylamidase, cysteine arylamidase, acid phosphatase, naphtol-AS-BI-phosphohydrolase, β-glucosidase, *N*-acetyl-β-glucosaminidase. They do not exhibit activity of alkaline phosphatase, esterase lipase (C8), lipase (C14), trypsin, α-chymotrypsin, α- and β-galactosidase, β-glucuronidase, α-glucosidase, α-mannosidase and α-fucosidase. Strain dependent and differential phenotypic characteristics between cluster 1 isolates and *L. apis* LMG 26964^T are shown in Table 2.

Cluster 2 isolates and *W. hellenica* LMG 15125^T were found to produce acid from L-arabinose, D-glucose, D-fructose, D-mannose, methyl-α-D-glucopyranoside, *N*-acetylglucosamine, esculin ferric citrate, D-maltose, sucrose, D-trehalose and potassium gluconate but not from glycerol, erythritol, D-arabinose, D- and L-xylose, methyl-β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-α-D-mannopyranoside, amygdalin, inulin, D-melezitose, starch, glycogen, xylitol, D-tagatose, D- and L-fucose or L-arabitol. LMG 28290^T and *W. hellenica* LMG 15125^T exhibit activity of acid phosphatase, naphtol-AS-BI-phosphohydrolase, β-galactosidase and α-glucosidase. They do not exhibit activity of esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cysteine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, β-glucosidase,

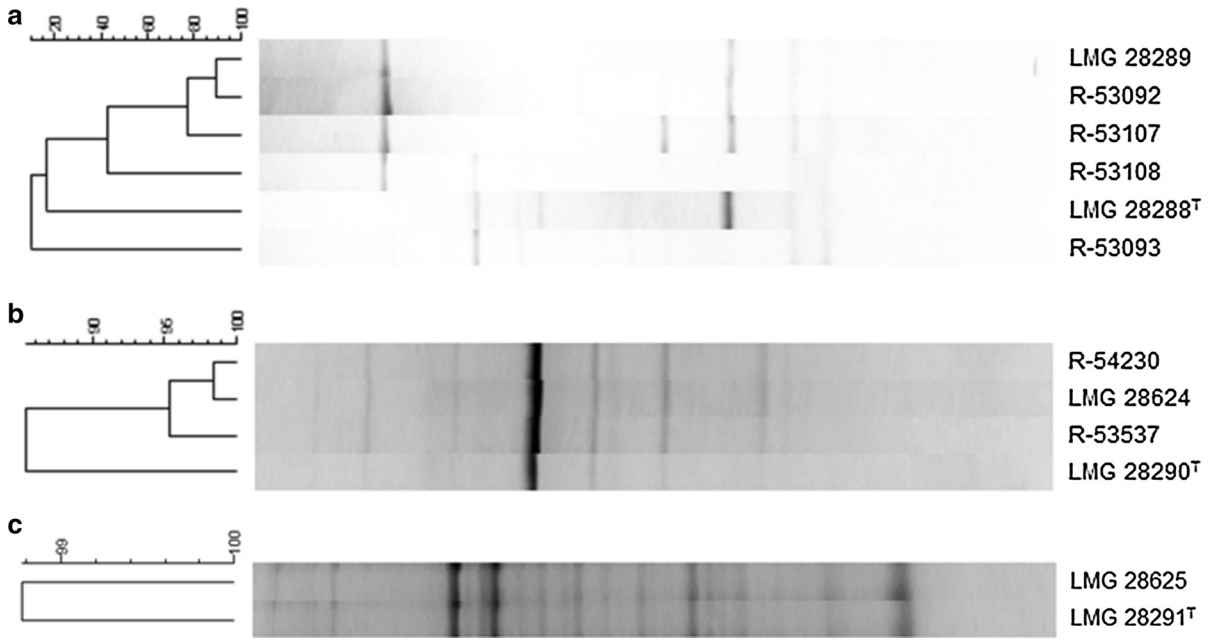

Fig. 3 $(GTG)_5$-PCR profiles of different *Lactobacillus bombicola* (**a**), *Weissella bombi* (**b**) and *Convivina intestini* (**c**) isolates. Cluster analysis was performed by Pearson correlation and the hierarchical clustering method UPGMA

N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Strain dependent characteristics and differential phenotypic characteristics between cluster 2 isolates and *W. hellenica* LMG 15125^T are shown in Table 2.

Biochemical characteristics of cluster 3 and 4 isolates are listed below. An electron microscopy picture of LMG 28291^T cells is shown in Fig. 4.

Discussion

In the present study, LAB were isolated from the gut of bumble bees of *B. pascuorum*, *B. terrestris*, *B. vestalis*, *B. hypnorum* and *B. lapidarius*. Comparative MALDI-TOF MS analysis using an in-house database of LAB MALDI-TOF MS spectra allowed the assignment of most isolates to well-known LAB species such as *F. tropeaoli*, *L. mesenteroides, L. kunkeei*, *E. faecalis* and *L. lactis*, but revealed four clusters of spectra of isolates that could not be identified (Table 1).

The six cluster 1 isolates were obtained from six bumble bees representing four different species, i.e. *B. pascuorum*, *B. terrestris*, *B. vestalis* and *B. lapidarius* and were collected at different locations in the region of Ghent, Belgium (Table 1). The 16S rRNA gene and *pheS* sequences of strain LMG 28288^T (Fig. 2 and S5) revealed *L. apis* LMG 26964^T as the nearest neighbour (97.0 and 81.5 % sequence similarity, respectively). The value of the 16S rRNA gene sequence similarity is well below the species delineation cut-off values of 98 % (Stackebrandt et al. 2014) or 98.65 % (Kim et al. 2014) indicating that cluster 1 represented a novel *Lactobacillus* species. This novel species has most likely been observed earlier in a bumble bee gut metagenomic dataset of Koch and Schmid-Hempel (2011) as revealed by a 16S rRNA gene sequence similarity of 99.9 % between strain LMG 28288^T and *Lactobacillus* clone Q05008Plasm3. It is noteworthy that several additional *Lactobacillus* phylotypes have been reported in the gut microbiota of honey bees and bumble bees, of which only some have been formally named (i.e. *L. kunkeei*, *Lactobacillus bombi, L. apis*, *Lactobacillus apinorum*, *Lactobacillus mellifer*, *Lactobacillus mellis*, *Lactobacillus melliventris*, *Lactobacillus kimbladii*, *Lactobacillus helsingborgensis* and *Lactobacillus kullabergensis*) (Endo et al. 2012; Killer et al. 2014, 2013; Olofsson et al. 2014). The DNA G+C content of LMG 28288^T (34.5 mol%) differs from that of *L. apis* (41.3 mol%). Although

Table 2 Differential phenotypic characteristics of *Lactobacillus bombicola* LMG 28288^T and *Lactobacillus apis* LMG 26964^T and *Weissella bombi* LMG 28290^T and *Weissella hellenica* LMG 15125^T

Phenotypic characteristic	LMG 28288^T	LMG 26964^T	LMG 28290^T	LMG 15125^T
Production of acid from				
L-Arabinose	+	−	+	+
D-Ribose	+	+	+ (0/3)[a]	−
D-Adonitol	−	−	− (2/3)[a]	−
D-Galactose	−	−	+	w
Arbutin	+	+	+	−
Salicin	+	+	+ (2/3)[a]	−
D-Cellobiose	+	+*	+	−
D-Lactose	−	−	+ (2/3)[a]	−
D-Melibiose	−	−	+	−
D-Raffinose	−	−	+	−
Gentiobiose	+	+	+	−
D-Turanose	−	−	+	w
D-Lyxose	w	−	−	−
D-Tagatose	w	−	−	−
D-Arabitol	−	−	−	+
Potassium 2-ketogluconate	−	−	+ (1/3)[a]	−
Potassium 5-ketagluconate	w	−	+	−
Alkaline phosphatase	−	−	−	+
Esterase (C4)	−	+	−	−
DNA G+C content (mol%)	34.5	41.3	37.2	39.4
Growth at pH 3–9	pH 3–7	pH 5–9	pH 3–9	pH 3–9
Growth at 10–45 °C	28–37 °C	28–37 °C	10–37 °C	10–28 °C

The API 50CHL and APIZYM test kits and the D-/L-lactic acid test (Megazyme) were performed to obtain the data

+ positive, − negative, *w* weakly positive

[a] Number of isolates with a reaction identical to that of the type strain

* This result does not correspond with published data (Collins et al. 1993)

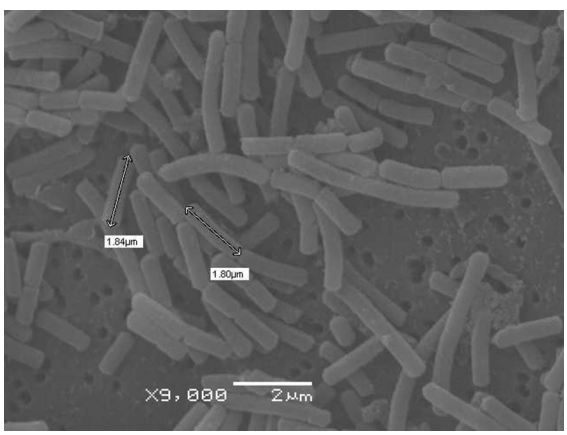

Fig. 4 Electron microscopy picture of LMG 28291^T cells

phenotypically coherent, the *pheS* sequences and $(GTG)_5$-PCR fingerprints revealed some genotypic differences among the six cluster 1 isolates indicating that multiple strains were isolated and studied. The present novel taxon can be distinguished from its nearest neighbour species, *L. apis,* both phenotypically (Table 2; Supplementary Fig. 1a) as well as genotypically (Fig. 3a). We therefore propose to classify the six cluster 1 isolates into the novel species *Lactobacillus bombicola* sp. nov., with strain LMG 28288^T as the type strain and present its description below.

The four cluster 2 isolates originated from four bumble bees which represented two different species (*B. terrestris* and *B. hypnorum*) collected at three different locations (Table 1). Analysis of the 16S rRNA gene sequence of a representative isolate (LMG 28290^T) revealed its closest relatedness to *W. hellenica* LMG 15125^T with 99.6 % gene sequence similarity (Fig. 2). DNA–DNA hybridization between LMG 28290^T and *W. hellenica* LMG 15125^T revealed a value of 37.2 ± 4.5 % which indicated that LMG 28290^T belongs to a novel species. The *pheS* gene sequences of all four isolates are identical and showed 89.2 % similarity to that of *W. hellenica* LMG 15125^T. However, the $(GTG)_5$-PCR profile of LMG 28290^T

differed from those of the other three isolates. The DNA G+C content of LMG 28290^T is 37.2 mol%, which differs from that of *W. hellenica* LMG 15125^T (39.4 mol%). We are not aware of 16S rRNA gene sequences detected in metagenomic or other data sets of honey bees or bumble bees which are highly similar to the 16S rRNA gene sequences of the *Weissella* isolates of the present study. The cluster 2 isolates can be distinguished phenotypically as well as genotypically from its nearest neighbour species, *W. hellenica*. Therefore, we propose to classify them into the novel species *Weissella bombi* sp. nov., with strain LMG 28290^T as the type strain.

Finally, the cluster 3 and 4 strains proved to represent a single taxon as revealed by their identical 16S rRNA gene and *phe*S sequences. This 16S rRNA gene sequence is 99 % similar to that of an uncultured Firmicutes phylotype (murgBL2to) detected by Vásquez et al. (2012) in honey bees. The nearly complete 16S rRNA gene sequences demonstrated that this taxon occupies a phylogenetic position intermediate to that of the genera *Leuconostoc* and *Fructobacillus* (Fig. 2). While EzTaxon-e analysis of the 16S rRNA gene sequence revealed a *Leuconostoc* as nearest neighbour species (i.e. *L. mesenteroides* subsp. *mesenteroides* LMG 6893^T; Fig. 2), MEGA5 analysis of the *phe*S sequence revealed a *Fructobacillus* as the nearest neighbour species (i.e. *F. ficulneus* LMG 21928^T, Fig. S7). However, the clearly rod-shaped cellular morphology (Fig. 4) demonstrated that this taxon does not conform to the characteristics of *Leuconostoc* species, while the absence of a fructophilic metabolism differentiated it from the members of the genus *Fructobacillus* (Table 3). Indeed, *Fructobacillus* species prefer fructose as a carbon source and can only use glucose in an anaerobic atmosphere if an electron acceptor (such as fructose) is present (Endo and Salminen 2013; Endo et al. 2012). Strains LMG 28291^T and LMG 28625, in contrast, grow rapidly with glucose as a carbon source in both aerobic as well as anaerobic conditions, and do not require fructose or an alternative electron acceptor for cultivation in an anaerobic atmosphere. Furthermore, the DNA G+C content of LMG 28291^T is 31.9 mol% which is considerably different from the DNA G+C content range within the genera *Leuconostoc* (37–44 mol%; Vos et al. 2009) and *Fructobacillus* (42–45 mol%; Endo and Okada 2008). Given the considerable phylogenetic divergence between this novel taxon and the genera *Leuconostoc* and *Fructobacillus,* and the differential genotypic and phenotypic characteristics, we feel it is most appropriate to classify the taxon represented by strains LMG 28291^T and LMG 28625 as a novel species of a novel LAB genus, for which we propose the name *Convivina intestini* gen. nov., sp. nov. with LMG 28291^T as the type strain of the type species.

Description of *Lactobacillus bombicola* sp. nov

Lactobacillus bombicola [bom.bi'co.la. L. n. *bombus* a boom, a deep hollow noise, buzzing, also the zoological genus name of the bumble bee; L. suf. -cola (derived from *incola*, inhabitant) dwelling, occurring in; N.L. n. *bombicola* occurring in *Bombus*].

Grows on MRS agar at 37 °C and is facultative anaerobic, Gram-stain positive, non-motile and oxidase- and catalase-negative. Does not form endospores. The cells are rod shaped (0.5–1 μm wide and 3 μm long) and the colonies are brown, shiny, undulate and 2 mm after 2 days of incubation. Grows at 25 and 37 °C but not at 10, 15 and 45 °C. Grows at pH 3–7 and does not produce gas from glucose. Only D-lactic acid is produced from glucose. No growth is observed at the tested NaCl concentrations (5–8 % NaCl). The type strain produces acid from D-cellobiose, L-arabinose, D-ribose, D-glucose, D-fructose, D-mannose, amygdalin, arbutin, D-trehalose, *N*-acetylglucosamine, esculin ferric citrate, salicin, D-lyxose (although weakly), D-tagatose (although weakly), potassium 5-ketogluconate (although weakly) and gentiobiose. The type strain does not produce acid from glycerol, erythritol, D-arabinose, D-xylose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-raffinose, inulin, D-melezitose, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, D-maltose, D-lactose, D-melibiose, sucrose, starch, glycogen, xylitol, D-turanose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate and potassium 2-ketogluconate. The type strain exhibits activity of leucine arylamidase, valine arylamidase, cysteine arylamidase, acid phosphatase, naphtol-AS-BI-phosphohydrolase, β-glucosidase, *N*-acetyl-β-glucosaminidase. The type strain does not exhibit activity of alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), trypsin, α-chymotrypsin, α- and β-galactosidase, β-glucuronidase, α-glucosidase, α-mannosidase and α-fucosidase.

Table 3 Differential characteristics of the genera *Fructobacillus, Leuconostoc* and *Convivina*

Phenotypic characteristic	*Fructobacillus*[a]	*Leuconostoc*[b]	*Convivina*
Cell morphology	Rod shaped	Coccus	Rod shaped
Fructophilic metabolism	Yes	No	No
% G+C	42–45 %	37–44 %	31.9 %

[a] Data obtained from Endo and Okada (2008)

[b] Data obtained from Vos et al. (2009)

The DNA G+C content of the type strain is 34.5 mol%. The type strain, LMG 28288^T (=DSM 28793^T), was isolated from the gut of a *Bombus lapidarius* bumble bee in 2013 in the region of Ghent, Belgium. The accession numbers for the 16S rRNA gene and *pheS* sequences of *L. bombicola* LMG 28288^T are LK054485 and LM999917, respectively.

Description of *Weissella bombi* sp. nov.

Weissella bombi (bom'bi. L. n. *bombus* a boom, a deep hollow noise, buzzing, also the zoological genus name of the bumble bee; N.L. gen. n. *bombi* of *Bombus*, of a bumble bee).

Strains are facultative anaerobes and are Gram-stain positive, non-motile and oxidase- and catalase-negative. Do not form endospores. The cells are elongated cocci (0.5–1 μm wide and 2 μm long) which occur in pairs or chains and the colonies are white, undulate, shiny and 1–2 mm after 2 days of incubation. The cells tend to precipitate in MRS broth. Growth is observed at pH 3–9 and 10–37 °C but not at 45 °C. Grows in the presence of the tested NaCl concentrations (5–8 % NaCl) and produces gas, D-lactic acid and acetic acid as end products from glucose fermentation. The type strain produces acid from L-arabinose, D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, arbutin, D-trehalose, methyl-α-D-glucopyranoside, *N*-acetyl-glucosamine, esculin ferric citrate, D-maltose, D-melibiose, D-raffinose, gentiobiose, D-lactose, sucrose, salicin, D-turanose, D-cellobiose, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate. Acid is not produced from glycerol, erythritol, D-arabinose, D- and L-xylose, D-adonitol, methyl-β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, amygdalin, inulin, D-melezitose, D-sorbitol, methyl-α-D-mannopyranoside, starch, glycogen, xylitol, D-tagatose, D-fucose, L-fucose, D-arabitol and L-arabitol. The type strain exhibits activity of acid phosphatase, naphtol-AS-BI-phosphohydrolase, β-galactosidase and α-glucosidase. The type strain does not exhibit activity of alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cysteine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The DNA G+C content of the type strain is 37.2 mol%.

The type strain LMG 28290^T (=DSM 28794^T), was isolated from the gut of a *B. terrestris* bumble bee in 2013 in the region of Ghent, Belgium. The accession numbers for the 16S rRNA gene and *pheS* sequences of *W. bombi* LMG 28290^T are LK054487 and LM999920, respectively.

Description of *Convivina* gen. nov

Convivina (Con.vi.vi'na. L. n. *conviva* guest, table companion; N.L.fem. n. *Convivina* a commensal bacterium)

Cells are rod shaped, catalase- and oxidase-negative, Gram-stain positive, produce D-lactic acid and acetic acid as end products from glucose fermentation and do not form endospores. Non-fructophilic. *C. intestini* is the type species of the genus.

Description of *Convivina intestini* sp. nov

Convivina intestini (in.tes.ti'ni. L. gen. n. *intestini*, of the gut)

The characteristics are as described for the genus with the following additional properties. The colonies are white, round, 1–2 mm, convex and shiny after 2 days. The cells are 0.5–1 μm wide and 2 μm long. Grows at pH 3–9 and at 10–45 °C, although growth at 45 °C is weak. Produces gas from glucose and grows weakly at the tested NaCl concentrations (5–8 % NaCl) after 5 days of incubation. Acid is produced

from D-ribose, D-glucose, D-fructose, D-mannitol, D-trehalose, esculin ferric citrate, sucrose and potassium gluconate. Acid is not produced from glycerol, erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, D-galactose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, amygdalin, arbutin, D-raffinose, inulin, D-melezitose, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, *N*-acetylglucosamine, salicin, D-maltose, D-lactose, D-melibiose, starch, glycogen, xylitol, gentiobiose, D-raffinose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, D-cellobiose, potassium 2-ketogluconate and potassium 5-ketogluconate. The type strain exhibits activity of alkaline phosphatase, leucine arylamidase, acid phosphatase and naphtol-AS-BI-phosphohydrolase. The type strain does not exhibit activity of esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, cysteine arylamidase, trypsin, α-chymotrypsin, α- and β-galactosidase, β-glucuronidase, α- and β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The DNA G+C content of the type strain is 31.9 mol%.

The type strain LMG 28291^T (=DSM 28795^T) was isolated from the gut of a *B. terrestris* bumble bee in 2013 in the region of Ghent, Belgium. The accession numbers for the 16S rRNA gene and *pheS* sequences of *C. intestini* LMG 28291^T are LK054488 and LM999919, respectively.

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Compliance with Ethical Standards The present research involved sampling of bumble bees for which no permission was required as bumble bees are not included in the "Decree of Species (het Soortenbesluit (http://codex.vlaanderen.be/Zoeken/Document.aspx?DID=1018227&param=informatie])" of the Flemish government with inception on 01/09/2009. The authors do not have a conflict of interest.

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