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Characterization of *Bifidobacterium* species in feaces of the Egyptian fruit bat: Description of *B. vespertilionis* sp. nov. and *B. rousetti* sp. nov.

Running title: Description of *B. vespertilionis* sp. nov. and *B. rousetti* sp. nov. from *Rousettus aegyptiacus*

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The GenBank accession number for the 16S rRNA partial gene sequence for the novel isolated strains RST 7, RST 8, RST 9^T, RST 11, RST 16^T, RST 17, RST 19, RST 27 are MK722390, MK722393, MK722392, MK722391, MK722394, MK722395, MK722396, MK722397, respectively.

The GenBank accession numbers for the genomes of the novel isolated strains RST 7, RST 8, RST 9^T, RST 11, RST 16^T, RST 17, RST 19, RST 27 are RZUI00000000, RZOA00000000, PEBH00000000, RZUJ00000000, RZNZ00000000, RZUH00000000, RZUG00000000, RZJP00000000, respectively.

The deposit accession numbers for the isolated strains are the following: RST 7 (BCRC 81134, NBRC 113376, DSM 106022), RST 8 (BCRC 81135, NBRC 113377); RST 11 (BCRC 81136, NBRC 113379, DSM 106024); RST 17 (BCRC 81139, NBRC 113381, DSM 106026); RST 19 (BCRC 81140, NBRC 113382, DSM 106027); RST 27 (BCRC 81141, NBRC 113383, DSM 106028)

Fifteen bifidobacterial strains were obtained from faeces of *Rousettus aegyptiacus;* after grouping them by RAPD PCR only eight were selected and characterized. Analysis of 16S rRNA and of five housekeeping (*hsp*60, *rpo*B, *clp*C, *dna*J, *dna* G) genes revealed that these eight strains were classified into five clusters: Cluster I (RST 8 and RST 16^T), Cluster II (RST 9^T and RST 27), Cluster III (RST 7 and RST 11), Cluster IV (RST 19), Cluster V (RST 17) were closest to *Bifidobacterium avesanii* DSM 100685^T (96.3%), *Bifidobacterium callitrichos* DSM 23973^T (99.2% and 99.7%), *Bifidobacterium tissieri* DSM 100201^T (99.7 and 99.2%), *Bifidobacterium reuteri* DSM 23975^T (98.9%) and *Bifidobacterium myosotis* DSM 100196^T (99.3%), respectively. Strains in Cluster I and strain RST 9 in Cluster II could not be placed within any recognized species while the other ones were identified as known species. The average nucleotide identity values between two novel strains, RST 16^T and RST 9^T and their closest relatives were lower than 79% and 89%, respectively. *In silico* DNA–DNA hybridization values for those closest relatives were 32.5 and 42.1%, respectively. Phenotypic and genotypic tests demonstrated that strains in Cluster I and RST 9^T in Cluster II represent two novel species for which the names *Bifidobacterium vespertilionis* sp. nov. (RST 16^T =BCRC 81138^T =NBRC 113380^T =DSM 106025^T ; RST 8=BCRC 81135=NBRC 113377) and *Bifidobacterium rousetti* sp. nov. (RST 9^T =BCRC 81136^T=NBRC 113378^T =DSM 106027^T) are proposed.

Keywords: New species, *Bifidobacterium*, *Bifidobacterium vespertilionis* sp. nov., *Bifidobacterium rousetti* sp. nov., *Rousettus aegyptiacus*

Introduction

Of all living mammals, bats are extraordinary and unique given their peculiar adaptations [28]. They are the only mammals that can perform real active flight, which is essential for their biology [14] and this characteristic gave the bat order its scientific name – *Chiroptera*, or hand-wing in Greek [11] [8]. They have evolved to thrive in diverse ecological niches across the globe [28] and can feed on insects, small mammals, fish, blood, nectar, fruit, and pollen [27].

Bats perform key ecosystem services, pollinating crop species in the tropics (e.g., bats pollinate the flowers of agave, making possible the production of tequila) as well as dispersing seeds [1,14]. [1,28]. Bats are able to migrate over long distances, creating opportunities for diverse exposure and widespread dissemination of microbes [29].

The Egyptian fruit-bat *Rousettus aegyptiacus* (body mass: 140–160 g) is widely distributed in tropical and subtropical regions of Africa, and Asia. Its northernmost distribution reaches southern Turkey. This bat consumes a variety of wild and cultivated fruits such as *Ficus retusa* and *F. sycomorus* (fig), *Melia azedarach* (chinaberry), *Phoenix dactylifera* (date), mango and banana and therefore is a potential seed disperser [13] 5].

From the anatomical standpoint, *Rousettus aegyptiacus* has a relatively short intestine, not differentiated into small and large parts and with no observed cecum or appendix; consequently, the duration of the intestinal pass is approximately 40 min [11,12].

Studies on the microbial ecology of gut microbiota in bats are limited and such information is necessary in determining the ecological significance of these hosts [11].

There are relatively fewer studies investigating the bacterial microbiota in bats with respect to dietary habits of bats. Except a few [11,12], majority of the studies have used culture based approach to study the bacterial communities associated with the bat intestinal tract. These have led to the identification of different bacteria such as *Salmonella*, *Shigella*, *Enterobacter*, *Yersinia* and

many other enteric pathogens from the bat digestive tract. Different sources used for bacterial isolation and identification includes urine, guano and intestinal content or intestine [1][7][11].

At the time of writing, there is no study investigating the presence and the distribution of *Bifidobacterium* species in fruit bats. Several studies have suggested the importance of isolating and identifying novel strains of the genus *Bifidobacterium* from various animals, including humans, in order to understand how they are mostly distributed [17,19] and, especially, which are their phenotypic and genotypic characteristics, thus allowing the reconstruction of a more robust bifidobacterial phylogeny.

Therefore, the objective of this study was the characterization of bifidobacterial isolates derived from the faeces of Egyptian fruit bat (*Rousettus aegyptiacus).*

In March 2017, fresh guanos from the captive colony of the Egyptian fruit bat housed under semi-natural conditions in Parco Natura Viva - Garda Zoological Park (Bussolengo, Verona, Italy), were collected from the ground using a sterile spoon by the animal-care staffs (keepers) during their routine cleaning of the enclosure, put into a sterile plastic tube and stored under anaerobic conditions in an anaerobic jar (Merck) at 4 °C, and were taken promptly to the laboratory (within 2 h). The anaerobic atmosphere was obtained using the GasPak EZ Anaerobic Pouch system (BD).

Animals were free from intestinal infections and did not receive antibiotics or probiotics for two months before samples were collected. Their diet consisted mostly of fruit.

For isolation and enumeration of bifidobacteria, aliquots of approximately 1–2 g of faecal sample were serially 10-fold diluted with Peptone Water (Merck) supplemented with L-cysteine hydrochloride (0.5 g/L). Aliquots of 1 ml from each dilution (from 10^{-1} down to 10^{-9}) were inoculated onto MRS (Difco) agar supplemented with L-cysteine hydrochloride (0.5 g/L), acetic acid (1 ml/L) (Merck) and mupirocin (100 mg/L) (Applichem) [21]. Plates were incubated in anaerobic conditions, at 37 °C for 48–72 hours. The anaerobic atmosphere was obtained using the GasPak EZ Anaerobic Pouch system. After incubation, results showed the presence of

bifidobacteria, with a colony count number of 4.6 $\log_{10}$ CFU g^{-1} faeces. Therefore, morphologically different colonies were randomly picked and re-streaked for several generations in order to isolate purified individual bacterial strains.

Isolates were suspended in a 10% (w/v) sterile skim milk solution, supplied with lactose (3%) and yeast extract (0.3%), and kept both freeze dried and frozen at –120 °C until further analysis.

A total of 15 strains were obtained, namely: RST 1, RST 2, RST 3, RST 4, RST 5, RST 7, RST 8, RST 9, RST 11, RST 12, RST 16, RST 17, RST 19, RST 26 and RST 27. For discrimination of the isolates, RAPD-PCR fingerprintings were carried out by using BOXA1R primer (5′-CTACGGCAAGGCGACGCTGACG-3′), ERIC1 (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) primers, as previously described [18,19]. Based on the resulting BOX-PCR and ERIC banding profiles, the isolates were categorized into six groups: Group I (RST 1, RST 2, RST 3, RST 4, RST 5, RST 8, RST 12, RST 16 and RST 26), Group II (RST9^T), Group III (RST 7 and RST 11), Group IV (RST 19), Group V (RST 17) and Group VI (RST 27) (Supplementary Figs. S1, S2). One or wherever possible two representative strains for each group were selected and a total of eight strains were chosen (RST 8 and RST 16^T for Group I, RST 9^T for Group II, RST 7 and RST 11 for Group III, RST 19 for Group IV, RST 17 for Group V and RST 27 for Group VI) for 16S rRNA gene sequencing as described by Michelini *et al*. [16] .

The 16S rRNA gene sequences (1400 bp) of strains and of their closest relatives retrieved from the DDBJ/GenBank/EMBL databases were aligned using CLUSTAL Omega in a CLC Sequence Viewer (1328 nt). A phylogenetic tree based on a total of 81 partial 16S rRNA gene sequences, including those of members of the genus *Bifidobacterium* was reconstructed with the maximum-likelihood method [3] and the evolutionary distances were computed by nucleotide model of GTR CAT. The tree was constructed using RaxML version 8.2.7 [25] and rooted with *Scardovia inopinata* JCM 12537^T. The statistical reliability of the tree was evaluated by the bootstrap analysis

of 1000 replicates with the algorithm Bootstrap rapid hill climbing algorithm. The tree was visualized using FigTree [22].

Comparative analysis of the 16S rRNA gene sequence revealed that these eight strains were classified into five clusters: Cluster I (RST 8, RST 16^T), Cluster II (RST 9^T and RST 27), Cluster III (RST 7, RST 11), Cluster IV (RST 19), Cluster V (RST 17). Strains in Cluster I (RST 8, RST 16^T) were the closest to *Bifidobacterium avesanii* (96.30 % similarity), strains in Cluster II (RST 9^T and RST 27) were closest to *Bifidobacterium callitrichos* DSM 23973^T (99.2 % and 99.7%, respectively), strains in Cluster III (RST 7 and RST 11) were closest to *Bifidobacterium tissieri* DSM 100201^T (99.7 % and 99.2 %), strain in Cluster IV (RST 19) was closest to *Bifidobacterium reuteri* DSM 23975^T (98.9 %), strain in Cluster V (RST 17) was closest to *Bifidobacterium myosotis* DSM 100196^T (99.3 %) (Fig. 1).

In order to assess the genetic diversity of the eight strains as compared to the other currently recognized *Bifidobacterium* species, multilocus sequence analysis (MLSA) based on the concatenated five housekeeping genes (*hsp*60, *rpo*B, *clp*C, *dna*J, *dna*G) was carried out. Thus, the phylogenetic location of the novel strains was verified by the analysis of genes which have proven to be discriminative for the classification of the genus *Bifidobacterium* [10,30].

For this purpose, a phylogenetic tree for 79 bifidobacterial type strains was constructed by joining the five coding sequences in the following order: *hsp*60 (662 bp), *rpo*B (500 bp), *clp*C (720 bp), *dna*J (477 bp) and *dna*G (992 bp). The resulting in-frame concatenated gene sequences (3154 bp) were aligned with the MAFFT program at CBRC (http://mafft.cbrc.jp/alignment/software/) [9]. The evolutionary distances were computed by nucleotide model GTR CAT, and the phylogenetic tree was constructed by RaxML (version 8.2.7, maximum-likelihood method) [25] with *Scardovia inopinata* JCM 12537^T as an outgroup. The statistical reliability of the tree was evaluated by bootstrap analysis (rapid hill climbing) of 1000 replicates. The visualization was performed with FigTree.

The MLSA analysis confirmed the close phylogenetic relatedness with the nearest neighbours of all the strains (Fig. 2).

Furthermore, the level of similarity for the partial housekeeping gene sequences of strains in relation to the type strains of their closest phylogenetic relatives was calculated using EMBOSS Water web-based program (https://www.ebi.ac.uk/Tools/psa/emboss_water/nucleotide.html): the values of similarity for the *hsp*60, *rpo*B, *clp*C, *dna*J and *dna*G gene sequences were calculated and reported in Supplementary Table S1.

The genome of all 8 strains was decoded through a next generation sequencing (NGS) approach, using a MiSeq platform (Illumina) at BioFab Research (Roma, Italy). The generated data were depleted of low quality reads using FASTQ/A Trimmer in FASTX-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), assembled by SPADES version 3.13.0 [29] and annotated through DFAST program [26].

The draft genome size of strains RST 8 and RST 16^T (Cluster I), RST 9^T and RST 27 (Cluster II), RST 7 and RST 11 (Cluster III), RST 19 (Cluster IV) and RST 17 (Cluster V) ranged between 2.80 and 3.28 Mbp as indicated in Table 1 together with their other genomic features. In *silico* analysis of all the sequenced genomes allowed the estimation of their G + C contents, which ranged from 60.39 to 64.55 mol%, falling in the range indicated for the genus *Bifidobacterium*, i.e., 52–67 mol%. The project outline has been submitted to the BioProject PRJNA415181 and the GenBank accession numbers were reported in Table 1.

In order to reconfirm the above phylogenetic analysis, we also constructed the phylogenetic tree based on the core genome of *Bifidobacterium* spp. Total 76 type strains of *Bifidobacterium* were annotated with the DFAST program [26], and 355 orthologous genes were identified as the core retained in all genomes. The amino acid sequences of core genes from each genome were

concatenated and aligned using the MAFFT program (version 7.313) [9]. The alignments were trimmed using trimAl with -automated1 option [2].

The phylogenomic tree (Fig. 3) based on the core genome (355 genes) confirmed the positioning of the eight isolated strains within the genus *Bifidobacterium* as observed in the phylogenetic analyses based on 16S rRNA and housekeeping gene sequences (Figs. 1 and 2).

In addition, the genetic similarity at genomic level of RST 16^T and RST 8 (Cluster I), RST 9 ^{T} and RST 27 (Cluster II), RST 7 and RST 11 (Cluster III), RST 19 (Cluster IV) and RST 17 (Cluster V) isolates with respect the other currently recognized bifidobacterial species was evaluated based on average nucleotide identity (ANI) analysis by using the web server JSpeciesWS (http://jspecies.ribohost.com/jspeciesws/). This analysis (Supplementary Table S2) showed that the highest sequence identity value between RST 16^T and RST 8 was 78.66 % and 78.49 % when compared to the chromosome sequence of *Bifidobacterium reuteri* DSM 23975^T, the highest sequence identity value between RST 9^T and RST 27 when compared to *Bifidobacterium callitrichos* DSM 23973^T were 89.43 % and 96.06 % respectively, the highest sequence identity value between RST 7 and RST 11 was 95.10% and 94.78 % when compared to *Bifidobacterium tissieri* DSM 100201^T, the highest sequence identity value between RST 19 was 96.94 % when compared to *Bifidobacterium reuteri* DSM 23975^T and that of RST 17 was 95.98 % when compared to *Bifidobacterium myosotis* DSM 100196^T.

ANI with closest neighbours supported an independent phylogenetic position, i.e. ANI value of ≤ 95% for strains in Clusters I (RST 16^T and RST 8) and in Cluster II (RST 9^T). Therefore, strain RST 16^T and strain RST 9^T were selected as the type strains of the two novel species. However, RST 9^T showed high sequence similarity with *B. callitrichos* DSM 23973^T for their 16S rRNA gene sequences.

To verify their low ANI values, an *in silico* DDH (isDDH) was also carried out using Genome-to-Genome Distance Calculator (GGDC) version 2.1 formula 2 [15], the most accurate known tool for calculating DDH-analogous values, developed at DSMZ and available at www.ggdc.dsmz.de. The

threshold value of ≤ 70% is generally accepted for separated prokaryote species [46]. When comparing strains RST 16^T and RST 9^T with their nereast neighbours, the values achieved were 32.5 and 42.1%, respectively. The analysis was also performed among the 8 isolated strains and obtained results were in the range of 65.2 –79.5% (Supplementary Table S3).

Evaluation of the above phylogenetic relationships of RST 16^T and RST 8, RST 9^T, RST 7 and RST 11, RST 19, RST 17 and RST 27 isolates with respect to other (sub)species clearly recognized two putative novel species, namely RST 16^T and RST 9^T.

Furthermore, this work describe the morphological, biochemical and molecular characterizations of the remaining strains placed on Clusters II, III, IV and V belonging to the known species *B. callithricos*, *B. tissieri*, *B. myosotis* and *B. reuteri*, respectively, all of which have been described as isolates from *Rousettus aegyptiacus*.

The eight strains, selected as representatives from identified clusters, showed rod-shaped cells, frequently forming filaments, with irregular contractions along the cells and bifurcations. They were cultivated under anaerobic conditions and maintained in TPY broth [24] pH 6.9, at 37 °C, unless indicated otherwise.

Morphological, cultural and biochemical characterization of the strains were performed at 37 °C unless otherwise stated, as described previously [18].

The morphology of cells of strains RST 16^T and RST 9^T, as revealed by phase-contrast microscopy, is shown in Fig. 4.

Optimal growth conditions of the strains were determined in TPY broth after 24 h of incubation at 37 °C in anaerobic condition. Growth at 22, 25, 30, 35, 37, 40, 42, 45, 48 °C was tested. Sensitivity to low pH was screened at 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5 values of pH. The ability of

the strains to grow under aerobic and microaerophilic conditions (CampyGen; Oxoid) was also verified in TPY broth after 48 h of incubation at 37 °C. Particularly, for strains RST 16^T, RST 8 and RST 9^T best growth conditions were obtained in TPY broth pH 7 at 37 °C and they were able to survive and grow in microaerophilic and in aerobic conditions. All results are showed in Table 2. Haemolytic activity was determined in Columbia blood agar (Biolife) at 37 °C under anaerobic conditions for 48 h [27].

Gram staining, motility assay, catalase and oxidase activities were performed as described previously [24].

Strains RST 16^T and RST 8, RST 9^T, RST 7 and RST 11, RST 19, RST 17 and RST 27 and related species *B. avesanii* DSM 100685^T, *B. reuteri* DSM 23975^T, *B. myosotis* 100196^T, *B. tissieri* DSM 100201^T, and *B. callitrichos* DSM 23973^T were also investigated for substrates utilization and enzymes production with API 50 CHL and Rapid ID 32 A test kits (BioMérieux). Results are summarized in Table 2.

Bifidobacteria and members of related genera possess fructose-6-phosphate phosphoketolase (F6PPK), the enzyme degrading hexose via the F6PPK pathway, which is considered a taxonomic marker for identification of *Bifidobacterium* and related genera [15]. Detection of F6PPK activity was carried out according to the method described by Orban & Patterson [26]. All studied strains possessed the F6PPK activity.

For analysis of amino acid composition, the cell-wall peptidoglycan of strains RST 16^T and RST 9^T was prepared and hydrolysed as described previously [6]. Cell-wall amino acids were analysed by HPLC (model LC-20AB; Shimadzu) equipped with a Wakopak wakosil-PTC column (200×4.0 mm i.d.; Wako Pure Chemical Industries), as their phenyl isothiocyanate derivatives (Wako). Amino acid isomers in the cell-wall hydrolysate were analysed as described previously using a liquid chromatograph-mass spectrometer (model LCMS-2020 and LC-20AB; Shimadzu) equipped with a Shim-Pack FC-ODS column (150 × 2.0 mm i.d.; Shimadzu) [20]. The peptidoglycan of strain

RST16^T contained glutamic acid (Glu), serine (Ser), alanine (Ala) and ornithine (Orn) in a molar ratio of 4:1:2:1. Enantiomeric analysis of the peptidoglycan amino acids revealed the presence of L-Orn-L-Glu3-D-Ser-Glu3.

The peptidoglycan of strain RST 9^T contained glutamic acid (Glu), alanine (Ala) ornithine (Orn) and lysine (Lys) in a molar ratio of 2:2:1:1. Enantiomeric analysis of the peptidoglycan amino acids revealed the presence of L-Orn(L-Lys)-D-Glu.

Whole-cell fatty acids were analysed as fatty acid methyl esters (FAMEs) with Sherlock phospholipid fatty acid software (midi). The cultures of all studied strains were incubated on MRS agar added with 0.05% cysteine plates at 37 °C for 48 h under anaerobic conditions. FAMEs were extracted and prepared as described previously [23]. Results are shown in Table 3.

On the basis of the phenotypic and chemotaxonomic characterization as well as the molecular-based methods phylogenetic analysis based on the 16S rRNA gene sequences, MLSA based on the concatenated five housekeeping gene sequences, and the whole-genome-based sequence comparisons *in silico*, strains RST 16^T and RST 9^T were genetically and phenotypically discernible from the currently recognized species of bifidobacteria; thus, according to Minimal Standard guidelines [16], they represent two novel taxa for which the name *Bifidobacterium vespertilionis* sp. nov. and *Bifidobacterium rousetti* sp. nov. are proposed.

Description of *Bifidobacterium vespertilionis* sp. nov.

Bifidobacterium vespertilionis (ve.sper.ti.lio'nis. L. gen. n. *vespertilionis* of a bat).

Cells are Gram-positive-staining, non-motile, asporogenous, non-haemolytic, F6PPK-positive, catalase- and oxidase-negative, indole-negative, and when growing in TPY broth are rods of various shapes forming a branched structure with 'Y' at both sides. The well isolated colonies grown on the

surface of TPY agar under anaerobic conditions are white, opaque, smooth and circular with entire edges, while the embedded colonies are lens-shaped or elliptical. Colonies reach 1.0–2.0 mm in diameter after 3 days of incubation. Cells can grow in the range 22–48°C. Cells grow at pH 4.0–7.5. Optimal conditions of growth occur at pH 7 and 37 °C. Using API 50 CHL system acids are produced from D-glucose, L-arabinose, lactose, D-fructose, D-mannitol, D-mannose, maltose, sucrose, raffinose, turanose, D-xylose, gluconic acid, and produced weakly from D-galactose, D-mannose, Methyl-α-D-glucopyranoside, starch, 5-ketogluconate but not from other carbohydrates. Activity was observed for α- and β-galactosidase, α-glucosidase, α-arabinosidase, *N*-acetyl-β-glucosaminidase, alkaline phosphatase, arginine arylamidase, proline arylamidase, leucyl glycine arylamidase, phenylalanine arylamidase, leucine arylamidase, pyroglutamic acid arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, serine arylamidase. Activity was also observed weakly for arginine dihydrolase, β-glucosidase, glutamyl glutamic acid arylamidase. Aesculine is not hydrolysed. No reduction of nitrates was recognized. Cells are positive for urease. The peptidoglycan type is L-Orn-L-Glu3-D-Ser.

The type strain RST 16^T (= BCRC 81138^T = NBRC 113380^T = DSM 106025^T) was isolated from the faeces of the Egyptian fruit bat *Rousettus aegyptiacus*. The DNA G + C content of the type strain is 64.20 mol%.

The taxonumber of digital protologue is TA00874.

Description of *Bifidobacterium rousetti* sp. nov.

Bifidobacterium rousetti (rou.set'ti. N.L. gen. n. *rousetti* of *Rousettus aegyptiacus*, the Egyptian fruit bat).

Cells are Gram-positive-staining, non-motile, asporogenous, non-haemolytic, F6PPK-positive, catalase- and oxidase-negative, indole-negative, and when growing in TPY broth are rods of various shapes forming a branched structure with 'Y' at both sides. The well isolated colonies grown on the

surface of TPY agar under anaerobic conditions are white, opaque, smooth and circular with entire edges, while the embedded colonies are lens-shaped or elliptical. Colonies reach 1.0–2.0 mm in diameter after 3 days of incubation. Cells can grow in the range 22–48 °C. Cells grow at pH 4.0–7.5. Optimal conditions of growth occur at pH 7 and 37 °C. Using API 50 CHL system acids are produced from D-glucose, L-arabinose, D-fructose, D-mannitol, D-mannose, raffinose, turanose, D-galactose, sorbitol, gluconic acid and produced weakly from D-ribose, maltose, lactose, starch and 5-ketogluconate but not from other carbohydrates. Activity was observed for α- and *β*-galactosidase, *α*-glucosidase, *α*-arabinosidase, glutamic acid decarboxylase, arginine arylamidase, proline arylamidase, phenylalanine arylamidase, leucine arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, serine arylamidase. Activity was also observed weakly for L-arginine dihydrolase, *β*-glucosidase. Aesculine is hydrolysed. No reduction of nitrates was recognized. Cells are positive for urease. The peptidoglycan type is L-Orn(L-Lys)-D-Glu.

The type strain RST 9^T (=BCRC 81136^T = NBRC 113378^T = DSM 106027^T) was isolated from the faeces of the Egyptian fruit bat *Rousettus egyptiacus*. The DNA G + C content of the type strain is 64.55 mol%.

The taxonumber of digital protologue is TA00875.

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Captions

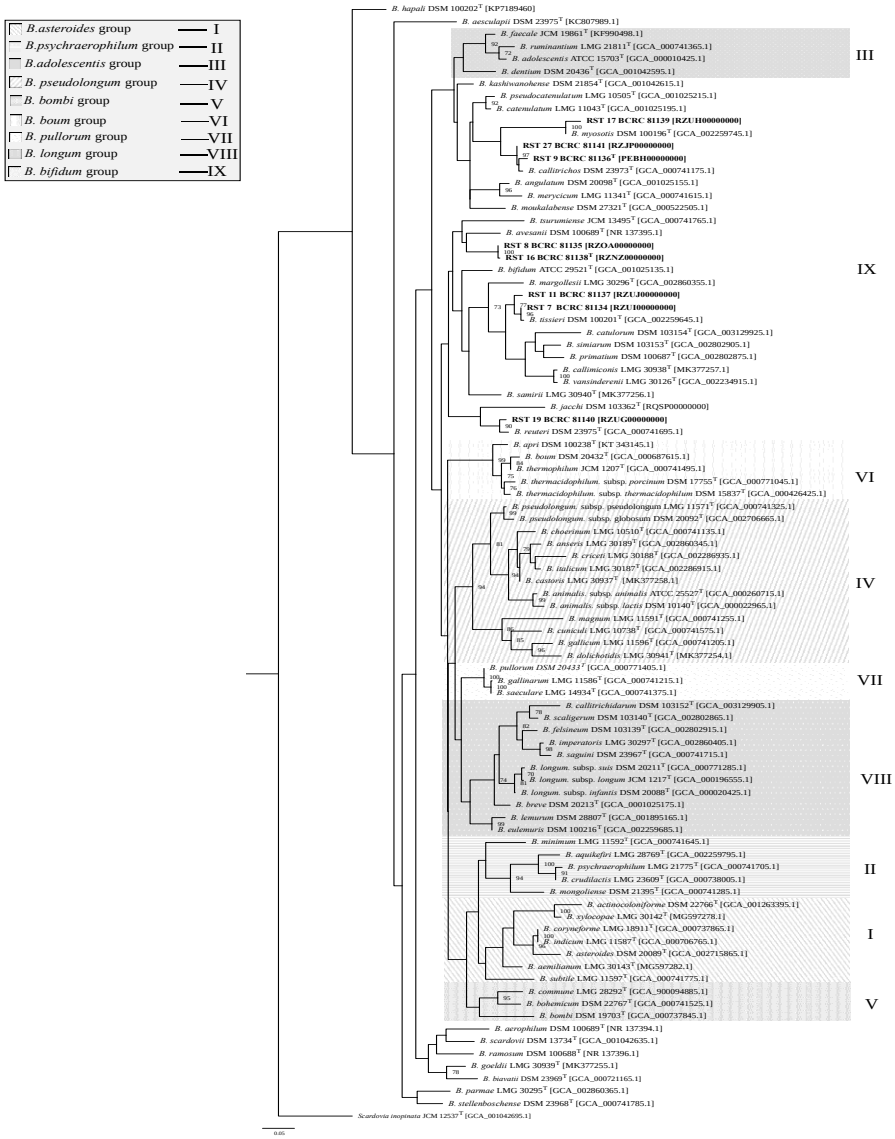

Figure 1. Phylogenetic tree of the *Bifidobacterium* genus based on 16S rRNA gene sequences, showing the relationship between strains isolated from Egyptian fruit bats and other members of the *Bifidobacterium* genus. The 16S rRNA gene-based tree was constructed by the maximum likelihood method; the corresponding sequence of *Scardovia inopinata* JCM 12537^{T} was used as outgroup. Bootstrap percentages above 70 are shown at node points, based on 1000 replicates of the phylogenetic tree.

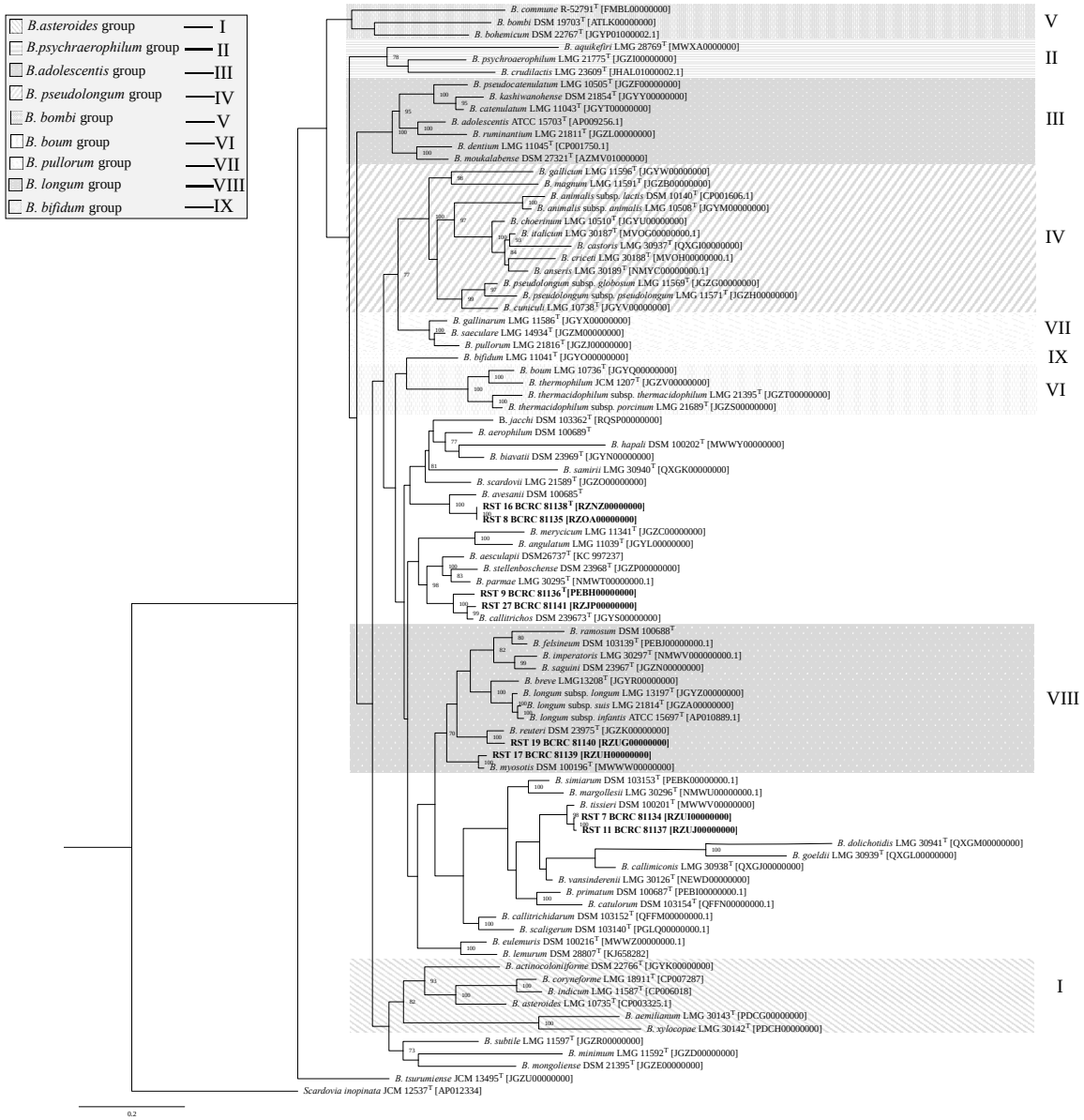

Figure 2. Phylogenetic three based on the concatenation of proteins sequences deduced from the housekeeping genes *hsp*60, *rpo*B, *clp*C, *dna*J *and dna*G, showing the phylogenetic relationships between strains isolated from Egyptian fruit bats and members of the *Bifidobacterium* genus. The housekeeping gene-based tree was constructed by the maximum likelihood method, with corresponding sequences of *Scardovia inopinata* JCM 12537^{T} being used as outgroup. Bootstrap percentages above 70 are shown at node points, based on 1000 replicates of the phylogenetic tree.

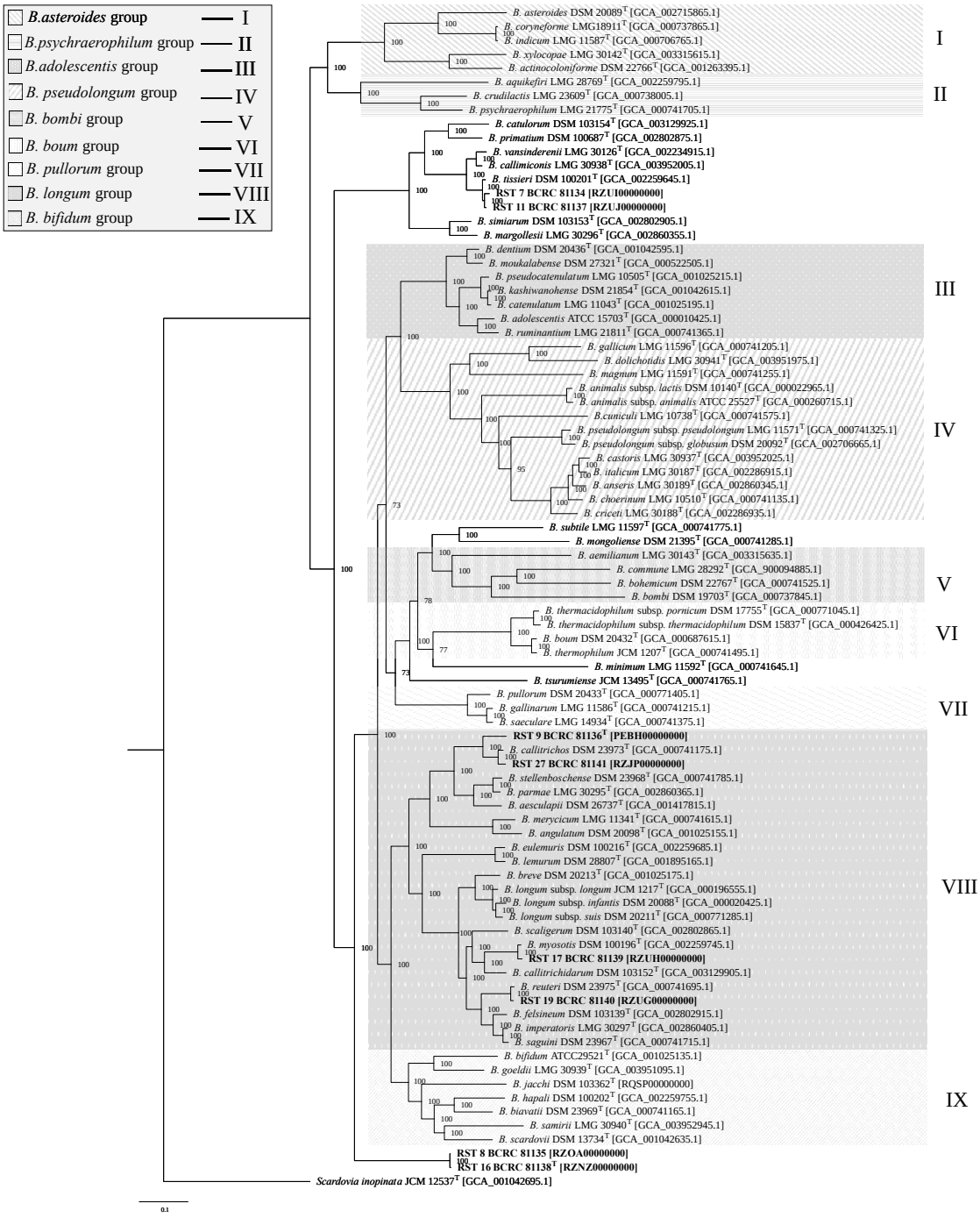

Figure 3. Phylogenetic tree of the *Bifidobacterium* genus based on the concatenated amino acid sequences of 355 core genes of strains isolated from Egyptian fruit bats and members of the *Bifidobacterium* genus. The core genes-based tree shows the subdivision of the seven phylogenetic groups of the *Bifidobacterium* genus represented with different colors. The phylogenetic tree was built by the maximum likelihood method with corresponding sequences of *Scardovia inopinata*

JCM 12537^{T} being used as outgroup. Bootstrap percentages above 70 are shown at node points, based on 1000 replicates of the phylogenetic tree.

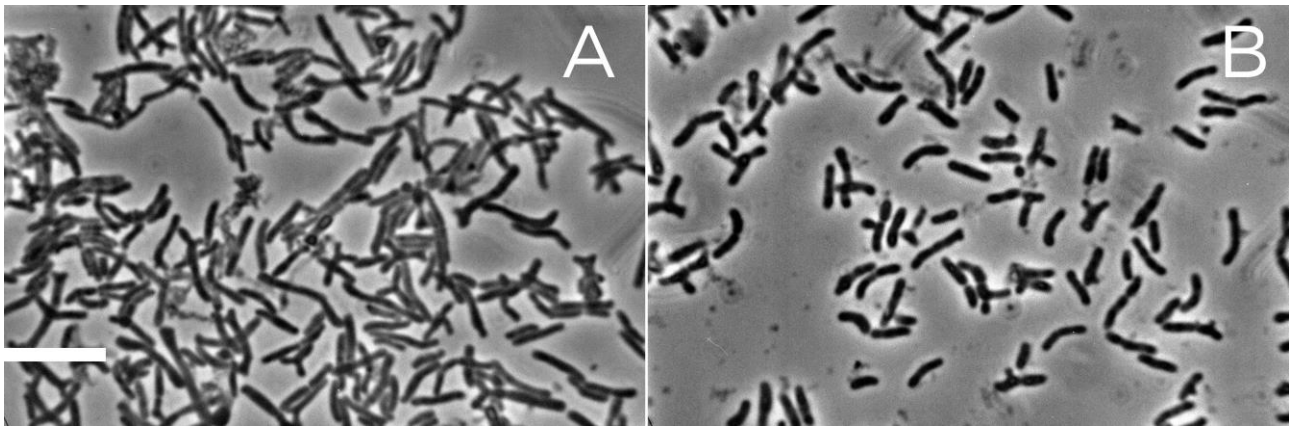

Figure 4. Phase-contrast photomicrographs of cells of *B. rousetti* sp. nov. RST 9^{T} (A) and B. *vespertilionis* sp. nov. RST 16^{T} (B) grown in TPY broth showing cellular morphology. Bar, 10 μm.

Table 1. Genomic and phylogenetic features of novel bifidobacterial strains isolated from the Egyptian bats

Strain	**RST 16^T**	**RST 8**	**RST 9^T**	**RST 7**	**RST 11**	**RST 17**	**RST 19**	**RST 27**
Accession Number	RZNZ00000000	RZOA000000000	PEBH00000000	RZUI00000000	RZUJ00000000	RZUH00000000	RZUG00000000	RZJP00000000
GC content	64.20 %	64.20 %	64.55 %	60.96 %	60.76 %	63.16 %	60.39 %	63.58 %
Contigs	49	74	37	49	46	60	80	11
Length (bp)	3075992	3067389	3053799	3032244	2986510	3275217	2833112	2797830
No. of ORF	2577	2594	2712	2526	2540	2759	2436	2320
tRNA	57	62	64	61	62	62	59	61
rRNA	5	3	5	2	2	5	3	3
Coverage	118	165	189	107	145	209	140	155

Table 2. Differential phenotypic characteristics of strains isolated from the Egyptian fruit bat and their phylogenetic related species *B. tissieri* DSM 100201^T, *B. myosotis* DSM 100196^T, *B. callitrichos* DSM 23973^T, *B. reuteri* DSM 23975^T, *B. avesanii* DSM 100685^T. Phenotypic data are from this study.

	RST 9	**RST 7**	**RST 11**	**RST 8**	**RST 16**	**RST 17**	**RST 19**	**RST 27**	***B. tissieri***	***B. myosotis***	***B. callitrichos***	***B. reuteri***	***B. avesanii***
FERMENTATION													
Glycerol	-	-	-	-	-	-	-	-	+	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	w	-	-	-	-	-
L-Arabinose	+	+	+	+	+	+	+	+	+	+	+	-	+
Ribose	w	+	+	-	-	w	w	w	-	-	-	-	-
D-Xylose	-	w	+	+	+	+	+	+	+	+	+	+	+
L-Xylose	-	-	-	-	-	-	-	-	-	-	-	-	+
Methyl-β-D-xylopyranoside	-	-	-	-	-	w	-	-	-	w	-	-	-
Galactose	+	+	+	+	w	+	+	+	+	w	+	-	w
Fructose	+	w	+	+	+	+	+	+	+	w	+	+	+
Mannose	+	+	+	w	w	-	+	+	+	w	+	-	+
Sorbose	-	-	-	-	-	w	-	w	-	-	-	-	-
Rhamnose	-	w	w	-	-	-	-	w	+	-	-	-	-
Inositol	-	-	-	-	-	-	-	+	-	-	-	-	-
Mannitol	+	+	+	w	+	-	+	+	w	w	+	-	-
Sorbitol	+	-	-	-	-	-	+	+	-	-	+	-	-
Methyl-α-D-mannopyranoside	-	-	-	-	-	-	-	+	-	-	-	-	-
Methyl-α-D-glucopyranoside	-	+	+	w	w	+	+	+	+	-	w	-	-
N-Acetyl glucosamine	-	w	w	-	-	-	-	+	w	-	w	-	-
Amygdalin	-	+	+	-	-	+	+	w	-	w	-	w	-
Arbutin	-	+	+	-	-	+	+	+	-	-	+	w	-
Aesculin	+	+	+	-	-	+	+	+	+	+	+	-	-
Salicin	-	+	+	-	-	+	+	+	+	+	+	+	-
Cellobiose	-	+	+	-	-	w	w	+	+	+	+	w	-

Maltose	W	W	+	+	+	+	+	+	+	+	+	+	-
Lactose	W	+	+	+	+	+	+	+	+	+	+	+	-
Melebiose	+	+	+	+	+	+	+	+	-	+	+	+	-
Sucrose	-	+	+	+	+	+	+	+	+	+	+	+	+
Trehalose	-	+	+	-	-	-	+	+	+	+	+	w	-
Inulin	-	-	-	-	-	-	w	-	-	-	-	-	-
Melezitose	-	+	+	-	-	-	+	-	w	w	+	w	-
Raffinose	+	+	+	+	+	+	+	+	+	-	+	+	+
Starch	W	w	w	w	w	+	w	w	-	-	-	+	-
Glycogen	-	-	-	-	-	-	-	-	-	-	-	+	-
Xylitol	-	-	-	-	-	-	w	w	-	-	-	-	-
Gentiobiose	-	+	+	-	-	+	+	+	-	w	w	+	-
Turanose	+	+	+	+	+	+	+	+	-	w	w	w	-
Lyxose	-	-	-	-	-	-	-	w	-	-	+	-	-
L-Fucose	-	-	-	-	-	-	-	+	-	-	+	-	-
Gluconate	+	-	-	+	+	+	+	+	-	-	w	w	-
2-keto-gluconate	-	+	+	-	-	w	-	-	+	-	-	-	w
5-keto-gluconate	w	+	+	w	w	w	w	w	-	-	-	-	-
ENZYMATIC ACTIVITY													
Urease	+	+	+	+	+	-	-	+	+	+	+	-	-
L-arginine dihydrolase	w		+	-	w	w	+	+	+	+	+	-	-
α -Glucosidase	+	+	+	+	+	+	+	+	-	w	+	+	+
β-Glucosidase	w	+	+	w	w	+	+	+	-	w	+	+	-
α -Arabinosidase	+	-	+	w	+	+	+	-	-	-	+	-	
β-Glucuronidase	+	-	+	-	+	-	-	+	-	-	w	-	w
N-Acetyl-β-glucosaminidase	-	-	-	-	+	-	-	-	-	-	-	-	-

Glutamic acid decarboxylase	+	w	w	-	-	-	+	-	-	-	+	-	-
ALkaline phosphatase	-	w	+	+	+	-	-	+	-	-	-	+	+
Arginine arylamidase	+	+	+	+	+	+	+	+	-	-	+	+	+
Leucyl glycine arylamidase	-	-	-	-	+	+	+	+	+	+	w	w	+
Phenylalanine arylamidase	+	+	+	+	+	+	+	+	+	+	+	w	+
Leucine arylamidase	+	w	+	+	+	+	+	+	+	+	+	+	+
Pyroglutamic acid arylamidase	-	-	-	+	+	-	-	-	-	-	-	-	+
Tyrosine arylamidase	+	w	+	+	+	+	-	+	+	+	+	+	+
alanineaArylamidase	+	-	-	-	+	+	+	+	+	+	w	-	+
Glycine arylamidase	+	+	+	w	+	+	+	+	+	+	w	-	+
Histidine arylamidase	+	+	+	+	+	+	+	+	+	+	+	+	+
Glutamyl glutamic acid arylamidase	-	-	-	-	W	-	-	-	-	-	-	-	w
Serine arylamidase	+	+	+	+	+	+	+	+	-	+	w	-	+
Temperature range for growth	22-48°C	22-48°C	22-48°C	22-48°C	22-48°C	22-48°C	22-48°C	22-48°C	20-44 °C	20-48°C	26-42°C	35-37°C	25-50 °C
Optimum temperature	37 °C	37 °C	37 °C	37 °C	37 °C	37 °C	37 °C	37 °C	35 °C	42 °C	37 °C	37 °C	40 °C
pH range for growth	3.5-7.5	3.5-7.5	3.5-7.5	3.5-7.5	3.5-7.5	3.5-7.5	3.5-7.5	3.5-7.5	5.5-7.5	4-7.5	5.0-8.0	5.0-7.0	4.0 – 7.5
Optimum pH	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	6.5	7.0	7.0	7.0	6.0
DNA GC content (mol%)	64.5*	60.96*	60.76*	64.20*	64.20*	63.16*	60.39*	63.58*	63.7*	65.1*	64.3*	61.3§	65.9^
Peptidoglycan type	L-Orn(L-Lys)-D-Glu	L-Glu–L-Ala–L-Lys	L-Glu–L-Ala–L-Lys	L-Orn-L-Glu3-D-Ser	L-Orn-L-Glu3-D-Ser	L-Glu– L-Ala– L-Lys	l-Lys-Gly	l-Lys(l-Orn)-d-Asp	L-Glu–L-Ala–L-Lys	L-Glu– L-Ala– L-Lys	l-Lys(l-Orn)-d-Asp	l-Lys-Gly	l-Orn(Lys)–d-Ser–d-Glu.

+, positive; –, negative; w, weakly positive. All strains ferment glucose. No strains ferment erytrol, L-xylose, D-adonitol, dulcitol, glycogen, tagatose, D-fucose, D and L-arabitol. No strains show *β*-galactosidase-6-phophatase, *α*-fucosidase, no reduction of nitrates or indole production.

*Data are from this study. § Data are from Endo *et al*. [4]; ^data are from Michelini et al. [16].

Table 3. Cellular fatty acid profiles of strains isolated from the Egyptian fruit bat.

Fatty acid	Cluster I		Cluster II		Cluster III		Cluster IV	Cluster V
	RST 8	RST 16^T	RST 9^T	RST 27	RST 7	RST 11	RST 19	RST 17
C16:0	**51.15 ± 0.30**	**39.02 ± 0.20**	**34.82 ± 1.04**	**39.89 ± 0.30**	**56.21 ± 0.67**	**43.46 ± 0.54**	**24.29 ± 0.43**	**37.78 ± 0.27**
C14:0	5.80 ± 0.12	**16.3 ± 0.47**	**24.96 ± 1.75**	**23.07 ± 0.72**	**10.57 ± 0.39**	**11.80 ± 0.45**	**21.68 ± 0.62**	**19.68 ± 0.17**
C18:1 ω9c	**18.15 ± 0.64**	8.14 ± 0.2	8.17 ± 0.71	8.74 ± 0.34		**11.13 ± 0.28**	**15.15 ± 0.62**	7.20 ± 0.34
C16:1 ω9c	2.45 ± 0.10	6.07 ± 0.56	2.83 ± 0.10	2.15 ± 0.03	1.62 ± 0.31	1.54 ± 0.16	8.18 ± 0.23	3.30 ± 0.10
C18:0	1.99 ± 0.01	1.64 ± 0.04	1.30 ± 0.07	2.04 ± 0.07	9.76 ± 0.29	6.15 ± 0.29		1.64 ± 0.01
C12:0		1.35 ± 0.02	1.85 ± 0.30	1.74 ± 0.12	1.84 ± 0.10		2.84 ± 0.20	1.87 ± 0.09
C13:1 at 12-13		2.19 ± 0.30	2.03 ± 0.26	1.65 ± 0.14	2.19 ± 0.01	1.10 ± 0.07		2.32 ± 0.05
C19:0 iso I					2.37 ± 0.05	1.14 ± 0.04		
C20:0					1.91 ± 0.23			
C17:1 ω9c					1.49 ± 0.09			
C10:0					1.26 ± 0.08			
Summed features*								
1	2.30 ± 0.07	6.85 ± 0.44	6.53 ± 0.39	5.34 ± 0.16	6.68 ± 0.29	3.32 ± 0.22	2.74 ± 0.09	6.86 ± 0.26
4	1.49 ± 0.09				2.41 ± 0.08	1.55 ± 0.10		
7	**12.53 ± 0.40**	**13.80 ±0.90**	**11.38 ± 1.00**	**10.05 ± 0.34**		**13.62 ± 0.10**	**18.19 ± 0.91**	**13.70 ± 0.28**
8	1.76 ± 0.14	2..34 ± 0.29	3.12 ± 0.11	2.26 ± 0.06		3.27 ± 0.30	2.12 ± 0.03	2.44 ± 0.16

In bold the main fatty acids produced