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Bifidobacterium mellis sp. nov., isolated from the honey stomach of the honey bee *Apis mellifera*

Tobias C. Olofsson[1,2], Monica Modesto[3], Stefano Pascarelli[4], Donatella Scarafile[3], Paola Mattarelli[3,*] and Alejandra Vasquez[1,2]

Abstract

A novel *Bifidobacterium* strain, Bin7N^T, was isolated from the honey stomach of the honey bee *Apis mellifera*. Cells are Gram-positive, non-motile, non-sporulating, facultative anaerobic and fructose 6-phosphate phosphoketolase-positive. Their optimal growth is at 37 °C in anaerobiosis in MRS (De Man, Rogosa and Sharpe) added with cysteine. The honey bee microbiota was composed of several phylotypes of *Bifidobacterium* and *Lactobacillus*. Comparative analysis of 16S rRNA gene sequence similarity revealed that strain Bin7N^T grouped with *Bifidobacterium* species originating from honey bees and was closely related to *Bifidobacterium asteroides* DSM 20089^T (99.67% similarity). However, the highest average nucleotide identity and digital DNA–DNA hybridization values of 94.88 and 60.6%, respectively, were obtained with *Bifidobacterium choladohabitans* JCM 34586^T. The DNA G+C content of the type strain is 60.8mol%. The cell-wall peptidoglycan is of the A4β L-Orn–D-Asp type. The main cellular fatty acids of strain Bin7N^T are $C_{18:1}$ ω9*c*, $C_{16:0}$, $C_{18:1}$ ω7*c* and $C_{18:0}$. Phenotypic characterization and genotyping based on the genome sequences clearly show that this strain is distinct from the type strains of the so far recognized *Bifidobacterium* species. Thus, *Bifidobacterium mellis* sp. nov. (Bin7N^T=DSM 29108^T=CCUG 66113^T) is proposed as novel *Bifidobacterium* species.

The Western honey bee subspecies *Apis mellifera mellifera* [1] was named by Carl von Linné in 1758 at a time when it was free living in Europe. Today this subspecies is protected since it is threatened by extinction. A previous study on beneficial bacteria from honey bees described a symbiotic lactic acid bacterial (LAB) microbiota in the honey stomach of the Western honey bee *Apis mellifera* [1]. The honey stomach involved in the honey bee's food production is used for the collection of nectar and its transport to the hive. In both honey stomach, which plays a key role in the production of honey [1, 2], and bee bread [3], stored long-term, for both adult honey bees and larvae, a previously unknown microbiota composed of several phylotypes belonging to the genera *Lactobacillus* and *Bifidobacterium* was found [4]. This LAB microbiota is consistent across the native and introduced *A. mellifera* range [1, 5, 6] and it is present similarly in all recognized honey bee (Apini) species plus in stingless bee species (Meliponini) [2].

Many authors from different countries have found different *Bifidobacterium* phylotypes in the stomach of honey bees and their food. Besides its importance in the honey bee's food production and preservation, this highly co-evolved microbiota has shown a protective action against severe bee pathogens [2, 7] and bacteria present in nectar by producing active proteins [8]. Bifidobacteria are Gram-positive, anaerobic, non-spore- forming, lactate- and acetate-producing bacteria belonging to the class Actinobacteria. Together with lactobacilli, they are one of the core gut members of the honey bee [9]. At the time of writing, nine bifidobacterial species have been characterized from the guts of different bee species of the Apidae family; the most recently described honey bee species are *Bifidobacterium apousia*, *Bifidobacterium choladohabitans*, *Bifidobacterium mizhiense* and *Bifidobacterium polysaccharoliticum* [10, 11].

Author affiliations: [1]Department of Laboratory Medicine, Medical Microbiology, Lund University, Medicon Village, SE-223 81 Lund, Sweden; [2]ConCellae AB, Bårslövsvägen 3, 25373 Helsingborg, Sweden; [3]Department of Agricultural and Food Sciences, University of Bologna, Viale Fanin 42, 40127 Bologna, Italy; [4]Protein Engineering and Evolution Unit, Okinawa Institute of Science, Technology Graduate University, Okinawa, Onna, Japan.
***Correspondence:** Paola Mattarelli, paola.mattarelli@unibo.it
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Abbreviations: ANI, average nucleotide identity; dDDH, digital DNA–DNA hybridization.
The GenBank (EMBL) accession numbers for the 16S rRNA gene sequence and genome of *Bifidobacterium mellis* are JX099565 and JWMF01000000, respectively.
One supplementary figure and two supplementary tables are available with the online version of this article.

The isolation of novel strains may increase the knowledge of the presence and diversity of cultivable bifidobacteria in the honey bee stomach. It is well known that members of bifidobacteria are beneficial to their host. Furthermore, novel strains might show potential as beneficial bacteria for pollinator insects.

The aim of the present study was the identification of the novel bifidobacterial isolate, Bin7N^T, described in the work of Ellegaard *et al.*[12], based on a polyphasic taxonomic approach including 16S rRNA, whole genome sequencing and phenotypic analysis.

The genome of strain was decoded and described in Ellegaard *et al.*[12]. Using *in silico* analysis of the sequenced genome, G+C content has been determined to be 60.8 mol%, which is coherent with the average G+C content of *Bifidobacterium* species, i.e., 52–67% [13].

We investigated the phylogenetic relatedness of the strain with the other recognized bifidobacterial taxa by inferring the nucleotide sequences of 16S rRNA as well as the genes constituting the core genome of *Bifidobacterium* species.

The 16S rRNA gene sequence (1529 bp) of strain Bin7N^T and those of its closest relatives retrieved from the DDBJ/GenBank/EMBL databases were aligned using MAFFT [14] with default parameters. A phylogenetic tree based on a total of 109 16S rRNA gene sequences, including those of members of the genus *Bifidobacterium* described to date, was reconstructed with the neighbour-joining method [15] and the evolutionary distances were computed using the nucleotide model of Jukes and Cantor [13]. The tree was reconstructed using MEGAX [16] and rooted with *Scardovia inopinata* JCM 12537^T. The statistical reliability of the tree was evaluated by bootstrap analysis of 1000 replicates and the algorithm of Jukes and Cantor [17] was used. The tree was visualized using iTOL (https://itol.embl.de/; (Fig. S1, available in the online version of this article).

The tree topology was also confirmed with the maximum-likelihood method. A tree was reconstructed with IQ-TREE [18] by using the model GTR+F+I+G4 [15], with *Scardovia inopinata* JCM 12537^T as the outgroup. The statistical reliability of the tree was evaluated by bootstrap analysis of 1000 replicates. The tree was visualized using iTOL (Fig. 1).

Comparative analysis of the 16S rRNA gene sequences revealed that strain Bin7N^T was closely related to *Bifidobacterium asteroides* DSM 20089^T (99.67%). Similarity values were obtained using the EzBioCloud [19]. The maximum-likelihood analysis confirmed the phylogenetic relatedness of strain Bin7N^T with its closest neighbour (Fig. 1).

Strain Bin7N^T revealed a high 16S rRNA gene sequence similarity to *B. asteroides* DSM 20089^T. Indeed, the obtained value (99.67%) was higher than the suggested cut-off for species delineation, i.e. 98.7%. Therefore, the genetic similarity at genomic level of the isolate Bin7N^T with respect to its nearest neighbour and the other strains isolated from honey bees was evaluated based on average nucleotide identity (ANI) analysis, which was calculated by using JSpeciesWS: a web server for prokaryotic species circumscription based on pairwise genome comparison (http://jspecies.ribohost.com/jspeciesws; Table S1). Results revealed that strain Bin7N^T showed an ANI value of 94.88% with respect to *B. choladohabitans* JCM 34586^T. This value is in the range of the cut-off value for species delineation corresponding to 95–96% [20], however, due to the high 16S rRNA gene sequence identity and the ANI value obtained, digital DNA–DNA hybridization (dDDH) was also carried out using the Genome-to-Genome Distance Calculator (version 2.1), the most accurate known tool for calculating DDH-analogous values, developed at DSMZ and available at https://ggdc.dsmz.de/ggdc.php. The threshold value of ≤70% DNA–DNA is generally accepted for separated prokaryote species [20]. The value achieved with formula 2 for Bin7N^T with respect to *B. choladohabitans* JCM 34586^T was 60.6% (Table S1), below of the recommendations of a threshold value for the definition of bacterial species [21]. Based on these results, Bin7N^T was characterized further as representing a novel species.

In order to assess the genetic diversity of Bin7N^T as compared to the other currently recognized *Bifidobacterium* species, a phylogenetic tree based on the core genome of the genus *Bifidobacterium* was reconstructed. A phylogenomic analysis of 109 *Bifidobacterium* species, including 10 genomes from honey bee and *S. inopinata* JCM 12537^T as an outgroup, was performed using the PGAP [22]. The Cluster of Orthologous Genes (COGs) was obtained using the Pan Genome Analysis Toolkit [22]. The threshold used for COG detection was 0.5 sequence identity and 0.9 min coverage, resulting in 92 COGs. We aligned each single COG fasta file using MAFFT [14], and built a concatenated alignment. To reconstruct the phylogenetic tree, IQ-TREE [18], ModelFinder [23] and UFBoot [24] were used. The model for the maximum-likelihood tree reconstruction was LG+F+R8, the LG matrix [25], empirical rate of amino acid frequency, and free rate heterogeneity [25, 26].

The phylogenomic tree (Fig. 2) based on the concatenated amino acid sequences of 92 core genes confirmed the positioning of the strain Bin7N^T in the *B. asteroides* cluster, as observed in the phylogenetic analyses based on 16S rRNA gene sequences (Fig. 1).

Thus, these findings clearly supported the genetic diversity of strain Bin7N^T from any other currently recognized bifidobacterial species.

Cells were cultivated under anaerobic conditions in mMRS broth supplemented with 2% fructose and 0.1% L-cysteine at pH 6.9 and 35 °C, unless indicated otherwise. Morphological, cultural and biochemical characterization of the strains was performed at 35 °C unless otherwise stated.

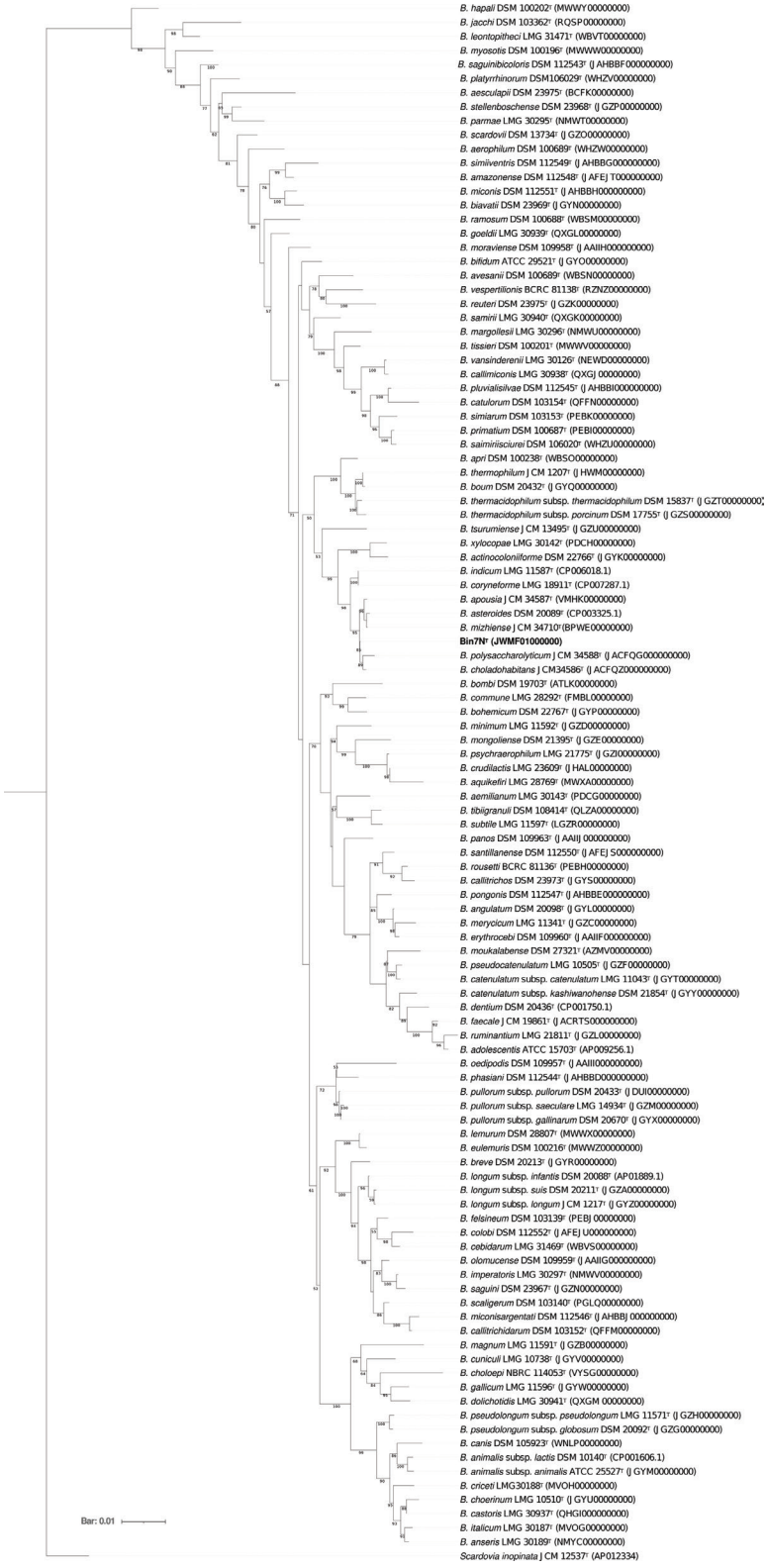

Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequence analyses, showing the relationship of the novel species to members of the genus *Bifidobacterium*. The 16S rRNA gene-based tree was reconstructed by the maximum-likelihood method. The sequence of *Scardovia inopinata* DSMZ 10107^T served as outgroup. Approximately 1397 nt from each sequence were used for the alignment. Bar: 0.01 substitutions per nucleotide position. Numbers indicate bootstrap values for branch points.

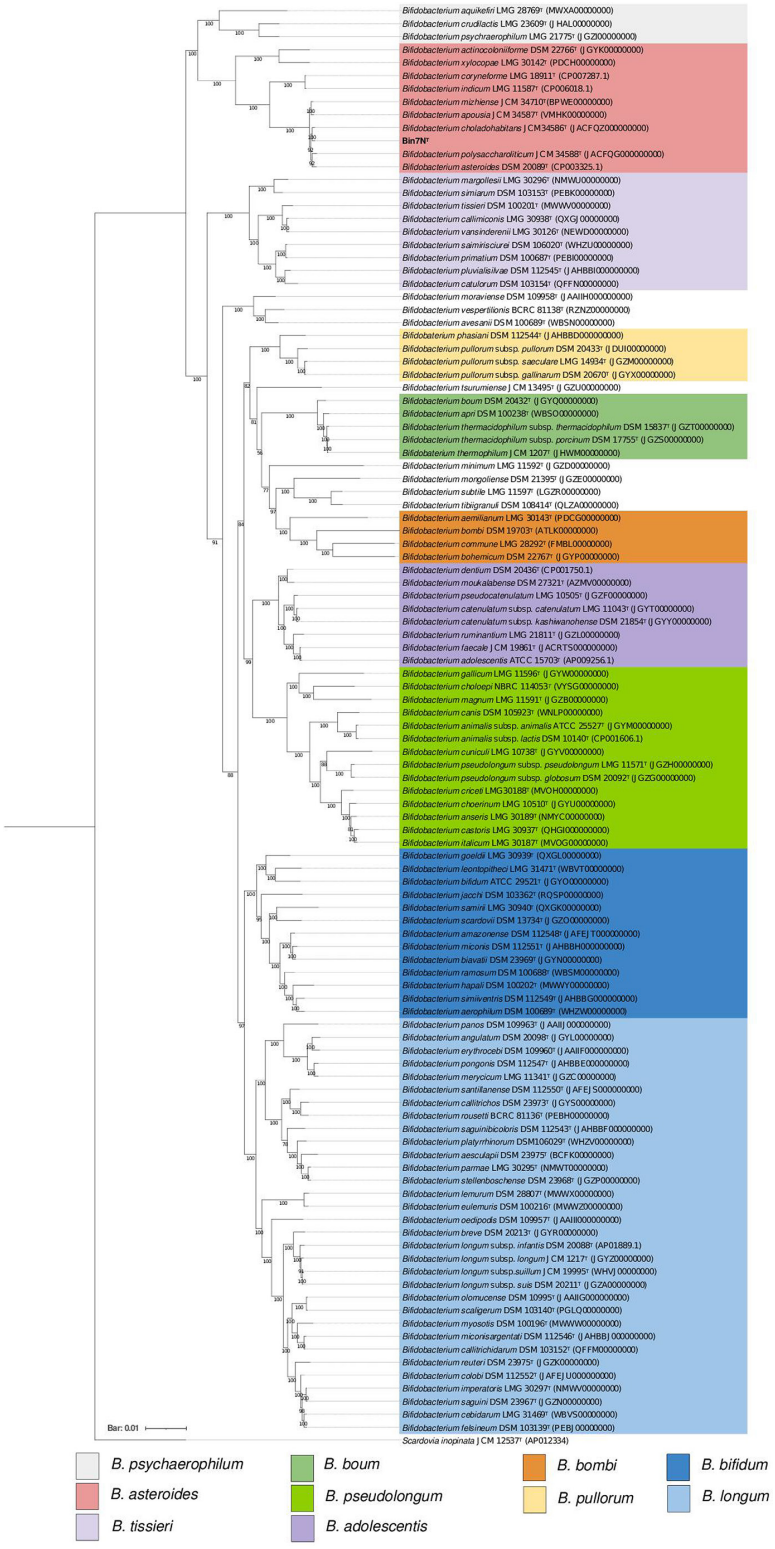

Fig. 2. Phylogenomic tree of the genus *Bifidobacterium* based on the concatenation of 92 core protein sequences from genomes of the novel strain Bin7N^T characterized in this study and the 108 type strains of the genus *Bifidobacterium*. Different colours show the division into 10 phylogenetic groups of which *B. asteroides* group comprise the novel strain (highlighted in bold). The phylogenetic tree was reconstructed by the maximum-likelihood method with the genome sequence of *Scardovia inopinata* DSMZ 10107^T as an outgroup. Bootstrap values above 50% are shown at node points, based on 1000 replications of the phylogenetic tree.

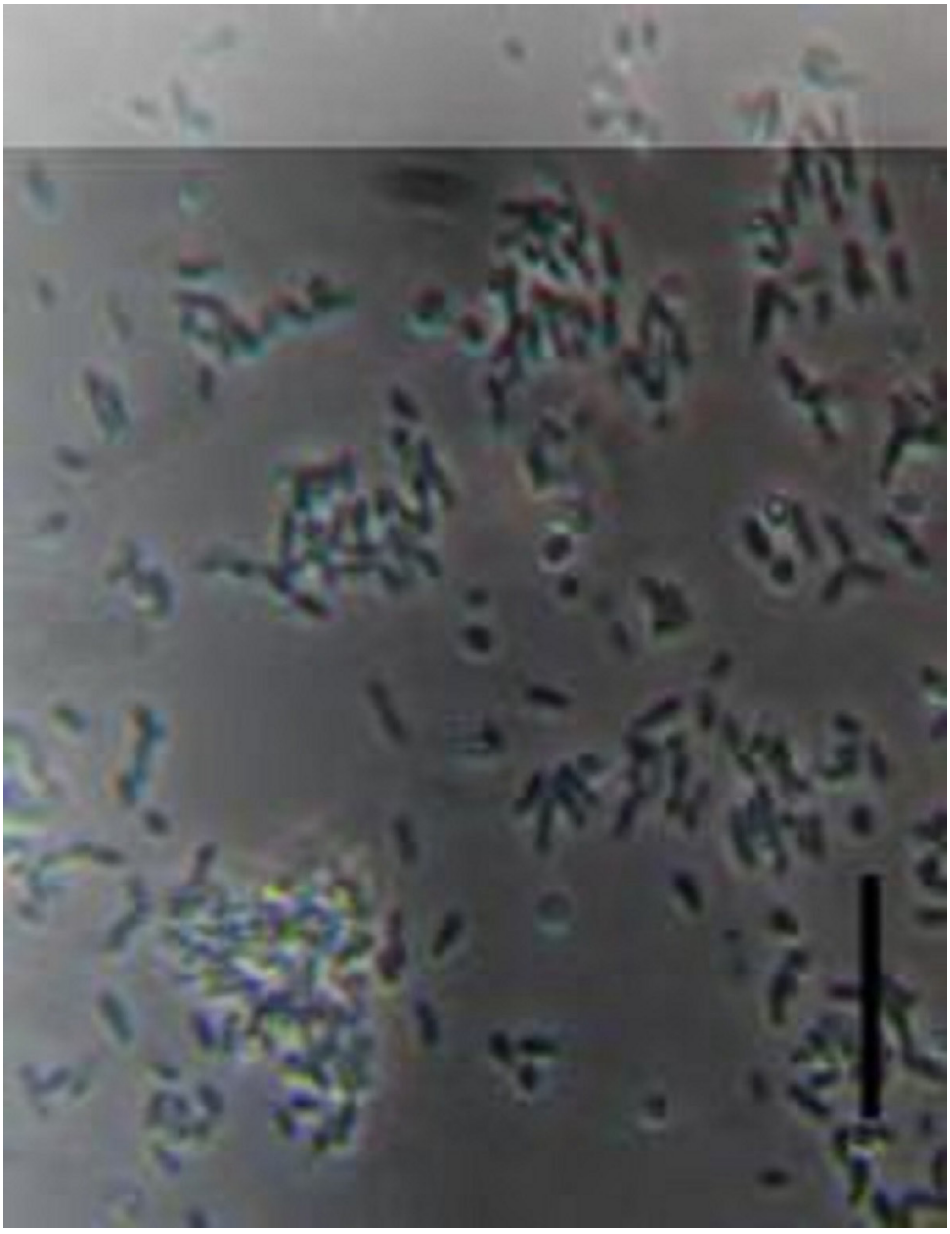

Fig. 3. Cell morphology of strain Bin7N^T. Cells of the strain were grown on mMRS agar plates for 48 h at 35 °C under anaerobic conditions. Phase-contrast photomicrograph. Bar, 10 mm.

Cell morphology and spore-forming ability of the isolated strain were examined by phase contrast microscopy [2]. Strain Bin7N^T showed rod-shaped cells occurring singly or in pairs (Fig. 3). Determination of Gram-stain reactions was performed using a Gram-staining kit (bioMérieux). Catalase activity was determined by transferring fresh colonies from mMRS agar to a glass slide and adding 5% H_2O_2 (bioMérieux). Growth at various pH values and temperatures was determined by adjusting mMRS broth with HCl and NaOH and cultivation of the bacteria on mMRS plates at various temperatures [27]. For Bin7N^T, growth conditions were examined in mMRS broth at pH 6 and at 35 °C.

The ability of the strain to grow under aerobic and microaerophilic conditions (CampyGen; Oxoid) was tested on mMRS plates after 48 h of incubation at 35 °C. It was found to be able to survive and grow in microaerophilic and in aerobic conditions.

Sugar fermentation patterns and aesculin hydrolysis were assessed using the API 50 CHL system (bioMérieux) in duplicate after 5 days incubation at 35 °C. Enzyme activities of the strain were measured using the API-ZYM strip (bioMérieux) as described by the manufacturer. The strain was grown on mMRS agar at 35 °C for 72 h prior to the test. The results are summarized in Table 1.

Table 1. Characteristics that differentiate Bin7N^T from closely related phylogenetic relatives

Strains: 1, Bin7N^T; 2, *Bifidobacterium choladohabitans* JCM 34586^T; 3, *Bifidobacterium asteroides* DSM 20089^T. Biochemical tests were performed using API CH50 and API ZYM systems (API bioMérieux). +, Positive reaction; w, weak positive reaction; –, negative reaction; ND, not determined.

Characteristic	1	2*	3
Production of acid from:			
Glycerol	w	–	–
D-Xylose	–	+	+
D-Galactose	–	+	w
D-Fructose	+	w	+
D-Mannose	+	+	w
D-Sorbitol	+	–	–
Methyl α-D-mannoside	ND	_	ND
Methyl α-D-glucoside	w	+	–
Amygdalin	+	+	–
Arbutin	+	+	–
Cellobiose	+	+	+
Maltose	+	+	w
Lactose	+	w	–
Sucrose	+	w	+
Trehalose	+	–	–
Melezitose	+	–	–
Gentiobiose	+	+	–
Turanose	w	–	–
Gluconate	w	–	+
Enzyme activities:			
Esterase (C4)	+	ND	–
Valine arylamidase	w	ND	+
Cysteine arylamidase	+	ND	w
Acid phosphatase	w	ND	+
α-Galactosidase	+	ND	–
α-Mannosidase	+	ND	w
α-Fucosidase	w	+	–

*Data from [10].

Analysis of cellular fatty acids was carried out by DSMZ according to previously described methods [28–30]. Cellular fatty acids composition showed the presence of major components of $C_{18:1}$ ω9*c*, $C_{16:0}$ and $C_{18:1}$ ω7*c* (Table S2).

Peptidoglycan analysis was performed following the protocol of Schumann [31]. The cell-wall peptidoglycan was of the A4*β* L-Orn–D-Asp type.

On the basis of the phenotypic and chemotaxonomic characterization as well as molecular-based methods, phylogenetic analysis based on 16S rRNA gene sequences, and phylogenomic analysis based on the concatenated core genome sequences, strain Bin7N^T was genetically and phenotypically discernible from the currently recognized species of bifidobacteria; thus, according to minimal standard guidelines [32] it represents a novel taxon for which the name *Bifidobacterium mellis* sp. nov. is proposed.

DESCRIPTION OF *BIFIDOBACTERIUM MELLIS* SP. NOV.

Bifidobacterium mellis (mel'lis. L. gen. n. *mellis* of honey).

Cells are Gram-positive, non-motile, non-spore-forming, catalase-positive rods (1.25×2.0–3.0 mm) that occur singly or in pairs. After anaerobic growth on supplemented mMRS agar (0.1% L-cysteine and 2.0% fructose) at 35 °C for 72 h, colonies appear white, translucent, with a smooth to rough surface, circular, with a convex elevation, moist and punctiform, with a diameter of approximately 2–3 mm. Cells are facultatively anaerobic and grow well on mMRS agar under microaerophilic and aerobic conditions. On mMRS agar, growth occurs at 15–50 °C, with an optimum at 35 °C. Growth occurs in mMRS broth at pH 3.0–8.0, with an optimum at pH 6.0. Results from API 50CH and API ZYM tests show production of acid from L- arabinose, ribose, glucose, fructose, mannose, sorbitol, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, melezitose, raffinose and gentibiose, and weak production from glycerol, methyl α-glucoside, turanose and gluconate. Aesculin is hydrolysed. Enzyme activities are shown for esterase (C4), leucine arylamindase, cysteine arylamidase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, *β*-galactosidase, α-glucosidase, *β*-glucosidase and α-mannosidase, and weak activity for valine arylamidase, acid phosphatase and α-fucosidase.

The major fatty acids detected are $C_{18:1}$ ω9*c*, $C_{16:0}$ and $C_{18:1}$ ω7*c*. The cell-wall peptidoglycan is of the A4*β* L-Orn–D-Asp type.

The type strain, Bin7N^T (= DSM 29108^T=CCUG 66113^T), was isolated from the honey stomach of the honey bee *A. mellifera*. The DNA G+C content of the type strain is 60.8%.

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Conflicts of interest
A.V. and T.C.O. are the founders of and hold stocks in ConCellae AB, a university spin-off company that develops and markets products for humans and bees. A.V. and T.C.O. are inventors of three patent applications related to the applications of *Lactobacillus* and *Bifidobacterium* strains isolated from honey bees. However, the present study was not funded by ConCellae AB.

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