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# *Bifidobacterium xylocopae* sp. nov. and *Bifidobacterium aemilianum* sp. nov., from the carpenter bee (*Xylocopa violacea*) digestive tract

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Social bees harbor a community of gut mutualistic bacteria, among which bifidobacteria occupy an important niche. Recently, four novel species have been isolated from guts of different bumblebees, thus allowing to suppose that a core bifidobacterial population may be present in wild solitary bees. To date there is sparse information about bifidobacteria in solitary bees such as *Xylocopa* and *Osmia* spp., this study is therefore focused on the isolation and characterization of bifidobacterial strains from solitary bees, in particular carpenter bee (*Xylocopa violacea*), builder bee (*Osmia cornuta*), and red mason bee (*Osmia rufa*). Among the isolates from *Osmia* spp. no new species have been detected whereas among *Xylocopa* isolates four strains (XV2, XV4, XV10, XV16) belonging to putative new species were found. Isolated strains are Gram-positive, lactate- and acetate-producing and possess the fructose-6-phosphate phosphoketolase enzyme. Full genome sequencing and genome annotation were performed for XV2 and XV10. Phylogenetic relationships were determined using partial and complete 16S rRNA sequences and hsp60 restriction analysis that confirmed the belonging of the new strains to *Bifidobacterium* genus and the relatedness of the strains XV2 and XV10 with XV16 and XV4, respectively. Phenotypic tests were performed for the proposed type strains, reference strains and their closest neighbor in the phylogenetic tree. The results support the proposal of two novel species *Bifidobacterium xylocopae* sp. nov. whose type strain is XV2 (=DSM 104955$^T$ = LMG 30142$^T$), reference strain XV16 and *Bifidobacterium aemilianum* sp. nov. whose type strain is XV10 (=DSM 104956$^T$ = LMG 30143$^T$), reference strain XV4.

*Keywords:* New species *Bifidobacterium xylocopae* sp. nov. *Bifidobacterium aemilianum* sp. nov. Phylogenetics *Xylocopa violacea* *Osmia* spp.

## Introduction

Bifidobacteria are commensal anaerobic bacteria of the human and animal gut, where they exert important functions for the host. In-depth studies of bifidobacteria type-strain genomes support the hypothesis of co-evolution with the host with both DNA acquisition and deletion events [47]. This taxon constitutes one of the most numerous groups of beneficial bacteria in the gut contributing to the intestinal microbiota in different percentages depending on the host species, age and diet [69]. Different molecules have been described and characterized to date as mediators of the *Bifidobacterium* cross-talk with the host [57,21,71] and accountable for a number of positive effects in host development and physiology [10,64]. As mammals, insects rely on a mutualistic gut microbial community. In some insects, such as several ant species, the microbiota seems to be acquired from the food and the environment [18], whereas in honey bees and bumble bees it appears to be more host-specific [36]. These specialized bacteria may influence host nutrition, as they contain genes involved in carbohydrate digestion [17], and contribute to host defense and physiology [50,23]. Whether gut microbes are environmentally acquired or host-specific, they are extremely important for the host health status and bifidobacteria represents an important gut taxon to be investigated for its beneficial properties. Bifidobacterial population has been characterized in the sixties in some pollinating insects, including the honeybees *Apis mellifera* and *Apis cerana*, and isolated strains were classified as new species: *Bifidobacterium coryneforme*, *Bifidobacterium asteroides* and *Bifidobacterium indicum* [59]. Later studies, aimed at the identification of dominant and recurring honey bee-associated gut microorganisms, confirmed bifidobacteria as stable colonizers/inhabitants of honey bee gut [58]. Honey bees are not the only pollinators harboring bifidobacteria: in the last decade four novel species have been identified from *Bombus* spp. gut: *Bifidobacterium bombi*, *Bifidobacterium actinocoloniiforme*

and *Bifidobacterium bohemicum* from the digestive tract of *Bombus lucorum* and *Bifidobacterium commune* from *Bombus lapidarius* [30,31,53]. However, no information is available in the literature on the presence of bifidobacteria in solitary bees such as *Xylocopa* spp., known as carpenter bees for their ability to burrow into hard plant material, and *Osmia* spp., known as mason bees for their habit of using mud or other "masonry" products to construct their nests. These bees exert a highly efficient pollination service [74]. *Xylocopa* species are known to pollinate several crops, such as legumes, eggplants, broccoli etc. [68,73] and several fruit crops, especially *Prunus* spp. [73,15], thus assuming great value for crop pollination strategies. *Osmia* spp., especially *Osmia cornuta*, are also of utmost importance for orchard pollination, especially for *Rosaceae* family plants, like pear and apple and also for almond [42,9].

The health status of solitary bees is crucial for the maintenance of the pollination service. In the last century, many wild bee populations, including solitary bees, have become reduced in number and the loss of their genetic diversity makes them more vulnerable to infectious diseases and other stressors such as pesticides [20,62]. Moreover, the spread of parasitic infections from managed bees to wild bees has been reported and recently reviewed by Graystock et al. [20] and represents a potential threat for wild bees' population. The gut of insects may harbor one of the largest reservoirs of a yet unexplored microbial diversity. A deeper knowledge of the gut microorganisms of alternative pollinators, such as *Osmia* and *Xylocopa*, could be of great importance, since their health status depends on the presence and activity of commensal microorganisms as for any other animal. Novel strains might show a potential as beneficial bacteria for pollinator insects, reinforcing attempts to establish a beneficial bacteria strategy for bee health [6,1,2]. This work is therefore aimed at increasing the knowledge on the presence and diversity of cultivable bifidobacteria in solitary bees, in particular in the genera *Xylocopa* and *Osmia*.

## Materials and methods

### *Samples collection and microorganisms isolation*

In spring 2016, 2 worker carpenter bees were collected in a flowery meadow in Spilamberto (Modena, Italy), whereas 10 builder and 10 red mason bees were collected in a rearing field located in Cadriano (Bologna, Italy) and promptly transferred to the laboratory. All solitary bees were anesthetized and sacrificed, and their gut content was extracted, weighted, serially diluted and plated on two different media for bifidobacteria isolation: Tryptone, Phytone, and Yeast extract (TPY) agar medium [44] supplemented with mupirocin (200 mg $L^{-1}$) and de Man Rogosa Sharpe (MRS) agar medium (Scharlau Chemie, Gato Perez, Spain) supplemented with fructose (10 g$L^{-1}$), cysteine (1 g$L^{-1}$) [51] and cycloheximide (0.01 mg $L^{-1}$). Plates were incubated 5 days at 35 °C, in both anaerobic and microaerophilic atmosphere generated in jars with GasPak™ EZ systems (Becton, Dickinson Co., Sparks, MD, USA). The choice of the two different oxygen concentrations was done considering that often *Bifidobacterium* strains isolated from insects show a faster growth time in microaerophilic conditions [10] than in strict anaerobiosis.

Morphologically different colonies were randomly picked, restreaked, checked for purity and prepared for cryoconservation at −80 °C. For each isolated strain, the anaerobic/microaerophilic growing conditions were tested to define the best culture condition.

### *DNA isolation from microorganisms and fingerprinting analyses*

Chromosomal DNA was extracted from each isolate with Wizard® Genomic DNA Purification Kit (Promega, Madison, Wi, USA). Isolates were characterized with two PCR-dependent fingerprinting techniques. Randomly amplified polymorphic DNA (RAPD) PCR was performed with primer M13 according to Andrighetto et al. [4]. Enterobacterial repetitive intergenic consensus (ERIC) sequence PCR was carried out with primers ERIC-1 and ERIC-2 according to Versalovic et al. [72]. Obtained fingerprinting profile patterns were analyzed with GelCompar II 6.6 (Applied Maths, Kortrijk, Belgium) using the DICE coefficient and the UPMGA clustering algorithm.

### *16S rRNA gene sequencing*

DNA amplification of the 16S rRNA gene was performed with primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACT-3′) [37] according to Gaggìa et al. [19]. Amplicons were purified and sent to commercial sequencing facility (Eurofins MWG, Ebersberg, Germany). Sequence chromatograms were analyzed, manually edited and classified using BLAST tool from NCBI [3]. Sequences were deposited in GeneBank nucleotide database (accession numbers MG597261–MG597286, MH043274 and MH043277) (Table 1).

### *Whole-genome sequencing and GC content of newly proposed species*

Genomic DNA from XV2 and XV10 strains was sequenced on the Illumina MiSeq NGS platform by BMR Genomics facilities (www.bmr-genomics.it), according to Illumina protocol for Nextera XT DNA library preparation. Reads were assembled into contigs with SPAdes 3.7 according to Bankevich et al. [7] and assembly quality assessed with QUAST [22]. Average Nucleotide Identity (ANI) values were calculated with PYANI [54], a python3 module, using different methods: ANIb (based on BLAST algorithm), ANIm (based on MUMmer algorithm) and TETRA (Tetranucleotide Signature Frequency Correlation Coefficient) as described by Richter and Rosselló-Móra [56]. Two annotation programs based on different algorithms were used for the identification of the ORFs and subsequent functional annotation: (i) PROKKA – rapid prokaryotic genome annotation software – [61] implemented with HMMER package for ribosomal RNA profile annotation [16] and (ii) Blast KOALA (KEGG tools) [27]. Full-length sequences of 16S rRNA of XV2 and XV10 were obtained from the whole-sequencing results and further analyzed with the web tool EMBOSS-Water (EMBL-EBI release) that uses the Smith-Waterman algorithm to calculate the local alignment of two sequences. This in-depth analysis allowed to calculate similarity percentages between XV2, VX10 and the 16S rRNA full-sequences of their closest relatives (obtained from type strains repository) (the accession numbers of full-16S rRNA sequences of XV2 and XV10 are MH043275 and MH043276).

Estimation of the G + C content was performed by DSMZ Identification Service (Braunschweig, Germany) according to the DSMZ protocol [11,46,66].

### *Phylogenetic trees and core and pan-genomes*

Evolutionary analyses were conducted in MEGA7 [34] and exported in Newick format. The iTOL web-based software [38] has been used for the annotation and management of the published phylogenetic trees. Partial 16S rRNA sequences were used to verify the relatedness of XV2 and XV10 with XV16 and XV4 respectively, the tree was inferred by using the Maximum Likelihood method [12] based on the Tamura-Nei model [67]. On the other hand, fifty-nine complete 16S rRNA and *hsp60* sequences were retrieved from whole genome sequencing repository database of NCBI (ftp://ftp.ncbi.nlm.nih.gov/genomes/refseq/bacteria/) to infer the phylogeny of the new species. For 16S rRNA, the evolutionary history was

**Table 1**
Identification of isolates based on partial 16S rRNA gene sequences and NCBI blast tool.

| Isolated Strain | Isolation source | GenBank | Microorganism (RDP classifier) | GenBank | % identity NCBI |
|---|---|---|---|---|---|
| OCN2 | *Osmia cornuta* | MG597261 | *Cutibacterium acnes* | NR_040847.1 | 100.00 |
| OCN10 | *Osmia cornuta* | MG597263 | *Cutibacterium acnes* | NR_040847.1 | 99.925 |
| OCV2 | *Osmia cornuta* | MG597265 | *Cutibacterium namnetense* | NR_151943.1 | 99.777 |
| OCV7 | *Osmia cornuta* | MG597266 | *Cutibacterium granulosum* | NR_118646.1 | 99.851 |
| OCV18 | *Osmia cornuta* | MG597267 | *Cutibacterium acnes* | NR_040847.1 | 99.629 |
| OCV20 | *Osmia cornuta* | MG597268 | *Paenibacillus peoriae* | NR_117743.1 | 99.156 |
| OCV21 | *Osmia cornuta* | MG597269 | *Cutibacterium acnes* | NR_040847.1 | 99.926 |
| OCV26 | *Osmia cornuta* | MG597270 | *Cutibacterium granulosum* | NR_118646.1 | 99.851 |
| OCV28 | *Osmia cornuta* | MG597271 | *Bifidobacterium coryneforme* | NR_115978.1 | 99.925 |
| OCV32 | *Osmia cornuta* | MG597272 | *Vagococcus entomophilus* | NR_133886.1 | 99.927 |
| ORV8 | *Osmia rufa* | MG597273 | *Bifidobacterium coryneforme* | NR_115978.1 | 99.852 |
| ORV20 | *Osmia rufa* | MG597274 | *Cutibacterium acnes* | NR_040847.1 | 100.00 |
| ORV27 | *Osmia rufa* | MG597276 | *Bifidobacterium coryneforme* | NR_115978.1 | 99.854 |
| ORV30 | *Osmia rufa* | MG597277 | *Bifidobacterium coryneforme* | NR_115978.1 | 99.926 |
| XV2 | *Xylocopa violacea* | MG597278 | *Bifidobacterium bifidum* | NR_113873.1 | 95.573 |
| XV2L | *Xylocopa violacea* | MG597279 | *Lactobacillus bombi* | NR_134065.1 | 98.763 |
| XV4 | *Xylocopa violacea* | MH043274 | *Bifidobacterium coryneforme* | NR_115978.1 | 96.935 |
| XV5L | *Xylocopa violacea* | MG597280 | *Lactobacillus bombi* | NR_134065.1 | 98.692 |
| XV8L | *Xylocopa violacea* | MG597281 | *Lactobacillus bombi* | NR_134065.1 | 98.695 |
| XV10 | *Xylocopa violacea* | MG597282 | *Bifidobacterium coryneforme* | NR_115978.1 | 96.952 |
| XV11 | *Xylocopa violacea* | MG597283 | *Bifidobacterium actinocoloniiforme* | NR_108438.1 | 99.048 |
| XV13L | *Xylocopa violacea* | MG597284 | *Lactobacillus apis* | NR_125702.1 | 96.906 |
| XV16 | *Xylocopa violacea* | MG597285 | *Bifidobacterium bifidum* | NR_113873.1 | 95.997 |
| XV16L | *Xylocopa violacea* | MH043277 | *Lactobacillus bombi* | NR_134065.1 | 98.697 |
| XV17L | *Xylocopa violacea* | MG597286 | *Lactobacillus bombi* | NR_134065.1 | 98.694 |

inferred by using the Maximum Likelihood method based on the Tamura-Nei model [67]. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3807)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 65.2412% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 1512 positions in the final dataset. For *hsp60* gene the Maximum Likelihood method based on the General Time Reversible model (a discrete Gamma distribution was used to model evolutionary rate differences among sites, +G) was used according to the result obtained with MEGA7 after calculation of the best model on *hsp60* sequences.

The bifidobacteria core- and pan-genome were analyzed using BPGA (Bacterial Pan Genome Analysis tool) [14]. For the core-tree, after evaluation of core-genes with BPGA, the amino acid sequences of 273 genes were concatenated and aligned to generate the phylogenetic tree using the Neighbor-Joining method.

*PCR-RFLP*

The restriction of XV2 and XV10 *hsp60* gene, as well as of closely related species, was performed according to Stenico et al. [65]. An *in-silico* analysis was preliminary done to obtain the theoretical restriction profiles for the species that were not previously reported in Stenico et al. [65], using the *hsp60* sequences retrieved from the GenBank and RefSeq databases and using Webcutterb2.0 (http://rna.lundberg.gu.se/cutter2/) (Table S1).

*Metabolic profiles, peptidoglycan and fatty acids analysis*

Enzymatic activities and substrate fermentation capabilities for the novel strains and the species closely related to them (listed in Table 2) were obtained with API 50 CHL and Rapid ID 32A kits (bioMérieux, Lyon, France) according to the manufacturer's instructions with a slight modification: 10 $\mu L^{-1}$ of a cysteine sterile solution (1 $gL^{-1}$) were added to the API 50 CHL medium. API 50 CHL inoculated galleries were incubated in anaerobiosis, except for XV10 that was incubated in microaerophilic conditions. Catalase and Oxidase tests were performed according to Modesto et al. [49]. The cell wall murein composition was determined by DSMZ Identification Service, according to the protocol described by Schumann [60].

Analyses of cellular fatty acids were carried out at DSMZ Identification Service according to Miller [48] and Kuykendall et al. [35] with minor modifications described in the DSMZ protocol published online.

*Hemolytic activity test and analysis on related proteins*

Hemolytic activity of XV2 and XV10 was tested in three different media: Columbia Blood Agar (BIOLIFE, Milan, Italy), MRS and TPY supplemented with 5% of defibrinated sheep blood.

Sequence related to hemolytic activity have been identified in the genome annotation file and an UniProt BLAST analysis has been performed searching for Hemolysin III related proteins in bifidobacterial species. The first 47 hits were downloaded, aligned in MEGA7 with MUSCLE and the best ML substitution model was evaluated (WAG + I + F) to generate the tree.

## Results and discussion

*Microorganisms isolation, fingerprinting and cluster analysis*

A total of 33, 34 and 24 bifidobacteria isolates were obtained from *X. violacea*, *O. cornuta* and *O. rufa* (synonym *Osmia bicornis*), respectively. Five clusters were obtained in fingerprinting profiles of microbial isolates from *X. violacea* (Fig. S1), and nine clusters in fingerprinting profiles of isolates from *O. cornuta* and *O. rufa* (data not shown). A representative strain for each cluster was further processed.

**Table 2**
Characteristic that differentiate the proposed novel species of *Bifidobacterium* from the closest relatives. Strains: (1) *Bifidobacterium* sp. XV2; (2) *Bifidobacterium* sp. XV16; (3) *Bifidobacterium* sp. XV10; (4) *Bifidobacterium* sp. XV4; (5) *B. bombi* DSM 19703$^T$; (6) *B. asteroides* DSM 20089$^T$; (7) *B. coryneforme* DSM 20216$^T$; (8) *B. indicum* DSM 20214$^T$; (9) *B. actinocoloniiforme* DSM 22766$^T$; (10) *B. bohemicum* DSM 22767$^T$; (11) *B. commune* LMG 28292 $^T$; (12) *B. minimum* ATCC 27538$^T$; (13) *B. subtile* DSM 20096$^T$. Biochemical test were performed using API CH50 and Rapid ID32 tests (bioMérieux). +, Positive; w, weakly positive; −, negative; ND, not determined;.

| Characteristics | Sp. nov. | | | | Insect core bifidobacteria | | | | | | | Other bifidobacteria species | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | XV2 | XV16 | XV10 | XV4 | *B. bombi* | *B. asteroides* | *B. coryneforme* | *B. indicum* | *B. actinoco-loniiforme* | *B. bohemicum* | *B. commune* | *B. minimum* | *B. subtile* |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| Temperature range for growth (°C) | 25–37 | 25–37 | 25–37 | 25–37 | 10–37 | 21–42 | 22–43 | 22–43 | 25–37 | 10–37 | 10–37 | 15–35 | ND |
| DNA G + C content (mol%) | 61.3 | ND | 61.9 | ND | 50.5 | 59 | 55 | 60 | 52.7 | 51.2 | 54.3 | 61.5 | 61.5 |
| Growth under microaerophilic conditions | − | + | + | + | − | + | + | + | + | − | + | − | − |
| Peptidoglycan structure | A4α L-Lys–D-Asp | ND | A4α L-Lys–L-Ala$_2$–Gly[a] | ND | ND | A3α L-Lys–Gly | A4α L-Lys–D-Asp | A4α L-Lys–D-Asp | ND | ND | ND | A3α L-Lys–L-Ser[a] | A4α L-Lys–D-Asp |
| Acid production from | | | | | | | | | | | | | |
| L-Arabinose | w | w | w | w | w | − | w | + | − | + | − | − | − |
| D-Ribose | w | + | + | + | + | + | + | + | w | + | − | − | w |
| D-Xylose | − | − | + | + | − | − | − | − | − | w | − | − | − |
| D-Galactose | − | − | − | − | + | − | + | w | − | + | + | − | + |
| D-Glucose | + | − | + | + | + | + | + | + | + | + | + | + | + |
| D-Fructose | + | + | + | + | w | + | + | + | − | − | − | + | + |
| D-Mannose | − | − | + | + | w | − | − | w | − | + | − | − | − |
| D-Lactose | − | − | − | − | − | − | + | w | − | − | − | − | w |
| D-Sorbitol | − | − | − | − | − | − | w | − | − | − | − | − | − |
| Methyl α-D-glucopyranoside | − | − | w | − | w | − | − | w | − | + | + | − | w |
| Arbutin | w | w | + | w | + | + | w | + | w | + | + | − | − |
| Aesculin | + | + | + | + | + | + | + | + | + | + | + | − | − |
| Salicin | + | + | + | + | + | + | + | + | + | + | + | − | − |
| Amygdalin | − | − | − | − | + | + | + | − | + | − | + | − | − |
| D-Cellobiose | − | − | − | − | + | + | − | + | + | − | − | − | − |
| D-Maltose | − | − | − | − | w | − | + | w | − | w | − | + | + |
| D-Melibiose | − | + | w | − | + | − | + | + | w | + | + | − | + |
| D-Sucrose | + | + | + | + | w | + | + | + | − | − | w | − | + |
| D-Trehalose | − | − | − | − | − | − | − | − | − | − | − | − | − |
| Inulin | − | − | w | − | − | − | − | − | − | − | − | − | − |
| D-Raffinose | − | − | + | + | + | − | + | + | − | + | + | − | + |
| Starch | − | − | w | − | − | − | − | − | − | − | − | + | + |
| Glycogen | − | − | − | − | − | − | − | − | − | − | − | + | + |
| D-Turanose | − | − | w | − | − | − | − | w | − | − | − | + | + |
| Gentiobiose | − | − | − | − | + | + | + | + | + | + | − | − | − |
| L-Fucose | − | − | − | − | − | − | − | w | − | − | − | − | − |
| Potassium Gluconate | − | − | − | − | − | − | − | − | − | w | − | − | − |
| Potassium 5-ketogluconate | − | − | w | − | − | − | − | − | − | w | − | − | − |
| Enzymatic activity | | | | | | | | | | | | | |
| α-Galactosidase | + | + | − | − | + | + | + | + | + | − | + | − | + |
| β-Galactosidase | + | + | + | − | + | + | + | + | + | − | + | − | + |
| α-Glucosidase | − | + | + | + | + | + | + | + | + | + | + | + | + |
| β-Glucosidase | + | + | + | + | + | + | + | + | + | + | + | − | + |
| α-Arabinosidase | + | + | + | + | + | + | + | + | + | + | + | − | + |
| N-Acetyl-β-glucosaminidase | − | − | + | + | − | − | − | − | + | − | − | − | − |
| α-Fucosidase | + | + | − | − | − | + | − | − | − | − | − | − | − |
| Alkaline phosphatase | − | − | − | − | − | − | − | − | − | + | − | − | + |
| Proline arylamidase | + | + | + | + | + | + | + | + | + | + | − | + | + |
| Leucyl glycine arylamidase | + | − | w | − | − | + | + | − | − | − | − | + | + |
| Tyrosine arylamidase | + | + | + | + | + | + | + | + | − | + | + | + | + |
| Alanine arylamidase | + | − | w | − | − | + | + | − | − | − | + | + | − |
| Glycine arylamidase | + | + | + | + | + | + | + | + | − | + | + | + | + |
| Histidine arylamidase | + | + | + | + | + | + | + | + | − | + | + | + | + |
| Serine arylamidase | + | + | + | − | + | + | + | − | − | − | + | + | + |

[a] α-Carboxyl group of D-Glu substituted by glycine.

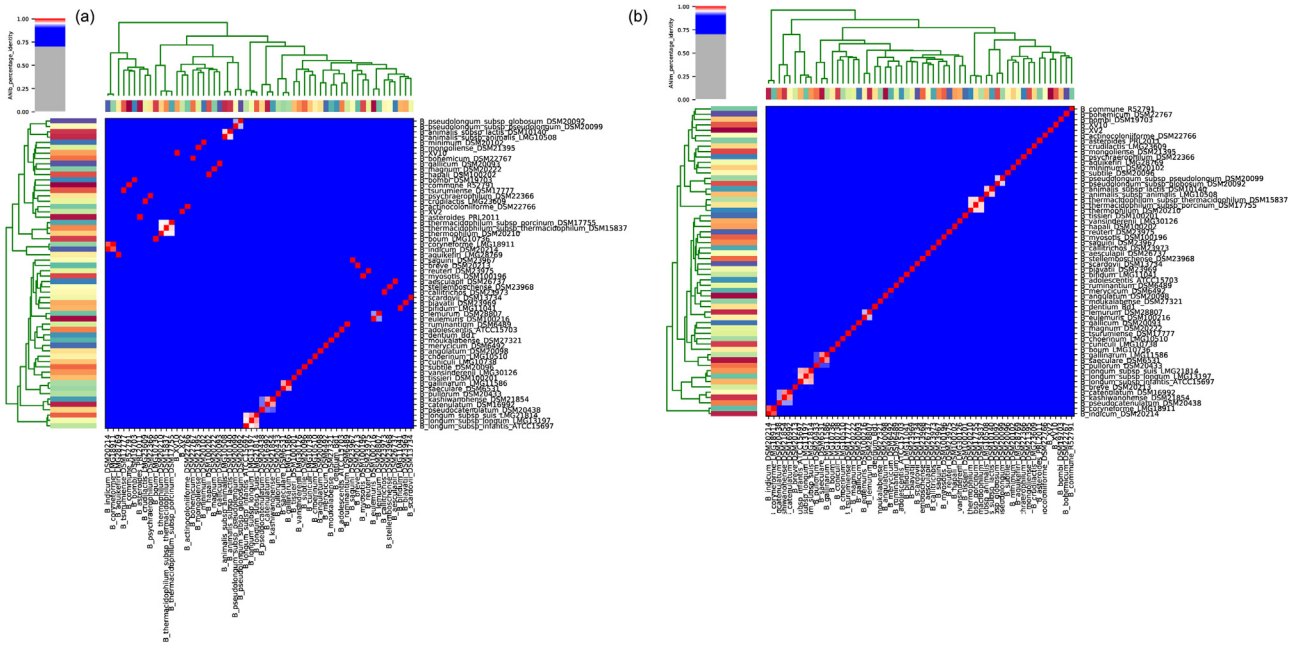


**Fig. 1.** Average nucleotide identity. Grafical output of PYANI for average nucleotide identity analyses (a) ANIb (BLAST algorithm) (b) ANIm (MUMmer algorithm). ANI values of less than 0.95 are colored in blue, showing that the strains are different species. Red squares with ANI values greater than 0.95 indicate that theses strains belong to the same species. Color intensities fade as the ANI values approach 0.95.

*16S sequencing and microorganisms identification*

Recent studies show how gut microbial population of honey bees (*A. mellifera*) is transmitted horizontally through contact with nurse bees, fresh faces or hive surfaces [43], therefore it can be defined as a "socially transmitted gut microbiota". On the contrary, *Osmia* and *Xylocopa* genera consist of solitary bees, therefore social transmission at the time of edging is hardly occurring as also evidenced by Lozo et al. [39]. In recent studies [39,28] *Bifidobacterium* species were not detected in mason bees alimentary tract. In our study, on the contrary, isolates from *Osmia* spp. in TPY agar medium were identified as *Cutibacterium acnes*, *Cutibacterium granulosum, B. asteroides* and *B. coryneforme* (Table 1), showing a similarity with *A. mellifera* bifidobacteria core species. An acquisition of bifidobacteria from food sources (flowers) that might be occasionally shared between solitary and honey bees [45,33] can be hypothesized. Novel species were not detected in *Osmia* spp. gut.

The analyses of partial 16S rRNA sequences of TPY and MRS isolates (Table 1) showed that XV2 and XV10 strains from *X. violacea* deserved an in-depth analysis on the whole 16S rRNA gene sequence retrieved from full genome sequencing NCBI repository files (described in the following paragraph). The in-depth analysis performed with EMBOSS-Water (Smith-Waterman algorithm) allowed to compare XV2 and XV10 strains with their closest type strains. XV2 analysis gave a similarity of 97.6% with *B. actinocoloniiforme* DSM 22766$^T$ and of 95.4% with *B. asteroides* PRL2011, *B. coryneforme* DSM 20216$^T$ and *B. indicum* DSM 20214$^T$. A similarity of 95.8% to *B. bohemicum* DSM 22767$^T$ and 95.7% to both *B. commune* LMG 28292$^T$ and *Bifidobacterium subtile* DSM 20096$^T$ was obtained for XV10. These scores are below the threshold of 98.5% similarity to be clustered in the same species [32]. Other isolates from *X. violacea* were assigned to *B. actinocoloniiforme*, *Lactobacillus apis and Lactobacillus bombi*, allowing us to assume a certain affinity for food sources between carpenter bee and *Bombus* spp. *Bifidobacterium* sp. XV2 and *Bifidobacterium* sp. XV10 are described and proposed as novel species.

*GC content and full genome sequencing*

The type strains XV2 and XV10 showed a G + C content of 61.3 and 61.9 mol% G + C, respectively. All values were within the G + C content range of the genus *Bifidobacterium* (52–67 mol%) [8,29].

The sequencing output of XV2 fastq counted 2,634,000 reads whereas XV10 fastq output counted 3,007,626 reads corresponding to an esteemed coverage of 350× and 400×, respectively. The reads assembly output consisted in 242 contigs (1.88 Mbp) for XV2 and 241 contigs (2.05 Mbp) for XV10. Average Nucleotide Identity (ANI) values, calculated with PYANI [54], are reported in Table S2a–c. XV2 and XV10 showed the highest identity values with each other of 79.3%, 86.9% and 95.4% for ANIb, ANIm and TETRA respectively. This percentages are far below the threshold of 96% for ANIb and ANIm and 99% for TETRA proposed by Richter and Rosselló-Móra [56] and mirroring the DDH value of 60–70% (Fig. 1a and b).

Annotation was carried out with Blast KOALA that performs KO (KEGG Orthology) assignments to characterize individual gene functions and reconstructs KEGG pathways, BRITE hierarchies and KEGG modules to infer high-level functions of the organism or the ecosystem [27] (Figs. 2 a and b, S2 and S3). Raw reads, contigs assembly and feature tables were deposited on the NCBI database (accession number PDCH00000000 and PDCG00000000). Genome annotations resulted in the prediction of 1556 genes of which 1412 coding sequences (CDSs), 51 genes for RNA and 93 pseudo genes for XV2 strain, whereas for XV10 strain the annotation resulted in 1719 genes of which 1531 CDSs, 52 genes for RNA and 136 pseudo genes (Table S3). Among detected genes, discussion has been focused on genes that might be of interest for the future use of the strains as beneficial bacteria in animal feeding like antibiotic resistance and vitamin productions. Both strains possess ORFs for two different B vitamin pathways (B6 and B9) in their sequences. Pyridoxin (B6) pathway ORFs are involved only in the "salvage pathway" for pyridoxal 5′-phosphate recycling (through pyridoxal kinase, PdxK). ORFs annotated in the folate biosynthesis also regard a "savage pathway" because of the absence of enzymes for precursor formation (the pteridine ring and the *para*-aminobenzoic acid unit *p*ABA). Only XV10 strain contains ORFs annotated as biotin

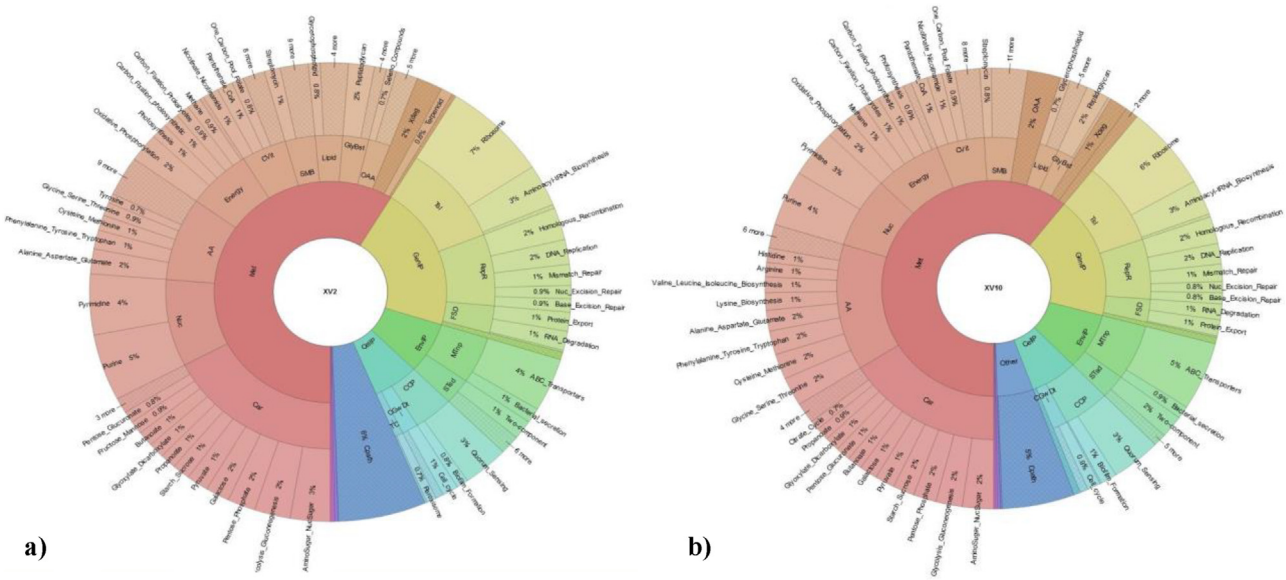


**Table of abbreviations for Krona charts**

| | | | | | |
|---|---|---|---|---|---|
| **Met** | **Metabolism** | **GenIP** | **Genetic_Information_Processing** | **Other** | **Other** |
| Car | Carbohydrate | Tsc | Transcription | Cpath | Common_Pathways |
| Energy | Energy | Tsl | Translation | DR | Drug_Resistance |
| Lipid | Lipid | FSD | Folding_Sorting_Degradation | RepR | Replication_Repair |
| Nuc | Nucleotide | RRep | Replication_Rerepai | IS | Immune_System |
| AA | Amino_acid | **EnvIP** | **Environmental_Information_Processing** | | |
| OAA | Other_Amino_acid | MTnp | Membrane_Transport | | |
| GlyBst | Glycan_biosynthesis | STsd | Signal_Transduction | | |
| CVit | Cofactors_Vitamins | **CellP** | **Cellular_Processe** | | |
| TeKe | Terpenoids_Polyketides | TC | Transport_Catabolism | | |
| SMB | Secondary_Metabolites_Biosynthesis | CGwDt | Cell_Growth_Death | | |
| Xdeg | Xenobiotics biodegradation | CCP | Cellular Community Prokaryotes | | |

**Fig. 2.** Krona charts. (a) *Bifidobacterium* sp. XV2 and (b) *Bifidobacterium* sp. XV10 annotated with BLAST KOALA and elaborated with KronaTools v2.7 [52].

(B7) biosynthesis, however the genes present are only involved in biotin uptake (i.e. BioY, component of biotin ECF transporter). Concerning ORFs for antibiotic resistance, a gene for tetracycline resistance has been annotated both in XV2 and XV10 strains (a ribosome protection type tetracycline resistance gene) and 4-fluoroquinolone resistance mutations in the *parC*, *parE*, *gyrA* and *gyrB* genes have been found. Some annotated ORFs for sugar utilization have been chosen for the discussion in order to compare phenotypes investigated with API tests and genotype results and clarify discrepancies occurred during the strain characterization. In the fermentation tests, negative results were obtained for lactose fermentation while the presence of β-galactosidase activity was observed with the use of RAPID ID32A. However, this apparent contradiction is explained by the absence, in both strains, of *lacS* gene coding for the lactose–galactose permease, as shown in genome annotations. Some genes related to maltose and maltodextrin utilization are present in XV10 strains but also in this case not all genes for the production of the maltose/maltodextrin transporters were shown and indeed fermentation did not occur with this carbon source (Table 2). Genes for arabinose utilization using AraBAD pathway are present in both XV2 and XV10 strains. L-arabinose residues are widely distributed among many heteropolysaccharides of different plant tissues. In *Bifidobacterium* sp. XV10 a full ribose utilization operon and ribose-5-phosphate isomerase enzyme have been annotated. Moreover, the strain has a xylose transport system permease (protein XylH) together with genes of the XylAB pathway, therefore it is capable of fermenting xylose. As already reported and fully annotated in *B. asteroides* PRL2011 [10], a simplified respiratory metabolism was also evidenced in *Bifidobacterium* sp. XV2 and XV10 together with enzymes involved in the antioxidant activity such as catalase and superoxide dismutase (the latter present only in XV2 strain). This is consistent with the description of the bee gut as an environment with a higher oxygen concentration compared to humans and other mammals.

### *Hemolytic activity and analysis on related proteins*

Since the discovery in *Bifidobacterium scardovii* of a weak hemolytic activity [24], hemolysis should always be checked in new *Bifidobacterium* species. XV2, XV16, XV10 and XV4 strains showed α-hemolytic activity in all media as a greenish discoloration that surrounds bacterial colonies in the tested media. Genome annotation with Prokka as well as the annotation provided by the NCBI pipeline during sequence submission highlighted two distinct ORFs annotated as Hemolysin and Hemolysin III. Hemolysin sequence is a membrane protein of the CCB3/YggT family (IPR003425) and UniProt BLAST results showed its presence in several bifidobacterial strains with identity scores ranging from 85.6% to 57.9% (taking into consideration only the first 47 hits). An YggT protein characterized in *Escherichia coli* seems to be related to osmotic shock protection [26], even if the length of *E. coli* YggY protein is 180 amino acids while bifidobacteria sequences are about 100 aminoacid-long. Concerning the annotation of Hemolysin III, this is an integral membrane protein component of the AdipoR/Haemolysin-III-related (IPR004254) family. This family, among others, groups proteins from pathogenic and non-pathogenic bacteria, including pore-forming proteins. A phylogenetic tree has been generated (Fig. S4) using 47 sequences related to Hemolysin III sequence from UniProt database and newly characterized species. This protein seems to have a high intra-species conservation. Proteins from bee species clustered together, including XV2, whereas XV10 strain clustered with its closest 16S rRNA gene neighbor. Being hemolysin-III protein poorly characterized, it is not possible to infer a correlation with the hemolytic activity. However, a correlation between

hemolytic activity and iron availability in the intestine can be discussed. Iron is essential for both host [63] and bacterial physiological processes and microorganisms have developed different strategies for iron uptake such as reduction of ferric iron with subsequent transport, iron acquisition from heme or iron-containing proteins of the host and production of siderophores [13]. Differently from mammals that rely on erythrocytes to transport oxygen, insects transport oxygen via the hemolymph, a fluid that contains free proteins named hemocyanins (copper as oxygen carrier). The use of copper cofactor as oxygen binder does not exclude the presence in the hemolymph of other proteins binding iron, such as ferritin, holoferritin and apoferritin. Ferritin accumulates in the smooth endoplasmic reticulum of the midgut cells of insects [25,55] and it can be speculated that iron contained in these cells can be used by bacteria once intestinal cells lyse. Moreover, genome annotations reported the presence of a ferrous iron transport protein and an iron transporter permease both in *Bifidobacterium* sp. XV2 and XV10. Therefore, a first hypothesis of the hemolytic phenotype is that hemolysins can act in synergy with other proteins to uptake iron from the surrounding environment, including insects gut cells containing ferritin. This peculiar weak hemolytic activity is therefore not a pathogenic trait [41], but it may be a way to improve iron uptake. Further investigations are necessary to confirm this hypothesis. A second hypothesis of the detected hemolytic activity could be related to the effect of $H_2O_2$ produced by SOD, whose sequence was found in XV2 genome. However, this phenotype was also found in the XV10 strain even if the SOD was not annotated by Prokka or Blast KOALA programs.

*Phylogenetic trees*

Two phylogenetic trees, with complete (Fig. 3) and partial (Fig. S5) 16S rRNA gene sequences were constructed. The phylogenetic tree with partial 16S rRNA gene sequences confirms the relatedness of XV2 and XV10 with XV16 and XV4, respectively. The phylogenetic tree with full length 16S rRNA genes includes fifty-nine sequences from type strains. Seven *Bifidobacterium* species (*B. aerophilum* DSM 100689$^T$; *B. aesculapii* DSM 26737$^T$; *B. apri* CCM 8605$^T$; *B. avesanii* DSM 100685$^T$; *B. callitrichidarum* DSM 103152$^T$; *B. faecale* JCM19861; *B. ramosum* DSM 100688$^T$) were excluded from the 16S rRNA based phylogenetic tree due to only partial 16S rRNA sequence available. There were a total of 1512 bases in the final dataset. XV2 clustered close to *B. actinocoloniiforme* in the *B. asteroides* group while *Bifidobacterium* sp. XV10 clustered with *B. subtile* DSM 20096, a bacterium isolated from sewage. Phylogenetic tree constructed with *hsp60* genes (Fig. 4) confirmed the relatedness of XV2 with *B. actinocoloniiforme* and other bifidobacteria from honeybees. XV10 falls just outside the same cluster showing a minor relatedness to XV2 and other bifidobacteria isolated from *Apoidea*.

*Core and pan-genome analysis*

The BPGA pipeline calculates shared genes after stepwise addition of each individual genome and plots the trend as core and pan-genome profile curve (data not shown). According to this curve, the bifidobacteria pan-genome can be considered as "open", as also recently evidenced by Lugli et al. [40]. Moreover, the pipeline generates a phylogenetic tree based on pan-matrix data (Fig. 5a). Fig. 5b shows the core-tree calculated with 273 amino acid sequences. To better evidence the relationship among *Bifidobacterium* species, no outgroup was used for the pan-genome tree. The phylogenetic analysis of pan-genome highlighted distinct groups (Fig. 5a) that were, however, not confirmed in the core-genome tree. In the latter, the presence of an outgroup species restricts the core-genes of the *Bifidobacterium* taxon and probably underestimates peculiarities linked to the adaptation to different ecological niches including horizontal gene transfer events [70,71,75].

*PCR-RFLP*

PCR-RFLP analysis of *hsp60* gene allows a rapid and accurate identification of common species of the genus *Bifidobacterium* [5,65]. The *in silico* analysis showed two new and distinct restriction profiles for XV2 and XV10 confirming the high discriminating ability of HaeIII enzyme. Moreover, restriction profiles were confirmed on agarose gel for species in bold in Table S1 and for the XV4 and XV16 strains. The restriction profiles of XV4 and XV16 were identical to those of XV10 and XV2 respectively (data not shown).

*Metabolic profiles, peptidoglycan and fatty acids analysis*

Metabolic profiles and fatty acids analysis are reported in Table 2. XV2 and XV10 strains tested negative for catalase and oxidase activities; however, the genome annotation underlined the presence of a catalase-related coding sequence both in XV2 and XV10 strains. In XV2 a superoxide dismutase CDS was also evidenced, even if XV2 strain resulted more affected by oxygen presence than XV10. It is possible to speculate that the diverse phenotypes can be related to different regulatory mechanisms. Peptidoglycan type resulted different for the two strains and is reported in Table 2. Analyses of cellular fatty acids are summarized in Table 3.

**Description of *Bifidobacterium xylocopae* sp. nov.**

*Bifidobacterium xylocopae* [xy.lo.co'pae. N.L. gen. n. *xylocopae* of *Xylocopa*, a wood cutter, the genus name of the insect from which the strain was isolated]. Cells are Gram-positive, non-motile, non-sporulating, F6PPK-positive, catalase- and oxidase-negative, indole-negative. XV2 strain grows in anaerobic conditions and cannot survive in microaerophilic conditions. Colonies, grown on the surface of TPY agar plate, are white and circular. The diameter of each colony ranges from 0.5 to 1.0 mm. Strain XV2 grows in the temperature range 25–40 °C; no growth occurs at or below 20 °C. Cells grow in the pH range 4.5–9.0. Optimal conditions for growth occur at pH 6.5 and 35 °C. Fermentation profiles of *B. xylocopae* XV2 show that it is able to ferment a narrow range of mono and di-saccharides: D-glucose, D-fructose and D-sucrose. Moreover, the strain hydrolyzes aesculin and salicin, while it displays scarce growth on L-arabinose, D-ribose and arbutin. Positive enzymatic activity is observed for α- and β-galactosidase, β-glucosidase, α-arabinosidase, α-fucosidase, arginine arylamidase, proline arylamidase, leucyl-glycine arylamidase, phenylalanine arylamidase, leucine arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase and serine arylamidase. Whereas, α-glucosidase, *N*-acetyl-β-glucosaminidase and alkaline phosphatase activities are negative (Table 2). The major fatty acids identified are palmitic acid, oleic and linoleic acid plus a mixture of unresolved fatty acids (referred to as summed features 7 in the related Table 3). The peptidoglycan type is A4α L-Lys–D-Asp. The DNA G + C content is 61.3%. The type strain XV2 (=DSM 104955$^T$ = LMG 30142$^T$) and the reference strain XV16 (=DSM 106832 = LMG 30564) were isolated from gut samples of carpenter bees (*X. violacea*). The formal proposal of the new species "*Bifidobacterium xylocopae* sp. nov." is given in Table S4 with the Taxonumber TA00367.

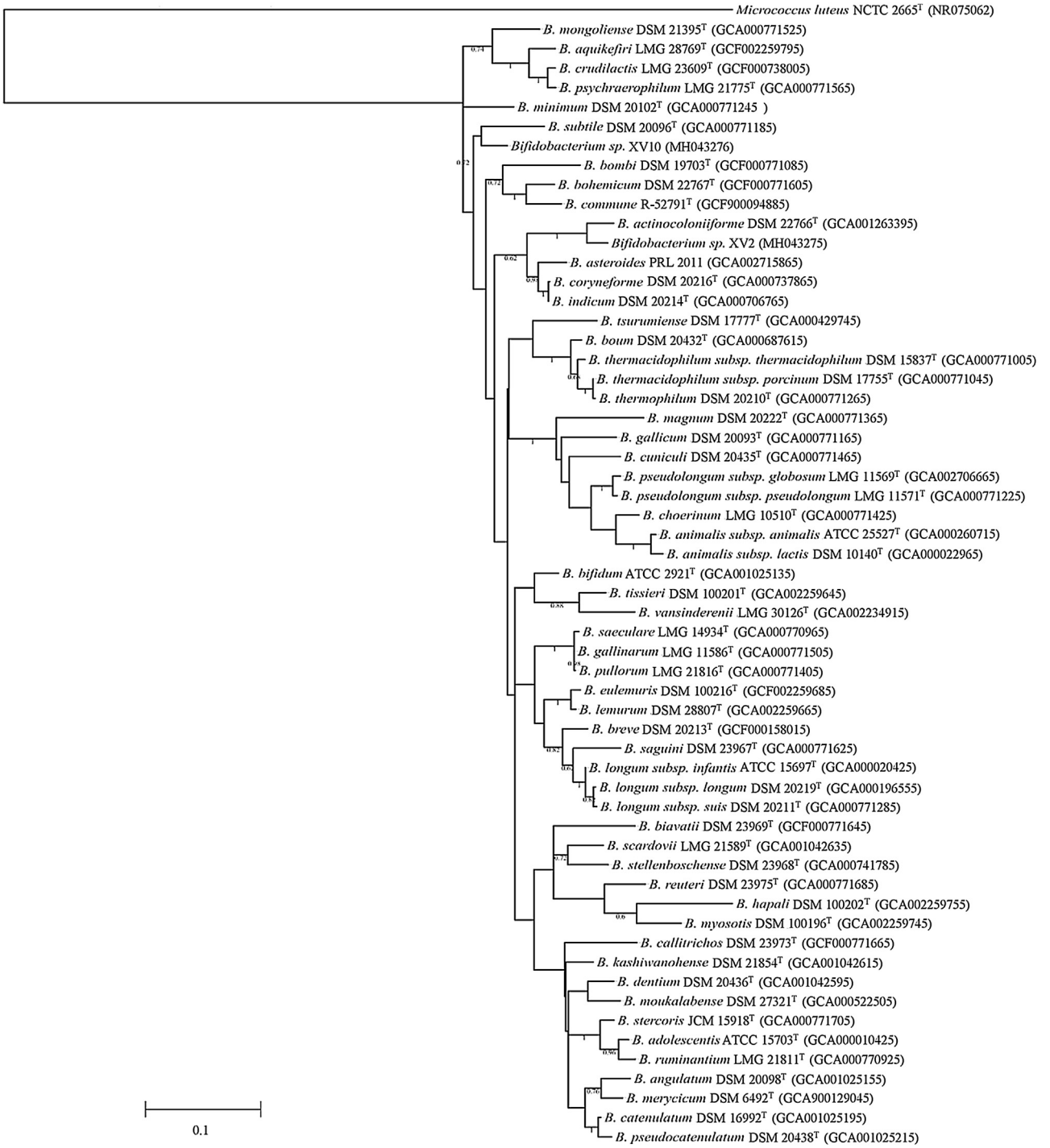


**Fig. 3.** Phylogenetic tree on 16S rRNA gene. 16S rRNA gene complete sequences of recognized *Bifidobacterium* species, *Bifidobacterium* sp. XV2 and *Bifidobacterium* sp. XV10. The analysis involved 58 nucleotide sequences with the *Micrococcus luteus* NCTC 2665$^T$ strain as outgroup.

## Description of *Bifidobacterium aemilianum* sp. nov.

*Bifidobacterium aemilianum* [ae.mi.li.a'num. L. neut. adj. *aemilianum* from the Emilia region, referring to the Italian region where the bacterium was first isolated]. Cells are Gram-positive, non-sporulating, F6PPK-positive, catalase- and oxidase-negative, indole-negative. XV10 strain grows in microaerophilic conditions but it cannot survive in both anaerobic and aerobic conditions. Colonies, grown on the surface of MRS agar plates supplemented with cysteine and fructose, are white and circular. The diameter of each colony ranges from 0.2 to 0.5 mm. Strain XV10 grows in the temperature range 20–40 °C; no growth occurs below 20 °C. The strain grows in the pH range 5.0–9.0. Optimal conditions for growth occur at pH 6.5 and 35 °C. Fermentation profiles of *B. aemilianum* XV10 reveal that it is able to ferment a wide range of mono and di-saccharides: D-ribose, D-xylose, D-glucose, D-fructose D-mannose, D-raffinose and D-sucrose. The strain hydrolyzes aesculin, arbutin and salicin, while it displays scarce growth on L-arabinose, Methyl α-D-glucopyranoside, D-melibiose, inulin, methadone, D-turanose and potassium 5-ketogluconate. Positive enzymatic activity is observed for β-galactosidase, α- and β-glucosidase, α-arabinosidase, *N*-acetyl-β-glucosaminidase, argi-


**Fig. 4.** Phylogenetic tree on *hsp60* gene. *hsp60* gene complete sequences of recognized *Bifidobacterium* species, *Bifidobacterium* sp. XV2 and *Bifidobacterium* sp. XV10. The analysis involved 58 nucleotide sequences with the *Micrococcus luteus* NCTC 2665$^{T}$ strain as outgroup.

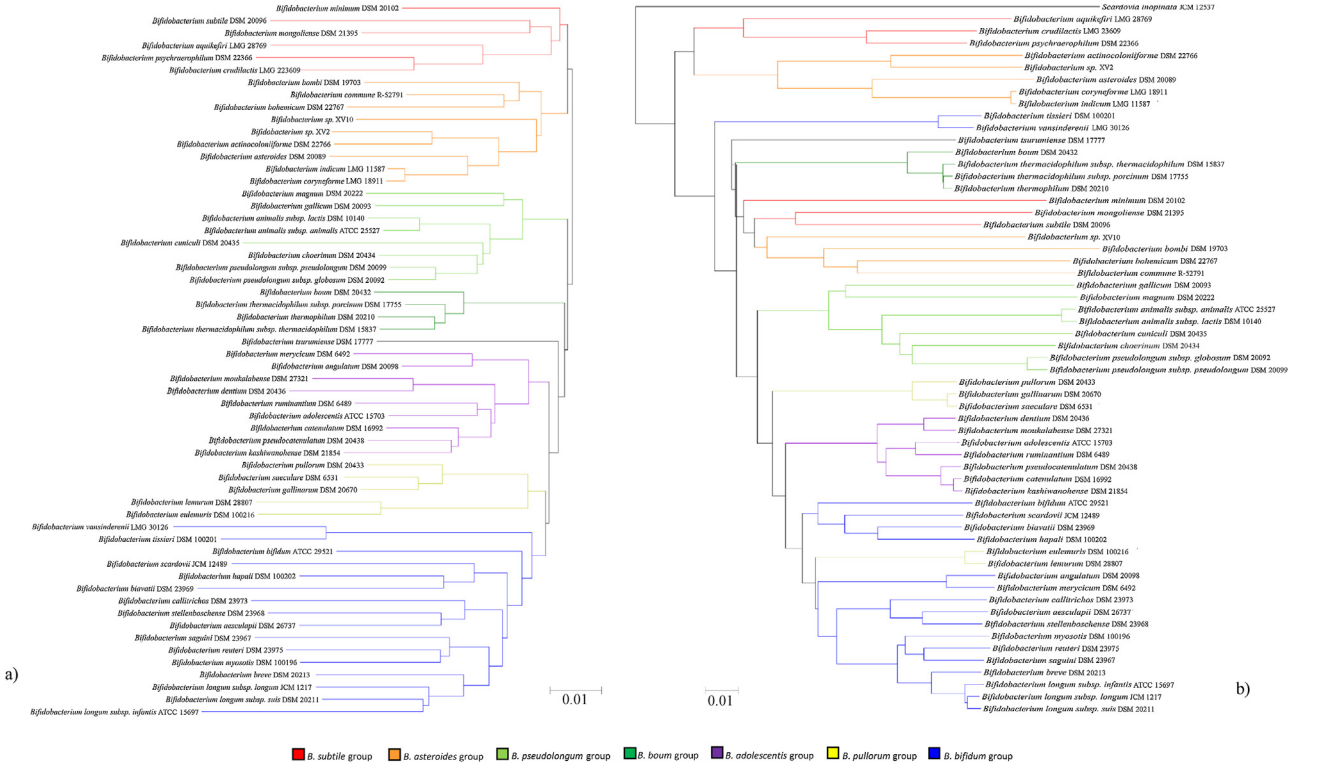


**Fig. 5.** Core- and pan-genome. (a) Phylogenetic tree based on pan-genome analysis. (b) Phylogenetic tree based on the concatenation of 273 core amino acid sequence genes of XV2, XV10 and members of the *Bifidobacterium* genus.

**Table 3**
Cellular fatty acid profiles of insect related *Bifidobacterium*. Strains: (1) *Bifidobacterium* sp. XV2; (2) *Bifidobacterium* sp. XV10; (3) *B. bombi* DSM 19703$^T$; (4) *B. actinocoloniiforme* DSM 22766$^T$; (5) *B. bohemicum* DSM 22767$^T$. Data are from this study and a previous study of Killer et al. [30]. Relative concentrations (%) of total fatty acids were calculated.

| Fatty acid | UPAC name | Common name | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|---|
| C10: 0 | Decanoic acid | Capric acid | 0.11 | 0.06 | – | – | – |
| C12: 0 | Dodecanoic acid | Lauric acid | 0.71 | 0.46 | – | – | – |
| C14: 0 | Tetradecanoic acid | Myristic acid | 1.28 | 1,43 | – | 1.97 | 2.86 |
| C15: 0 | Pentadecanoic acid | Pentadecylic acid | – | – | – | – | – |
| Iso-C15: 0 | 13-Methyltetradecanoic acid | Methylmyristic acid | – | – | – | – | – |
| C16: 0 | Hexadecanoic acid | Palmitic acid | 39.68 | 21.29 | 7.14 | 20.17 | 15.97 |
| C16: 1 ω9c | (7Z)-7-Hexadecenoic acid | *cis*-7-Palmitoleic acid | 1.11 | 0.41 | – | – | – |
| C17: 0 | Heptadecanoic acid | Margaric acid | – | – | 4.21 | 2.11 | 2.56 |
| C17: 1 ω9c | (8Z)-8-Heptadecenoic acid | – | – | 1.10 | – | – | – |
| Cyclo-C17: 0 | *cis*-9,10-Methylene-Hexadecanoic acid | – | 0.13 | – | – | – | – |
| C18: 0 | Octadecanoic acid | Stearic acid | – | 3.85 | 5.91 | 7.05 | 6.56 |
| C18: 1 ω6c | *cis*-12-Oleic acid | – | 1.19 | – | – | – | – |
| C18: 1 ω7c | *cis*-Vaccenic acid | – | 2.58 | 6.6 | – | – | – |
| C18: 1 ω9c | (9Z)-9-Octadecenoic acid | Oleic acid | 19.3 | 57.92 | 7.49 | 9.99 | 4.69 |
| C18: 1 ω9c DMA | (9Z)-1,1-Dimethoxy-9-Octadecene | – | – | 8.47 | – | – | – |
| C18: 2 ω6c | (9Z,12Z)-9,12-Octadecadienoic acid | Linoleic acid | 6.05 | – | 7.34 | – | – |
| C20: 0 | Icosanoic acid | Arachidic acid | – | – | 7.18 | 3.25 | 4.62 |
| Iso-C19: 0 | 17-Methylstearic acid | – | 0.6 | 0.74 | – | – | – |
| C22: 0 | Docosanoic acid | Behenic acid | – | – | 5.87 | – | – |
| C23: 0 | Tricosanoic acid | Tricosylic acid | – | – | 5.38 | – | – |
| C24: 0 | Tetracosanoic acid | Lignoceric acid | – | – | 4.68 | – | – |
| C15: 1 ω$^n$c | ($^n$Z)-$^n$-Pentadecenoic acid | – | – | – | 2.47 | – | – |
| – | Summed features 3a[a] | NA | 0.49 | – | ND | ND | ND |
| – | Summed features 3b[a] | NA | – | 0.84 | ND | ND | ND |
| – | Summed features 7[a] | NA | 26.49 | 1.12 | ND | ND | ND |
| – | Summed features 8[a] | NA | 3.76 | – | ND | ND | ND |
| – | Summed features 10[a] | NA | – | 5.96 | ND | ND | ND |
| – | Summed features 12[a] | NA | – | 0.66 | ND | ND | ND |

[a] Summed features are groups of two or more fatty acids that cannot be separated by GLC (MIDI System). Summed feature 3a contained C16:1 ω6c and/or C16:1 ω7c; Summed feature 3b contained C16:1 ω7c and/or C15:0 ISO 2-OH. Summed feature 7 contained C19:0 CYCLO ω10c and/or C19:1 ω6c; Summed feature 8 contained C18:1 ω7c and/or C18:1 ω6c; Summed feature 10 contained C18:1 ω7c and/or unknown ECL 17.834; Summed feature 12 contained unknown ECL 18.622 and/or iso-C19:0.

nine arylamidase, proline arylamidase, phenylalanine arylamidase, leucine arylamidase, tyrosine arylamidase, glycine arylamidase, histidine arylamidase and serine arylamidase. On the contrary, enzymatic activity is negative for α-galactosidase, α-fucosidase, alkaline phosphatase, leucyl glycine arylamidase, alanine arylamidase (Table 2). The major fatty acids identified are palmitic

acid, oleic and stearic acid and (9Z)-1,1-dimethoxy-9-octadecene (Table 3). The peptidoglycan type is A4α L-Lys–L-Ala with a D-Glu at position 2 substituted by Gly. The DNA G+C content is 61.9%. The type strain XV10 (=DSM 104956$^T$ = LMG 30143$^T$) and the reference strain XV4 (=DSM 106832 = LMG 30565) were isolated from gut samples of carpenter bees (*X. violacea*). The formal proposal of the new species "*Bifidobacterium aemilianum* sp. nov." is given in Table S4 with the Taxonumber TA00369.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.syapm.2018.11.005.

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