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Fructilactobacillus cliffordii sp. nov., *Fructilactobacillus hinvesii* sp. nov., *Fructilactobacillus myrtifloralis* sp. nov., *Fructilactobacillus carniphilus* sp. nov. and *Fructobacillus americanaquae* sp. nov., five novel lactic acid bacteria isolated from insects or flowers of Kangaroo Island, South Australia

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Abstract

Six strains, KI11_D11^T, KI4_B1, KI11_C11^T, KI16_H9^T, KI4_A6^T and KI3_B9^T, were isolated from insects and flowers on Kangaroo Island, South Australia. On the basis of 16S rRNA gene phylogeny, strains KI11_D11^T, KI4_B1, KI11_C11^T, KI16_H9^T, KI4_A6^T were found to be closely related to *Fructilactobacillus ixorae* Ru20-1^T. Due to the lack of a whole genome sequence for this species, whole genome sequencing of *Fructilactobacillus ixorae* Ru20-1^T was undertaken. KI3_B9^T was found to be closely related to *Fructobacillus tropaeoli* F214-1^T. Utilizing core gene phylogenetics and whole genome analyses, such as determination of AAI, ANI and dDDH, we propose that these six isolates represent five novel species with the names *Fructilactobacillus cliffordii* (KI11_D11^T= LMG 32130^T = NBRC 114988^T), *Fructilactobacillus hinvesii* (KI11_C11^T = LMG 32129^T = NBRC 114987^T), *Fructilactobacillus myrtifloralis* (KI16_H9^T= LMG 32131^T = NBRC 114989^T) *Fructilactobacillus carniphilus* (KI4_A6^T = LMG 32127^T = NBRC 114985^T) and *Fructobacillus americanaquae* (KI3_B9^T = LMG 32124^T = NBRC 114983^T). Chemotaxonomic analyses detected no fructophilic characters for these strains of member of the genus *Fructilactobacillus*. KI3_B9^T was found to be obligately fructophilic, similarly to its phylogenetic neighbours in the genus *Fructobacillus*. This study represents the first isolation, to our knowledge, of novel species in the family *Lactobacillaceae* from the Australian wild.

INTRODUCTION

Lactic acid bacteria (LAB) represent a diverse group of microorganisms that can be found in a variety of niches and have been variously classified as free-living, nomadic and host adapted, reflective of their evolutionary history [1]. Flowers have previously been a common source for isolating diverse groups of LAB [2–10]. For example, *Fructilactobacillus florum* was isolated from flowers of peony (*Paeonia suffruticosa*) and bitou (*Chrysanthemoides monilifera*), *Fructilactobacillus ixorae* from West Indian jasmine (*Ixora coccinea* L.) and *Fructobacillus tropaeoli* from nasturtium (*Tropaeolum majus*) [3, 4, 10]. At these active hubs, the exchange of microorganisms through floral visitations by Hymenopterans (bees, wasps, sawflies and ants) has been readily observed [7, 11].

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Keywords: *Apis mellifera*; Australian lactic acid bacteria; *Blatta orientalis*; *Calytrix glaberrima*; European honey bees; FLAB; *Fructobacillus*; fructophilic lactic acid bacteria; *Fructilactobacillus*; Kangaroo Island; *Melaleuca gibbosa*; meat ant; *Iridomyrmex purpureus*; oriental cockroach; smooth fringe-myrtle; slender honey-myrtle.

Abbreviations: AAI, pairwise amino acid identity; ANI, pairwise average nucleotide identity; cAAI, pairwise amino acid identity of conserved genes; dDDH, digital DNA-DNA hybridization; gDNA, genomic DNA; GGDC, genome-to-genome distance calculator.

The GenBank accession numbers for the 16S rRNA gene sequences for *Fructilactobacillus cliffordii* KI11_D11^T, *Fructilactobacillus cliffordii* KI4_B1, *Fructilactobacillus hinvesii* KI11_C11^T, *Fructilactobacillus myrtifloralis* KI16_H9^T, *Fructilactobacillus carniphilus* KI4_A6^T and *Fructobacillus americanaquae* KI3_B9^T are ON545730, ON545726, ON545729, ON545727, ON545728 and ON545731 respectively. The GenBank accession numbers for the draft genome sequences of *Fructilactobacillus cliffordii* KI11_D11^T, *Fructilactobacillus cliffordii* KI4_B1, *Fructilactobacillus hinvesii* KI11_C11^T, *Fructilactobacillus myrtifloralis* KI16_H9^T, *Fructilactobacillus carniphilus* KI4_A6^T and *Fructobacillus americanaquae* KI3_B9^T are CP097117, CP097119–CP097120, CP097118, CP097116, CP097121 and CP097122 respectively.

Six supplementary figures and six supplementary tables are available with the online version of this article.

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Within insects, LAB host adaptation is ubiquitous, with honey bees being unique compared with other Hymenoptera because of their relatively consistent association with LAB from the genera *Apilactobacillus*, *Bombilactobacillus* and *Lactobacillus*, regardless of geography [1, 11–17]. Within these genera, species of fructophilic lactic acid bacteria (FLAB) are common, which are also associated with fruits, flowers, fermented foods and insects [18–22]. Species of the genus *Apilactobacillus* and all species of the genus *Fructobacillus* are LAB that represent FLAB due to changes in the *adhE* gene that encodes a bifunctional alcohol/acetaldehyde dehydrogenase [14, 20, 21, 23]. Furthermore, honey bees have been reported to form more transient relationships with some of the reported core LAB, such as the FLAB *Apilactobacillus kunkeei*, being isolated from a variety of sources [8, 24–27]. Due to the 'allochthonous' nature of LAB, caution is warranted when drawing conclusions about the evolutionary history of specific strains i.e., strain presence may be due to low sporadic and transient colonization [1]. Thus, in routine environmental isolations, the reported isolation source may be quite distant both spatially and physiochemically from the niche in which the strain evolved.

ISOLATION AND ECOLOGY

A sampling expedition in late 2019 sought novel isolates of lactic acid bacteria in remote Australian regions. Kangaroo Island in South Australia was selected due to its unique microclimates, undisturbed and biodiverse plant life and the beekeeping industry with intact biosecurity protocols [28–30]. Native flowers, insects, cultivated honey bees and honey samples were aseptically collected. Samples were either put directly into saline, de Man, Rogosa and Sharpe (MRS) broth (Oxoid), MRS broth supplemented with 20% (v/v) preservative free apple juice, (Golden Circle or Coles brand; MRSAJ), MRS broth supplemented with 20 g l^{-1} D-fructose (ChemSupply) and 0.1% (w/v) L-cysteine (MRSFC) or in empty tubes [15]. All media were adjusted to pH 6.2 using hydrochloric acid or sodium hydroxide prior to use. Insects were killed immediately by homogenization with 0.5–1 ml of sterile saline. Crude samples were serially diluted with saline and plated using an automatic spiral plater (Don Whitley Scientific) onto agar plates (20 g l^{-1}) of MRSAJ or MRSFC with 100 mg l^{-1} of natamycin (Natap, Handry) to prevent fungal growth. Plates were incubated in a 20% CO_2 atmosphere at 30 °C for 2–14 days (CellXpert C170; Eppendorf). Colonies were picked on the basis of morphological characteristics and at different time points from each medium and then placed into the liquid medium type of the source plate. After 4–7 days of growth, strains were cryopreserved (20% (w/v) glycerol, −80 °C). Preliminary identification of isolates was achieved by profiling on a MALDI Biotyper platform (Bruker) at the Mass Spectrometry and Proteomics at the Future Industries Institute (MSP@FII), University of South Australia, Adelaide, South Australia, Australia. Briefly, isolates were grown for 2–7 days, spotted and dried on an MSP 96 target polished steel BC plate prior to processing using the Extended Direct Transfer protocol (Bruker Daltonik). Prepared plates were analysed using an AutoFlex Speed MALDI-TOF/TOF mass spectrometer operated in linear positive mode under MALDI Biotyper 3.0 Real-time Classification (version 3.1) and FlexControl (version 3.4) software (Bruker Daltonik). Strains for which data gave poor confidence in identification, were re-identified by amplifying the 16S rRNA gene using primers 8F and 1492R [31]. Specifically, thawed glycerol stocks were diluted 1/8 using ultrapure water prior to 16S PCR. Initial Sanger sequencing involved sequencing the first approximately 1000 bp of the 16S rRNA sequence. Six strains, designated KI11_D11^T, KI4_B1, KI11_C11^T, KI16_H9^T, KI4_A6^T and KI3_B9^T displayed less than 99.7% 16S rRNA similarity to the most closely related type strain obtained from a BLAST search [32]. Confirmation sequencing of doubly re-purified isolates using both primers 8F and 1492R was conducted prior to submitting samples for whole-genome sequencing.

The six isolates of interest were recovered from various sites around Kangaroo Island. KI11_D11^T was from the homogenate of a European honey bee (*Apis mellifera*) from the Clifford's honey farm in Haines. KI4_B1 and KI4_A6^T were from the homogenates of two meat ants (*Iridomyrmex purpureus*) found in a private paddock in Haines. KI11_C11^T was isolated from a flower of the slender honey-myrtle (*Melaleuca gibbosa*) from Living Honey in Haines. KI16_H9^T was isolated from a flower of the smooth fringe-myrtle (*Calytrix glaberrima*) from the De-Tong Ling Buddhist Retreat Centre in Gosse. KI3_B9^T was from the homogenate of an oriental cockroach (*Blatta orientalis*) dwelling on Banksia at Sunrise on Falie in American River.

16S rRNA PHYLOGENY

Genomic DNA from KI11_D11^T, KI4_B1, KI11_C11^T, KI16_H9^T, KI4_A6^T and KI3_B9^T was purified and quantified as described previously [33]. Due to the high 16S rRNA similarity between KI11_D11^T, KI4_B1, KI11_C11^T, KI16_H9^T, KI4_A6^T and *Fructilactobacillus ixorae* Ru20-1^T and the lack of an available whole-genome assembly, whole-genome sequencing of *F. ixorae* Ru20-1^T was undertaken [10, 14]. DNA of *F. ixorae* Ru20-1^T was purified as detailed above. Genomic DNA of *F. ixorae* Ru20-1^T was sent to the AGRF Cancer Genomics Facility in the Centre for Cancer Biology (Adelaide, Australia). Genomic DNA of KI11_D11^T, KI4_B1, KI11_C11^T, KI16_H9^T, KI4_A6^T and KI3_B9^T was sent on dry ice to Maryland Genomics, Institute for Genome Sciences, University of Maryland School of Medicine, Baltimore, Maryland, USA.

Libraries for KI11_D11^T, KI4_B1, KI11_C11^T, KI16_H9^T, KI4_A6^T and KI3_B9^T were generated using SMRTbell Express Template Prep Kit 2.0 (Pacific Bioscience), and SMRT sequencing was conducted on a PacBio Sequel II as described previously [33].

For *Fructilactobacillus ixorae* Ru20-1^T, 1 µg of gDNA was sheared as described above to produce library sizes of approximately 6.2 kbp. The library was then prepared following guidelines in the PacBio Procedure and Checklist – Preparing Multiplexed

Microbial Libraries Using SMRTbell Express Template Prep Kit 2.0 (Pacific Biosciences). AMPure PB beads were used to remove SMRTBell Templates smaller that 3 kb. The PacBio Binding Kit 3.0 was used for the binding reaction, with calculations from SMRT Link v8.0, sequencing primer v4, 1 h polymerase binding and 1.2× AMPure PB beads complex clean-up. The library was sequenced on a PacBio Sequel I instrument with SMRT Cell 1M v3 LR for 10 h. Whole genome metrics for all strains are shown in Table 1.

As described previously [33], Genome assemblies were annotated using Prokka v1.14.6 [33, 34] All annotated 16S genes were extracted and uploaded to EZBioCloud's 16S-based ID App [35] and resulting 16S hits were downloaded for phylogenetic analysis (Table S1). The 16S-based ID App as well as the Pairwise Nucleotide Sequence Alignment For Taxonomy Tool were used to calculate 16S similarities [35]. A single 16S sequence was obtained for each of two species ('*Enterococcus massiliensis*' AM1 – NR_144723.2 and *Lactococcus chungangensis* CAU 28^T - NR_044357.1) to be used as outgroups. The corresponding genome assemblies were also obtained for these type strains (Table S1). The 16S sequences were processed to be used as input into RAxML v8.2.12 for performing phylogenetic reconstruction [33, 36, 37]. The phylogenetic analyses used the General Time Reversible (GTR) model of evolution across lineages and gamma distributed rates across sites (GTR+G) and 5000 bootstrap replicates (Figs 1 and 2).

KI11_D11^T displayed the highest 16S rRNA similarity to *Fructilactobacillus ixorae* Ru20-1^T (98.64%), *Fructilactobacillus lindneri* KPA^T (97.00%) and *Fructilactobacillus vespulae* DCY75^T (95.66%) [10, 14, 38–40]. Percentage similarities of the remaining strains to *Fructilactobacillus ixorae* Ru20-1^T, *Fructilactobacillus lindneri* KPA^T,and *Fructilactobacillus vespulae* DCY75^T were as follows: KI4_B1, 98.64%, 97.00% and 95.66%, respectively; KI11_C11^T, 98.58, 96.73 and 95.59%, respectively; KI16_H9^T, 99.73, 96.93 and 95.80%, respectively and KI4_A6^T, 98.71, 97.00 and 95.94%, respectively. Pairwise 16S comparisons between KI11_D11^T, KI4_B1, KI11_C11^T, KI16_H9^T and KI4_A6^T are provided in Table S2. Overall, all strains exhibited relatively moderate 16S pairwise similarities to the most closely related strain except for the higher values observed between KI16_H9^T and *F. ixorae* Ru20-1^T (99.73%) and between KI11_D11^T and KI4_B1 (99.84%). As shown in Fig. 1, KI11_D11^T, KI4_B1, KI11_C11^T, KI16_H9^T, KI4_A6^T form a clade with *Fructilactobacillus ixorae* Ru20-1^T (95% bootstrap support) and are confidently grouped with all other type strains of members of the genus *Fructilactobacillus* with 88% bootstrap support. Considering this and the high 16S pairwise similarities to other Fructilactobacilli, all strains can be confidently placed into this genus. However, it is unclear from 16S phylogeny alone what determinations should be made below genus level classification.

Strain KI3_B9^T displayed the highest 16S rRNA similarity to *Fructobacillus tropaeoli* F214-1^T (99.66%), *Fructobacillus pseudoficulneus* DSM 15468^T (99.12%) and *Fructobacillus ficulneus* JCM 12225^T (98.03%) [4, 41–43]. This is supported by the short evolutionary distances observed in the 16S phylogenetic tree for these operational taxonomic units (OTUs), forming a small clade (Fig. 2). KI3_B9^T can be confidently placed in the *Fructobacillus* clade (100% bootstrap support). Although, its precise position is not completely resolved, it is clear it occupies a more basal position within the genus *Fructobacillus*.

WHOLE-GENOME PHYLOGENY

A phylogenetic tree was reconstructed from concatenated multiple sequence alignments of 81 core bacterial genes using the UBCG2 pipeline, as described previously (Figs 3 and 4) [33].

The concatenated gene phylogenetic tree of KI11_D11^T, KI4_B1, KI11_C11^T, KI16_H9^T, KI4_A6^T almost fully resolves the phylogenetic relationships of the strains of members of the genus *Fructilactobacillus* with all but one internal branch supported by 100% GSI (Fig. 3). It is now clear that *Fructilactobacillus ixorae* Ru20-1^T and KI16_H9^T have a sister relationship to each other, and together they have a sister group relationship to KI11_D11^T, KI4_B1^T, KI4_A6^T and KI11_C11^T.

The concatenated gene phylogenetic tree of KI3_B9^T (Fig. 4) clarifies the branch ordering of the 16S rRNA based tree (Fig. 2). The position of KI3_B9^T is now fully resolved within the *Fructobacillus* clade with all internal branches having 100% GSI. It forms a clade together with *Fructobacillus tropaeoli* F214-1^T, *Fructobacillus pseudoficulneus* DSM 15468^T and *Fructobacillus ficulneus* JCM 12225^T.

To assist in assignment to the appropriate taxonomic level, average nucleotide identity (ANI) and average amino acid identity (AAI) was calculated for every pairwise combination of genome, as described previously (Fig. S1–S4, available in the online version of this article) [33, 44]. Digital DNA–DNA hybridization (dDDH) values were calculated first by uploading the genome sequences to the Type (Strain) Genome Server (TYGS) (https://tygs.dsmz.de) [45]. dDDH values and confidence intervals associated with distance formula d_4 were calculated using the recommended settings of the GGDC 3.0 and downloaded (Table S3) [46, 47]. ANI, AAI and dDDH data from the top six closely related type strains are shown in Table 2.

KI11_D11^T, KI4_B1, KI11_C11^T, KI16_H9^T and KI4_A6^T display AAI values exceeding 66.5% to type strains of species of the genus *Fructilactobacillus* (Table 2, Fig. S1). This along with the results from the 16S rRNA and concatenated gene phylogenetic trees reaffirm that all five strains represent members of the genus *Fructilactobacillus* [14, 48]. Furthermore, all strains display ANI and dDDH values to the most closely related type strain that fall below 95 and 70%, the respective ANI and dDDH thresholds to assign a strain to a published species [46, 47, 49–53] (Table 2, Fig. S3, Table S3). KI11_D11^T, KI4_B1, KI11_C11^T and KI4_A6^T

Table 1. Genome features for strains KI11_D11^T, KI4_B1, KI11_C11^T, KI16_H9^T, KI4_A6^T, KI3_B9^T and *Fructilactobacillus ixorae* Ru20-1^T.

Species	*Fructilactobacillus cliffordii*	*Fructilactobacillus cliffordii*	*Fructilactobacillus hinvesii*	*Fructilactobacillus myrtifloralis*	*Fructilactobacillus carniphilus*	*Fructobacillus americanaquae*	*Fructilactobacillus ixorae*
Strain	KI11_D11^T	KI4_B1	KI11_C11^T	KI16_H9^T	KI4_A6^T	KI3_B9^T	Ru20-1^T
Sample Origin (Species)	*Apis mellifera*	*Iridomyrmex purpureus*	*Melaleuca gibbosa*	*Calytrix glaberrima*	*Iridomyrmex purpureus*	*Blatta orientalis*	Culture Collection Sample (LMG 29008)
Sample Origin (Material)	Homogenate	Homogenate	Flower	Flower	Homogenate	Homogenate	
Accession Number	CP097117	CP097119–CP097120	CP097118	CP097116	CP097121	CP097122	CP097478–CP097479
Genome Size	1459401	1457211	1465046	1458675	1491353	1414204	1422963
Number of Contigs	1	2	1	1	1	1	2
Contig 1 Size (Chromosome)	1459401	1416177	1465046	1458675	1491353	1414204	1374918
Contig 2 Size (Plasmid 1)	0	41034	0	0	0	0	48045
Coverage (×)	12945.29	12992.45	14600.86	10761.07	15666.59	9666.79	100.00
DNA G+C Content (mol %)	44.44	44.16	43.88	46.96	44.49	43.64	46.71
# N's per 100 kbp	0	0	0	0	0	0	0
Check M Completeness (%)	99.01	99.01	99.01	99.06	99.01	97.99	99.06
Check M Contamination (%)	0.63	0.63	0.63	0.63	0.63	0.00	0.63

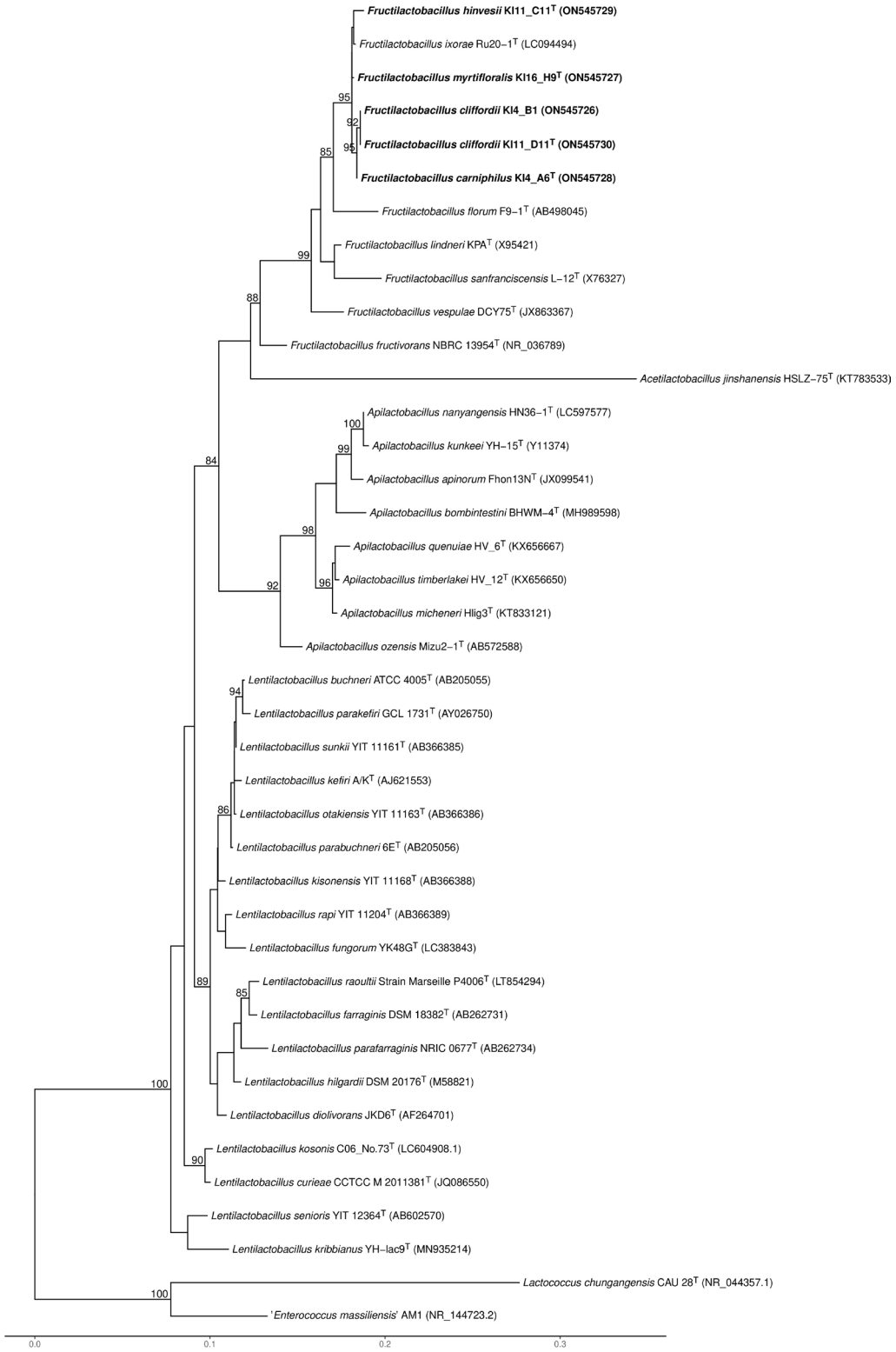

Fig. 1. Maximum-likelihood phylogenetic tree reconstructed from 16S rRNA gene sequences relating to whole-genome extracted 16S sequences for strains KI11_D11^T, KI4_B1, KI11_C11^T, KI16_H9^T and KI4_A6^T. GenBank accession numbers for the sequences are provided in parentheses. The phylogenetic analysis used the GTR+G model with 5000 bootstrap replicates. Bootstrap values below 80 are not shown. An outgroup consisting of '*Enterococcus massiliensis*' and Lactococcus chungangensis was used to root the tree. The scale bar shows the mean number of nucleotide substitutions per site.

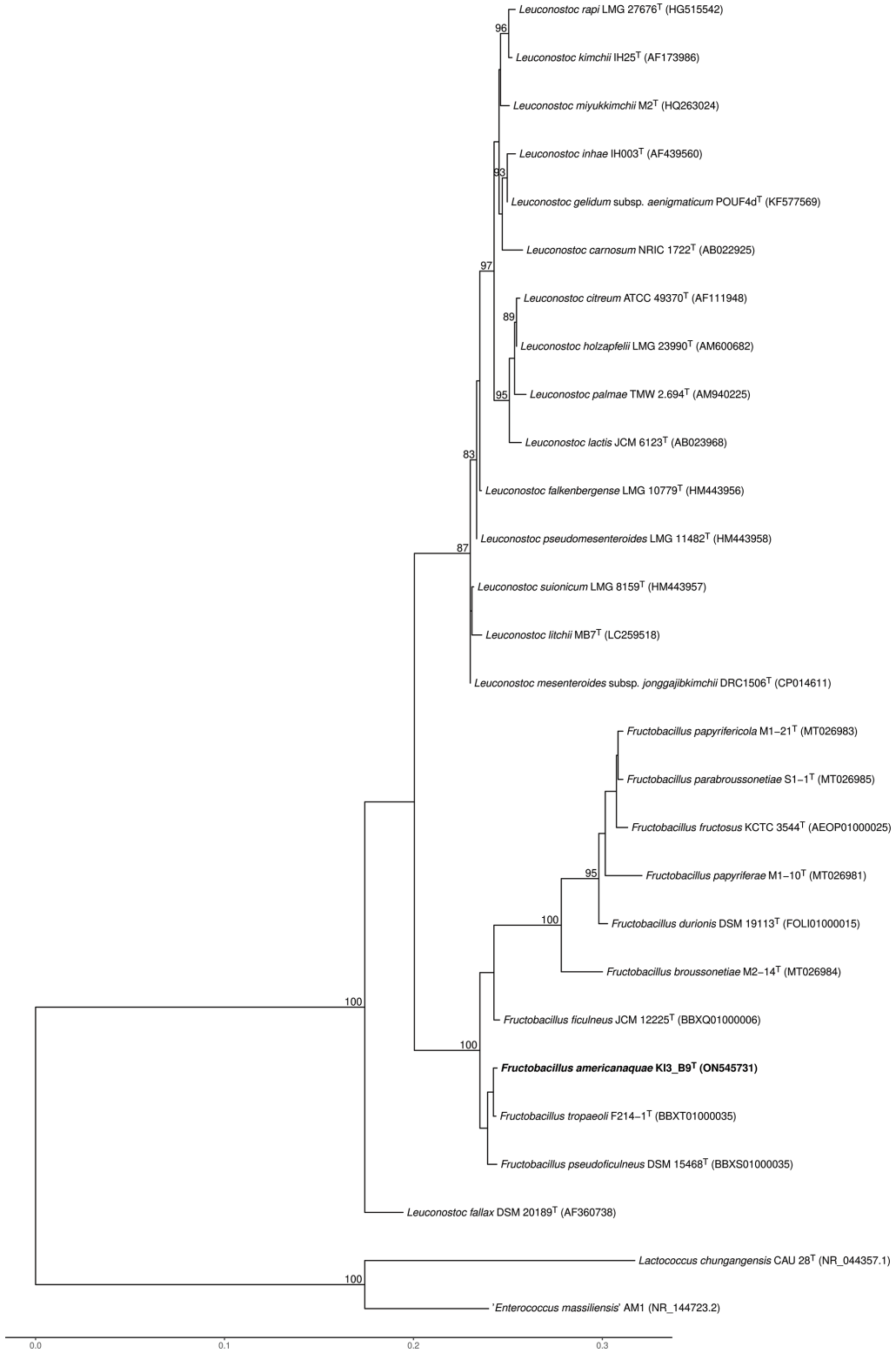

Fig. 2. Maximum-likelihood phylogenetic tree reconstructed from 16S rRNA gene sequences relating to whole-genome extracted 16S sequences for KI3_B9^T. GenBank accession numbers for the sequences are provided in parentheses. The phylogenetic analysis used the GTR+G model with 5000 bootstrap replicates. Bootstrap values below 80 are not shown. An outgroup consisting of '*Enterococcus massiliensis*' and *Lactococcus chungangensis* was used to root the tree. The scale bar shows the mean number of nucleotide substitutions per site.

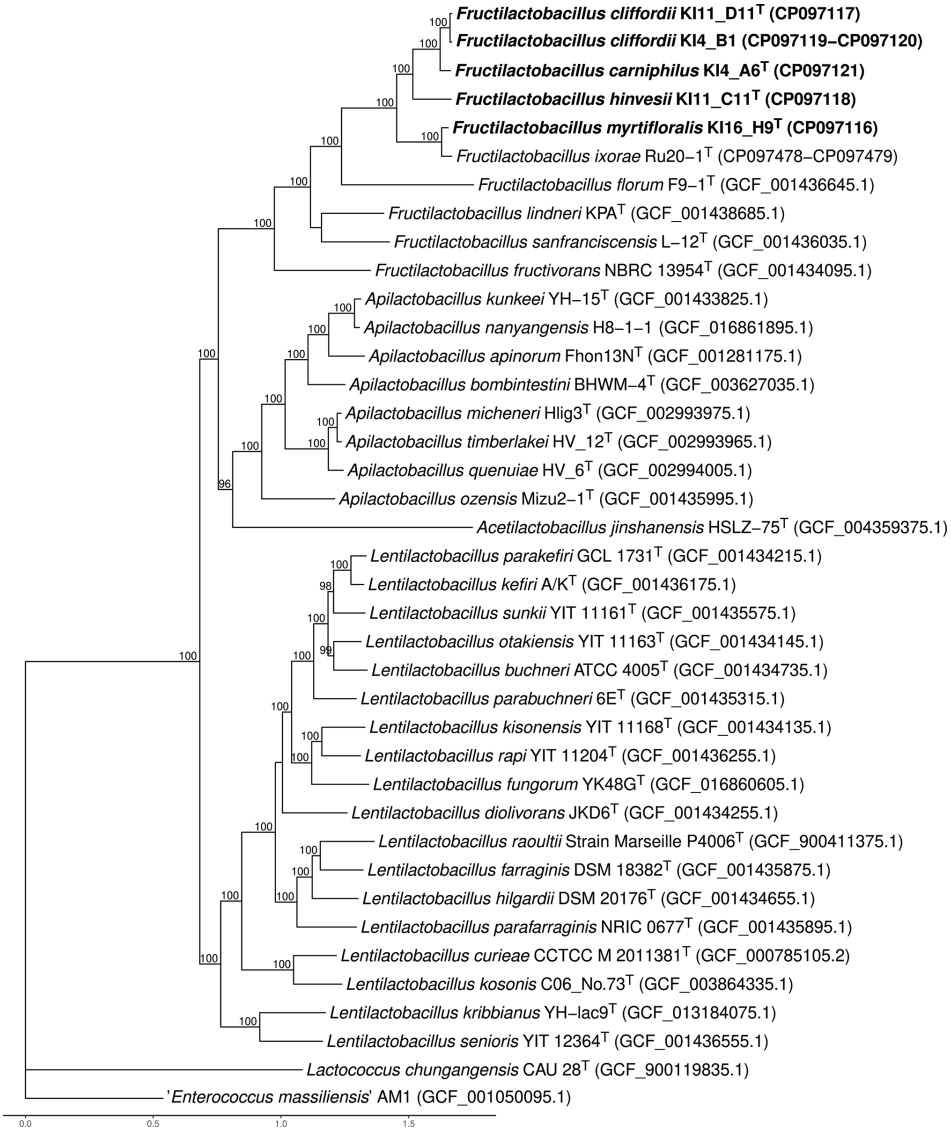

Fig. 3. Maximum-likelihood phylogenetic tree reconstructed from the concatenated multiple sequence alignments of 81 core bacterial genes according to UBCG2 relating to strains KI11_D11^T, KI4_B1, KI11_C11^T, KI16_H9^T and KI4_A6^T. GenBank accession numbers for the genome assemblies are provided in parentheses. The phylogenetic analysis used the GTR+CAT model and Gene Support Index (GSI) values below 80 are not shown. An outgroup consisting of '*Enterococcus massiliensis*' and *Lactococcus chungangensis* was used to root the tree. The scale bar shows the mean number of nucleotide substitutions per site.

display a relatively narrow range of AAI, ANI and dDDH values of 82.6–82.8%, 76.7–77.2% and 20.0–20.3%, respectively, to *Fructilactobacillus ixorae* Ru20-1^T. Furthermore, pairwise comparisons between all isolates bolsters the two sister groups shown in Fig. 3, highlighting the close relationship between KI11_D11^T, KI4_B1, KI11_C11^T and KI4_A6^T and between KI16_H9^T and *Fructilactobacillus ixorae* Ru20-1^T. The results of pairwise comparisons between all isolates except KI11_D11^T and KI4_B1, indicate that each strain represents a unique species with ANI and dDDH values below the 95 and 70% thresholds, respectively, for assignment to a species with a validly published name [46, 47, 49–53]. KI11_D11^T and KI4_B1 display ANI and dDDH values of 98.1 and 83.1%, respectively. Due to this, KI4_B1 is classified as a strain of the species with the type strain KI11_D11^T.

Strain KI3_B9^T displays AAI values exceeding 68% to all strains of members of the genus *Fructobacillus*, which range from 72.3 to 98.0%, placing it in the genus *Fructobacillus* with a high level of confidence (Table 2, Fig. S2) [14, 48]. KI3_B9^T displays the highest AAI, ANI and dDDH values of 94.0, 89.7 and 38.7%, respectively, to *Fructobacillus tropaeoli* F214-1^T. High AAI, ANI and dDDH values are also observed between KI3_B9^T and *Fructobacillus pseudoficulneus* DSM15468^T and *Fructobacillus ficulneus* JCM 12225^T (Table 2, Fig. S2 and S4, Table S3). Overall, these values are below the 95 and 70% respective ANI and dDDH values to assign KI3_B9^T to a species with a validly published name [46, 47, 49–53].

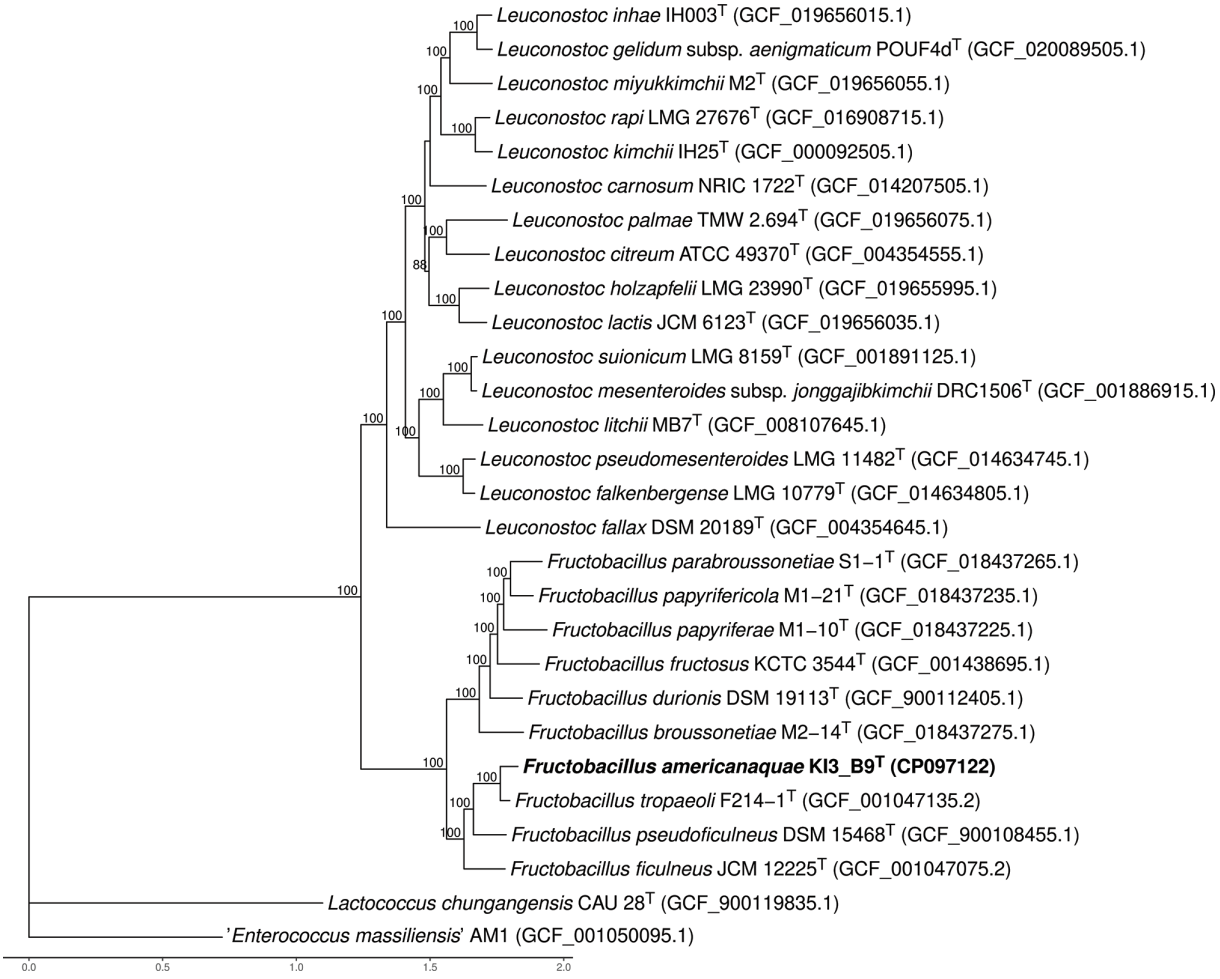

Fig. 4. Maximum-likelihood phylogenetic tree reconstructed from the concatenated multiple sequence alignments of 81 core bacterial genes according to UBCG2 relating to strain KI3_B9^T. GenBank accession numbers for the genome assemblies are provided in parentheses. The phylogenetic analysis used the GTR+CAT model and Gene Support Index (GSI) values below 80 are not shown. An outgroup consisting of '*Enterococcus massiliensis*' and *Lactococcus chungangensis* was used to root the tree. The scale bar shows the mean number of nucleotide substitutions per site.

PHYSIOLOGY AND CHEMOTAXONOMY

Since these strains were phylogenetically close to several known facultatively and obligately fructophilic LAB, their fructophilic properties were assessed as described previously [3, 43] with modifications [33]. Briefly, strains were grown in various media based on supplemented variations of MRS or GYP (Fig. S5) [43]. Triplicated trials were performed in 1 ml of each medium in 2 ml 96-well plates, sealed with breathable cloth (BF-400-S, Axygen and incubated statically under both anaerobic and aerobic conditions. OD_{600} was assessed after 6 days (Infinite 200 Pro, Tecan) and values reduced by the negative control. All phenotypic experiments were conducted similarly unless otherwise noted. Only KI3_B9^T grew to higher densities on fructose, whereas KI11_D11^T, KI4_B1, KI11_C11^T, KI16_H9^T, KI4_A6^T grew to higher densities on glucose.

To further understand the fructophilic nature of KI3_B9^T and the potential for facultatively fructophilic characteristics in strains KI11_D11^T, KI4_B1, KI11_C11^T, KI16_H9^T, KI4_A6^T and *Fructilactobacillus ixorae* Ru20-1^T, growth was assessed in GYP, FYP, FGYP and GYP+P, each with 10 g l^{-1} of each carbohydrate [2, 3, 43]. Replicated plates allowed for sacrificial sampling to determine growth kinetics (OD_{600}), and for KI3_B9^T sugar consumption and mannitol production (Fig. 5 and S6). KI3_B9^T displayed a similar phenotype to obligately fructophilic LAB, that is, poor growth on D-glucose (GYP) and enhanced growth on D-fructose (FYP) or D-glucose with the addition of electron acceptors such as; pyruvate (GYP+P) or D-fructose (FGYP) [2, 23] (Fig. 5). Exhaustion of fructose also led to a greatly decreased consumption of glucose and cessation of growth, presumably due to exhaustion of an external electron acceptor for continued glycolysis. Mannitol was formed from fructose, consistent with other obligate FLAB [21]. KI11_D11^T, KI4_B1, KI11_C11^T, KI16_H9^T, KI4_A6^T and *Fructilactobacillus ixorae* Ru20-1^T displayed no kinetic differences with regard to growth on D-glucose or D-fructose or enhanced growth on D-glucose with the addition of D-fructose (Fig. S6).

Table 2. ANI, AAI and dDDH values between the strains and six of the most similar strains based on AAI. Pairwise ANI, AAI,and dDDH values from Figs. S1–S4 and Table S3, between our strains and the six most similar strains based on AAI.

	KI11_D11^T			**KI11_C11^T**			**KI4_B1**			**KI16_H9^T**			**KI4_A6^T**		
Closely Related Species	**AAI (%)**	**ANI (%)**	**dDDH (%)**	**AAI (%)**	**ANI (%)**	**dDDH (%)**	**AAI (%)**	**ANI (%)**	**dDDH (%)**	**AAI (%)**	**ANI (%)**	**dDDH (%)**	**AAI (%)**	**ANI (%)**	**dDDH (%)**
KI4_B1	**98.7**	**98.1**	83.1	88.0	80.8	23.1	100.0	100.0	100.0	83.5	77.2	19.9	**95.2**	**92.0**	45.9
KI4_A6^T	95.2	91.9	46.1	**88.1**	**81.0**	23.2	95.2	92.0	45.9	83.3	77.2	20.5	100.0	100.0	100.0
KI11_C11^T	87.7	80.7	23.0	100.0	100.0	100.0	88.0	80.8	23.1	83.1	77.2	20.1	88.1	81.0	23.2
KI11_D11^T	100.0	100.0	100.0	87.7	80.7	23.0	**98.7**	**98.1**	83.1	83.2	77.5	20.0	95.2	91.9	46.1
KI16_H9^T	83.2	77.5	20.0	83.1	77.2	20.1	83.5	77.2	19.9	100.0	100.0	100.0	83.3	77.2	20.5
Fructilactobacillus ixrorae Ru20-1^T	82.6	76.7	20.2	82.7	76.9	20.3	82.7	76.8	20.0	**95.2**	**92.6**	48.8	82.8	77.2	20.3
Fructilactobacillus lindneri KPA^T	72.3	71.2	17.7	72.0	71.4	18.2	72.5	71.5	17.6	71.8	70.9	18.0	72.5	71.6	18.4
All *Fructilactobacillus*	66.8–98.7	69.3–98.1	17.6–83.1	66.7–88.1	69.3–81.0	17.3–23.2	66.9–98.7	69.3–98.1	17.5–83.1	66.5–95.2	68.3–92.6	17.1–48.8	67.0–95.2	69.1–92.0	17.6–45.9

	KI3_B9^T		
Closely Related Species	**AAI (%)**	**ANI (%)**	**dDDH (%)**
Fructobacillus tropaeoli F214-1^T	**94.0**	**89.7**	38.7
Fructobacillus pseudoficulneus DSM15468^T	82.6	77.4	20.7
Fructobacillus ficulneus JCM 12225^T	79.6	75.8	20.0
Fructobacillus papyriferae M1-10^T	72.6	71.9	21.1
Fructobacillus durionis DSM 19113^T	72.5	72.6	20.1
Fructobacillus broussonetiae M2-14^T	72.4	72.0	20.1
All *Fructobacillus*	72.3–98.0	71.9–89.7	20.0–38.7

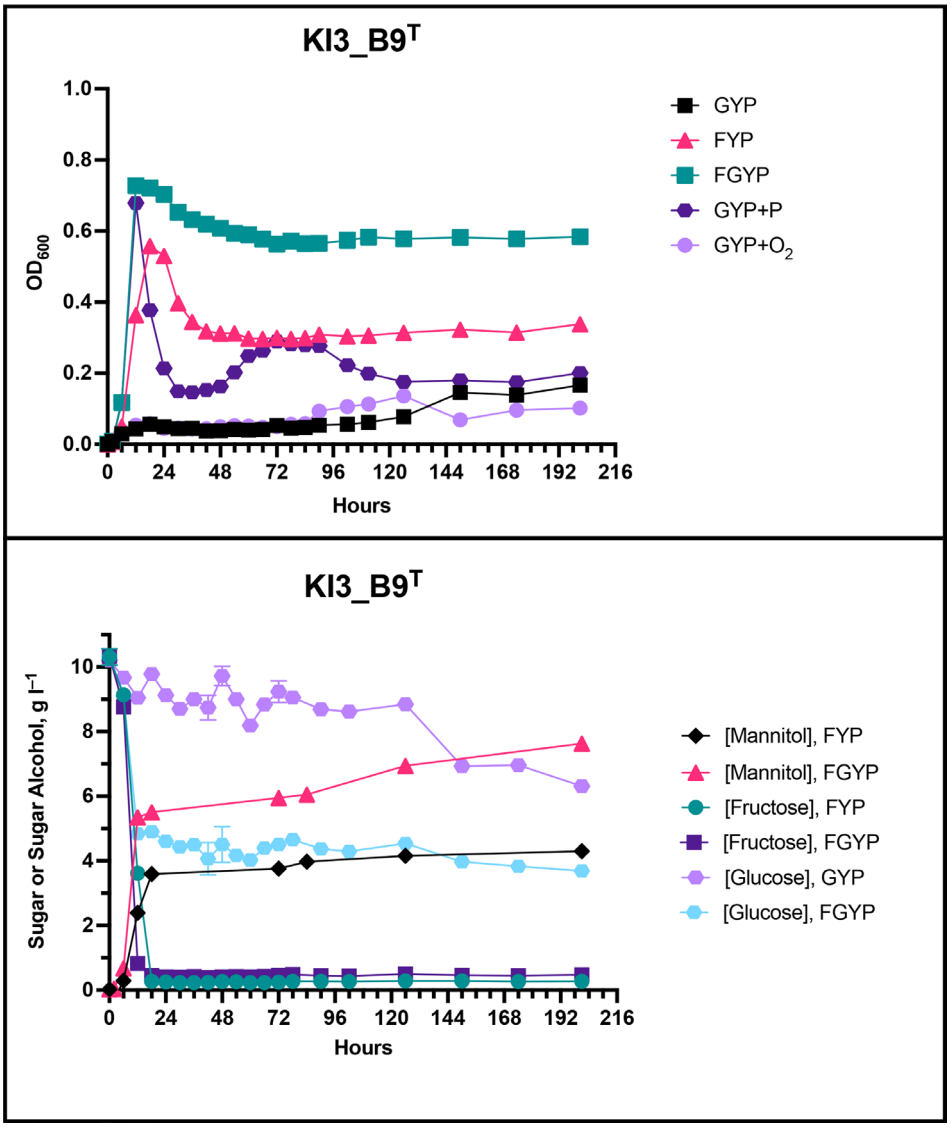

Fig. 5. Growth, sugar consumption and mannitol production of KI3_B9^T over time.

Furthermore, the effect of oxygen was negligible, however, the inclusion of pyruvate did enhance growth on D-glucose, similarly to *Fructilactobacillus florum* F9-1^T. Overall, this distinct clade of several species of the genus *Fructilactobacillus* is not fructophilic.

To determine the utilisation of sole carbohydrates, API 50 CH strips (bioMériuex) were used following the manufacturer's protocol. Assays were incubated aerobically with a sterile mineral oil overlay at 30 °C, with observations made daily for 14 days. API ZYM strips (bioMériuex) allowed assessment of enzyme activity. Data for the API 50CH and API ZYM panels are shown in Table S4A and B [10, 22, 40]. KI4_A6^T, KI11_D11^T and KI4_B1 were unique with respect to other Fructilactobacilli due to their ability to utilise D-mannose (Table S4A). KI11_C11^T, KI11_D11^T and KI4_B1 were unique in their positive or weak utilisation of sucrose. KI16_H9^T was unique given its ability to utilise glycerol, arbutin and salicin. Within our strains, each displayed a distinguishing fermentation profile. In terms of their enzymatic API ZYM profiles, our strains could not be distinguished from each other. Furthermore, no strain provided any unique enzymatic activities except for the presence of β-glucosidase for KI16_H9^T. KI3_B9^T displayed a unique carbohydrate utilisation profile with regards to other *Fructobacilli* (Table S4B). The weak enzymatic activities of KI3_B9^T made it difficult to assess appropriate distinction between it and other isolates.

For the following phenotypic experiments, all strains were grown in MRSF. Temperature dependence of growth was assessed using 2 ml 96-well plates placed in a BD GasPak EZ container system with a BD BBL anaerobic GasPak at 4, 10, 17, 23, 28, 30, 37, 40, 45 and 50 °C and sealed with breathable cloth (BF-400-S, Axygen). Growth with added NaCl (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3, 4, 5, 7.5, 10, 12.5, 15 or 20%) was also similarly assessed. The influence of pH (2, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.2, 6.5, 6.8, 7, 7.5, 8, 8.5, 9 and

10) was assessed in 15 ml sealed Eppendorf tubes filled with 14 ml of media. Catalase activity was evidenced by gas production after placing fresh colonies into 3% (v/v) H_2O_2 on a glass slide. Gram-staining and spore staining were conducted using the Gram Staining Kit and the Schaeffer and Fulton Spore Stain Kit, respectively, (Sigma-Aldrich). Cells were imaged and measured using a DM300 microscope (Leica). Lactic acid stereoisomers were determined by the D-/L-Lactic Acid Rapid Assay Kit (Megazyme). All chemotaxonomic data are detailed in the relevant species descriptions.

Fermentation type was assessed through HPLC measurement of end products from the fermentation of GYP, FYP and FGYP [54] (Table S5A and B). KI11_D11^T, KI4_B1, KI11_C11^T, KI16_H9^T, KI4_A6^T displayed similar fermentation profiles to each other and *Fructilactobacillus ixorae* Ru20-1^T (Table S5A). That is, no detectable acetic acid production from D-glucose and increased acetic acid production from D-fructose. These profiles are distinct from that of *Fructilactobacillus florum*, where acetic acid is formed from D-glucose. Overall, all strains were heterofermentative. KI3_B9^T consumed D-glucose poorly, with small amounts of lactic acid and ethanol produced (Table S5B). Inclusion of fructose increased glucose consumption, consistent with the data presented in Fig. 5, due to fructose being a primary electron acceptor. Inclusion of fructose also increased production of acetic acid relative to ethanol. With regard to other obligate FLAB, KI3_B9^T produced larger amounts of ethanol. A possible explanation could be the presence of an NADH-dependent butanol dehydrogenase, which has some homology to the alcohol dehydrogenase domain (ADH) of *adhE* (data not shown).

The Identification Service of the DSMZ was used to determine peptidoglycan structures by established protocols [55] and cellular fatty acids using the Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID), before comparison to relevant taxa (Tables S6A–C). GC-MS was used for identity confirmation and to resolve summed features of the MIDI analysis (Tables S6B and C). Major fatty acids reported in species descriptions were determined by these GC-MS mediated corrections. Table S6A details the fatty acid profiles of KI11_D11^T, KI4_B1, KI11_C11^T, KI16_H9^T, KI4_A6^T and other Fructilactobacilli. Regarding the major fatty acids, KI11_D11^T, KI4_B1, KI11_C11^T, KI16_H9^T, KI4_A6^T and to a lesser extent *Fructilactobacillus ixorae* Ru20-1^T, displayed a larger amount of arachidic acid ($C_{20:0}$) compared with other Fructilactobacilli. Regarding KI3_B9^T, the fatty acid profile was similar to those of phylogenetically related strains such as *Fructobacillus tropaeoli* F214-1^T and *Fructobacillus pseudoficulneus* DSM 15468^T with a higher quantity of $C_{18:1}\omega7c$ than $C_{18:1}\omega9c$ compared with other *Fructobacilli* (Table S6C).

DESCRIPTION OF *FRUCTILACTOBACILLUS CLIFFORDII* SP. NOV.

Fructilactobacillus cliffordii sp. nov. (clif.for'di.i. N.L. gen. n. *cliffordii*, named after the Clifford family, who own Clifford's Honey Farm where the host of the bacterium was found)

Cells are Gram-stain-positive, non-motile, non-spore forming, catalase-negative rods measuring 1.3–6.9×0.7–1.0 µm, occurring singly or in chains. D-lactic acid and L-lactic acid are produced from glucose. Heterofermentative, with the ratio of lactic acid, ethanol and acetic acid of 1:0.84:0.00 with respect to glucose fermentation and 1:0.44:0.52 with respect to glucose and fructose fermentation. After 48 h anaerobic growth on MRSF agar (MRS with 20 g l^{-1} fructose), colonies appear ivory, circular, umbonate, undulate, glistening and opaque, with diameters of 1–2 mm. Facultatively anaerobic. Growth in MRSF occurs at between pH 4.0 and 8.5, 10–45 °C and with 0–7.5% added NaCl and optimally at pH 6.8, 17 °C and with 0.0% added NaCl. Acid is produced from D-glucose, D-fructose, D-mannose and D-mannitol and weakly from sucrose and potassium gluconate. Positive for leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase, weakly positive for cystine arylamidase. The major fatty acids are $C_{20:0}$, $C_{20:1}\omega7c$, $C_{19:0}$cyclo $\omega7c$ and $C_{19:0}$cyclo $\omega9c$. The cell wall peptidoglycan structure is A4α L-Lys–D-Asp.

The type strain (KI11_D11^T= LMG 32130^T = NBRC 114988^T) was isolated from the homogenate of a European honeybee (*Apis mellifera*) in the suburb of Haines on Kangaroo Island, South Australia in 2019. The DNA G+C content of the type strain is 44.44 mol%. The GenBank accession numbers for the 16S rRNA gene sequence and draft genome sequence are ON545730 and CP097117, respectively.

DESCRIPTION OF *FRUCTILACTOBACILLUS HINVESII* SP. NOV.

Fructilactobacillus hinvesii sp. nov. (hin.ve'si.i. N.L. gen. n. *hinvesii*, named after the Hinves family, who own the honey farm Living Honey where the host of the bacterium was found)

Cells are Gram-stain-positive, non-motile, non-spore forming, catalase-negative rods measuring 1.8–4.7×0.8–1.1 µm, occurring singly or in chains. D-lactic acid and L-lactic acid are produced from glucose. Heterofermentative, with the ratio of lactic acid, ethanol and acetic acid of 1:0.7:0.00 with respect to glucose fermentation and 1:0.51:0.46 with respect to glucose and fructose fermentation. After 48 h anaerobic growth on MRSF agar (MRS with 20 g l^{-1} fructose), colonies appear ivory, circular, umbonate, entire, glistening and opaque, with diameters of 1–2 mm. Facultatively anaerobic. Growth in MRSF occurs at between pH 3.5 and 8.0, 4–50 °C and with 0–7.5% added NaCl and optimally at pH 5.0, 30 °C and with 0.0% added NaCl. Acid is produced from D-glucose, D-fructose, D-mannitol and sucrose and weakly from potassium gluconate. Positive for leucine arylamidase, valine

arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase, weakly positive for cystine arylamidase. The major fatty acids are $C_{20:0}$, $C_{18:1}\omega9c$, $C_{20:1}\omega7c$, $C_{19:0}$cyclo ω7*c* and $C_{19:0}$cyclo ω9*c*. The cell wall peptidoglycan structure is A4α L-Lys–D-Asp.

The type strain (KI11_C11^T = LMG 32129^T = NBRC 114987^T) was isolated from a flower of a slender honey-myrtle (*Melaleuca gibbosa*) in the suburb of Haines on Kangaroo Island, South Australia in 2019. The DNA G+C content of the type strain is 43.88 mol%. The GenBank accession numbers for the 16S rRNA gene sequence and draft genome sequence are ON545729 and CP097118, respectively.

DESCRIPTION OF *FRUCTILACTOBACILLUS MYRTIFLORALIS* SP. NOV.

Fructilactobacillus myrtifloralis (myr.ti.flo.ra'lis, L. fem. or masc. n. *myrtus*, myrtle tree; L. masc. adj. *floralis*, belonging to flowers; N.L. masc. adj. *myrtifloralis*, pertaining to the myrtle flower where the bacterium was isolated).

Cells are Gram-stain-positive, non-motile, non-spore forming, catalase-negative rods measuring 1.6–4.7×0.7–1.1 µm, occurring singly or in chains. D-lactic acid and L-lactic acid are produced from glucose. Heterofermentative, with the ratio of lactic acid, ethanol and acetic acid of 1:0.83:0.00 with respect to glucose fermentation and 1:0.42:0.53 with respect to glucose and fructose fermentation. After 48 h anaerobic growth on MRSF agar (MRS with 20 fructose), colonies appear ivory, circular, umbonate, undulate, dull and opaque, with diameters of 0.5–1.0 mm. Facultatively anaerobic. Growth in MRSF occurs at between pH 3.5 and 9.0, 10–40 °C and with 0–10.0% added NaCl and optimally at pH 4.0, 17 °C and with 0.0% added NaCl. Acid is produced from glycerol, D-glucose, D-fructose, D-mannitol, arbutin, aesculin ferric citrate and salicin and weakly from potassium gluconate. Positive for leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and β-glucosidase and weakly positive for cystine arylamidase. The major fatty acids are $C_{20:0}$, $C_{18:1}\omega7c$, $C_{20:1}\omega7c$, $C_{19:0}$cyclo ω7*c* and $C_{19:0}$cyclo ω9*c*. The cell wall peptidoglycan structure is A4α L-Lys–D-Asp.

The type strain (KI16_H9^T = LMG 32131^T = NBRC 114989^T) was isolated from a flower of a smooth fringe-myrtle (*Calytrix glaberrima*) in the suburb of Gosse on Kangaroo Island, South Australia in 2019. The DNA G+C content of the type strain is 46.96 mol%. The GenBank accession numbers for the 16S rRNA gene sequence and draft genome sequence are ON545727 and CP097116, respectively.

DESCRIPTION OF *FRUCTILACTOBACILLUS CARNIPHILUS* SP. NOV.

Fructilactobacillus carniphilus (car.ni'phi.lus, L. fem. n. *caro*, *carnis*, meat, flesh; N.L. masc. adj. *philus*, loving; N.L. masc. adj. *carniphilus*, flesh-loving, referring to the attraction to meat of the host of the bacterium).

Cells are Gram-stain-positive, non-motile, non-spore forming, catalase-negative rods measuring 1.4–5.1×0.6–0.9 µm, occurring singly or in chains. D-lactic acid and L-lactic acid are produced from glucose. Heterofermentative, with the ratio of lactic acid, ethanol and acetic acid of 1:0.69:0.00 with respect to glucose fermentation and 1:0.57:0.38 with respect to glucose and fructose fermentation. After 48 h anaerobic growth on MRSF agar (MRS with 20 g l^{-1} fructose), colonies appear beige, circular, umbonate, entire, dull and opaque, with diameters of 0.5–1.5 mm. Facultatively anaerobic. Growth in MRSF occurs at between pH 4.0 and 8.0, 10–45 °C and with 0–7.5% added NaCl and optimally at pH 6.0, 30 °C and with 0.0% added NaCl. Acid is produced from D-glucose, D-fructose, D-mannose and D-mannitol and weakly from potassium gluconate. Positive for leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase, weakly positive for cystine arylamidase. The major fatty acids are $C_{18:0}$, $C_{20:0}$, $C_{18:1}\omega7c$, $C_{18:1}\omega9c$, $C_{20:1}\omega7c$ and $C_{19:0}$cyclo ω7*c*. The cell wall peptidoglycan structure is A4α L-Lys–D-Asp.

The type strain (KI4_A6^T = LMG 32127^T = NBRC 114985^T) was isolated from the homogenate of a meat ant (*Iridomyrmex purpureus*) in the suburb of Haines on Kangaroo Island, South Australia, in 2019. The DNA G+C content of the type strain is 44.49 mol%. The GenBank accession numbers for the 16S rRNA gene sequence and draft genome sequence are ON545728 and CP097121, respectively.

DESCRIPTION *FRUCTOBACILLUS AMERICANAQUAE* SP. NOV.

Fructobacillus americanaquae (a.me.ri.can.a'quae. L. fem. n. *aqua*, water; N.L. gen. n. *americanaquae*, of American River, the suburb where the host of the isolate bacterium was found).

Cells are Gram-stain-positive, non-motile, non-spore forming, catalase-negative rods measuring 2.1–5.8×0.5–0.9 µm, occurring singly or in chains. D-lactic acid is produced from glucose. Heterofermentative, with the ratio of lactic acid, ethanol and acetic acid of 1:1.09:0.00 with respect to glucose fermentation and 1:0.13:0.85 with respect to glucose and fructose fermentation. After 48 h anaerobic growth on MRSF agar (MRS with 20 g l^{-1} fructose), colonies appear beige, circular, umbonate, entire, glistening and opaque, with diameters of 1–2 mm. Facultatively anaerobic. Growth in MRSF occurs at between pH 4.0 and 8.5, 17–37 °C and with 0–7.5% added NaCl and optimally at pH 5.5, 30 °C and with 4.0% added NaCl. Acid is produced from D-glucose, D-fructose, D-mannitol and D-trehalose. Weakly positive for leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. The major fatty acids are $C_{16:0}$, $C_{18:1}\omega7c$ and $C_{18:1}\omega9c$. The cell wall peptidoglycan structure is A4α L-Lys–L-Ala–L-Glu.

The type strain (KI3_B9^T = LMG 32124^T = NBRC 114983^T) was isolated from the homogenate of an oriental cockroach (*Blatta orientalis*) in the suburb of American River on Kangaroo Island, South Australia, in 2019. The DNA G+C content of the type strain is 43.64 mol%. The GenBank accession numbers for the 16S rRNA gene sequence and draft genome sequence are ON545731 and CP097122, respectively.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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