

Apilactobacillus apisilvae sp. nov., *Nicolia spurrieriana* gen. nov. sp. nov., *Bombilactobacillus folatiphilus* sp. nov. and *Bombilactobacillus thymidiniphilus* sp. nov., four new lactic acid bacterial isolates from stingless bees *Tetragonula carbonaria* and *Austroplebeia australis*

Scott A. Oliphant¹, Nathan S. Watson-Haigh^{2,3}, Krista M. Sumby^{1,4,*}, Jennifer Gardner¹, Scott Groom⁵ and Vladimir Jiranek^{1,4}

Abstract

Four strains, SG5_A10^T, SGEP1_A5^T, SG4_D2^T, and SG4_A1^T, were isolated from the honey or homogenate of Australian stingless bee species *Tetragonula carbonaria* and *Austroplebeia australis*. Based on 16S rRNA gene phylogeny, core gene phylogenetics, whole genome analyses such as determination of amino acid identity (AAI), cAAI of conserved genes, average nucleotide identity (ANI), and digital DNA–DNA hybridization (dDDH), chemotaxonomic analyses, and the novel isolation sources and unique geography, we propose three new species and one genus with the names *Apilactobacillus apisilvae* sp. nov. (SG5_A10^T = LMG 32133^T = NBRC 114991^T), *Bombilactobacillus thymidiniphilus* sp. nov. (SG4_A1^T = LMG 32125^T = NBRC 114984^T), *Bombilactobacillus folatiphilus* sp. nov. (SG4_D2^T = LMG 32126^T = NBRC 115004^T) and *Nicolia spurrieriana* sp. nov. (SGEP1_A5^T = LMG 32134^T = NBRC 114992^T). Three out of the four strains were found to be fructophilic, where SG5_A10^T and SGEP1_A5^T belong to obligately fructophilic lactic acid bacteria, and SG4_D2^T representing a new type denoted here as kinetically fructophilic. This study represents the first published lactic acid bacterial species associated with the unique niche of Australian stingless bees.

INTRODUCTION

Lactic acid bacteria (LAB) are beneficial in the suppression of spoilage agents in food and pathogenic organisms in many animals [1–4]. Fructophilic LAB (FLAB) are a group within the LAB that grow in fructose-rich environments such as fruit or flowers, but also the insects that depend on these as food sources [5–8]. Along with many Hymenoptera, bees utilize the pollen and nectar of flowers as a food source, resulting in these species harbouring common types of LAB and FLAB from a wide range of environments [9, 10]. *Apilactobacillus*, *Bombilactobacillus*, *Lactobacillus* and, to a lesser extent, *Fructobacillus* are the main genera

Author affiliations: ¹Department of Wine Science, School of Agriculture, Food & Wine, The University of Adelaide, Waite Campus, Urrbrae, SA 5064, Australia; ²South Australian Genomics Centre (SAGC), SAHMRI, Adelaide, SA 5000, Australia; ³Australian Genome Research Facility, Victorian Comprehensive Cancer Centre, Melbourne, VIC 3000, Australia; ⁴Australian Research Council Training Centre for Innovative Wine Production, Glen Osmond, SA 5064, Australia; ⁵Department of Agricultural Science, School of Agriculture, Food & Wine, The University of Adelaide, Waite Campus, Urrbrae, SA 5064, Australia.

***Correspondence:** Krista M. Sumby, krista.sumby@adelaide.edu.au

Keywords: *Apilactobacillus*; *Austroplebeia australis*; *Bombilactobacillus*; FLAB; *Fructobacillus*; fructophilic lactic acid bacteria; LAB; stingless bees; sugarbag bees; *Tetragonula carbonaria*.

Abbreviations: AAI, amino acid identity; ANI, average nucleotide identity; cAAI, amino acid identity of conserved genes; dDDH, digital DNA–DNA hybridization; FGYP, D-glucose with D-fructose; FLAB, fructophilic lactic acid bacteria; FYP, D-fructose; GSI, gene support index; GYP, D-glucose; GYP+P, D-glucose with pyruvate; LAB, lactic acid bacteria; MRS, de Man, Rogosa and Sharpe; MRSAJ, MRS broth supplemented with preservative-free apple juice; MRSAJM, MRS broth supplemented with preservative-free apple juice and malic acid; MRSF, MRS broth supplemented with D-fructose; MRSFC, MRS broth supplemented with D-fructose and L-cysteine; MRSFCFa, MRS broth supplemented with D-fructose, L-cysteine and folic acid; MRSFNucFa, MRSF supplemented with Embryomax nucleosides and folic acid; MSA, multiple sequence alignment.

The GenBank accession numbers for the 16S rRNA gene sequences for *Apilactobacillus apisilvae* (SG5_A10^T), *Nicolia spurrieriana* (SGEP1_A5^T), *Bombilactobacillus thymidiniphilus* (SG4_A1^T), *Bombilactobacillus folatiphilus* (SG4_D2^T) are OM986478, OM986479, OM986476 and OM986477, respectively. The GenBank accession numbers for the draft genome sequences for *Apilactobacillus apisilvae* (SG5_A10^T), *Nicolia spurrieriana* (SGEP1_A5^T), *Bombilactobacillus thymidiniphilus* (SG4_A1^T) and *Bombilactobacillus folatiphilus* (SG4_D2^T) are CP093362–CP093364, CP093360–CP093361, CP093365 and CP093366–P093367, respectively.

Eleven supplementary figures and four supplementary tables are available with the online version of this article.

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associated with bees, with several *Apilactobacillus* and all *Fructobacillus* species representing FLAB due to changes in the *adhE* gene [7, 8, 11, 12]. Species of the eusocial corbiculate bees (*Apidae: Apinae*) also harbour a distinct suite of host-specific LAB from these genera [11, 13, 14]. The honey stomach, honey, bee pollen and bee bread of all nine recognized honeybee and three stingless bee species harbour an abundance of LAB species [4], although relatively few bacteria have been found to colonize the honey stomach compared to the rectum in honey bees [15, 16], which consistently contains the majority of core honey bee *Lactobacillaceae* [17]. While stingless bees have been shown to harbour the same core strains shared across the corbiculates, they also tend to show greater shifts in population composition, reflecting their broad range of geographical and resource use [14]. In Australia, stingless bee microbiomes have also been found to differ between colonies reflecting this dynamism [18]. Relevant taxa that have been found to be associated with Australian stingless bee species are the historic 'Firm-4' clade or *Lactobacillus mellifer* group, containing the *Bombilactobacillus* species, the 'Firm-5' or *Lactobacillus melliventris* clade, containing *Lactobacillus* species, and a currently unclassified sister clade to an unidentified Halictidae-associated cluster [11, 18, 19]. Stingless bee operational taxonomic units from this sister clade do not significantly match any currently described species using BLAST (data not shown) [20]. Further, the LAB content of Australian stingless bee honey has not been described, but FLAB species in the genus *Fructobacillus* have been found in *Heterotrigona itama* honey [21]. Here we describe three new fructophilic and one glucophilic lactic acid bacterial isolates from the Australian stingless bee species *Tetragonula carbonaria* and *Austroplebeia australis*.

ISOLATION AND ECOLOGY

The sugar-rich niche of hives of Australian stingless bees belonging to the species *T. carbonaria*, *T. hockingsi* and *A. australis* were sampled for fermentative micro-organisms in the Brisbane, Queensland area. During December 2019–June 2021 honey, resin pots, worker bees and bee bread samples were collected aseptically in sterile tubes and transported to the Wine Microbiology Laboratory, Waite Campus, Adelaide, South Australia. Bees were killed immediately by homogenization in 100–400 µl sterile saline while resin pots were homogenized with several millilitres of sterile saline. Crude homogenates and honey were serially diluted in sterile saline and spiral plated using a Whitley automatic spiral plater (Don Whitley Scientific) on the following media. MRSAJ, MRS (de Man, Rogosa and Sharpe) broth (Oxoid) supplemented with 20% preservative-free apple juice (Golden Circle or Coles brand); MRSAJM, MRSAJ broth supplemented with 2 g l⁻¹ malic acid; and MRSFC, MRS broth supplemented with 20 g l⁻¹ D-fructose (ChemSupply) and 0.1% L-cysteine [22]. Solid media contained 2% agar and 100 mg l⁻¹ natamycin (Natap, Handry) to prevent fungal growth. The pH of all media was adjusted to pH 6.2 with either hydrochloric acid or sodium hydroxide. Agar plates were placed into a 20% CO₂ atmosphere at 30 °C for 1–7 days (Eppendorf CellXpert C170). Colonies were picked from plates at various time points depending on growth and colony morphology and placed into a liquid form of the corresponding medium without natamycin. After 4–7 days of growth, cultures were supplemented to 20% glycerol and stored at –80 °C. The 16S rRNA gene was amplified directly from 1/8 ultrapure water diluted glycerol stocks with primers 8F and 1492R [23]. For the first round of Sanger sequencing, only the first ~1000 bp of the 16S rRNA gene were sequenced. Several strains, designated SG5_A10^T, SGEP1_A5^T, SG4_A1^T and SG4_D2^T, appeared to have lower than 99.7% 16S rRNA gene similarity to type strains obtained from a BLAST search [20]. These were purified a further two times, and re-sequenced to obtain ~1420 bp of the 16S rRNA gene. Strains that maintained lower than 99.7% similarity to the nearest type strain were prepared for whole genome sequencing and basic phenotyping.

Strain SG5_A10^T was isolated from the homogenate of *A. australis* worker bees. Strains SGEP1_A5^T, SG4_A1^T and SG4_D2^T were isolated from the honey and homogenates, respectively, of *T. carbonaria* worker bees. Here, we propose the description of three novel species and one novel genus in the family *Lactobacillaceae* based on the novel isolation sources, unique geographical locale, 16S rRNA gene analyses, whole genome-based phylogeny, and chemotaxonomic analyses.

16S rRNA GENE PHYLOGENY

For this current study, genomic DNA was extracted from 2-day-old liquid cultures of SG5_A10^T and SGEP1_A5^T and ~7-day-old liquid cultures of SG4_D2^T and SG4_A1^T, using the MagAttract HMW DNA Kit (Qiagen). The protocol for Gram-positive bacteria was adopted with the addition of 100 U mutanolysin (Sigma-Aldrich) during the cell lysis step. DNA quantity was assessed through fluorometry using the Quant-iT dsDNA broad range assay kit (Invitrogen). DNA quality was assessed on 0.75% agarose gels by electrophoresis, and a NanoDrop One (Thermo Scientific). Genomic DNA samples were then sent on dry ice to Maryland Genomics at the Institute for Genome Sciences (University of Maryland School of Medicine, USA).

Sequencing libraries were generated for SMRT sequencing on the PacBio Sequel II instrument. Briefly, genomic DNA samples were fragmented to an average size of ~13 kbp with a g-Tube (Covaris). Library construction was performed by using SMRTbell Express Template Prep Kit 2.0 (Pacific Bioscience) with barcoded overhang adapters. Fragments shorter than 10 kbp were removed through size-selection on BluePippin instrument (Sage Science). The final library pool was sequenced with Sequel II Sequencing 2.0 chemistry and SMRT Cell 8M on the Sequel II instrument. All reads of each sample were assembled using the microbial assembly module of the SMRTLink9.0 pipeline and default parameters. Resulting contigs were polished using Arrow software with default parameters to generate final contig consensus. Overlapping regions from the ends of uncircularized contigs were trimmed

Table 1. Genome features for strains SG5_A10^T, SGEP1_A5^T, SG4_A1^T and SG4_D2^T

Species	<i>Apilactobacillus apisilvae</i>	<i>Nicola spurrieriana</i>	<i>Bombilactobacillus thymidiniphilus</i>	<i>Bombilactobacillus folatiphilus</i>
Strain	SG5_A10 ^T	SGEP1_A5 ^T	SG4_A1 ^T	SG4_D2 ^T
Sample origin (species)	<i>Austroplebeia australis</i>	<i>Tetragonula carbonaria</i>	<i>Tetragonula carbonaria</i>	<i>Tetragonula carbonaria</i>
Sample origin (material)	Whole bee homogenate	Honey	Whole bee homogenate	Whole bee homogenate
Accession No.	CP093362-CP093364	CP093360-CP093361	CP093365	CP093366-CP093367
Genome size	1550949	2053587	1494436	1637944
No. of CDSs	1494	1646	1452	1550
No. of contigs	3	2	1	2
Contig 1 size (chromosome)	1469670	1709727	1494436	1622785
Contig 2 size (plasmid 1)	42663	343860	0	15159
Contig 3 size (plasmid 2)	38616	0	0	0
Coverage (x)	11482	6375	3628	7404
G+C content (mol%)	30.98	42.11	36.42	38.38
N50	1469670	1709727	1494436	1622785
N75	1469670	1709727	1494436	1622785
No. of unspecified bases (N's) per 100 kbp	0	0	0	0
Check M completeness	97.86	97.14	97.91	99.13
Check M contamination	0.63	0.78	0.26	0

using minimus2 from the AMOS package with default parameters (<http://amos.sourceforge.net/wiki/index.php/Minimus2>). Final circularized and polished contigs were evaluated and assessed using QUAST [24]. Contamination and the completeness of each genome assembly was assessed through CheckM [25]. Whole genome metrics are shown in Table 1.

Genome assemblies were annotated using Prokka version 1.14.6 [26]. All annotated 16S rRNA genes were extracted and uploaded to EZBioCloud's 16S-based ID App [27]. All resulting 16S rRNA gene hits were downloaded for phylogenetic analysis and shown in Table S1. The 16-based ID App as well as the Pairwise Nucleotide Sequence Alignment For Taxonomy Tool was used to calculate 16S rRNA gene similarities [27]. A single 16S rRNA gene sequence was obtained for each of two species [*Enterococcus massiliensis* AM1^T (NR_144723.2) and *Lactococcus chungangensis* CAU 28^T (NR_044357.1)] to be used as an outgroup. The corresponding genome assemblies were also obtained for these type strains (Table S1).

The 16S rRNA gene sequences were aligned, unreliably aligned regions were masked and then converted to FASTA-formatted multiple sequence alignments using SSU-ALIGN version 0.1.1 with default parameters [28]. Masked multiple sequence alignments from SSU-ALIGN were used to calculate pairwise sequence identity matrices using the SeqinR version 4.2_8 package and as input into RAXML version 8.2.12 for performing phylogenetic reconstruction [29, 30]. 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).

As shown in Fig. 1, strain SG5_A10^T is phylogenetically placed well within *Apilactobacillus* clade with 94% bootstrap support. SG5_A10^T displayed the highest 16S rRNA gene identities to *Apilactobacillus quenuiae* HV_6^T (98.94%) *A. micheneri* 10H^T (98.93%), and *A. timberlakei* HV_26^T (98.81%) [31]. Further, SG5_A10^T exhibited 1S rRNA gene identities higher than 94.5% to type species of *Apilactobacillus*, supporting its appropriate placement within this genus [11, 32]. The phylogenetic position of strain SGEP1_A5^T is firmly outside of the *Apilactobacillus* and *Fructilactobacillus* clades as each of these have strong ($\geq 94\%$) bootstrap support. However, poor (<80%) bootstrap support means the branching order is not well defined using the 16S rRNA gene alone. SGEP1_A5^T has the highest 16S rRNA gene percent identities to *Lentilactobacillus curieae* CCTCC M 2011381^T (93.64%), *L. kisonensis* YIT 11168^T (93.37%) and *Apilactobacillus ozensis* Mizu2-1^T (93.35%) [33–35]. These values are at the limit of the 94.5% threshold for correct genus-level assignment based on 16S rRNA gene sequence similarity [11, 32]. Further, due to the lack of integral clustering to the genera *Lentilactobacillus* and *Apilactobacillus*, it was expected that SGEP1_A5^T would not be assigned to either genus.

SG4_A1^T displayed the highest 16S rRNA gene identities to SG4_D2^T (96.61%), *Bombilactobacillus bombi* BTLCH M1/2^T (95.28%) and *B. mellis* Hon2N^T (95.22%) [36, 37]. SG2_D2^T similarly matched these two strains, albeit with lower identities for *B. bombi*

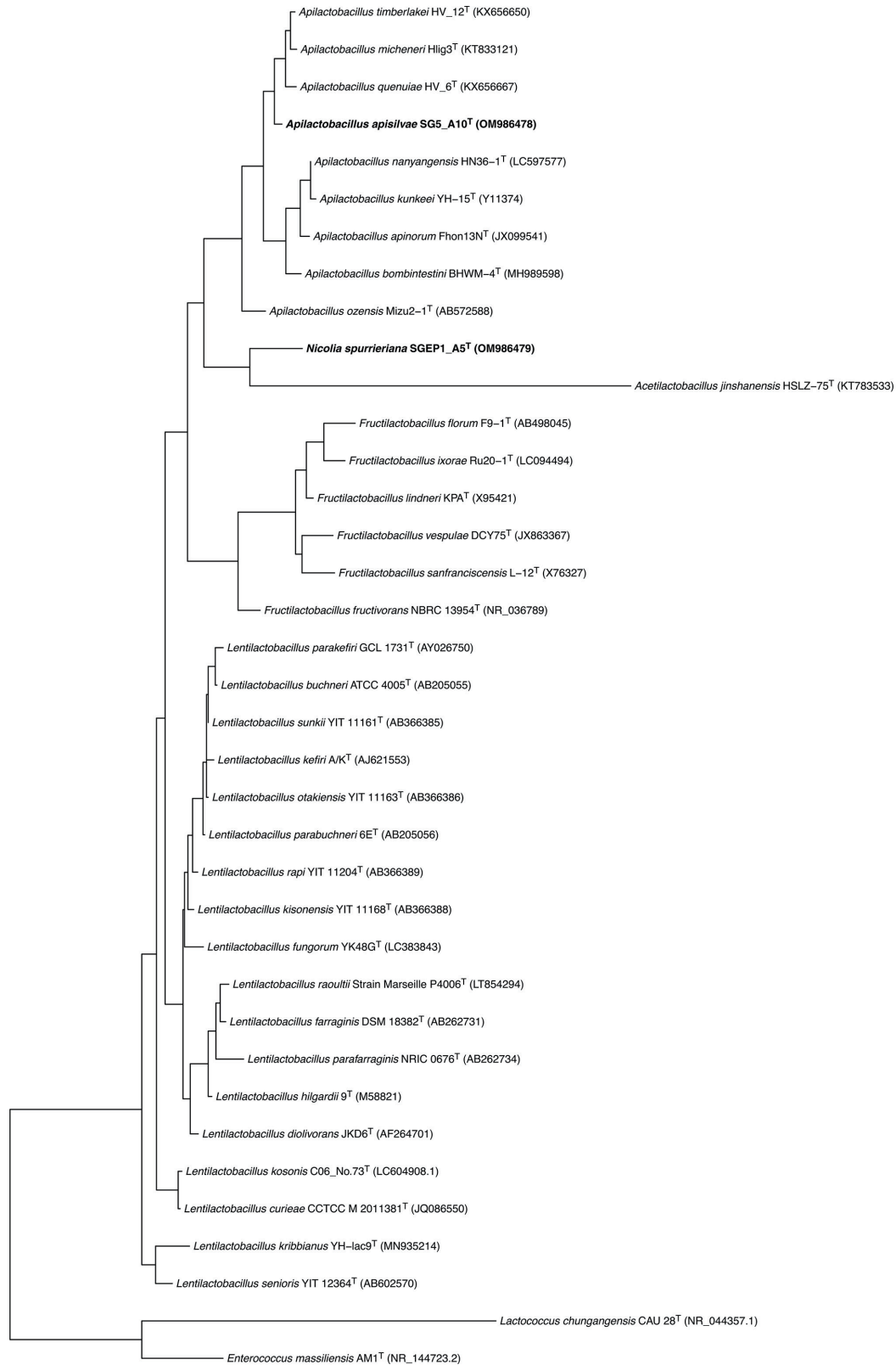


Fig. 1. A maximum-likelihood phylogenetic tree reconstructed from 16S rRNA gene sequences relating to whole genomes extracted 16S rRNA gene sequences for strains SG5_A10^T and SGEP1_A5^T. GenBank accession numbers for the sequences are provided in parentheses. The phylogenetic analysis used the GTR-G model with 5000 bootstrap replicates. Bootstrap values below 80 are not shown. An outgroup consisting of *Enterococcus massiliensis* and *Lactococcus chungangensis* type strain sequences were used to root the tree. The scale bar shows the mean number of nucleotide substitutions per site.

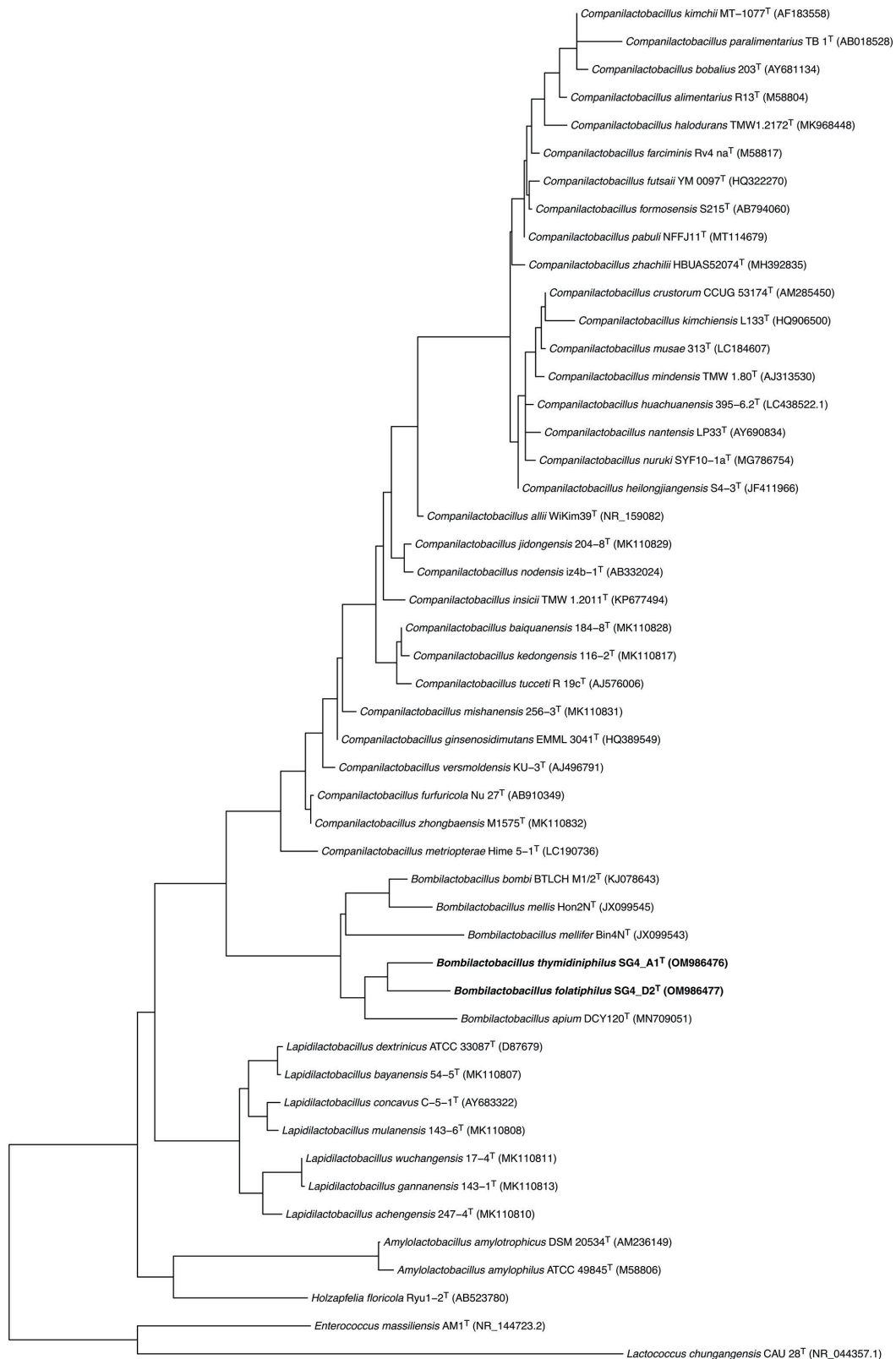


Fig. 2. A maximum-likelihood phylogenetic tree reconstructed from 16S rRNA gene sequences relating to whole genome extracted 16S rRNA gene sequences for strains SG4_A1^T and SG4_D2^T. GenBank accession numbers for the sequences are provided in parentheses. The phylogenetic analysis used the GTR-G model with 5000 bootstrap replicates. Bootstrap values below 80 are not shown. An outgroup consisting of *Enterococcus massiliensis* and *Lactococcus chungangensis* type strain sequences were used to root the tree. The scale bar shows the mean number of nucleotide substitutions per site.

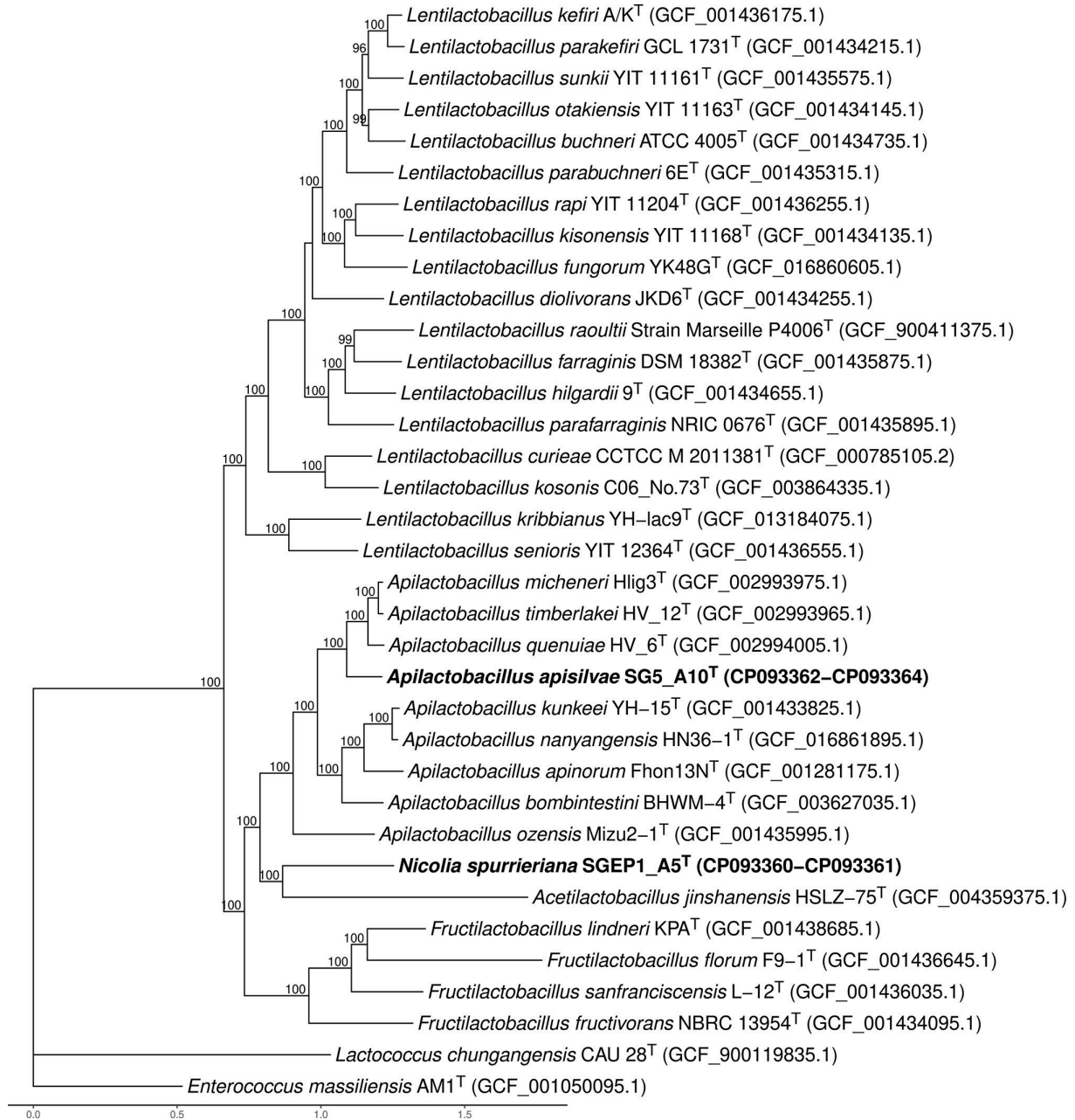


Fig. 3. A maximum-likelihood phylogenetic tree reconstructed from the concatenated multiple sequence alignments of 81 core bacterial genes according to UBCG2 relating to strains SG5_A10^T and SGEP1_A5^T. GenBank accession numbers for the genome assemblies are provided in parentheses. The phylogenetic analysis used the GTR+CAT model and gene support index values below 80 are not shown. An outgroup consisting of *Enterococcus massiliensis* and *Lactococcus chungangensis* type strain sequences were used to root the tree. The scale bar shows the mean number of nucleotide substitutions per site.

BTLCH M1/2^T (94.25%) and *B. mellis* Hon2N^T (94.28%). While the 16S rRNA gene similarities are near the threshold for appropriate genus-level assignment, as shown in Fig. 2, strains SG4_A1^T and SG4_D2^T are placed strongly within the *Bombilactobacillus* clade with 100% bootstrap support.

GENOME-BASED PHYLOGENY

A phylogenetic tree was reconstructed from concatenated multiple sequence alignments of 81 core bacterial genes (Figs. 3 and 4). We processed the genome assemblies of the present species and the identified type strains through the UBCG2 pipeline [38].

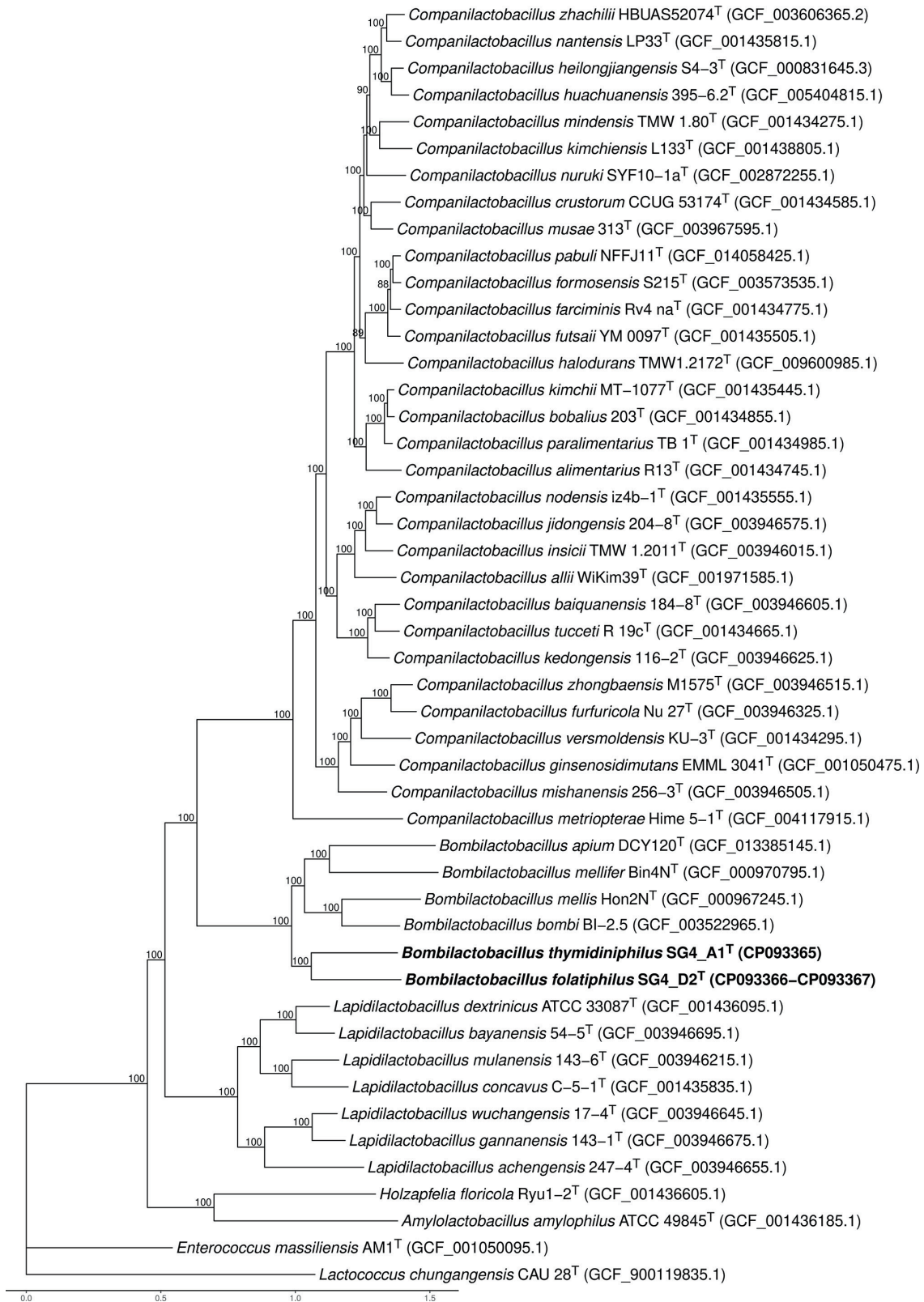


Fig. 4. A maximum-likelihood phylogenetic tree reconstructed from the concatenated multiple sequence alignments of 81 core bacterial genes according to UBCG2 relating to strains SG4_A1^T and SG4_D2^T. GenBank accession numbers for the genome assemblies are provided in parentheses. The phylogenetic analysis used the GTR+CAT model and gene support index values below 80 are not shown. An outgroup consisting of *Enterococcus massiliensis* and *Lactococcus chungangensis* type strain sequences were used to root the tree. The scale bar shows the mean number of nucleotide substitutions per site.

In summary, this pipeline uses Prodigal and HMMER to identify and extract the sequences of 81 core bacterial genes, MAFFT to perform multiple sequence alignments of the genes and RAXML to perform phylogeny reconstruction using the GTR+CAT model. Bipartitions of the resulting phylogeny show the gene support index (GSI) values reported by UBCG2 of $\geq 95\%$.

The phylogenetic position of SG5_A10^T is clearly resolved by the concatenated gene tree and is placed well within the *Apilactobacillus* clade with 100% GSI (Fig. 3). Strain SGEP1_A5^T is fully resolved and is placed with *Acetilactobacillus jinshanensis* HSLZ-75^T as a sister clade to *Apilactobacillus*. Strains SG4_D2^T and SG4_A1^T are most closely related to each other and together form a sister group relationship to the *Bombilactobacillus* clade with 100% GSI (Fig. 4). Overall, this clade appears to represent three groups with the addition of SG4_A1^T and SG4_D2^T.

To assist in the appropriate taxonomic level assignment, average nucleotide identity (ANI) was calculated for every pairwise combination of genomes sequences using OrthoANU version 1.2 with USEARCH version 11.0.667 [39, 40]. The resulting output files were parsed to generate an adjacency list of ANI values for all pairs of genomes. Average amino acid identity (AAI) was calculated for every pairwise combination of genomes sequences using EzAAI version 1.2 with prodigal version 2.6.3 and MMSeqs2 version 13.45111 [41–43]. ANI and AAI values were plotted as heatmaps using the ComplexHeatmap version 2.10.0 package (Figs. S1–S4, available with the online version of this article) [44]. Digital DNA–DNA hybridization (dDDH) values were calculated by the Genome-to-Genome Distance Calculator (Table S2) [45, 46]. To further clarify and support the genus-level assignment of our strains, AAI of conserved genes (cAAI) was calculated using the set of 530 orthologous genes defined by Zheng *et al.* [11]. First, multiple sequence alignments (MSAs) for each of the 530 sets of orthologous genes were generated using MUSCLE version 5.1 and a consensus sequence generated using a profile HMM generated by HMMer version 3.3.2 [47]. A representative HMM from the Prokaryotic Genome Annotation Pipeline was extracted for each consensus sequence and used as input to UBCG2 to generate MSAs for each of 384 genes from the genomes used in this study [48]. The cAAI for a pair of genomes was calculated by averaging the amino acid identity calculated for each of the 384 genes from their corresponding MSAs using dist.alignment from the seqinr version 4.2.8 package [29]. cAAI values were plotted as heatmaps using the ComplexHeatmap version 2.10.0 package (Figs. S5, S6) [44]. ANI, AAI, dDDH and cAAI data from the top six closely related type strains are shown in Table 2.

Strain SG5_A10^T displayed AAI values that ranged from 60.5 to 82.9% for all type strains analysed, with the highest values associated with type strains of *Apilactobacillus* (Table 2, Fig. S1). Along with the 16S rRNA gene and concatenated gene analyses, this strongly places it within the genus *Apilactobacillus*. Further, the ANI and dDDH values ranged from 67.6 to 82.0% and 17.4 to 25.4%, respectively, for all type strains analysed in *Apilactobacillus* and other closely related genera within the family *Lactobacillaceae* (Fig. S3, Table S2, Table 2). These values are well below 95 and 70% respective ANI and dDDH thresholds for the assignment to published species [45, 46, 49–53]. While cAAI analyses are not required in this scenario, the intra-genus identities in our analysis ranged from 74.6–98.1% for apilactobacilli (Fig. S5). SG5_A10^T falls within this range, exhibiting cAAI values of 75.5–88.3% with respect to other apilactobacilli.

Strain SGEP1_A5^T displayed AAI values ranging from 60.8 to 66.0% for all type strains analysed (Table 2, Fig. S1). While the highest values were associated with other apilactobacilli (65.2–66.0%), these fall below the 68% threshold to confidently assign this strain to a particular genus [11, 54]. Based on the cAAI and concatenated gene analyses, the genus *Apilactobacillus* can be split into two core groups (Fig. S5). *Apilactobacillus ozensis* Mizu2-1^T appears distant to these groups, but maintains high intra-genus cAAI values that range from 74.6–75.9%. The highest inter-genus cAAI values are between *Apilactobacillus* and *Lentilactobacillus*, which reach 69.5%. SGEP1_A5^T maintains low cAAI values between itself and other apilactobacilli, ranging from 69.8–70.4%. Slightly lower cAAI values can be seen between SGEP1_A5^T and *Lentilactobacillus* reaching 68.3%. This data is consistent with the phylogenetic trees, where SGEP1_A5^T is observed as a sister clade to *Apilactobacillus*. Consistent with this is the presence and absence of signature genes. Three of the eight signature genes defined by Zheng *et al.* [11] are missing in SGEP1_A5^T (see supplementary data for signature gene files). The remaining five genes support a common evolutionary history of SGEP1_A5^T and other apilactobacilli. However, due to a lack of closely related species, a signature gene analysis for SGEP1_A5^T remains impossible. Additional species published in the future will likely clarify this. Other major differences between SGEP1_A5^T and currently published type strains of apilactobacilli are genome size and G+C content (Fig. S7).

Overall, ANI and dDDH values ranged from 66.6 to 71.3% and 17.8 to 25.5%, respectively, between SGEP1_A5^T and all analysed type strains, far below the species limit threshold (Fig. S3, Table S2, Table 2) [45, 46, 49–53]. Further, the low separation of intra vs. inter genus cAAI values, low AAI values between SGEP1_A5^T and other established species supports the establishment of a novel genus within the family *Lactobacillaceae*.

Strains SG4_A1^T and SG4_D2^T displayed AAI values of 60.7–70.7% for all type strains analysed, with the highest values associated between these strains and other bombilactobacilli (Fig. S2, Table 2). AAI values ranged from 67.8 to 70.7% between SG4_A1^T and SG4_D2^T and other bombilactobacilli, which is at the threshold for appropriate genus-level assignment based on this metric. However, the strong clustering from the 16S rRNA gene and concatenated gene analyses strongly place these two strains within the genus *Bombilactobacillus*. Further support is bolstered with the higher intra vs. inter-genus cAAI values with the inclusion of these two species in the *Bombilactobacillus* clade (Fig. S6, Table 2). Both strains exhibited ANI and dDDH values of 67.9–72.8 and 19.2–37.1%, respectively, far below the thresholds for the correct assignment to any published species (Fig. S4, Table S2, Table 2) [45, 46, 49–53].

Table 2. Pairwise average nucleotide identity (ANI), average nucleotide identity (AAI), amino acid identity of conserved genes (cAAI) and digital DNA–DNA hybridization (dDDH) values from Figs. S1–S6 and Table S2, between our strains and six most similar strains based on cAAI

SG5_A10 ^T					
Reference genome	Closely related species	ANI (%)	AAI (%)	cAAI (%)	dDDH (%)
GCF_002993975.1	<i>Apilactobacillus micheneri</i> Hlig3 ^T	82.0	82.9	88.3	24.3
GCF_002993965.1	<i>Apilactobacillus timberlakei</i> HV_12 ^T	81.5	82.0	88.1	23.7
GCF_002994005.1	<i>Apilactobacillus quenuiae</i> HV_6 ^T	81.3	82.4	87.9	23.7
GCF_003627035.1	<i>Apilactobacillus bombintestini</i> BHW-4 ^T	76.0	74.5	80.3	19.6
GCF_001433825.1	<i>Apilactobacillus kunkeei</i> YH-15 ^T	74.5	73.8	80.1	18.5
GCF_016861895.1	<i>Apilactobacillus nanyangensis</i> HN36-1 ^T	74.3	73.5	80.1	18.4
All species analysed		67.6–82.0	60.5–82.9	63.0–88.3	17.4–25.4
SGEP1_A5 ^T					
Reference genome	Closely related species	ANI (%)	AAI (%)	cAAI (%)	dDDH (%)
GCF_001435995.1	<i>Apilactobacillus ozensis</i> Mizu2-1 ^T	70.1	65.6	70.4	20.7
GCF_002993975.1	<i>Apilactobacillus micheneri</i> Hlig3 ^T	70.2	65.8	70.4	18.5
GCF_003627035.1	<i>Apilactobacillus bombintestini</i> BHW-4 ^T	70.4	66.0	70.3	21.9
GCF_002993965.1	<i>Apilactobacillus timberlakei</i> HV_12 ^T	70.3	65.5	70.2	18.4
GCF_001433825.1	<i>Apilactobacillus kunkeei</i> YH-15 ^T	69.4	65.6	70.2	19.1
CP093362–CP093364	<i>Apilactobacillus apisilvae</i> SG5_A10 ^T	71.3	65.6	70.1	19.3
All species analysed		66.6–71.3	60.8–66.0	63.4–70.4	17.8–25.5
SG4_A1 ^T					
Reference genome	Closely related species	ANI (%)	AAI (%)	cAAI (%)	dDDH (%)
CP093366–CP093367	<i>Bombilactobacillus folatiphilus</i> SG4_D2 ^T	72.8	70.7	76.1	20.5
GCF_003522965.1	<i>Bombilactobacillus bombi</i> BI-2.5	72.0	68.9	74.9	19.6
GCF_000967245.1	<i>Bombilactobacillus mellis</i> Hon2N ^T	71.4	68.5	74.2	19.2
GCF_000970795.1	<i>Bombilactobacillus mellifer</i> Bin4N ^T	70.4	68.0	73.6	19.2
GCF_013385145.1	<i>Bombilactobacillus apium</i> DCY120 ^T	70.7	67.8	72.7	19.4
GCF_003573535.1	<i>Companilactobacillus formosensis</i> S215 ^T	68.0	60.8	63.5	23.7
All species analysed		67.2–72.8	58.9–70.7	59.3–76.1	19.2–37.1
SG4_D2 ^T					
Reference genome	Closely related species	ANI (%)	AAI (%)	cAAI (%)	dDDH (%)
CP093365	<i>Bombilactobacillus thymidiniphilus</i> SG4_A1 ^T	72.8	70.7	76.1	20.5
GCF_003522965.1	<i>Bombilactobacillus bombi</i> BI-2.5	71.3	68.6	74.9	21.4
GCF_000967245.1	<i>Bombilactobacillus mellis</i> Hon2N ^T	70.8	68.5	74.1	20.0
GCF_000970795.1	<i>Bombilactobacillus mellifer</i> Bin4N ^T	70.4	67.9	73.4	20.4
GCF_013385145.1	<i>Bombilactobacillus apium</i> DCY120 ^T	70.2	67.8	73.0	20.5
GCF_003573535.1	<i>Companilactobacillus formosensis</i> S215 ^T	67.9	60.7	63.3	23.9
All species analysed		66.3–72.8	58.7–70.7	58.9–76.1	20.0–30.7

PHYSIOLOGY AND CHEMOTAXONOMY

Due to the close phylogeny of the strains to several known obligately FLAB, all strains were assessed for their potential fructophilic properties as originally outlined by Endo and Okada [55]. As a preliminary test, all strains were assessed for simple end-point growth in various media based on supplemented variations of MRS or GYP (Fig. S8) [55]. Briefly, strains were inoculated in triplicate into

1 ml of each liquid medium in deep (2 ml) 96-well plates and sealed with breathable cloth (BF-400-S, Axygen) prior to static anaerobic incubation. After 6 days, growth was assessed by optical density at 600 nm with an Infinite 200 Pro spectrophotometer (Tecan) and values were subtracted from a negative control. All phenotypic experiments were conducted similarly unless otherwise noted. All strains exhibited a fructophilic phenotype, except SG4_D2^T. Interestingly, this strain could not be grown to appreciable densities without the inclusion of 20% preservative free apple juice to MRSF (i.e., MRSF vs MRSF+AJ). Strain SG4_A1^T also appeared to grow better with the inclusion of apple juice. To decipher what compounds may have supported more luxuriant laboratory growth, Biolog phenotypic microarray plates were employed (Biolog). Both strains were first tested with plate PM5, where strains SG4_A1^T and SG4_D2^T were inoculated into MRSF and MRSFC respectively, and 200 µl were added to each well of the plate. This plate was tested first due to known vitamin auxotrophies for some LAB [56]. SG4_D2^T grew to a higher optical density with the inclusion of folic acid, and to a lesser extent, thymidine (Fig. S9). No sole compound enhanced growth of SG4_A1^T from plate PM5, thus plates PM1–8 were tested. Supplementation with compounds such as dipeptides, folic acid and nucleosides enhanced growth (Fig. S9). Taking this into account, SG4_A1^T grew to significantly higher densities in MRSF supplemented with the commercially available Embryomax nucleosides (100×; 0.73 g l⁻¹ cytidine, 0.85 g l⁻¹ guanosine, 0.73 g l⁻¹ uridine, 0.8 g l⁻¹ adenosine, 0.24 g l⁻¹ thymidine; Sigma-Aldrich) and 1 mg l⁻¹ folic acid (MRSFNucFa) over MRSF alone or MRSF with apple juice (Fig. S10). Similarly, SG4_D2^T grew to significantly higher densities in MRSF supplemented with 0.1% L-cysteine and 1 mg l⁻¹ folic acid (MRSFCFa) over MRSF alone or MRSF with apple juice. Additionally, Embryomax nucleosides (100×) could be added to MRSFCFa to enhance growth, but was found not to be necessary to obtain harvestable quantities of this strain within 2–3 days (Fig. S10). While the inclusion of fructose and L-cysteine was used in the isolation and growth of other bombilactobacilli, SG4_A1^T and SG4_D2^T represent extraordinarily fastidious new additions to the genus [37].

To further understand the fructophilic nature of strains SG5_A10^T and SGEP1_A5^T, growth was assessed in D-glucose (GYP), D-fructose (FYP), and D-glucose plus pyruvate (GYP+P) or D-fructose (FGYP), each with 10 g l⁻¹ of each carbohydrate [55, 57]. Strains SG4_A1^T and SG4_D2^T were grown in the same four media with the inclusion of their respective MRSF growth factors. Growth (OD₆₀₀) was monitored every 6 h from sacrificial samples (Fig. 5). SG5_A10^T and SGEP1_A5^T displayed similar phenotypes to obligately FLAB, that is, poor growth in GYP, and enhanced growth in FYP or GYP+P or FGYP. While oxygen did stimulate growth on D-glucose, its effect was much smaller than reported for other obligately FLAB [12, 57]. This is likely due to the effect of agitation in previous studies, which can enhance bacterial growth irrespective of the presence of oxygen [58]. SG4_A1^T displayed a reduced growth rate in FYP compared to GYP, while the addition of fructose, pyruvate, or oxygen either did not change or decreased growth. SG4_D2^T displayed a reduced rate of growth in GYP compared to FYP and FGYP. Oxygen was inhibitory to growth by this species in all growth conditions tested. To further understand the phenotypes of these species, sugar consumption and mannitol production were assessed using D-fructose/D-glucose and D-mannitol assay kits (Megazyme; Fig. S11). SG5_A10^T and SGEP1_A5^T behaved similarly, with mannitol being the major product during the consumption of fructose, consistent with other FLAB [8]. Both strains fully consumed fructose in both FYP and FGYP. In FGYP, all glucose was consumed (by 56 h) in comparison to only slow or incomplete consumption in GYP alone. This, along with the greater concentration of mannitol produced in FGYP supports the fact that fructose is being used as an electron acceptor to aid in the oxidation of glucose [8, 12]. No mannitol was produced by either SG4_A1^T or SG4_D2^T. SG4_D2^T consumed more fructose than glucose in FGYP. Overall, SGEP1_A5^T and SG5_A10^T were obligately FLAB similar to other apilactobacilli [12]. This is further supported by their lack of an ADH domain of *adhE* (data not shown). However, SG4_D2^T represents a novel fructophilic phenotype, denoted here as kinetically fructophilic.

To determine the utilization of sole carbohydrates, API 50 CH strips (bioMérieux) were used following the manufacturer's protocol with the following modifications. The API 50 CHL medium was used for SG5_A10^T and SGEP1_A5^T. For SG4_A1^T, the API 50 CHL medium was supplemented with Embryomax nucleosides (100×) and 1 mg l⁻¹ folic acid. For SG4_D2^T, it was supplemented with 0.1% L-cysteine and 1 mg l⁻¹ folic acid. SG5_A10^T and SGEP1_A5^T were incubated aerobically with a sterile mineral oil overlay at 30 °C, while strains SG4_A1^T and SG4_D2^T were incubated anaerobically with a sterile mineral overlay at 30 °C. Observations were recorded daily for 14 days for SG5_A10^T and SGEP1_A5^T and 30 days for SG4_A1^T and SG4_D2^T. Enzyme activity was assessed using API ZYM (bioMérieux) strips with no modification to the manufacturer's protocol. Data for the API 50CH and API ZYM panels are shown in Tables S3A and S3B. Strain SG5_A10^T was not easily distinguished from other apilactobacilli due to the similarity of both its carbohydrate utilization and enzymatic patterns to type strains of the genus. However, strain SGEP1_A5^T was easily distinguished by the unique presence of α-glucosidase and the ability to ferment methyl α-D-glucopyranoside compared to all apilactobacilli (Table S3A). Strains SG4_A1^T and SG4_D2^T had the greatest similarity to *B. bombi* BTLCH M1/2^T. SG4_D2^T was unique with respect to other bombilactobacilli with the ability to ferment L-sorbose, and weakly ferment D-mannitol, D-sorbitol and potassium 5-ketogluconate and the lack of β-glucosidase activity (Table S3B).

For the following phenotypic experiments SG5_A10^T and SGEP1_A5^T were grown in MRSF, SG4_A1^T in MRSFNucFa, and SG4_D2^T in MRSFCFa. Growth at various temperatures was assessed using 2 ml deep 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. Growth with 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3, 4, 5, 7.5, 10, 12.5, 15 and 20% added NaCl to each medium was also assessed. Growth at 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 13 ml of media. Catalase activity was checked by placing fresh colonies into 3% (v/v) H₂O₂ on a glass slide and observing for gas production. Catalase activity was compared with RAST annotations of the genomes to search for the presence of NADH/NADPH peroxidase or catalase genes [59]. Gram-staining was conducted using the

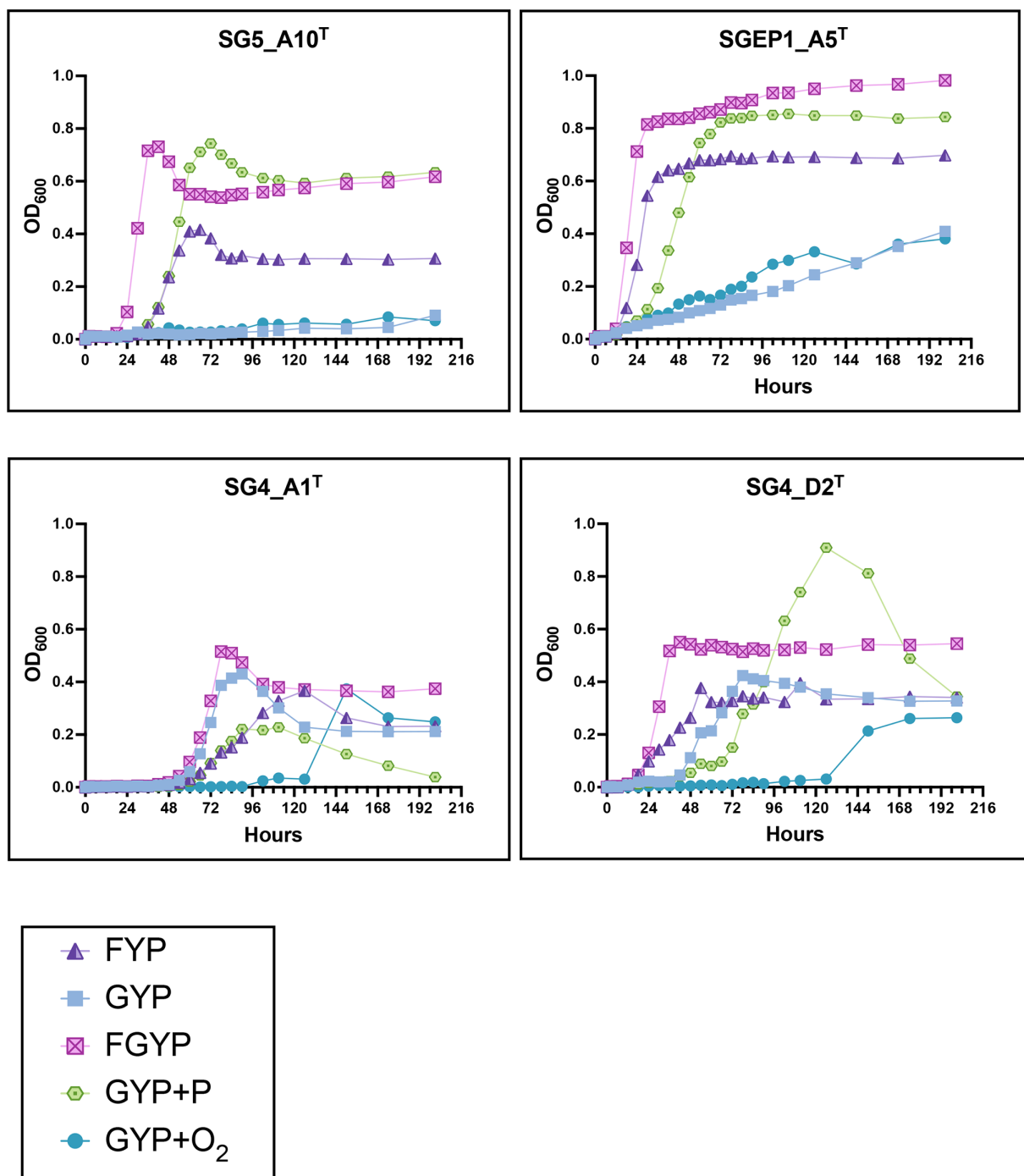


Fig. 5. Growth, as assessed by optical density at 600 nm, of strains SG5_A10^T, SGEP1_A5^T, SG4_A1^T, and SG4_D2^T in GYP, FYP, FGYP, and GYP+P broth under anaerobic conditions, as well as GYP broth under aerobic conditions.

Gram-staining kit (Sigma-Aldrich), and spore stain using the Schaeffer and Fulton spore stain kit (Sigma-Aldrich). Cells were imaged and measured using a Leica DM300 microscope. Lactic acid stereoisomers were determined by the D-/L-lactic acid rapid assay kit (Megazyme). Fermentation type was assessed through HPLC measurement of end products from the fermentation of glucose as described by Sumby *et al.* [60]. Peptidoglycan structures were determined through the Identification Service of the DSMZ. Isolation and structure determination were performed using established protocols [61]. All aforementioned chemotaxonomic data are detailed in the relevant species descriptions. Cellular fatty acids were determined through the Identification Service of the DSMZ using the Sherlock Microbial Identification System (MIDI) and compared to relevant taxa (Tables S4A and B). For identity confirmation and to

resolve summed features of the MIDI analysis, the analysis was supplemented by GC-MS (Table S4C). Major fatty acids reported in species description were determined by these GC-MS-mediated corrections.

DESCRIPTION OF *NICOLIA* GEN. NOV.

Nicolia (Ni.co'li.a N.L. fem. n. *Nicolia* named in honour of Dr Nicola Spurrier, Chief Public Health Officer of South Australia, who went above and beyond the call of duty in managing the COVID-19 pandemic in South Australia).

Cells of the only described species are Gram-stain-positive, non-motile, non-spore-forming, catalase-negative rods measuring 2.4–6.3×0.9–1.2 µm, occurring singly or in pairs. Catalase and NADH peroxidase genes are present. After anaerobic growth on MRSF agar (MRS with 20 g l⁻¹ fructose), colonies appear beige, circular, raised, entire, glistening, opaque, with a diameter of 1–3 mm. The type species is *Nicolia spurrieriana*.

DESCRIPTION OF *NICOLIA SPURRIERIANA* SP. NOV.

Nicolia spurrieriana (spur.rier.i.a'na. N.L. fem. adj. *spurrieriana* named in honour of the Spurrier family of South Australia).

Cells are rods measuring 2.4–6.3×0.9–1.2 µm, occurring singly or in pairs. D-Lactic acid and L-lactic acid are produced from glucose. Heterofermentative, with the ratio lactic acid, ethanol, and acetic acid 1:0.17:0.36 with respect to glucose fermentation. Facultatively anaerobic and obligately fructophilic. Growth in D-glucose is enhanced with pyruvate, fructose and, to a minimal extent, oxygen. Growth in MRSF occurs between pH 4.0–7.0, 17–37 °C and 0–0.5% added NaCl, and optimally at pH 6.8, 30 °C, and 0.0% added NaCl. Acid is produced from D-glucose, D-fructose, methyl α-D-glucopyranoside, maltose and sucrose, and weakly for D-mannitol and raffinose. The major fatty acids are C_{16:0} and C_{19:0} cyclo ω7c. Positive for leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, alpha-glucosidase and weakly for valine arylamidase. The cell-wall peptidoglycan structure is A4α L-Lys-D-Asp.

The type strain (SGEP1_A5^T=LMG 32134^T=NBRC 114992^T) was isolated from the honey of the sugarbag bee (*Tetragonula carbonaria*) collected by Tim Heard in the suburb of West End, Brisbane, Queensland, Australia, in 2020. The DNA G+C content of the type strain is 42.11 mol%.

DESCRIPTION OF *APILACTOBACILLUS APISILVAE* SP. NOV.

Apilactobacillus apisilvae (a.pi.sil'vae. L. fem. n. *apis*, bee; L. fem. n. *silva*, forest; N.L. gen. n. *apisilvae*, forest bee, pertaining to the bush bee *Austroplebeia australis*).

Cells are Gram-stain-positive, non-motile, non-spore-forming catalase-negative rods measuring 1.3–2.4×0.7–1.1 µm, occurring singly or in pairs. Catalase and NADH peroxidase genes are present. D-Lactic acid and L-lactic acid are produced from glucose. Heterofermentative, with the ratio lactic acid, ethanol and acetic acid 1:0.21:0.55 with respect to glucose fermentation. After anaerobic growth on MRSF agar (MRS with 20 g l⁻¹ fructose), colonies appear white, circular, slightly umbonate, entire, glistening, opaque, with a diameter of 1–2 mm. Facultatively anaerobic and obligately fructophilic. Growth in D-glucose is enhanced with pyruvate, D-fructose and, to a minimal extent, oxygen. Growth in MRSF occurs between pH 4.5–7.0, 17–30 °C, 0–0.5% added NaCl, and optimally at pH 5.0, 28 °C and 0.5% added NaCl. Acid is produced from D-glucose, D-fructose and sucrose. The major fatty acids are C_{16:0}, C_{18:1} ω9c and a double peak of C_{19:0} cyclo ω9c and C_{19:0} cyclo ω7c. Positive for leucine arylamidase and weakly for esterase (C4), valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. The cell-wall peptidoglycan structure is A4α L-Lys-D-Asp.

The type strain (SG5_A10^T=LMG 32133^T=NBRC 114991^T) was isolated from the homogenate of the bush bee (*Austroplebeia australis*) collected by Tim Heard in the suburb of West End, Brisbane, Queensland, Australia in 2020. The DNA G+C content of the type strain is 30.98 mol%.

DESCRIPTION OF *BOMBILACTOBACILLUS THYMINIPHILUS* SP. NOV.

Bombilactobacillus thymidiniphilus [thy.mi.di.ni'phi.lus. N.L. neut. n. *thymidinum*, thymidine; N.L. masc. adj. *philus* (from Gr. masc. adj. *philos*), loving; N.L. masc. adj. *thymidiniphilus*, thymidine loving, referring to the preference for thymidine in the growth medium].

Cells are Gram-stain-positive, non-motile, non-spore-forming catalase-negative rods, 1.3–4.3×0.8–1.1 µm, occurring singly or in chains. Catalase gene is present. D-Lactic acid and L-lactic acid are produced from glucose. Homofermentative, with lactic acid as the sole fermentation end-product. After anaerobic growth in MRSFNucFa (MRS with 20 g l⁻¹ fructose, 100× nucleosides and 1 mg l⁻¹ folic acid), colonies appear ivory, circular, flat slightly undulate, glistening, translucent, with a diameter of 0.5–1.0 mm. Facultatively anaerobic, with growth greatly enhanced either by apple juice, or a combination of folic acid and nucleosides. Glucophilic. Growth in MRSFNucFa occurs between pH 5.0–7.5, 17–40 °C and 0.0–5.0% added NaCl, and optimally at pH 5.0, 23 °C and 0.0% added NaCl. Acid is produced from D-glucose, D-fructose, D-mannose,

N-acetylglucosamine, arbutin, aesculin, salicin, cellobiose, melibiose, sucrose, raffinose and gentiobiose and very weakly from *L*-arabinose and *D*-ribose. The major fatty acids are $C_{16:0}$, $C_{18:1}$ $\omega 9c$ and $C_{19:0}$ cyclo $\omega 9c$. Positive for leucine and valine arylamidase and naphthol-AS-BI-phosphohydrolase, and weakly positive for cystine arylamidase, acid phosphatase, α -galactosidase, α -glucosidase and β -glucosidase. The cell-wall peptidoglycan structure is A4 α L-Lys-D-Asp.

The type strain (SG4_A1^T=LMG 32125^T=NBRC 114984^T) was isolated from the homogenate of the sugarbag bee (*Tetragonula carbonaria*) collected by Tim Heard in the suburb of West End, Brisbane, Queensland, Australia in 2020. The DNA G+C content of the type strain is 36.42 mol%.

DESCRIPTION OF *BOMBILACTOBACILLUS FOLATIPHILUS* SP. NOV.

Bombilactobacillus folatiphilus [fo.la.ti'phi.lus. N.L. masc. n. *folas* (gen. *folatis*), folate; N.L. masc. adj. *philus* (from Gr. masc. adj. *philos*), loving; N.L. masc. adj. *folatiphilus*, folate loving, referring to the requirement for folate (folic acid) in the growth medium].

Cells are Gram-stain-positive, non-motile, non-spore forming catalase-negative rods, 2.1–7.3×0.8–1.1 μ m, occurring singly or in chains. NADH peroxidase gene is present. *D*-Lactic acid and *L*-lactic acid are produced from glucose. Homofermentative, with lactic acid as the sole fermentation end-product. After anaerobic growth in MRSFCFa (MRS with 20 g l⁻¹ fructose, 0.1% *L*-cysteine and 1 mg l⁻¹ folic acid), colonies appear white, irregular, slightly umbonate, undulate, glistening, opaque, with a diameter of 1–2 mm. Very poor growth aerobically, and will only grow with apple juice or folic acid supplementation. Kinetically fructophilic. Growth in *D*-glucose is at a delayed rate compared to *D*-fructose, and growth in *D*-glucose is enhanced with *D*-fructose and pyruvate, but not oxygen. Growth in MRSFCFa occurs between pH 4.5–7.0, 23–40 °C and 0.0–3.0% added NaCl, and optimally at pH 7.0, 30 °C and 1.0% added NaCl. Acid is produced from *D*-glucose, *D*-fructose, *D*-mannose, *L*-sorbose, *L*-rhamnose, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, melibiose, sucrose, trehalose, raffinose and gentiobiose, and very weakly from *D*-ribose, *D*-mannitol, *D*-sorbitol and potassium 5-ketogluconate. The major fatty acids are $C_{16:0}$, $C_{18:1}$ $\omega 7c$, $C_{18:1}$ $\omega 9c$ and $C_{19:0}$ cyclo $\omega 7c$. Positive for leucine and valine arylamidase, and weakly positive for esterase (C4), cystine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. The cell-wall peptidoglycan structure is A4 α L-Lys-D-Asp.

The type strain (SG4_D2^T=LMG 32126^T=NBRC 115004^T) was isolated from the homogenate of the sugarbag bee (*Tetragonula carbonaria*) collected by Tim Heard in the suburb of West End, Brisbane, Queensland, Australia in 2020. The DNA G+C content of the type strain is 38.38 mol%.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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