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A comparative genomic analysis of *Fructobacillus evanidus* sp. nov. from bumble bees

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ABSTRACT

The increase in studies on bee microbiomes is prompted by concerns over global pollinator declines. Bumble bees host core and non-core microbiota which may contribute to increased lifetime fitness. The presence of *Fructobacillus* in the gut microbiomes of bumble bee workers, or the replacement of core symbionts with *Fructobacillus* bacteria, has been considered a marker of dysbiosis. A phylogenomic analysis and functional genomic characterization of the genomes of 21 *Fructobacillus* isolates from bumble bees demonstrated that they represented four species, i.e. *Fructobacillus cardui*, *Fructobacillus fructosus, Fructobacillus tropaeoli*, and the novel species *Fructobacillus evanidus* sp. nov. Our results confirmed and substantiated the presence of two phylogenetically and functionally distinct *Fructobacillus* species clades that differ in genome size, percentage G + C content, the number of coding DNA sequences and metabolic characteristics. Clade 1 and clade 2 species differed in amino acid and, to a lesser extent, in carbohydrate metabolism, with *F. evanidus* and *F. tropaeoli* genomes featuring a higher number of complete metabolic pathways. While *Fructobacillus* genomes encoded genes that allow adhesion, biofilm formation, antibacterial activity and detoxification, other bacteria isolated from the bumble bee gut appeared better equipped to co-exist with the bumble bee host. The isolation and identification of multiple *Fructobacillus* species from several bumble bee gut samples in the present study also argued against a specific partnership between *Fructobacillus* species and their bumble bee hosts.

Introduction

There is a surge in studies of the bee microbiome that is driven primarily by a growing concern for global bee decline (Potts et al., 2010, Zattara and Aizen, 2021, Ghisbain et al., 2023). This decline could be partly explained by health issues associated to dysbiosis (Engel et al., 2016, Voulgari-Kokota et al., 2019, Rothman et al., 2019). Social bees such as honey bees, bumble bees and stingless bees, host core and non-core microbiota that were reported to contribute to food digestion, detoxification, protection against parasites and pathogens, as well as to the provision of essential nutrients that support host fitness and homeostasis (Zhang and Zheng, 2022). While bacteria are present in the crop, midgut and hindgut of their bee hosts, around 99 % of bacterial colonization occurs in the hindgut where undigested remnants of pollen, sugars and nitrogenous waste products serve as nutrient sources (Zheng et al., 2017, Martinson et al., 2012). Compared to honey bee symbionts, bumble bee symbionts such as *Gilliamella* spp., *Bifidobacterium* spp. and *Lactobacillus sensu lato* have a more restricted polysaccharide-degrading enzyme repertoire, and therefore several roles of bumble bee symbiont bacteria remain speculative (Hammer et al., 2021, Ellegaard et al., 2019, Zheng et al., 2019).

Fructobacillus bacteria are generally considered non-core microbiota

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in social bees, but have been reported in bumble bee (Hammer et al., 2021), honey bee (Filannino et al., 2016) and carpenter bee (Handy et al., 2023) samples including the larval gut (Vojvodic et al., 2013, Rokop et al., 2015), brood cells and bee bread of honey bees (Rokop et al., 2015), honey of stingless bees (Andrade-Velasquez et al., 2023) and they dominated the microbial community in *Brassica rapa* floral nectar (Russell and McFrederick, 2022). Additionally, *Fructobacillus fructosus* has been reported as a core symbiont in another social insect, the Asian hornet (Hettiarachchi et al., 2023). Shifts in the gut microbiomes of bumble bee workers have been reported to be characterized by the replacement of core symbionts by *Fructobacillus* bacteria (Krams et al., 2022, Zhang et al., 2021, Villabona et al., 2023) and such shifts have been considered a form of dysbiosis (Hammer et al., 2021). While some bumble bee workers with *Fructobacillus*-dominated microbiomes have been collected from habitats with higher levels of anthropogenic disturbances which may have provoked gut dysbiosis (Villabona et al., 2023), others were collected in forest meadows, which represent a habitat with the least anthropogenic impact (Krams et al., 2022).

Fructobacilli are fructophilic lactic acid bacteria (FLAB), which highlights their preference for fructose over glucose as a growth substrate. They are obligate FLAB, which implies that they prefer aerobic growth conditions and grow poorly on glucose unless an external electron acceptor such as pyruvate, oxygen or fructose is present (Endo et al., 2018). Although they are heterofermentative lactic acid bacteria, FLAB do not produce ethanol from glucose, because of a complete or partial deletion of the *adhE* gene that encodes a bifunctional alcohol/acetaldehyde dehydrogenase (Endo et al., 2014, Endo et al., 2018). To date, twelve *Fructobacillus* species have been formally named, of which *F. fructosus* is the type species (Endo and Okada, 2008). The latter was first described in 1956 and has been reported in a large variety of sources including insect samples, flowers, fruits and fermented foods (Mesas et al., 2011, Koch and Schmid-Hempel, 2011, Praet et al., 2018, Yaacob et al., 2018, He et al., 2011, Thaochan et al., 2010, Rodriguez et al., 2019, Veron et al., 2017, Antunes et al., 2002, Endo and Okada, 2008). Most other *Fructobacillus* species were described more recently and these too have been isolated from insect samples, flowers, fruits and fermented foods (Endo and Okada, 2008, Leisner et al., 2005, Rodriguez et al., 2019, Antunes et al., 2002, Chambel et al., 2006, Endo et al., 2009, Janashia and Alaux, 2016, Endo et al., 2011, Praet et al., 2016, Snauwaert et al., 2013, Lin et al., 2022, Gallus et al., 2022, Chen et al., 2022, Oliphant et al., 2023).

There is limited information on the diversity and functional role of *Fructobacillus* isolates in their insect hosts. Metagenetic studies based on the analysis of partial 16S rRNA gene fragments generally fail to provide species level discrimination, and several groups of closely related *Fructobacillus* species have virtually identical 16S rRNA gene sequences (Endo et al., 2011, Gallus et al., 2022, Oliphant et al., 2023). A comparative genomic and phylogenetic analysis of publicly available *Fructobacillus* genome sequences showed that these genomes represented two clades that differed considerably in amino acid biosynthetic potential (Mohamed et al., 2023). *F. fructosus* isolates from stingless bees and honey bees exhibited antagonistic activity against several pathogenic bacteria (Yaacob et al., 2018, Zeid et al., 2022). Heptyl 2-methylbutyrate, di-isobutyl phthalate, d-turanose, heptakis (trimethylsilyl), di-isooctyl phthalate, and hyodeoxycholic acid compounds were identified in fractions with biological activity against *Paenibacillus larvae* (Zeid et al., 2022). *F. fructosus* isolates from honey bee gut samples metabolized phenolic acids commonly found in pollen (Filannino et al., 2016) and *Fructobacillus* sp. isolates from honey bee hives promoted growth of honey bee core bacteria (Rokop et al., 2015). In the present study, we generated draft genome sequences of 21 *Fructobacillus* isolates previously isolated from five bumble bee species (Praet et al., 2018) and three flowers (unpublished data) and performed a comprehensive functional genomic analysis.

Materials and methods

Fructobacillus isolates and cultivations conditions

Bombus hypnorum, Bombus lapidarius, Bombus lucorum, Bombus pascuorum and *Bombus terrestris* bumble bees were collected while foraging in 2013 and 2014 in Belgium (Supplementary Table S1) (Praet et al., 2018). Cell suspensions from whole gut samples were prepared and plated on a range of isolation media. A single *Fructobacillus* isolate per bumble bee gut sample was included in the present study unless isolates represented different species. In addition, cell suspensions of a range of flower samples collected in 2021 were plated on De Man, Rogosa and Sharpe (MRS) agar (Oxoid) supplemented with 200 ppm cycloheximide and incubated aerobically at 28 °C for the isolation of LAB. *Fructobacillus* isolates were tentatively identified by means of MALDI-TOF mass spectrometry as described previously (Dumolin et al., 2019). A single isolate from *Calibrachoa* sp., *Papaver* sp. and *Symphytum officinale* flowers was included in the present study (Supplementary Table S1).

DNA extraction, sequence analysis and genome assembly

Genomic DNA of isolates R-53140, R-53653, LMG 30234, LMG 30235, LMG 30237, LMG 32999^T, LMG 33331, LMG 33332, LMG 33333, LMG 33334, LMG 33335, LMG 33336, LMG 33337, LMG 33338, LMG 33339, LMG 33340 was extracted using the Maxwell Tissue DNA kit (Promega, USA) and the Maxwell RSC instrument according to the manufacturer's instructions. Genomic DNA of isolates R-55227, R-82265, R-82291 and R-82641 was extracted using the Maxwell Cultured Cells DNA kit (Promega, USA). Genomic DNA of isolate LMG 30238 was extracted as described by Pitcher et al. (1989). DNA extracts were treated with RNase (2 mg/mL, 5 μL per 100 μL of extract) and incubated at 37 °C for one hour. DNA quality was checked using 1 % agarose gel electrophoresis and DNA quantification was performed using the QuantiFluor ONE dsDNA system and the Quantus fluorometer (Promega, USA). Whole-genome sequencing was conducted using the Illumina NovaSeq 4000 for all isolates, except for the genomes of LMG 30238 and R-53140 which were sequenced using the HiSeq 4000 platform at the Oxford Genomics Centre (Oxford, UK).

A quality check of the reads was performed using FastQC v0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and the results were compiled into a single report using MultiQC 1.9 (Ewels et al., 2016). To remove low-quality sequences, reads were filtered using fastp v0.20.1 (Chen et al., 2018) under default settings. Specifically, reads with a quality score below 20, a length less than 15 nucleotides, or the presence of adapters were systematically excluded from further analysis. De novo assemblies were obtained with Shovill v1.0.4 (https://github.com/tseemann/shovill) (Bankevich et al., 2012) with disabled error correction and default settings. Contigs shorter than 500 bp were removed from the final assemblies. Reads were mapped to the assemblies using BWA v0.7.17 (Li and Durbin, 2009) and the resulting summary statistics, including mapped reads and coverage, were calculated with SAMtools v1.11 (Li et al., 2009) and Qualimap v2.2.1 (Okonechnikov et al., 2016).

Genome assemblies of the type strain of each established *Fructobacillus* species were downloaded from the NCBI database (February 20, 2023) by using the E-utilities Command (Kans, 2022) (Supplementary Table S2).

CheckM v1.2.2 was used to estimate genome completeness and contamination (Parks et al., 2015). The percentage G + C content and genome size were calculated using QUAST v5.0.2 (Gurevich et al., 2013). The 16S rRNA gene sequences were extracted from the draft genomes using the BAsic Rapid Ribosomal RNA Predictor software (Barrnap) (https://github.com/tseemann/barrnap) and were submitted to the EzBiocloud identification tool (Yoon et al., 2017a).

Phylogenomic analysis

The whole-genome sequences of the 21 *Fructobacillus* isolates and the 12 *Fructobacillus* type strains were used to construct a phylogenomic tree based on 107 single-copy genes using bcgTree (Ankenbrand and Keller, 2016) and a partitioned maximum-likelihood analysis in RAxML v8.2.12 (Kozlov et al., 2019). *Convivina intestini* LMG 28291^T and *Leuconostoc mesenteroides* ATCC 8293^T were used as outgroup taxa (Supplementary Table S2). Visualization and annotation of the tree were performed using iTOL (Letunic and Bork, 2019). To verify taxonomy, genomes were submitted to the Type Strain Genome Server (TYGS) (Meier-Kolthoff et al., 2022) and digital DNA-DNA hybridization (dDDH, formula d4) values were calculated using the Genome-to-Genome Distance Calculator (GGDC 2.1) with recommended settings (Meier-Kolthoff et al., 2013). Average Nucleotide Identity (ANI) values were calculated by using the OrthoANIu algorithm using the standalone tool from EzBioCloud (Yoon et al., 2017b).

Ecological analysis

The 16S rRNA gene sequence and the genome assembly of LMG 32999^T were analyzed using the Protologger tool V1.0 in quick mode. The 16S rRNA gene sequence was compared to sequences in 19,000 amplicon sequencing datasets and the genome assembly was compared with more than 49,000 metagenome-assembled genomes (Hitch et al., 2021).

Annotation

Prokka v1.14.5 was used to perform structural annotation of all *Fructobacillus* genomes (Seemann, 2014). The protein-coding DNA sequences (CDS) predicted by Prokka were annotated using eggNOG-mapper v2.1.9 and the eggNOG database v5.0 (Huerta-Cepas et al., 2019, Cantalapiedra et al., 2021, Buchfink et al., 2021). If applicable, each CDS was assigned to its respective KEGG orthology (Kanehisa and Sato, 2020, Kanehisa et al., 2022), KEGG module, KEGG reaction, KEGG pathway and Pfam. KEGG annotation was used for the calculation of the KEGG module completeness fraction (mcf) by using the KO_mapper script from MicrobeAnnotator (Ruiz-Perez et al., 2021). MicrobeAnnotator was updated to use the latest KEGG module definitions (June 2023). KEGGREST V1.38.0 was used to determine the reactions from the KEGG numbers for the identification of carbohydrate-utilizing enzymes and dbCAN3 was used for the identification of glycoside hydrolases (GHs) (Zheng et al., 2023). Blast + v2.14.1 (Camacho et al., 2009) was used to annotate *Fructobacillus* mannitol dehydrogenase genes. PlasmidHunter was used to annotate plasmids (Tian and Imanian, 2023) and Phastest was used to annotate phages (Wishart et al., 2023). AntiSMASH web version (February 2024) was used to detect putative bacteriocins in defined clusters (Blin et al., 2023). Finally, Anvi'o was employed to conduct a comparative genomics analysis of the novel species with a Markov clustering algorithm threshold set at 10 (Eren et al., 2021).

Data analysis

EggNOG-mapper, MicrobeAnnotator, dbCAN3, ANIu and dDDH results were imported in R 4.2.3 and analyzed using tidyverse, imputeTS, matrixStats, ggnewscale, KEGGREST, venn, and ComplexHeatmap packages.

Phenotypic tests

Phenotypic characteristics were determined for *Fructobacillus evanidus* sp. nov. LMG 32999^T. The temperature range for growth was examined aerobically and anaerobically in MRS medium at temperatures ranging from 4 °C to 45 °C (including 4, 15, 20, 28, 35, 37, 40, and 45 °C). The tolerance to NaCl was investigated under anaerobic and aerobic conditions by supplementing MRS broth with different concentrations of NaCl, ranging from 0 % to 10 %, in 1 % intervals. Similarly, the tolerance to pH was evaluated anaerobically and aerobically in MRS broth at pH 4.0 to 9.0, with intervals of 1 pH unit, using acetate buffer (pH 4.0 – 5.0), phosphate buffer (pH 6.0 – 8.0), and Tris-HCl (pH 9.0). Catalase and oxidase activity were verified using conventional procedures (MacFaddin, 2000). Fructophilic growth characteristics were investigated by growth on MRS without glucose supplemented with 2 % D-fructose in aerobic and anaerobic conditions. A slimy phenotype was evaluated on MRS agar supplemented with 20 % sucrose under aerobic conditions at 28 °C. Growth with 20 g/l chitin, D-fructose, D-glucose, D-melibiose, D-ribose, dextrin, galactose, sorbitol, sucrose and xylitol in MRS broth without glucose was measured after 0, 1, 2, 3, 4 and 7 days of incubation at 28 °C under aerobic and anaerobic conditions by measuring the optical density at 600 nm using a spectrophotometer (Tecan, Männedorf, Switzerland).

Lactate, acetate, gluconate, D-glucose, D-fructose, mannitol, sorbitol, and ethanol concentrations were determined after 4 days of incubation at 28 °C in MRS broth containing 20 g/l of D-glucose or D-fructose. To ensure accurate measurement of acetate, sodium acetate was excluded from the MRS media to prevent interference. For the determination of lactate, acetate, and gluconate, a solution was prepared by combining 100 µL of the sample, 400 µL of MQ water, and 500 µL of acetonitrile. Subsequently, the solution was centrifuged at 4 °C and 16,000 rpm for 10 min. These three organic acids were quantified using a Prominence LC2030C Plus 3D RoHS high-performance liquid chromatography system (Shimadzu) equipped with a photodiode array detector and an Aminex HPX-87H column (Bio-Rad, USA). The concentrations of D-glucose and D-fructose were determined using a Dionex ICS3000 high-performance anion exchange chromatography system (Thermo Fisher). Briefly, 100 µL of the sample was added to 900 µL of a deproteinization solution (25 % acetonitrile) containing 0.011328 g/L rhamnose as the internal standard. After centrifugation (10 min at 16,000 rpm at 4 °C), 50 µL of the supernatant was added to 950 µL of MQ water and centrifuged again. Then, 10 µL of this dilution was injected and separated in a CarboPac PA100 guard column (50 mm x 4 mm) followed by a PA100 analytical column (250 mm x 4 mm) at 30 °C. The eluent consisted of a gradient of 84 mM NaOH in ultrapure water (eluent A) and 84 mM NaOH with 250 mM sodium acetate (eluent B). An external standard curve was used for quantification. The concentrations of mannitol and sorbitol were determined using the same Dionex ICS3000 system. Samples were prepared by adding 100 µL of sample to 900 µL of a deproteinization solution. After centrifugation (10 min at 16,000 rpm at 4 °C), 100 µL of the supernatant was added to 900 µL of MQ water and centrifuged again. Then, 10 µL of this dilution was injected and separated in a CarboPac MA1 guard column (50 mm x 4 mm) followed by a MA1 analytical column (250 mm x 4 mm) at 30 °C. The eluent consisted of a gradient of 84 mM NaOH in ultrapure water (eluent A) and 1,000 mM NaOH (eluent B). Ethanol accumulation was assessed by filtering 45 mL of the sample and centrifuging it at 4 °C and 4200 rpm for 10 min. Subsequently, quantification of the sample was performed utilizing an Alcolyzer Plus (Anton Paar). Triplicate experiments were conducted to evaluate the production of these compounds as well as growth characteristics in different sugars, pH and concentrations of NaCl.

Results

Genomic characteristics

The characteristics of the 21 new *Fructobacillus* genomes determined in the present study are summarized in Supplementary Table S3. These genomes resulted in assemblies of 14 to 32 contigs. CheckM analysis revealed 98–99 % completeness and 0–0.02 % contamination using the *Lactobacillales* marker set. The genome size ranged from 1.3 Mb to 1.8 Mb. The percentage G + C content varied between 43.8 % and 45.0 % and the number of CDS varied from 1328 to 1677 (Supplementary

Figure S1).

Phylogenomic analysis

A phylogenomic analysis based on 107 single-copy core genes confirmed that the 21 isolates belong to the genus *Fructobacillus* (Fig. 1). The 21 genomes were distributed across four clusters. A first cluster of six bumble bee isolates grouped with the *F. fructosus* type strain within clade 1 *sensu* Mohamed et al. (2023). The remaining 15 isolates formed three clusters within clade 2 (Mohamed et al., 2023): four bumble bee isolates grouped with the *F. tropaeoli* type strain; three flower isolates and a single bumble bee isolate grouped with the *F. cardui* type strain; and seven bumble bee isolates represented a fourth cluster.

OrthoANIu and dDDH values were calculated between each pair of *Fructobacillus* genomes, including those of the 12 established type strains (Supplementary Figure S2). The four clusters described above each consisted of isolates that shared ANIu and dDDH values above the species delineation thresholds of 70 % dDDH (Meier-Kolthoff et al., 2013) and 95–96 % OrthoANIu (Chun et al., 2018), and therefore cluster 1, 2 and 3 isolates were identified as *F. fructosus*, *F. tropaeoli*, and *F. cardui*, respectively. Additionally, the dDDH and ANIu values between isolates of different clusters, or between isolates and type strains of other established *Fructobacillus* species were below or at the border of the species delineation thresholds, thus demonstrating that cluster 4 isolates represented a novel *Fructobacillus* species, for which we propose the name *Fructobacillus evanidus* sp. nov. below (Table 1).

The use of Protologger revealed no metagenome-assembled genomes corresponding with the *F. evanidus* LMG 32999^T genome. The 16S rRNA sequence strain of LMG 32999^T matched with 9.50 % of insect gut 16S rRNA amplicon data sets at a relative abundance of 1.36 %.

Core and accessory genomes of F. evanidus sp. nov.

The pangenome of *F. evanidus* consisted of 1670 gene clusters, which included a species core set of 1481 gene clusters (see Supplementary Figure S3). The genomes of strains LMG 32999^T and LMG 33336 contained 22 and 24 unique gene clusters, respectively, while the genomes of the remaining strains contained 3, 1 or none. The unique gene clusters of strain LMG 33336 corresponded to a multidrug resistance efflux pump EmrA (EmrA) exporter, a lysozyme [EC:3.2.1.17] (GH24), a gene cluster with a clfB domain and a bacteriocin/lantibiotic (K20344) gene; with the remaining being uncharacterized. Similarly, the unique gene clusters of strain LMG 32999^T consisted of three copies of glycosyltransferase (COG0438) (Supplementary Figure S3), while the rest remained unidentified. The unique annotated gene clusters for both strains were localized on contigs corresponding to plasmid regions. Strains LMG 33338 and LMG 33335 possessed 3 and 1 unique gene clusters with unknown functions, respectively, while strains LMG 33340, LMG 33337 and LMG 33339 lacked unique gene clusters. They each had unique combinations of accessory gene clusters. Within the accessory genes, 40 % were associated with phage genes, while the majority of the remaining genes were uncharacterized.

Metabolism

An analysis of metabolic pathways in KEGG revealed species-specific patterns and varying levels of completeness in several metabolic pathways (Fig. 2). All genes for the oxidative branch of the pentose phosphate pathway (M00006) were present, while the glycolysis (Embden-Meyerhof-Parnas), the oxidative phase of the pentose phosphate and the Entner-Doudoroff pathway were incomplete. All phosphoketolase

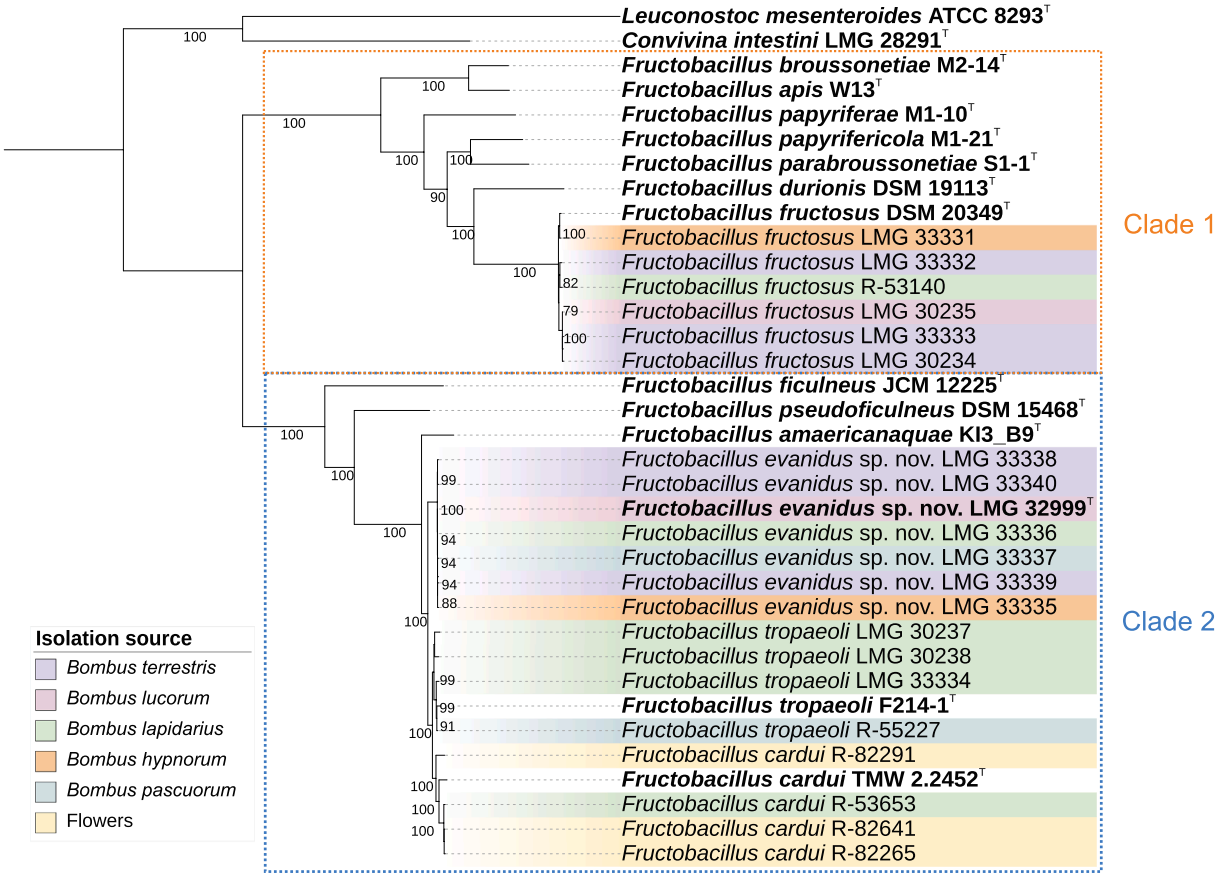

Fig. 1. Maximum likelihood bcgTree tree based on 107 core genes showing the phylogenomic relationships among *Fructobacillus* isolates, with *Convivina intestini* LMG 28291^T and *Leuconostoc mesenteroides* ATCC 8293^T as outgroup taxa. Bootstrap percentage (1000 replicates) are shown next to the branch points. The isolation sources are demarcated by a distinct background color. Clades 1 and 2 are as discussed in reference (Mohamed et al., 2023).

Table 1
Description of *Fructobacillus evanidus* sp. nov.

Guiding Code for Nomenclature [req]	**ICNP**
Nature of the type material [req]	Strain
Genus name [req]	*Fructobacillus*
Species name [req]	*Fructobacillus evanidus*
Specific epithet [req]	*evanidus*
Species status [req]	sp. nov.
Species etymology [req]	e.va'ni.dus. L. masc. adj. evanidus, vanishing, passing away, referring to its occurrence as a non-core microbiont in bumble bees.
Designation of the Type Strain [req under ICNP]	LMG 32999^T
Strain Collection Numbers [req under ICNP]	LMG 32999^T = CECT 30959^T
Designated Genome, MAG or SAG [req under SeqCode]	–
Type Genome, MAG or SAG accession Nr. [INSDC databases] [req under SeqCode]	GCA_963580165
Access to raw data (e.g. SRA accession) [opt under SeqCode]	ERZ21847234
Registry number [req under SeqCode]	
Genome status [opt]	incomplete
Genome size [opt]	1,728 kb
GC mol% [opt]	44.2
16S rRNA gene accession nr. [req]	OY759151
Description of the new taxon and diagnostic traits [req]	Gram-positive. Rod-shaped cells, 1–2 long μm and about 1 μm wide. Rods can be short or elongated and can occur in chains of 2 cells. Colonies on MRS medium are round, low convex and approximately 1 mm. Aerobic and anaerobic growth on MRS occurs at 15–40 °C. Grows at pH 4 to 9 anaerobically and at pH 4 to 8 aerobically. Grows on MRS broth and NaCl concentration of 0 % to 9 % in aerobic conditions; after prolonged incubation up to 7 days weak growth is obtained at 10 % NaCl. Weak growth on MRS broth and NaCl concentration of 0 % to 10 % is obtained under anaerobic conditions. Catalase and oxidase negative. Growth on MRS agar supplemented with 2 % fructose occurs in both aerobic and anaerobic conditions. Growth on MRS with D-glucose as single carbon source under aerobic conditions and weak growth with D-glucose under anaerobic conditions. Weak growth on MRS with galactose as single carbon source under aerobic and anaerobic conditions. No growth on chitin, D-cellobiose, D-melibiose, D-ribose, dextrin, sorbitol, sucrose or xylitol. No slimy phenotype on MRS agar supplemented with 20 % sucrose under aerobic conditions.
Country of origin [opt]	Belgium
Region of origin [opt]	Flanders (Ghent)
Date of isolation (dd/mm/yyyy) [opt]	2014
Source of isolation [opt]	The gut of a *Bombus lucorum* bumble bee
Sampling date (dd/mm/yyyy) [opt]	19/08/2013
Latitude (xx°xx′xx″N/S) [opt]	51° 0′ 30.96″ N
Longitude (xx°xx′xx″E/W) [opt]	3° 53′ 56.76″ E
Altitude (meters above sea level) [opt]	–
Number of strains in study [opt]	Seven
Source of isolation of non-type strains [opt]	*Bombus hypnorum*, *Bombus lapidarius*, *Bombus pascuorum* and *Bombus terrestris* gut samples
Information related to the Nagoya Protocol [req]	Flanders gives free access to genetic resources

pathway genes (heterolactic fermentation) were present, with the exception of *adhE* or *aldh* genes, implying a lack of ethanol production. *F. evanidus* genomes encoded genes for the production of acetate, D-lactate and L-lactate, and diacetyl and butane-2,3-diol. Differences in gene presence related to carbohydrate metabolism were observed in *F. evanidus*, *F. tropaeoli*, *F. cardui*, and *F. fructosus* genomes (Supplementary Figure S4). All *Fructobacillus* genomes encoded genes for the utilization of fructose, ribose, glucose, cellobiose and galactose. Fructose, ribose, and glucose are metabolized into fructose-6P, ribose-5P, and glucose-6P, respectively, through the phosphoketolase pathway. Cellobiose is transported into the cell via the phosphotransferase system, while galactose is enzymatically converted to α-D-galactose (K01785) as part of galactose metabolism. Some *F. fructosus* and *F. evanidus* genomes encoded a higher number of copies of the levansucrase gene. The GH66 gene (EC:3.2.1.11) was detected in five out of seven *F. evanidus* strains indicating the capacity to convert glucans to isomaltose. Endo-β1,4-galactanase (EC 3.2.1.89) (GH53) genes were found in four out of five *F. tropaeoli* genomes and *F. cardui* R-82265. Additionally, some *F. tropaeoli* genomes comprised pullulanase genes (GH13_14), which encode the hydrolysis of α-1,6-linked branches in starch-derived glucans. Four out of five *F. cardui* genomes contained the GH65 gene (EC:2.4.1.64), suggesting the ability to utilize trehalose and convert it into glucose. Finally, *Fructobacillus* genomes harbored hydrolase genes with lysozyme-like activity (i.e., GH73, GH25, and GH24) (Supplementary Figure S4); part of the GH25 genes were carried by phages (Supplementary Figure S3).

The presence of a mannitol dehydrogenase (*mdh*) gene was verified through a blastp search, in which all predicted protein sequences from *Fructobacillus* genomes were compared against a set of three mannitol dehydrogenase protein sequences from the *Lactobacillaceae* family (Supplementary Table S4). A gene with 84 % to 88 % amino acid identity towards the *Lactobacillaceae mdh* sequences was detected in each *Fructobacillus* genome. These gene sequences were functionally annotated as alcohol dehydrogenase with ADH_ZINC and GroES-like domains by eggNOG-mapper and were located adjacent to a putative fructose permease gene (*fruP*), together forming an operon for fructose utilization.

In the context of energy metabolism, *Fructobacillus* genomes lacked cytochrome genes. NADH dehydrogenase type II (EC: 1.6.5.9) was detected in *Fructobacillus* clade 2 genomes only, while H_2O-forming NADH oxidase (*nox*) (EC: 1.6.3.4) and NADH peroxidase (*npr*) (EC: 1.11.1.1) genes were detected in all examined *Fructobacillus* genomes.

The amino acid and vitamin metabolism exhibited pronounced variation among *Fructobacillus* species (Fig. 2), and between clade 1 and 2 species. *F. evanidus* and *F. tropaeoli* exhibited a higher number of complete pathways and encoded all genes for the biosynthesis of eight amino acids, while *F. cardui* genomes encoded genes for the biosynthesis of at least six amino acids. In clade 1, *F. fructosus* genomes encoded genes for two to four amino acids, while other members of clade 1 did not encode any complete amino acid modules (e.g., *F. papyriferae* and *F. parabroussonetiae*). Glutamine synthase (EC: 6.3.1.2) was identified in all *Fructobacillus* genomes.

Extracellular interaction

Five genes involved in surface adhesion and biofilm formation were identified in the *Fructobacillus* genomes. Multiple copies of the periplasmic zinc transport protein (*ZnuA*) gene were present in all *Fructobacillus* genomes while a putative adhesin gene (pfam 4097) was present in the majority of strains (Supplementary Figure S5). The genomes of *F. cardui, F. fructosus*, and *F. tropaeoli* encoded a surface-anchored protein gene that contains a collagen binding domain (pfam05737). in *Fructobacillus* clade 1, a higher copy number of this gene was observed, except in *F. fructosus*. Multiple copies of LysM (lysin motif) domain-containing genes and arabinose efflux permease (*AraJ*) genes were detected in all *Fructobacillus* genomes. A single gene cluster related to

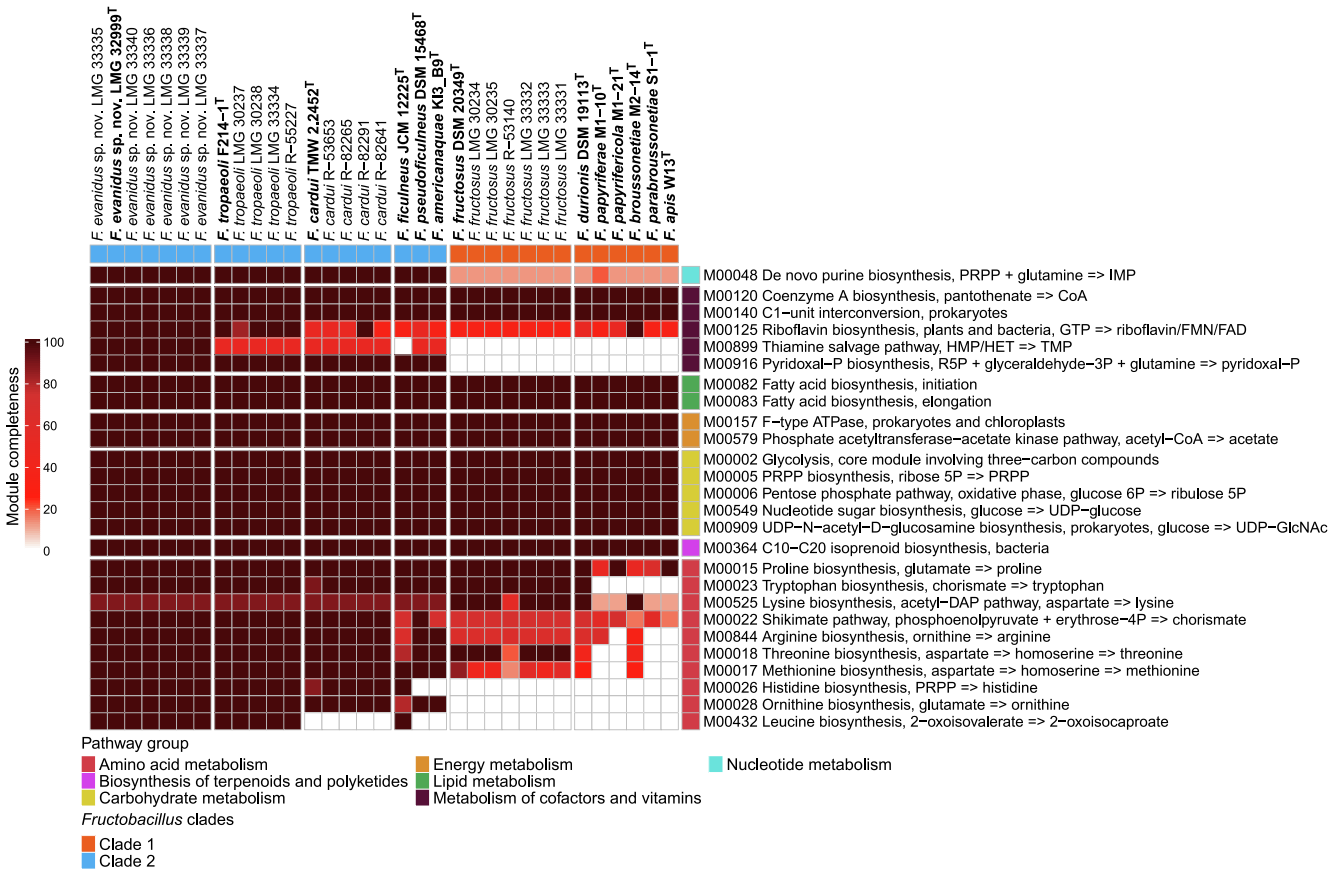

Fig. 2. Heatmap illustrating the level of completeness of KEGG metabolic modules annotated by MicrobeAnnotator, based on the presence and absence of genes. Modules that were 100% complete in at least one genome were included. The color scale ranges from 0 to 100, indicating the percentage of module completeness. Dark colors represent complete or highly complete modules; white and light colors refer to genomes in which a module is absent or highly incomplete.

bacteriocin gene clusters was identified in *F. evanidus* LMG 33336, *F. tropaeoli* LMG 33334 and in all *F. fructosus* strains. This gene cluster was identified as lactococcin G bacteriocin/lantibiotic exporter (K20344) (Supplementary Table S3).

Detoxification

Genes that contribute to tolerance to toxins (i.e., fusaric acid), nitroaromatics, and heavy metals (i.e. cadmium, selenium, cobalt, and manganese) were detected in all *Fructobacillus* genomes (Supplementary Figure S5). The nickel/cobalt efflux system (*hoxN*) and arsenate reductase (*arsC*) genes were identified in the genomes of *F. cardui*, *F. tropaeoli*, and *F. evanidus* (clade 2). The *arsC* and *arsR* (arsenic resistance transcriptional regulator) protein genes are responsible for the reduction of arsenate to arsenite; however, none of the genomes encoded a complete *arsRBC* operon.

Physiological characterization of F. evanidus LMG 32999^T

Strain LMG 32999^T grew well with glucose or fructose, but not with sucrose, D-ribose, D-cellobiose, D-melibiose, sorbitol, xylitol, chitin or dextrin, as sole carbon source. Growth with glucose or fructose as sole carbon source under anaerobic conditions was weaker than in aerobic conditions (Supplementary Figure S6). Weak growth was observed with galactose as the sole carbon source, especially in aerobic conditions (Supplementary Figure S6). Glucose or fructose fermentation by strain LMG 32999^T yielded primarily lactate and acetate, but no ethanol (Supplementary Table S5); mannitol was produced in the presence of fructose as the sole carbon source. The remaining phenotypic characteristics are summarized in Table 1.

Discussion

The genome analyses of 21 *Fructobacillus* isolates from different species of bumble bees and flowers allowed to classify them into three established (*F. tropaeoli*, *F. cardui* and *F. fructosus*) and one novel species (*F. evanidus* sp. nov.). Bumble bees that yielded isolates of this novel species were collected in 2013 and 2014 and represented five species, i.e. *B. lucorum, B. hypnorum, B. terrestris, B. lapidarius,* and *B. pascuorum* (Supplementary Table S1) which were caught at three locations in or near (15 km) the city of Ghent (Belgium) (location and coordinates were provided in Supplementary Table S1). None of these isolates shared 99.99 % OrthoANIu values suggesting they were non-clonal (Konstantinidis, 2023). While two isolates (LMG 33335 [from a *B. hypnorum* bumble bee caught on August 23, 2013 in Wetteren] and LMG 33339 [from a *B. terrestris* bumble bee caught on August 20, 2013 in Ghent]) shared an OrthoANIu value of 99.98 %, and while other pairs or sets of isolates were obtained from the same bumble bee species (Supplementary Table S1), they consistently had either unique gene clusters or unique combinations of gene clusters precluding clonal relationships (Supplementary Figure S3). Clade 1 *Fructobacillus* species which included *F. fructosus* generally had a smaller genome size (1.21 kb-1.50 kb), a smaller number of CDS (1184–1466) and a higher percentage G + C content (48.47 %-44.55 %) (Supplementary Figure S1). In contrast, clade 2 species which included *F. tropaeoli, F. cardui* and *F. evanidus* had genome sizes ranging from 1.55 to 1.78 kb, numbers of CDS varying between 1486 and 1677, and G + C percentages ranging between 43.64 % and 44.43 %. These genomic characteristics therefore confirmed the presence of two principal and distinct species clades in the genus *Fructobacillus* (Mohamed et al., 2023).

Fructobacillus amplicon sequence variants (ASVs) are commonly

(9.50 %) detected in low relative abundances (<2%) in insect gut 16S rRNA amplicon data sets, yet they are rarely detected -or discussed- in cultivation-independent studies of the bumble bee gut microbiota (Hammer et al., 2023); and when detected, ASVs are typically reported with an abundance of less than 1 % (Kwong et al., 2017, Praet et al., 2018, Zhang et al., 2021). A reanalysis of ASV sequences from earlier studies (data not shown) revealed *Fructobacillus* ASVs that were 100 % identical to 16S rRNA gene sequences of *Fructobacillus* clade 2 species in 4 % of *Bombus impatiens* samples in the USA (Powell et al., 2016a), 99.3 % identical to 16S rRNA gene sequences of *F. fructosus* in 3.3 % of *Bombus lepidus* samples from three provinces in China (Zhang et al., 2021), and 99.2 % identical to 16S rRNA gene sequences of *F. fructosus* in up to 60 % of *Bombus terrestris* samples from Latvia (Krams et al., 2022). Occasional dominance of bumble bee microbiota reads by *Fructobacillus* ASVs has been attributed to a decrease of normal bacterial symbionts and has been considered a marker of dysbiosis (Hammer et al., 2021). The isolation and identification of multiple *Fructobacillus* species from gut samples of several bumble bee species in the present study further argues against a specific partnership between *Fructobacillus* species and their bumble bee hosts and suggests that they are randomly picked up when foraging on different flower species.

The presence of carbohydrate-active enzyme genes in *Fructobacillus* genomes revealed some differences in the type of genes and the number of gene copies between species (Supplementary Figure S4). The presence of GH53 genes in some *F. tropaeoli* strains suggested a potential role in pectin degradation, while hydrolase genes with lysozyme-like activity (e.g., GH73, GH25, and GH24) may be involved in antibacterial activity and peptidoglycan degradation (Filannino et al., 2019). *F. evanidus* genomes encoded genes for the utilization of both monosaccharides and disaccharides, but growth tests revealed good growth only with the nectar monosaccharides glucose and fructose, and not with sucrose, ribose or cellobiose (Supplementary Figure S6). Weak growth was observed with galactose, which is toxic to bees (Barker, 1977). *Fructobacillus* species therefore appear to play a minor role in polysaccharide digestion, as reported for other lactic acid bacteria such as *Lactobacillus* and *Bombilactobacillus* (Zheng et al., 2019). The bacteria primarily responsible for degrading hemicellulose and pectin are *Bifidobacterium* and *Gilliamella*, whereas symbionts like *Commensalibacter* species encode genes for the digestion of less common sugars (Zheng et al., 2019, Botero et al., 2023).

The physiological characteristics observed in *F. evanidus* LMG 32999^T resembled those exhibited by other *Fructobacillus* species (Chen et al., 2022, Endo et al., 2011, Gallus et al., 2022, Oliphant et al., 2023, Endo and Okada, 2008, Lin et al., 2022). Strain LMG 32999^T utilized both oxygen and fructose as electron acceptors, as evidenced by its increased growth under aerobic conditions and its exclusive production of mannitol in fructose-containing media. The absence of cytochrome genes and the presence of dehydrogenases in *Fructobacillus* species have been reported previously (Endo et al., 2015, Endo et al., 2018, Mohamed et al., 2023). Four dehydrogenase genes, i.e. NADH dehydrogenase type II, *nox*, *npr*, and *mdh*, contribute to the regeneration of NAD^+ during heterolactic fermentation in *Fructobacillus* species. The *nox* enzyme serves as a water-forming NADH oxidase, reducing oxygen to water (Doi, 2019), while the *npr* enzyme functions as an NADH peroxidase, reducing hydrogen peroxide to water (Naraki et al., 2020). The gene encoding mannitol dehydrogenase (*mdh*) responsible for the conversion of fructose to mannitol exhibited domain characteristics shared with the *Lactobacillaceae mdh* protein sequences and with the putative *F. tropaeoli mdh* gene reported by Ruiz Rodriguez et al (2020).

Differences in amino acid and vitamin metabolism were noted among *Fructobacillus* species, with clade 2 retaining a higher number of genes in these pathways (Fig. 2). Bacteria can retain a larger set of complete metabolic pathways for amino acid biosynthesis when they inhabit environments where amino acids are scarce (Parish et al., 2022). The differences in amino acid metabolism between clade 1 (i.e. *F. fructosus*) and clade 2 (i.e. *F. tropaeoli*, *F. cardui*, and *F. evanidus*) species detected in the present study are striking, given that these species were all isolated from bumble bee gut samples. *F. evanidus* and *F. tropaeoli* appeared to be the most independent in terms of amino acid metabolism, whereas the lack of most amino acid biosynthetic pathways in *F. fructosus* and other clade 1 species implied their reliance on amino acids, proteins, and peptides sourced from the host or other microorganisms. Fructobacilli likely utilize ammonia from host waste that enters the hindgut to synthesize essential amino acids, as they encode the glutamine synthase gene (EC: 6.3.1.2). This enzyme plays a crucial role in recycling nitrogen waste products by converting ammonia into glutamine. Species of neither clades employ uric acid, as their genomes lacked genes associated with purine degradation (M00546).

F. evanidus and *F. tropaeoli* genomes encoded biosynthesis genes for eight amino acids, including six of the eight essential amino acids for insects (arginine, histidine, leucine, methionine, threonine and tryptophan), which may contribute to host fitness (Paoli et al., 2014, Stabler et al., 2015). However, when comparing this number of pathways to those in other bacteria isolated from bumble bees, it is evident that *Fructobacillus* encodes fewer amino acid biosynthesis pathways than *Gilliamella*, *Snodgrassella*, *Apibacter*, *Bifidobacterium*, *Bartonella* and *Commensalibacter* (Praet et al., 2016, Zheng et al., 2019). Furthermore, *Snodgrassella*, *Gilliamella*, and *Commensalibacter* not only encode genes for the utilization of host-derived ammonia but also for urea and uric acid (Li et al., 2022, Botero et al., 2023). Therefore, compared to other bacteria isolated from bees, the nutritional contribution of *Fructobacillus* to its host is likely not considerable.

The hindgut of bumble bees represents an environment characterized by a limited spectrum of metabolites, in which bacteria may encounter expulsion, be exposed to toxic substances, or be subjected to microbial assaults (Hammer et al., 2021). Bacteria use extracellular components to actively engage with their environment, interacting with host cells and other bacteria. Important factors in these interactions include the ability of bacteria to stick to surfaces and the formation of biofilms (Powell et al., 2016b). Five genes identified in *Fructobacillus* genomes encode extracellular components (pfam05737, LysM, DUF4097, *znuA* and *araJ*). Pfam05737 collagen-binding domain and LysM domain-containing genes contribute to the adhesion to matrix molecules (Kang et al., 2013); *znuA* enhances adhesion to epithelial cells (Gabbianelli et al., 2011); and *araJ* and levansucrase genes are involved in oligosaccharide production and biofilm formation (Endo et al., 2015, Alav et al., 2018). Genes encoding three of these proteins, i.e. LysM domain protein, *araJ*, and DUF4097 putative adhesin, have also been reported in genomes from other bee symbiont lactic acid bacteria (Butler et al., 2013, Martinson et al., 2014, Ellegaard et al., 2019). These findings suggest that the presence of adhesion and biofilm formation genes in *Fructobacillus* enables them to adhere to host epithelial cells. Finally, two bacteriocin gene clusters were earlier reported in *Fructobacillus durionis* (Mohamed et al., 2023). One of these, i.e. the lactococcin G gene cluster, was detected in the present study in several *F. evanidus*, *F. tropaeoli* and *F. fructosus* strains as well (Supplementary Table S3).

The presence of detoxification genes in all *Fructobacillus* genomes (Supplementary Figure S5) suggested that these bacteria can neutralize harmful substances. Various environmental toxins including cadmium, copper, hydrogen peroxide, and selenate can disturb the composition of the bumble bee microbiome. For instance, microbiome analyses showed that read abundances of *Serratia*, *Bombiscardovia*, *Commensalibacter* and *Lactobacillus* increased in the presence of environmental toxicants, while *Snodgrassella* reads decreased (Rothman et al., 2020). The accumulation of such toxins in flowers implies that they are likely present in the pollen reserves collected by bees (Gekiere et al., 2023) and in their digestive systems, particularly in areas where soils are contaminated (Rothman et al., 2020). The presence of genes associated with oxidative stress response and detoxification of copper, cadmium, or selenium ions has been reported earlier in both core and non-core symbionts of bumble bees (Rothman et al., 2020, Cornet et al., 2022). The mechanisms by which these bacteria residing in the hindgut may provide protection

against such toxic substances remain unclear, as heavy metals and other harmful substances are likely to be absorbed by the host within the midgut (Hammer et al., 2021).

Conclusion

The phylogenomic analysis and functional genomic characterization of 21 *Fructobacillus* isolate genomes in the present study demonstrated that bumble bee and flower isolates represent four species, including the novel species *F. evanidus* (Table 1 and Fig. 1). Our results confirmed and substantiated the presence of two phylogenetically and functionally distinct *Fructobacillus* species clades which differed in genome size, percentage G + C content, the number of coding DNA sequences, and metabolic characteristics. In the present study clade 1 (i.e. *F. fructosus*) and clade 2 (i.e. *F. evanidus*, *F. tropaeoli*, and *F. cardui*) species differed especially in amino acid and, to some extent, in carbohydrate metabolism, with *F. evanidus* and *F. tropaeoli* genomes featuring a higher number of complete metabolic pathways. While *Fructobacillus* genomes encoded genes that allow adhesion, biofilm formation, antibacterial activity, and detoxification, other gut symbionts appeared to possess a larger repertoire of genes to co-exist with the bumble bee host. Differences in genome size and in amino acid and vitamin metabolism may therefore be the result of adaptations to other hosts or ecosystems, rather than to the bumble bee gut ecosystem.

CRediT authorship contribution statement

Juliana Botero: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Charlotte Peeters:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology. **Evelien De Canck:** Investigation. **David Laureys:** Writing – review & editing, Methodology. **Anneleen D. Wieme:** Writing – review & editing, Methodology, Investigation. **Ilse Cleenwerck:** Writing – review & editing, Methodology, Investigation. **Eliza Depoorter:** Writing – review & editing, Methodology, Investigation. **Jessy Praet:** Writing – review & editing, Methodology, Investigation. **Denis Michez:** Writing – review & editing, Funding acquisition. **Guy Smagghe:** Writing – review & editing, Funding acquisition. **Peter Vandamme:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The annotated genome assemblies were submitted to the European Nucleotide Archive (ENA) and are publicly available under project PRJEB64171

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.syapm.2024.126505.

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