ORIGINAL ARTICLE

Convivina is a specialised core gut symbiont of the invasive hornet *Vespa velutina*

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Abstract

We provide a culturomics analysis of the cultivable bacterial communities of the crop, midgut and hindgut compartments, as well as the ovaries, of the invasive insect *Vespa velutina*, along with a cultivation-independent analysis of samples of the same nest through 16S rRNA amplicon sequencing. The *Vespa velutina* bacterial symbiont community was dominated by the genera *Convivina*, *Fructobacillus*, *Lactiplantibacillus*, *Lactococcus*, *Sphingomonas* and *Spiroplasma*. *Lactococcus lactis* and *Lactiplantibacillus plantarum* represented generalist core lactic acid bacteria (LAB) symbionts, while *Convivina* species and *Fructobacillus fructosus* represented highly specialised core LAB symbionts with strongly reduced genome sizes. *Sphingomonas* and *Spiroplasma* were the only non-LAB core symbionts but were not isolated. *Convivina* bacteria were particularly enriched in the hornet crop and included *Convivina intestini*, a species adapted towards amino acid metabolism, and *Convivina praedatoris* sp. nov. which was adapted towards carbohydrate metabolism.

KEYWORDS
Convivina, culturomics, gut symbiont, invasive insect, *Vespa velutina*

INTRODUCTION

The yellow-legged Asian hornet *Vespa velutina* is a eusocial wasp species, native to South-East Asia. It was first observed outside its native region in 2003 in South Korea, from where it spread further into Japan (Takeuchi et al., 2017). In 2004, the Asian hornet was first detected in Europe, that is, in the South-West of France. In both cases, *V. velutina* was accidentally introduced through shipments from China (Monceau et al., 2014). Today, this invasive species has spread throughout Europe and has been reported in Spain, Portugal, Italy, Belgium, the UK, Germany, The Netherlands and Switzerland (Espinosa et al., 2019; Keeling et al., 2017).

The strong reliance of *V. velutina* upon honey bees (*Apis mellifera*) and other native insects in invaded regions has negative ecological

and economic impacts. Recent studies suggest that insect predation by the Asian hornet can lead to disturbances in local ecological networks and potentially affect the provision of ecosystem services such as pollination (Rojas-Nossa & Calvino-Cancela, 2020). It may also affect native species such as the European hornet (*Vespa crabro*) through competition for the same resources (Cini et al., 2018). On an economical level, predation of honey bee workers may affect the beekeeping sector which is already subject to a range of different threats (Goulson, 2010; Potts et al., 2010; Steinhauer et al., 2018). The predatory activity of *V. velutina* could weaken honey bee colonies and accelerate their individual collapse (Laurino et al., 2020; Requier et al., 2019). Furthermore, the Asian hornet often establishes its nests in urban areas which may pose a threat to humans as well, as its venom may induce toxic and allergic reactions (Vidal et al., 2021).

Vespa velutina is a social insect inhabiting a monogynous colony (Monceau et al., 2014). The gut microbial communities of only a limited number of social insect species have been studied to date. These include the economically important honey bees and bumble bees (Martinson et al., 2011), but also termites and ant species (Anderson et al., 2012; Ohkuma & Brune, 2011). These social insects each display distinctive bacterial communities with specialised gut symbionts that are acquired through social transmission within the colony. The gut symbionts exhibit important functionalities within their host, including nutrient provisioning, insect development and pathogen resistance (Engel & Moran, 2013). The dietary behaviour of hornets is however unlike that of other social Hymenopterans such as the herbivorous and well-studied honey bees and bumble bees. Hornets acquire carbohydrates from plant derived liquids such as nectar, tree sap and ripening fruits, but obtain their proteins from animal prey (Monceau et al., 2014). Hornet workers prey on insects to feed them to larvae in the form of meat pellets. In return, the larvae secrete nitrogen-rich saliva which is consumed by adult hornets as these are unable to eat solid foods. This dietary behaviour is likely to influence the gut microbial communities as is seen in predatory and herbivorous ants (Anderson et al., 2012).

The rapid spread of hornets in invaded regions has prompted research which focused mainly on ecological aspects and development of control strategies. Few studies thus far analysed their gut microbial communities. A first study addressed the gut microbiome of *Vespa mandarinia* and *Vespa simillima* in Japan (Suenami et al., 2019) and reported that both species had simple but distinct gut microbiota communities, consisting of seven and eight core operational taxonomic units (OTUs), respectively. Core OTUs could be linked to diet and some core OTUs such as *Gilliamella* were indistinguishable from those associated with prey insects such as honey bees. A first study of the gut microbiota of *V. velutina* was performed in Korea (Kim et al., 2018). The latter study reported a gut microbiome mainly composed of the bacterial phyla Bacteroidota (previously called Bacteroidetes; Oren & Garrity, 2021) and Pseudomonadota (previously Proteobacteria; Oren & Garrity, 2021), which were represented by the genera *Flavobacterium*, *Aeromonas*, *Pseudomonas* and *Pedobacter*. The gut microbiome communities in *V. velutina* individuals of different castes and life stages, as well as colony samples, were investigated in Italy (Cini et al., 2020) which revealed that sample type shaped the community structure of both bacteria and

fungi. Overall, Bacillota (previously Firmicutes; Oren & Garrity, 2021), Pseudomonadota and Actinomycetota (previously Actinobacteria; Oren & Garrity, 2021) were the most abundant bacterial phyla, and Ascomycota, Basidiomycota and Zygomycota as the most abundant fungal phyla. The adult stages harboured mainly bacilli and gammaproteobacteria which revealed little similarities to the bacterial community reported in the Korean hornets (Kim et al., 2018). Recently, the midgut bacterial communities in queens, workers, and males of two subspecies of *V. velutina*, *V. velutina nigrithorax* and *V. velutina auraria*, were characterised in China (Zhang et al., 2022). The midgut bacterial communities of the two subspecies were similar and dominated by *Lactobacillus* and *Sphingomonas* reads.

In the present study, we analysed the cultivable gut bacterial communities of multiple hornets of a single nest of *V. velutina*, caught in Belgium in 2018. By combining these cultivation-based data with a 16S rRNA gene amplicon sequencing analysis of samples of the same nest we aimed to provide a more comprehensive image of the bacterial communities of the Asian hornet (Duthoo et al., 2022; Hong et al., 2009; Lagier et al., 2012; O'Callaghan et al., 2021) and to provide axenic cultures with accurate species level identification of key symbionts (Engel et al., 2016). In addition, we performed an in-depth functional genomic, metabolic and taxonomic characterisation of *Convivina* bacteria, a poorly known genus of lactic acid bacteria (Praet et al., 2015) and core symbiont of the Asian hornet.

MATERIALS AND METHODS

Collection of specimen and preparation of cell suspensions

Five male and five female Asian hornets were collected from a nest in Essene, Belgium (50°54′15.313″ N, 4°8′12.636″ E) upon nest destruction using a pyrethroid insecticide in November 2018. The samples were transported to the laboratory and immediately frozen at −80°C until further use. Specimens were dissected under sterile conditions after surface-sterilisation using Umonium38 Medical Spray. The gut was extracted, and crop, midgut and hindgut fractions were separated. Ovaries were collected as well. Gut fractions and ovaries were each homogenised in 250 μL of physiological saline (0.85% sodium chloride, 0.1% peptone, 0.1% Tween 80) using sterile micro-pestles. 125 μL of each cell suspension was stored at −80°C until DNA extraction. The remaining 125 μL cell suspension was mixed with an equal volume of 40% glycerol and stored at −80°C using a Nalgene® Mr. Frosty™ Freezing container until microbiological analyses.

Isolation of bacteria

Cell suspensions were thawed and diluted to 10^{-1} for crop and midgut samples and to 10^{-4} for hindgut and ovary samples. An aliquot of 50 μL of each dilution was plated onto multiple agar media in order to isolate a maximal bacterial diversity. The choice of isolation media and

growth conditions was based on results obtained during earlier microbiota analyses of hornets and other social bees (Cini et al., 2020; Kwong & Moran, 2016; Praet et al., 2018). Samples were incubated at 35°C micro-aerobically (80% N_2, 15% CO_2, 5% O_2) on several general purpose isolation media: All Culture (AC) agar (20 g/L tryptose, 3 g/L beef extract, 3 g/L yeast extract, 3 g/L malt extract, 5 g/L dextrose, 0.2 g/L ascorbic acid, 18 g/L agar); AC agar supplemented with 0.2% activated charcoal; Brain Heart Infusion agar (Oxoid) and Tryptone Soya Agar (Oxoid) supplemented with 5% sheep blood.

For the isolation of bifidobacteria, samples were incubated at 35°C anaerobically (80% N_2, 10% H_2, 10% CO_2) on LMG medium 144 (23 g/L special peptone, 1 g/L soluble starch, 5 g/L sodium chloride, 0.3 g/L cysteine hydrochloride, 5 g/L glucose, 15 g/L agar) and on de Man, Rogosa and Sharp (MRS) agar (Oxoid) supplemented with 0.1% L-cysteine. For the isolation of lactic acid bacteria (LAB), samples were incubated at 35°C on MRS agar (aerobically and anaerobically), MRS5 agar (Meroth et al., 2003) with sucrose instead of maltose (aerobically and anaerobically) and MRS agar supplemented with 2% and 20% fructose (aerobically). For the isolation of acetic acid bacteria (AAB), samples were incubated on LMG medium 404 and LMG medium 13 at 28°C aerobically (Li et al., 2017). An enrichment for acetic acid bacteria was performed by adding 50 μL of undiluted sample to 10 mL of enrichment medium 1 (EM1) and enrichment medium 2 (EM2) broth (Lisdiyanti et al., 2003) and incubating aerobically for 3 days at 28°C. The enrichment culture was plated on LMG medium 13, LMG medium 404, EM1 and EM2 agar after serial dilution. All media were supplemented with 10 ppm cycloheximide to inhibit fungal growth.

After 5 days of incubation, colonies were picked randomly and subcultivated twice using the respective isolation conditions.

MALDI-TOF MS dereplication

Third generation axenic cultures were used for protein extraction and were analysed via matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) as described before (Wieme et al., 2014). MALDI-TOF MS was performed on a Bruker Microflex LT/SH s-Smart platform (Bruker Daltonics, Bremen, Germany). For the identification of isolates, spectra were compared to the Bruker MSP library using MBT Compass Explorer software according to the manufacturer's settings (Bruker Daltonics). Mass spectra were converted to text files via the Flex Analysis batch processing software (Bruker Daltonics) and grouped into operational isolation units (OIUs) using the dereplication tool SPeDE with default settings (Dumolin et al., 2019). This dereplication step enabled the retention of only isolates with distinct mass spectra as a proxy to genetically distinct strains and as references for further analysis.

Identification of isolates

References of each OIU with a Bruker log score ≥ 2.0 were considered identified at the species level. References with a Bruker log score < 2.0 were further identified through sequence analysis (see below). To this end, DNA was extracted through alkaline lysis by suspending one colony in 20 μL of alkaline lysis buffer (0.25% (w/v) SDS, 0.05 M NaOH) and heating for 15 min at 95°C. After lysis, 180 μL of Milli-Q water was added and the suspension was collected after centrifugation for 5 min at 13,000 rpm at 4°C. The 16S rRNA gene was PCR amplified as described before (Coenye et al., 1999). For LAB, a fragment of the phenylalanine-tRNA ligase alpha subunit (pheS) gene was PCR amplified as described by Naser et al. (2007), as this gene has a higher taxonomic resolution than the 16S rRNA gene (Naser et al., 2007). The PCR products were purified using a NucleoFast 96 PCR clean-up kit (Macherey-Nagel, Eupen, Belgium) and were submitted for Sanger sequencing by a commercial company (Eurofins Genomics, Ebersberg, Germany). Complete 16S rRNA gene sequences were obtained using forward primers 16F358 (5′-CTCCTACGGGAGGCAGCAGT-3′) and 16F926 (5′-AACTCAAAGGAATTGACGG-3′) and the reverse primers 16R519 (5′-GTATTACCGCGGCTGCTGGCA-3′) and 16R1093 (5′-GTTGCGCTCGTTGCGGGACT-3′) (Coenye et al., 1999). A 361–428 nucleotide fragment of the pheS gene was determined using the PCR primers (Naser et al., 2007). Sequences were assembled using BioNumerics version 7.6 (Applied Maths, Sint-Martens-Latem, Belgium), and EzBioCloud (Yoon, Ha, Kwon, et al., 2017) and NCBI BLAST were used for the analysis of the 16S rRNA and pheS gene sequences, respectively. LAB isolates were considered identified at the species level based on a reported 3% pheS intra-species divergence (Naser et al., 2007). Bacterial isolates with 16S rRNA gene sequences that shared >98.65% sequence identity to only one type strain were considered tentatively identified at the species level (Kim et al., 2014). In case 16S rRNA gene sequences shared >98.65% sequence identity to multiple type strains, or when 16S rRNA gene sequences shared <98.65% sequence identity with established type strains, isolates were considered identified at the genus level only.

16S rRNA gene amplicon sequencing

DNA was extracted as described before (Snauwaert et al., 2016). Samples with DNA concentrations below 5 ng/μL were pooled per sample type for sequencing (Table S1). The resulting DNA samples were sent to Novogene (Cambridge, UK) for library preparation and amplicon sequencing. The 16S rRNA V3-V4 region was amplified using forward primer 341F (5′-CCTAYGGGRBGCASCAG-3′) and reverse primer 806R (5′-GGACTACNNGGGTATCTAAT-3′) containing Illumina adaptors. The library was sequenced on an Illumina NovaSeq 6000 platform generating 250 bp paired-end reads. Raw reads were analysed with the DADA2 pipeline version 1.14.1 (Callahan et al., 2017) following the default parameters with minor modifications. After read quality inspection, the forward and reverse reads were trimmed to a length of 226 bp and 223 bp, respectively. Upon modelling the error rates, a monotonicity was introduced (https://github.com/benjjneb/dada2/issues/791). Merged paired reads with a length between

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403 bp and 430 bp were retained for further analyses. Taxonomy was assigned to the resulting amplicon sequence variants (ASVs) using the DADA2 formatted training FASTA files of the SILVA SSU database version 132 (Quast et al., 2013). Sequences attributed to chloroplasts, mitochondria, Archaea, Eukarya and unclassified phyla were removed from further analyses. Taxonomic assignments of the 20 most abundant ASVs were reviewed through EzBioCloud (Yoon, Ha, Kwon, et al., 2017).

Genome sequence analysis

Two *Convivina intestini* isolates (i.e., R-77811 and R-78131) and three *Convivina* sp. isolates (i.e., LMG 32447[T], R-77815 and R-78138) were cultivated on their respective isolation media. DNA was extracted using an automated Maxwell DNA preparation Instrument (Promega). Genome sequences were determined using the Illumina HiSeq4000 platform (PE150) at the Oxford Genomics Centre (University of Oxford, UK). Read quality was assessed by FastQC. Data cleaning and assembly were performed using shovill version 1.1.0 containing SPAdes genome assembler version 3.14.0 (Bankevich et al., 2012). Contigs were filtered at a minimum length of 500 bp and reads were mapped back onto the contigs using BWA-MEM (Li, 2013). Quast was used to create quality reports of the resulting assemblies (Gurevich et al., 2013). Prokka version 1.14.5 was used for genome annotation (Seemann, 2014). Genome completeness and contamination were estimated with CheckM version 1.1.2 using gene markers specific to the *Leuconostocaceae* family (Parks et al., 2015). The genome sequence of *C. intestini* DSM 28795[T] was retrieved from GenBank (accession no. QEKT00000000) for further analyses.

Phylogenomic and comparative genomic analyses

All *Convivina* genomes were submitted to the Type (Strain) Genome Server (TYGS) for calculation of the digital DNA–DNA hybridization (dDDH) values using the recommended settings of the Genome-to-Genome Distance Calculator (Meier-Kolthoff & Goker, 2019). Average nucleotide identity (ANI) values of the genomes were calculated using the OrthoANIu algorithm, accessible via the EZBioCloud webserver (Yoon, Ha, Kwon, et al., 2017). The whole-genome sequence of strains LMG 32447[T] and DSM 28795[T], and of type strains of type species of closely related *Lactobacillaceae* genera were used to construct a phylogenomic tree based on 107 single-copy core genes using BcgTree (Ankenbrand & Keller, 2016). Visualisation and annotation of the tree were performed using iTOL (Letunic & Bork, 2021). A comparative functional genomics analysis of all *Convivina* genomes was performed using the anvi'o pangenomics workflow (Eren et al., 2021). Gene clusters, defined as clusters of translated DNA sequences from predicted open reading frames, were identified using NCBI's blastp with default settings (Camacho et al., 2009) and an MCL inflation parameter of 8 (Van Dongen & Abreu-Goodger, 2012). Genes were functionally annotated with Clusters of Orthologous Genes (COGs) using NCBI's

COG database (Galperin et al., 2021) and with eggNOG-mapper version 2.1.4 (Cantalapiedra et al., 2021). Pangenomes were visualised in the anvi'o interactive interface. Species-specific differences were analysed by identifying species-specific gene clusters and relative differences in COG classification.

Metabolic pathways were predicted via the KEGG Automatic Annotation Server (KAAS) version 2.1 (Moriya et al., 2007). Carbohydrate active enzymes (CAZy) were identified via the dbCAN2 meta server using tools HMMER, DIAMOND and Hotpep (Zhang et al., 2018). Enzymes belonging to CAZy families with hits in two or more of the employed tools were manually inspected in the eggNOG annotations to verify their presence.

Metabolic characterisation

Biochemical characteristics were determined for *C. intestini* LMG 28291[T] and *Convivina* sp. strain LMG 32447[T]. Growth tests were recorded after 1, 2, 3, 6 and 7 days. The temperature growth range was tested aerobically on MRS at 4, 15, 20, 28, 35, 37, 40 and 45°C. Anaerobic growth was tested on MRS at 28°C and 37°C. Tolerance to NaCl was investigated aerobically in MRS broth supplemented with different concentrations of NaCl ranging from 0% to 10% with 1% intervals. Tolerance to pH was evaluated aerobically in MRS broth at pH 4.0 to 9.0 at 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 was investigated using MRS supplemented with 2% D-fructose and incubation in aerobic and anaerobic atmospheres (Endo et al., 2018). Growth with 20 g/L D-glucose, D-fructose, sucrose, D-trehalose, D-maltose, D-ribose, D-fucose, D-melibiose, D-cellobiose, starch or chitin in MRS broth was measured after 0, 1, 2, 3, 4 and 7 days of aerobic incubation at 35°C by measuring the optical density at 600 nm using a spectrophotometer (Tecan, Männedorf, Switzerland). Production of lactate and acetate from D-glucose, D-fructose or sucrose metabolism was determined after 3 days of aerobic incubation at 35°C in MRS broth containing 20 g/L of the respective sugar using high-performance liquid chromatography combined with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA). Sodium acetate was excluded from the MRS media as this can interfere with acetate measurements. Ethanol accumulation was measured using an Anton Paar Alcolyzer Plus. Consumption of D-glucose, D-fructose and sucrose and mannitol production was determined using a Dionex ICS-3000 high-performance anion exchange chromatography coupled with pulsed amperometric detection system (Thermo Fisher Scientific, Waltham, MA, USA) and a Dionex CarboPac PA20 column. These experiments were performed in triplicate.

Statistical data analyses

Statistical analyses were performed in R version 4.1.0. Alpha diversity indices (Observed richness and Shannon diversity) were computed

and plotted using the phyloseq package version 1.36.0 (McMurdie & Holmes, 2013). The Kruskal–Wallis test was employed to compare the diversity indices between the different compartments and Wilcoxon rank sum test was used to perform pairwise comparisons. p values were adjusted for multiple hypothesis testing with the Benjamini-Hochberg method. Statistical tests were applied using the ggpubr package version 0.4.0.

RESULTS

Isolation of the bacterial community

A total of 861 isolates were collected from crop, midgut, hindgut and ovary samples of 5 female Asian hornets and were dereplicated into 197 OIUs, that is, mass spectrometry-defined independent strains (Dumolin et al., 2019). Reference isolates, hereafter referred to as 'references', of 116 OIUs had Bruker log scores ≥2.0 and were considered identified to the species level (Table S2). References of the remaining 81 OIUs were further identified via comparative sequence analysis (Table S3). The identification result for each of these references was then applied to all isolates of the same OIU and both sets of identification results were combined in Table 1.

Isolates belonging to 57 species were obtained (Table 1). Overall, the isolates belonged to the phyla Bacillota (81.6%), Pseudomonadota (14.4%), Actinomycetota (2.7%) and Bacteroidota (1.0%). The smallest number of species was isolated from hindgut ($n = 15$) and ovary ($n = 17$) samples, while crop and midgut samples yielded isolates of 31 and 28 species, respectively. LAB and AAB isolates dominated the cultivated fraction of all four sample types (79.1%). LAB isolates were identified as *Lactococcus lactis*, *Lactiplantibacillus plantarum*, *Fructobacillus fructosus*, *Leuconostoc* species, *Convivina* species and, sporadically, *Enterococcus* species and *Carnobacterium divergens*. *Acetobacter indonesiensis* was the most frequently isolated AAB, but also *Asaia platycodi*, *Gluconobacter cerinus* and *Commensalibacter* sp. were isolated.

Lactococcus lactis and *L. plantarum* were dominantly isolated from all four sample types, except for the ovaries where *L. plantarum* was isolated only sporadically (Figure 1). *Fructobacillus fructosus* was dominantly isolated from midgut and hindgut samples, whereas *Leuconostoc mesenteroides* and *A. indonesiensis* represented major fractions of the hindgut isolates. *Fructobacillus fructosus* and *Leuconostoc* species were not isolated from the crop, which contained major fractions of *Convivina* sp. and *Devosia submarina*. *Bacillus pumilus* and *Bacillus* sp. represented a major fraction of the ovaries and were only isolated from this sample type.

Bacterial community richness and composition

16S rRNA gene amplicon sequencing was performed on a total of 14 samples from different gut compartments of *V. velutina* (Table S1). A total of 1,939,107 demultiplexed paired-end reads of the 16S rRNA V3-V4 region were obtained. Analysis with the DADA2 pipeline and data cleaning resulted in 1,269,718 high quality reads which were assigned to

2727 ASVs. Each of the 14 samples contained between 71,431 and 113,987 reads and between 84 and 1270 ASVs. Rarefaction curves of all 14 samples reached a plateau indicating that a sufficient sampling depth was reached (data not shown). The observed number of ASVs and Shannon diversity of the samples were not significantly different between the analysed gut compartments ($p > 0.05$, Kruskal–Wallis test, Figure S1). Pairwise comparisons of both alpha diversity indices between the fractions and between individually sequenced and pooled samples within fractions indicated similar richness and diversity between bacterial communities ($p_{adj} > 0.05$, Wilcoxon test, data not shown).

The majority of the reads were classified through the SILVA SSU database as Bacillota (62.3% of total reads) and Pseudomonadota (28.0%), followed by Mycoplasmatota (previously Tenericutes; Oren & Garrity, 2021) (6.8%) and Actinomycetota (1.6%). The remaining 1.3% of the reads represented 25 phyla. The 20 most abundant ASVs comprised 1,213,094 reads and represented 95.5% of the total number of reads. Verification of the assigned SILVA taxonomy of these ASVs through EzBioCloud confirmed the majority of the assignments (Table 2). However, ASV4 and ASV5 were both assigned to the genus *Leuconostoc* via SILVA but appeared to be highly similar to *C. intestini* sequences upon EzBioCloud analysis, with 100% and 99.53% sequence similarity, respectively. Therefore, the taxonomy of ASV4 and ASV5 was adjusted to *Convivina*. Similarly, EzBioCloud analysis of *Serratia* ASVs ASV10, ASV12 and ASV13 revealed *Ewingella americana* as highest match and their identification was adjusted accordingly.

While individual variation between the samples of each fraction could be observed in the midgut, hindgut and ovaries (Figure 2), *Convivina* reads represented about 69% of all reads of the crop sample, followed by *Lactococcus* (about 13%), *Sphingomonas* (about 5%) and *Fructobacillus* (about 4%) reads. Midgut samples were dominated by *Lactococcus* reads (about 52%), followed by *Sphingomonas* (about 26%), *Spiroplasma* (about 9%), and *Convivina* (about 5%) reads. In hindgut samples, the relative proportion of *Lactococcus* reads was even higher (about 61%), followed by *Sphingomonas* (about 8%), *Spiroplasma* (about 9%), and *Convivina* (about 4%) reads. Finally, ovary samples had a strongly different composition that was characterised by *Lactococcus* and *Sphingomonas* reads, both representing about 47% of the reads. The presence of core microbiota in each gut compartment was determined as defined by Graystock et al. (2017), that is, core microbiota are present in at least half of the samples at a relative abundance greater than 1%. Six core ASVs were present in the crop, that is, *Convivina* (2 ASVs), *Lactococcus*, *Sphingomonas*, *Fructobacillus* and *Spiroplasma*. All of these except *Fructobacillus* were also identified as midgut core ASVs, while *Lactococcus* and *Sphingomonas* represented the core ASVs of both hindgut and ovary samples.

Genome characteristics of *Convivina* strains

Genome sequences of two *C. intestini* and three *Convivina* sp. isolates were determined and compared with the *C. intestini* DSM 28795[T] (a bumble bee isolate) genome sequence (Praet et al., 2015). As shown in Table 3, the OrthoANIu and dDDH values between isolates R-

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TABLE 1 Distribution of isolates over the crop, midgut, hindgut and ovary compartments.

Identification	Number of isolates from				Total number of isolates for a taxon	Taxon frequency among 861 isolates (%)	Isolation medium
	Crop	Midgut	Hindgut	Ovaries			
Bacillota							
Bacillus pumilus	0	0	0	27	27	3.1	ACC, BHI, M13, M404
Bacillus sp. 1	0	0	0	26	26	3.0	ACC, BHI, MRS5
Bacillus sp. 2	0	0	0	1	1	0.1	BHI
Bacillus subtilis	0	1	0	0	1	0.1	M404
Brevibacillus sp.	0	0	0	1	1	0.1	M13
Carnobacterium divergens	0	0	2	0	2	0.2	ACC
Convivina intestini	2	0	10	1	13	1.5	AC, MRS, MRSC, TSAB
Convivina sp.[a]	12	3	5	0	20	2.3	AC, M404, MRS, MRS5
Enterococcus casseliflavus	1	0	0	0	1	0.1	BHI
Enterococcus plantarum	2	0	0	0	2	0.2	AC
Fructobacillus fructosus	0	25	45	3	73	8.5	AC, ACC, M13, M144, M404, MRS, MRS5, MRSF20
Lactiplantibacillus plantarum	25	65	74	2	166	19.2	AC, ACC, EM1, EM2, M13, M144, M404, MRS, MRS5, MRSF20
Lactococcus lactis	45	46	53	122	266	30.8	AC, ACC, BHI, EM1, EM2, M13, M144, M404, MRS, MRS5, MRSC, TSAB
Leuconostoc citreum	1	0	2	0	3	0.3	BHI, MRS
Leuconostoc mesenteroides	0	0	60	0	60	7.0	ACC, MRS5, MRSC, MRSF
Leuconostoc pseudomesenteroides	0	3	1	2	6	0.7	ACC, BHI, M144, M404, MRS5
Leuconostoc suionicum	0	1	0	0	1	0.1	ACC
Lysinibacillus boronitolerans	3	0	0	0	3	0.3	BHI
Lysinibacillus sp.	2	0	3	0	5	0.6	BHI, M13
Lysinibacillus xylanilyticus	3	0	0	0	3	0.3	BHI
Mammaliicoccus sciuri	0	0	6	0	6	0.7	ACC
Mammaliicoccus sp.	1	1	0	0	2	0.2	BHI, TSAB
Paenibacillus typhae	0	0	0	6	6	0.7	MRS5
Psychrobacillus sp.	0	0	1	0	1	0.1	BHI
Staphylococcus capitis	0	1	0	0	1	0.1	M404
Staphylococcus epidermidis	0	0	0	3	3	0.3	MRS5
Staphylococcus pasteuri	0	0	0	1	1	0.1	M144
Staphylococcus warneri	0	0	0	5	5	0.6	MRS5
Alphaproteobacteria							
Acetobacter indonesiensis	6	2	34	4	46	5.3	AC, ACC, EM1, EM2, M13, M404
Asaia platycodi	0	3	0	8	11	1.3	M13, M404
Commensalibacter sp.	0	3	0	3	6	0.7	M13, M404
Devosia sp.	1	0	0	0	1	0.1	EM2
Devosia submarina	12	0	0	0	12	1.4	EM2
Gluconobacter cerinus	0	0	0	6	6	0.7	M13
Paenochrobactrum sp.	1	0	0	0	1	0.1	TSAB
Betaproteobacteria							
Achromobacter sp.	1	1	0	0	2	0.2	AC, TSAB

(Continues)

TABLE 1 (Continued)

Identification	Number of isolates from				Total number of isolates for a taxon	Taxon frequency among 861 isolates (%)	Isolation medium
	Crop	Midgut	Hindgut	Ovaries			
Advenella kashmirensis	1	0	0	0	1	0.1	TSAB
Alcaligenes faecalis	6	3	0	0	9	1.0	AC, BHI, TSAB
Comamonas serivorans	1	0	0	0	1	0.1	TSAB
Gammaproteobacteria							
Acinetobacter guillouiae	2	1	0	0	3	0.3	AC, ACC
Acinetobacter sp.	0	1	0	0	1	0.1	TSAB
Lelliottia amnigena	1	1	0	0	2	0.2	AC, BHI
Pseudomonas fulva	0	1	0	0	1	0.1	BHI
Pseudomonas protegens	4	1	0	0	5	0.6	AC, TSAB
Serratia liquefaciens	0	1	0	0	1	0.1	BHI
Serratia marcescens	0	0	5	0	5	0.6	BHI
Serratia sp.	1	0	0	0	1	0.1	AC
Stenotrophomonas rhizophila	2	2	0	0	4	0.5	M13, TSAB
Stenotrophomonas sp. 1	2	0	0	0	2	0.2	M13, M404
Stenotrophomonas sp. 2	0	1	0	0	1	0.1	TSAB
Actinomycetota							
Arthrobacter woluwensis	6	8	1	0	15	1.7	AC, BHI, TSAB
Cutibacterium acnes	0	1	0	0	1	0.1	M144
Leucobacter sp.	3	1	0	0	4	0.5	AC, ACC, TSAB
Microbacterium sp.	2	2	0	0	4	0.5	AC, BHI, TSAB
Oerskovia sp.	1	0	0	0	1	0.1	TSAB
Bacteroidota							
Flavobacterium ceti	1	2	0	0	3	0.3	TSAB
Sphingobacterium sp.	5	1	0	0	6	0.7	AC, TSAB

Note: Media used for isolation were All Culture agar (AC), AC agar supplemented with 0.2% activated charcoal (ACC), Brain Heart Infusion agar (BHI), enrichment medium 1 (EM1) and 2 (EM2) for acetic acid bacteria, LMG medium 13 (M13), LMG medium 144 (M144), LMG medium 404 (M404), de Man, Rogosa and Sharp agar (MRS), MRS5 agar with sucrose instead of maltose (MRS5), MRS agar supplemented with 0.1% L-cysteine (MRSC), and MRS agar supplemented with 2% (MRSF) and 20% fructose (MSRF20) and Tryptone Soya Agar supplemented with 5% sheep blood (TSAB).
[a]*Convivina praedatoris* sp. nov. reported in the present study.

77811 and R-78131 and *C. intestini* DSM 28795[T] were well above the species delineation thresholds of 70% dDDH (Meier-Kolthoff et al., 2013) and 95%–96% orthoANIu (Yoon, Ha, Lim, et al., 2017), and therefore identified these isolates as *C. intestini* (Table S2). Isolates LMG 32447[T], R-77815 and R-78138, in contrast, showed values below the species delineation thresholds towards *C. intestini* DSM 28795[T] but shared dDDH and orthoANIu values well above the species delineation thresholds, and thus represented a single novel species closely related to *C. intestini* (Table 3), for which we propose the name *Convivina praedatoris* sp. nov. (Table S4). The phylogenomic position of this novel species within the genus *Convivina* and among representatives of closely related *Lactobacillaceae* genera is shown in Figure S2. The genome features of all *Convivina* isolates were summarised in Table S4. All genomes displayed completeness values >95% and low contamination values (0.48%) after CheckM analysis. The genome size of the *C. intestini* isolates was approximately 1.6 Mbp with a G + C content of 40.9%. The *C. praedatoris* isolates LMG 32447[T], R-77815 and R-78138 displayed smaller genomes of approximately 1.5 Mbp with 40.2% G + C content. Accordingly, *C. intestini* genomes encoded on average 1616 genes, whereas *C. praedatoris* genomes encoded on average 1584 genes. Finally, a comparison of genome-extracted 16S rRNA gene sequences with ASV sequences revealed that *Convivina* ASV5 showed 100% sequence identity to the V3-V4 region of the *C. intestini* LMG 28291[T] 16S rRNA gene sequence, whereas *Convivina* ASV4 showed 1 nucleotide difference with the 16S rRNA V3-V4 region of *C. praedatoris* LMG 32447[T].

Convivina pangenome analysis

A *Convivina* comparative genomics analysis was performed using the anvi'o pangenomics workflow to identify functions that were over-
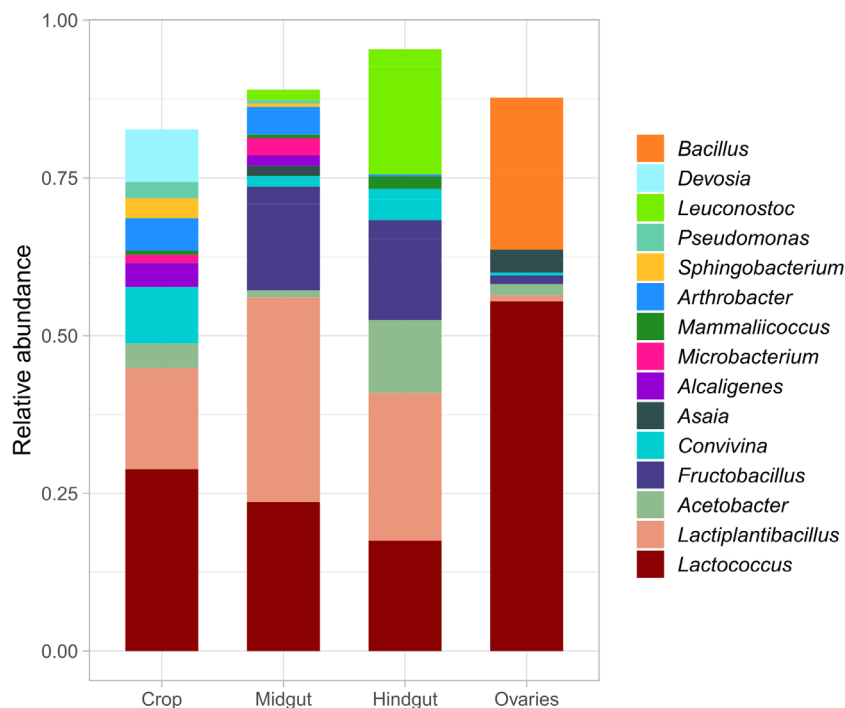
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FIGURE 1 Relative abundance of predominant bacteria in crop, midgut, hindgut and ovaries of the Asian hornet *Vespa velutina*, as revealed through cultivation experiments. The proportion of the 20 most abundant species is summarised at genus level. The gap between the bars and 100% represents the proportion of low abundant species not included in the figure.

and underrepresented between the two species. The pangenome of the six isolates was computed from a total of 9558 gene calls and contained 1901 gene clusters (Figure 3). The six genomes shared 1285 gene clusters (7854 genes) which represented the *Convivina* core genome. COG category assignments of the genes per isolate are summarised in Table 4 and were highly concordant within species. Between 21.9 and 23.1% of genes could not be assigned to COG categories. Genes involved in carbohydrate transport and metabolism (category G) accounted for 6.2 to 6.8% of genes assigned to a COG category and ranked 4th and 5th in *C. praedatoris* and *C. intestini*, respectively. The ratio of category E genes (amino acid transport and metabolism) was 9.8 to 11.4% and ranked 2nd within both species.

The species-specific gene clusters were defined as gene clusters exclusively present in all three analysed genomes per species and were composed of 95 gene clusters (including 293 genes) specific for *C. intestini* and 120 gene clusters (including 363 genes) specific for *C. praedatoris*. Species-specific gene clusters were analysed to verify relative differences in COG category assignments and species-specific characteristics (Figure 4 and Table S5). Up to 37.9% and 53.7% of *C. intestini* and *C. praedatoris* species-specific genes were not classified into COG categories, respectively. COG categories F (nucleotide transport and metabolism), N (cell motility), U (intracellular trafficking, secretion and vesicular transport) and X (mobilome: prophages, transposons) were not represented in the species-specific gene clusters.

Gene clusters specific for *C. intestini* were most notably enriched in category E (amino acid transport and metabolism), accounting for more than 21% of the species-specific genes (Figure 4). Closer

inspection of these genes revealed CDSs for a Na+/glutamate symporter as well as genes involved in the biosynthesis of methionine (m00017), histidine (m00026) and L-isoleucine from threonine (m00570). Analysis after KAAS prediction of pathways confirmed the presence of complete biosynthesis pathways for these amino acids in *C. intestini* strains, whereas the same pathways were incomplete in *C. praedatoris*. *Convivina intestini* specific gene clusters were furthermore enriched in COG categories K (transcription) and O (posttranslational modification, protein turnover, chaperones), whereas categories L (replication, recombination and repair) and V (defence mechanisms) were missing.

The *C. praedatoris* specific gene clusters were most prominently enriched in category G (carbohydrate transport and metabolism) with 12 gene clusters (36 genes). However, only five gene clusters had annotations that were exclusively found in this species, including aldose 1-epimerase (*galM*), maltose phosphorylase (*mapA*), fucose permease (*fucP*), EamA-like transporter family (*yicL*) and oligo-1,-6-glucosidase (*malL*). Categories I (lipid transport and metabolism) and J (translation, ribosomal structure and biogenesis) are missing from the *C. praedatoris* specific genes (Figure 4).

Carbohydrate metabolism analysis

All *Convivina* genomes were annotated via the KEGG database using the KAAS system for pathway prediction and via the bdCAN2 server to identify carbohydrate-active enzymes. The six *Convivina* isolates

TABLE 2 Taxonomic classification and percentage sequence similarity of the 20 most abundant ASVs in the Asian hornet microbiome based on SILVA and EzBioCloud 16S rRNA gene databases.

ASV number and SILVA taxonomic assignment	EzBioCloud closest neighbour and percentage identity	Crop* (%)	Midgut (%)				Hindgut (%)						Ovaries (%)		
			VV6m	VV10m	VVm1*	VVm2*	VV5h	VV6h	VV8h	VV9h	VV10h	VVh*	VV5o	VV6o	VVo*
Bacillota															
1—Lactococcus	Lactococcus lactis 100%	12.86	62.93	78.14	18.20	58.77	93.79	94.06	6.31	7.88	70.01	67.59	1.48	61.01	86.05
4—Leuconostoc	Convivina intestini[a] 100%	18.41	3.73	12.15	0.25	0.32	0.25	0.24	0.34	1.76	18.51	0.17	0.13	1.70	0.28
5—Leuconostoc	Convivina intestini[a] 99.53%	50.92	1.01	1.18	0.21	0.36	0.27	0.24	0.28	3.31	1.28	0.02	0.00	0.15	0.03
6—Fructobacillus	Fructobacillus fructosus 99.77%	3.35	0.50	0.62	0.69	0.12	0.10	0.09	0.23	12.95	1.27	0.10	0.05	0.38	0.22
17—Fructobacillus	Fructobacillus fructosus 100%	0.73	0.26	0.00	0.00	0.05	0.00	0.00	0.13	1.93	0.00	0.00	0.00	0.00	0.00
Alphaproteobacteria															
2—Sphingomonas	Sphingomonas spp. 100%	4.97	3.27	4.86	78.02	1.35	2.13	0.95	12.54	34.69	3.72	1.97	91.98	31.41	9.94
8—Sphingomonas	Sphingomonas hankookensis 100%	0.29	0.32	0.25	0.23	0.15	0.42	0.14	11.11	0.00	0.00	0.00	0.00	0.00	0.00
9—Methylobacterium—Methylorubrum	Methylobacterium spp. 100%	0.30	0.42	0.23	0.20	0.10	0.19	0.15	10.99	0.00	0.02	0.00	0.00	0.01	0.00
11—Acetobacter indonesiensis	Acetobacter indonesiensis 100%	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.18	0.16	7.17	0.17	0.21	0.16
16—Acidocella facilis	Acidocella facilis 99.75%	0.04	0.16	0.15	0.01	0.15	0.32	0.35	0.10	0.35	0.34	0.20	0.23	0.58	0.22
18—Sphingomonas	Sphingomonas spp. 100%	0.59	0.09	0.24	0.00	0.00	0.00	0.15	0.00	0.79	0.09	0.11	0.00	0.39	0.11
Gammaproteobacteria															
10—Serratia	Ewingella americana 98.37%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.16	10.08	0.11	0.11	0.11
12—Serratia	Ewingella americana and Yersinia spp. 98.37%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.12	0.12	5.97	0.08	0.07	0.08
13—Serratia	Ewingella americana 98.37%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.10	5.74	0.07	0.11	0.07
14—Stenotrophomonas	Stenotrophomonas maltophilia and Stenotrophomonas pavanii 99.30%	0.29	0.12	0.06	0.08	0.07	0.07	0.08	1.49	1.95	0.13	0.08	0.93	0.12	0.22
15—Stenotrophomonas maltophilia	S. maltophilia and S. pavanii 99.77%	0.21	0.10	0.03	0.10	0.07	0.05	0.06	1.18	1.63	0.13	0.04	0.72	0.10	0.11
Mycoplasmatota															
3—Spiroplasma	Spiroplasma monobiae 99.53%	2.81	1.52	0.88	0.57	35.36	0.60	0.68	44.93	9.84	0.11	0.04	0.04	0.09	0.08
20—Spiroplasma	Spiroplasma monobiae 99.29%	0.06	0.04	0.00	0.00	0.33	0.00	0.00	1.52	0.00	0.00	0.00	0.00	0.00	0.00
Actinomycetota															
7—Streptomyces	Streptomyces spp. 100%	0.96	0.32	0.10	0.25	0.26	0.14	0.22	2.77	7.86	0.25	0.11	0.75	0.24	0.30
19—Cutibacterium	Cutibacterium acnes 100%	0.10	0.05	0.03	0.03	0.04	0.08	0.07	0.15	1.37	0.11	0.05	0.11	0.13	0.13
Total relative abundance		96.89	74.83	98.94	98.83	97.47	98.42	97.50	94.08	86.98	96.52	99.33	96.83	96.80	98.12

Note: The relative abundance of the reads for each ASV within the analysed samples is given in percentage. Pooled samples are indicated with *.

[a] A comparison of genome-extracted 16S rRNA gene sequences revealed that *Convivina* ASV5 showed 100% sequence identity to the V3-V4 region of the *C. intestini* LMG 28291^T 16S rRNA gene sequence, whereas *Convivina* ASV4 showed 1 nucleotide difference with the 16S rRNA V3-V4 region of *C. praedatoris* LMG 32447^T.

CORE SYMBIONT *CONVIVINA* IN *VESPA VELUTINA*

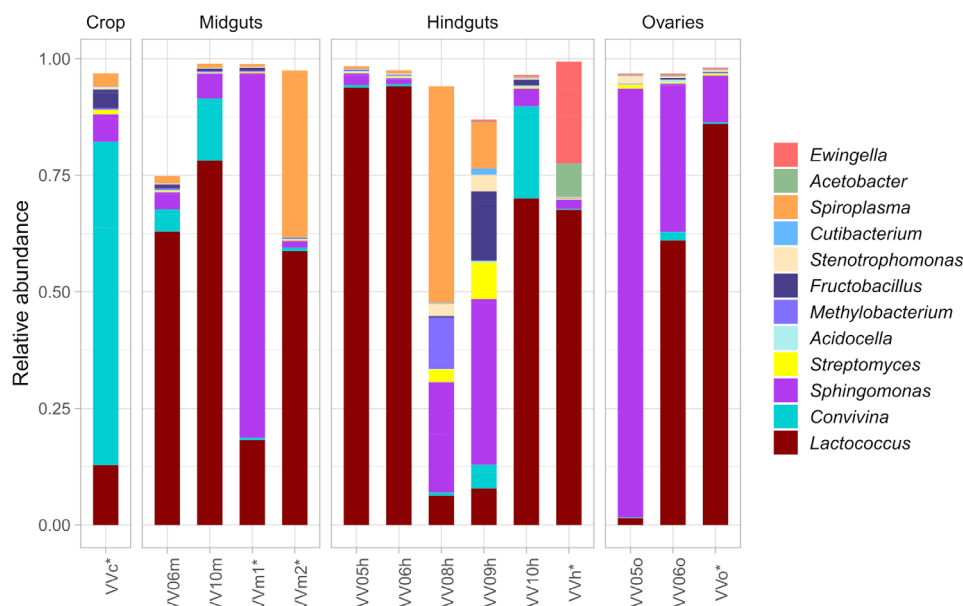Insect Molecular
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FIGURE 2 Relative abundance of predominant bacteria in crop, midgut, hindgut and ovaries of the Asian hornet *Vespa velutina* as revealed through 16S rRNA gene amplicon sequencing. The proportion of the 20 most abundant ASVs is summarised at genus level. The gap between the bars and 100% represents the proportion of low abundant ASVs not included in the figure. Pooled samples are indicated with *.

TABLE 3 OrthoANIu and digital DNA–DNA hybridization (dDDH) values of the *Convivina* genomes towards the genomes of the type strains *Convivina intestini* DSM 28795[T] and *Convivina praedatoris* LMG 32447[T].

	OrthoANIu value (%)		dDDH value (%)	
	C. intestini DSM 28795[T]	*C. praedatoris* LMG 32447[T]	*C. intestini* DSM 28795[T]	*C. praedatoris* LMG 32447[T]
C. intestini DSM 28795[T]	100	91.17	100	42.8
C. intestini R-77811	99.88	91.02	99.8	42.6
C. intestini R-78131	97.68	90.89	78.5	41.8
C. praedatoris LMG 32447[T]	91.17	100	42.8	100
C. praedatoris R-77815	91.21	99.88	42.9	99.6
C. praedatoris R-78138	91.09	99.75	42.7	98.3

did not contain all necessary genes to perform a complete glycolysis pathway. A phosphofructokinase enzyme was lacking for the conversion of D-fructose-6-phosphate to D-fructose-1,6-biphosphate. Therefore, metabolism of glucose initially occurs through the phosphoketolase pathway where glucose is converted into D-glyceraldehyde-3-phosphate (GAP) and acetyl-phosphate. GAP is further metabolised via the glycolysis pathway into pyruvate. As *Convivina* lacks several TCA cycle genes, pyruvate will be converted to lactate, acetyl-coA or acetyl-phosphate through the activity of lactate dehydrogenase, pyruvate dehydrogenase complex or pyruvate oxidase, respectively. Acetyl-phosphate can be converted to acetate via acetyl-phosphate kinase or to acetyl-coA through phosphate acetyltransferase. Acetyl-coA can be further reduced into acetaldehyde and ethanol via the bifunctional acetaldehyde/alcohol dehydrogenase.

Convivina can import and metabolise sucrose through the presence of sucrose phosphotransferase, sucrose phosphorylase and invertase/sucrase. dbCAN2 predicted genes for levansucrase (EC 2.4.1.10, CAZy family GH68) and inulosucrase (EC 2.4.1.9, CAZy family GH68) in both species, as well as a dextransucrase gene (EC 2.4.1.5, CAZy family GH70) only in *C. intestini*. This indicates that *Convivina* is able to produce polysaccharides while degrading sucrose. Furthermore, maltose phosphorylase (EC 2.4.1.8, CAZy family GH65) and oligo-1,6-glucosidase (EC 3.2.1.10, CAZy family GH13 subfamily 31) were identified in the genomes of *C. praedatoris* only. Other carbohydrate-active enzymes identified via the dbCAN2 server are summarised in Table S6.

Proteolytic metabolism analysis

KEGG and eggNOG annotations were investigated to map the proteolytic potential of *Convivina*. The *Convivina* genomes analysed lacked an
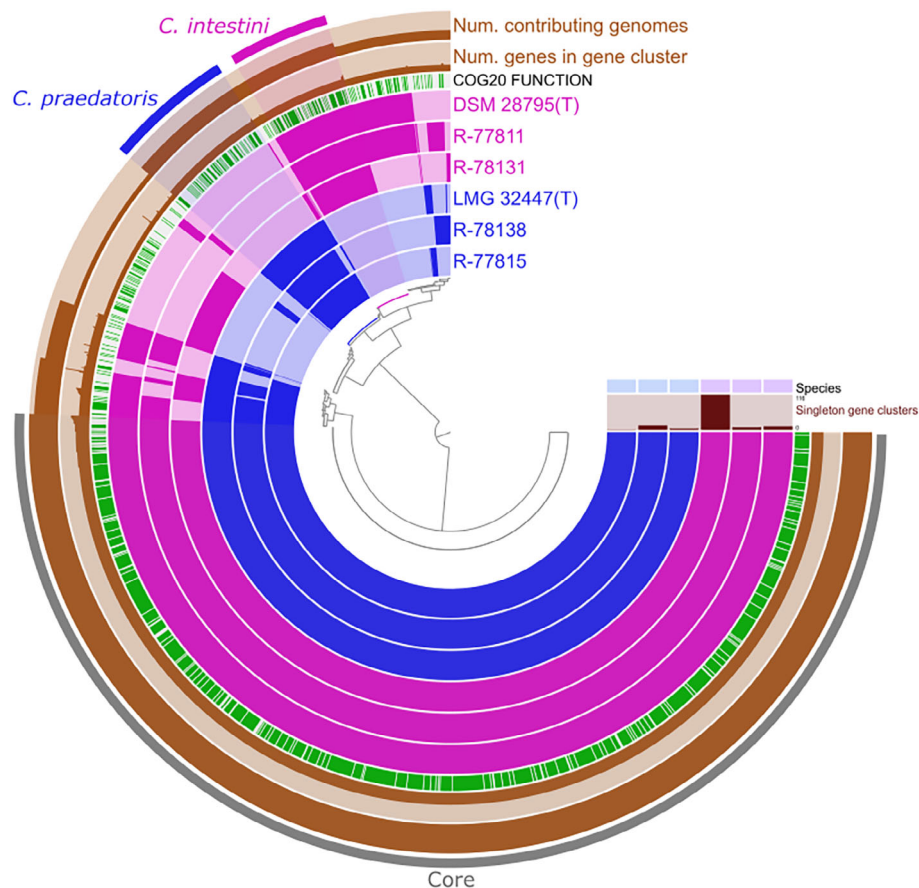
FIGURE 3 Pangenome analysis of *Convivina* strains computed through the anvi'o pangenomics workflow. Gene clusters ($n = 1901$) were ordered according to a hierarchical clustering of their presence/absence (inner dendrogram). Genomes were ordered based on the presence/absence of the gene clusters. The outermost ring indicates the number of genomes contributing to a gene cluster. The second ring indicates the number of genes that fall into a respective gene cluster. The third ring depicts for which gene clusters a COG20 function was assigned (dark green). The inner purple and blue rings display the presence (dark colour) or absence (light colour) of the gene clusters in each of the six genomes. The outer grey arc depicts the core genome composed of gene clusters present in all six *Convivina* genomes. The purple and blue arcs indicate gene clusters exclusively present in all three genomes of *C. intestini* or *C. praedatoris*, respectively.

extracellular proteinase for the initiation of proteolysis. However, several peptide and amino acid transporter systems were identified in the *Convivina* core genome such as the oligopeptide ABC transporter Opp and the di-/tripeptide transporter DtpT. *Convivina intestini* encodes an additional Na+/glutamate symporter not identified in *C. praedatoris*. Intracellular degradation of peptides is mediated by different peptidases located in the *Convivina* core genome: aminopeptidase (PepA), aminopeptidase N (PepN), tripeptidase (PepT), oligoendopeptidase F (PepF) and Xaa-proline aminopeptidase (PepP).

Phenotypic characterisation

Convivina intestini LMG 28291[T] and *C. praedatoris* LMG 32447[T] were catalase- and oxidase-negative and grew at pH values between 5 and 8. The latter tolerated higher temperatures and NaCl concentrations for growth, that is, 15–37°C and 0%–6% [weak growth at 7%] NaCl versus 15–35°C and 0%–5% NaCl. Both strains grew aerobically and anaerobically, both with and without 2% fructose. They showed no or very poor growth with D-ribose, D-fucose, D-melibiose, D-cellobiose, starch or chitin in MRS broth (Figure S3). Both strains showed similar growth with D-fructose and sucrose. *Convivina intestini* LMG 28291[T] did not grow with D-maltose as single carbon source, while *C. praedatoris* LMG 32447[T] did. In contrast, both strains grew well with D-glucose and D-trehalose as single carbon source, but much higher cell yields were obtained for *C. intestini* LMG 28291[T] compared to *C. praedatoris* LMG 32447[T]. Both strains showed a similar production of metabolites from D-glucose, except for acetate, which was accumulated at a higher concentration by *C. praedatoris* LMG 32447[T] (Table S7). D-Fructose and sucrose metabolism resulted in a higher production of mannitol and acetate by *C. intestini* LMG 28291[T] compared to *C. praedatoris* LMG 32447[T]. The latter produced significantly more lactate from growth on D-fructose, although the concentration of lactate accumulated by both strains was lower than with D-glucose or sucrose metabolization. Furthermore, sucrose was not fully utilised by *C. praedatoris* LMG 32447[T].

TABLE 4 Number of genes assigned to each COG category for all six analysed *Convivina* strains.

	C. intestini			C. praedatoris		
	DSM 28795[T]	R-77811	R-78131	LMG 32447[T]	R-77815	R-78138
C—Energy production and conversion	46	46	45	47	47	47
D—Cell cycle control, cell division, chromosome partitioning	31	31	31	31	31	30
E—Amino acid transport and metabolism	142	142	143	120	120	119
F—Nucleotide transport and metabolism	75	75	74	74	74	74
G—Carbohydrate transport and metabolism	77	78	79	83	83	83
H—Coenzyme transport and metabolism	64	64	64	64	64	64
I—Lipid transport and metabolism	52	52	52	50	50	50
J—Translation, ribosomal structure and biogenesis	181	182	182	180	180	180
K—Transcription	81	81	83	79	79	76
L—Replication, recombination and repair	91	92	86	92	90	91
M—Cell wall/membrane/envelope biogenesis	65	64	70	66	66	67
N—Cell motility	5	5	5	5	5	5
O—Posttranslational modification, protein turnover, chaperones	53	54	53	51	51	52
P—Inorganic ion transport and metabolism	55	56	51	48	47	48
Q—Secondary metabolites, biosynthesis, transport and catabolism	10	10	11	11	11	11
R—General functional prediction only	54	54	55	58	58	58
S—Function unknown	63	66	69	67	67	68
T—Signal transduction mechanisms	32	32	33	32	33	32
U—Intracellular trafficking, secretion and vesicular transport	14	14	14	14	14	14
V—Defence mechanisms	31	31	30	28	28	31
X—Mobilome: prophages, transposons	20	22	16	20	17	17
No category assigned	369	367	350	368	348	365
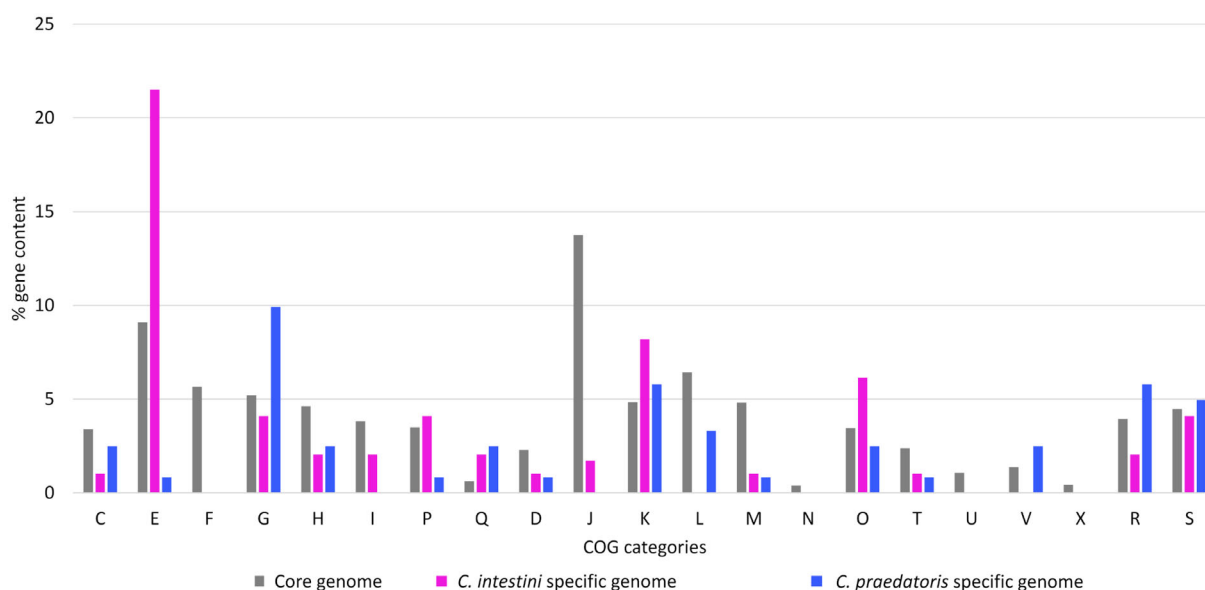
FIGURE 4 Relative COG category classification of the *Convivina* pangenome. Pangenome analysis, species-specific genome analysis and classification of genes into COG categories were performed through the anvi'o pangenomics pipeline. The core genome of *Convivina* was composed of genes present in all six analysed strains. The species-specific genomes of the two species were composed of genes present in all three genomes of each species. Function of each COG category is shown in Table 4.

DISCUSSION

We identified the cultivable bacterial communities of the crop, midgut, hindgut and ovaries in a single nest of the invasive hornet *V. velutina* through a large-scale isolation campaign. We subsequently compared the results obtained with those of a 16S rRNA amplicon sequencing analysis of samples of the same nest to provide a more complete image of these bacterial communities, as both approaches have intrinsic biases and limitations (Duthoo et al., 2022; Hong et al., 2009; Lagier et al., 2012; O'Callaghan et al., 2021). Both cultivation-independent and cultivation-dependent approaches revealed a dominant occurrence of Bacillota and Pseudomonadota in the hornet's gut microbiota, which was consistent with results from other insect species (Yun et al., 2014), other hornet species (Suenami et al., 2019) and two recent studies of *V. velutina* (Cini et al., 2020; Zhang et al., 2022). While 16S rRNA gene amplicon sequencing revealed a different gut microbiota composition in each of the sample types (i.e., gut fractions and ovaries), each sample type displayed a similar richness and diversity (Figure S1).

Gut symbiont community of the Asian hornet

16S rRNA gene amplicon sequencing allowed us to define bacterial core ASVs for each of the sample types and revealed three dominant *V. velutina* gut symbionts: *Lactococcus* and *Sphingomonas* were identified as bacterial core ASVs across all sample types and were the most abundant in the midgut, hindgut and ovaries, whereas *Convivina* ASVs dominated the crop. Other core ASVs were assigned to *Fructobacillus* and *Spiroplasma*. Isolates representing *Sphingomonas* and *Spiroplasma* core ASVs were not obtained during the cultivation campaign. While the former should be cultivable on several of the general-purpose isolation media used, the latter is a fastidious organism that requires highly specific isolation conditions (Bell-Sakyi et al., 2015). Isolates recovered during the cultivation campaign were primarily LAB and AAB, which are well-known members of the insect gut microbiota, especially in sugar-feeding insects (Crotti et al., 2010; Engel & Moran, 2013). Although the logistics of a large-scale isolation campaign did not allow to process all collected samples immediately after collection, the decision to immediately freeze freshly collected hornet specimens at −80°C until further analysis may have introduced a bias in our study. Nevertheless, a much higher species level diversity was detected through cultivation compared to 16S rRNA gene amplicon sequencing, as also observed in earlier studies (Audisio et al., 2011; Olofsson & Vasquez, 2008; Praet et al., 2018). We isolated representatives of 12 LAB and 4 AAB species while 5 LAB ASVs and a single AAB ASV were detected among the top 20 most predominant ASVs through 16S rRNA amplicon sequencing. Other LAB and AAB ASVs were only detected at an overall low relative abundance of less than 0.1%.

The dominantly isolated (Table 1) and detected (Table 2) LAB and AAB species in the present study differed from those reported in honey bees (Moran, 2015). *Lactococcus lactis, L. plantarum, Convivina*

spp., *F. fructosus* and *Leuconostoc* species were the most abundantly isolated LAB (Table 1). However, no representatives of the 'Firm 4' (i.e., the genus *Bombilactobacillus*; Zheng et al., 2020) or 'Firm 5' (i.e., the genus *Lactobacillus*; Zheng et al., 2020) phylotypes that are dominant in social bees, or the more sporadically detected 'Lacto-3' phylotype (i.e., the genus *Apilactobacillus*; Zheng et al., 2020) were isolated. We also did not isolate representatives of the 'Alpha 2.2' *Acetobacteraceae* phylotype (i.e., the genus *Bombella*; Li et al., 2015) but did obtain several *Commensalibacter* isolates (Table 1). The latter corresponds with the 'Alpha 2.1' phylotype which is detected sporadically in honey bees and fruit flies (Bonilla-Rosso & Engel, 2018; Martinson et al., 2011; Roh et al., 2008). We finally did not isolate representatives of the 'Bifido' or 'Alpha 1' phylotypes (i.e., *Bifidobacterium* and *Bartonella*, respectively) detected in honey bees. However, the compartment-specific bacterial community and similar alpha diversity indices between gut compartments corresponded with observations in honey bees (Callegari et al., 2021). The most predominant bacteria in our study were consistently present along the gut but varied in abundance in the gut compartments as *Convivina* dominance declined and *Lactococcus* abundance increased from crop towards the hindgut. A similar pattern was reported in the honey bee gut community where fermentative LAB was more dominant towards the distal compartments (Kwong & Moran, 2016; Martinson et al., 2012).

ASVs of honey bee-associated bacteria were previously detected in *V. velutina* (Cini et al., 2020), but also in the Japanese hornets *V. simillima* and *V. mandarinia* (Suenami et al., 2019), suggesting the detection of prey microbiota. Inspection of ASVs generated in the present study also revealed the sporadic occurrence of ASVs of honey bee-associated bacteria such as *Apilactobacillus kunkeei*, *Lactobacillus melliventris*, *Bifidobacterium*, *Bombella* and *Commensalibacter*, with less than 0.1% relative abundance (data not shown). This suggests that honey bee-associated bacteria can be detected upon honey bee predation but are likely not adapted for colonisation of the hornet gut. *Vespa velutina* hornets sampled in Korea showed a very distinctive bacterial community dominated by *Flavobacterium*, *Aeromonas*, *Pseudomonas* and *Pedobacter* (Kim et al., 2018). Most of the genera detected in the latter study were minimally represented in our ASV dataset, except for *Sphingomonas* and *Stenotrophomonas* which belonged to the 20 most abundant ASVs. While diet or environment-induced effects cannot be ruled out, this major difference in bacterial community is likely explained by the DNA extraction method employed by Kim et al. (2018) which led to the detection of predominantly Gram-negative bacteria. The DNA extraction procedure used by Cini et al. (2020) was more similar to ours, as were their results. Adult hornet microbiota samples in Italy were dominated by lactobacilli and *Lactococcus* (Cini et al., 2020). Sequence analysis of the most abundant ASVs retrieved from the amplicon sequencing data of the Italian study (Cini et al., 2020) revealed a 100% identity with the most abundant *Lactiplantibacillus* and *Lactococcus* ASVs in our dataset and furthermore had a 100% sequence identity to the 16S rRNA V3-V4 region of *L. plantarum* and *Lc. lactis* isolates of the present study (Table 1). In contrast, *Fructobacillus* and *Sphingomonas* were detected in a very low abundance and *Convivina* was not reported earlier (Cini

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et al., 2020). Re-examination of all *Leuconostoc* ASVs and *Lactobacillaceae* ASVs with unassigned genus status from the Cini et al. study identified two ASVs as *Convivina*: one with 100% sequence identity to *C. intestini* and one with 100% sequence identity to *C. praedatoris*. Together, these data confirmed the presence of *Lactococcus*, *Lactiplantibacillus*, *Fructobacillus*, *Sphingomonas* and *Convivina* in the Italian study (Cini et al., 2020). Furthermore, *Lactococcus*, *Lactiplantibacillus*, *Fructobacillus*, *Sphingomonas*, and *Spiroplasma* were also reported in the midgut microbiome of *V. velutina* in the native region China (Zhang et al., 2022).

The distinct LAB community in the Asian hornet that was dominated by *Lactococcus*, *Lactiplantibacillus*, *Convivina* and *Fructobacillus* in the two European populations examined thus far may assume functions similar to those provided by the LAB phylotypes in the social bee gut, but is likely to be tailored to the more ancestral carnivorous diet of its host. Consumption of sugar-rich plant-derived liquids as a carbon source is a common trait between hornets and bees, and various LAB have different carbohydrate processing affinities. LAB are bacteria with small genomes that range in size between 1.3 Mbp and 3.5 Mbp, highlighting processes of considerable gene loss in this phylogenetic lineage (Maeno et al., 2021). Analysis of LAB genomes suggested that the bulk of the genes lost were linked to adaptation to protein and carbohydrate-rich food environments (Schroeter & Klaenhammer, 2009). Some LAB, such as *Fructobacillus* species, are characterised by excessive gene loss (Endo et al., 2015; Maeno et al., 2021), while others, such as *L. plantarum* and *Lc. lactis*, have counterbalanced gene loss by retaining a larger number of sugar uptake systems and complete pathways for biosynthesis of most amino acids, and by the emergence of new genes via duplication and horizontal gene transfer (Makarova et al., 2006; Sun et al., 2015; Wels et al., 2019). Yet, the small LAB genomes typically encode a broad repertoire of transporters for efficient carbon and nitrogen acquisition from the nutritionally rich environments they live in to compensate for a limited biosynthetic potential (Makarova et al., 2006; Schroeter & Klaenhammer, 2009).

Ecology, metabolism, and functional genomics of *Convivina*

The Asian hornet thus hosts a community of LAB that are on the one hand well-known as broad generalists (i.e., *L. plantarum* and *Lc. lactis*), and others that have emerged as highly adapted specialists (*Fructobacillus* and *Convivina*). *Convivina* was the dominant LAB genus in the crop, with two ASVs comprising almost 70% of the reads of the pooled crop sample. So far only one species, isolated from the gut of the bumble bee *Bombus terrestris*, has been described (Praet et al., 2015). Apart from its subsequent detection in bumble bees and recently also in stingless bees (Figueroa et al., 2021), little is known about the habitat and functional role of these bacteria. A single '*Fructobacillus* sp.' isolate (16S rRNA accession no. JN167936) from *Hedera helix* flowers was reported in 2012 in a study in Sweden (Vasquez et al., 2012); its 16S rRNA sequence presented 99.85% identity with

the *C. intestini* type strain (data not shown). Similarly, a 16S rRNA gene sequence of a single '*Leuconostoc* sp.' isolate (16S rRNA accession no. JX863368.1) from a honey bee gut sample in South Korea was deposited in GenBank in 2012 (unpublished data) and presented 99.57% 16S rRNA gene sequence identity with the *C. intestini* type strain (data not shown), demonstrating that both strains belonged to *Convivina* as well. In the present study, we isolated a novel *Convivina* species, *C. praedatoris* sp. nov., from the three gut fractions of *V. velutina*. The two *Convivina* ASVs (Table 2) corresponded with the two species isolated from the hornet gut (Table 1) and with ASVs reported in the Italian study (Cini et al., 2020).

The genomic features of the *Convivina* strains were generally similar to those of *Fructobacillus* and *Leuconostoc* strains (Endo et al., 2015), their nearest phylogenetic neighbours. Category E genes (amino acid metabolism and transport) ranked 2nd in genomes of all three genera, whereas the ratio of category G (carbohydrate metabolism and transport) genes in *Convivina* genomes ranked higher than in *Fructobacillus* genomes but lower than *Leuconostoc* genomes (Table S5) (Endo et al., 2015). The comparative functional genome analyses presented here revealed that *C. intestini* has a genome that is more adapted towards amino acid metabolism, whereas *C. praedatoris* showed adaptation towards carbohydrate metabolism (Table 4; Tables S5 and S6). The lack of complete biosynthesis pathways for several amino acids in *C. praedatoris* indicates that this species is more dependent on the proteins, peptides and amino acids which *V. velutina* obtains through ingestion of nitrogen-rich larval saliva (Takashi et al., 1991). It seems plausible to speculate that *C. praedatoris* may have lost more of its amino acid biosynthetic potential as these are freely available in the hornet gut. Another striking difference between the two species is the presence of maltose phosphorylase and oligo-1,6-glucosidase in *C. praedatoris* (Table S6). These two enzymes can be directly linked to the capability of *C. praedatoris* to metabolise degradation products of starch, such as maltose, which is lacking in *C. intestini* (Figure S3). Maltose is a disaccharide formed by starch degradation during fruit ripening (Stone & Morell, 2009). As ripening fruits are a part of the hornet diet as a source of carbohydrates, this may highlight another adaptation of *C. praedatoris* towards the host diet.

While both *Convivina* species shared many biochemical characteristics, they differed in growth with D-glucose and D-trehalose as substrate (Figure S3). Inspection of the metabolites produced from D-glucose metabolism showed a significantly higher production of acetate by *C. praedatoris* (Table S7), possibly leading to a growth inhibition due to the increasing acidity of the environment. D-Fructose metabolism resulted in lower lactate concentrations than D-glucose metabolism (Table S7), as D-fructose can be used as both a fermentable substrate and as an electron acceptor: per mol of D-fructose metabolised, 2 mol of D-fructose must be reduced to mannitol to regenerate NAD^+ (Wisselink et al., 2002). The excess of electrons is a result of the conversion of acetyl-phosphate to acetate instead of ethanol, and explains the lower concentrations of ethanol for growth with D-fructose compared to growth with D-glucose. The two *Convivina* species showed significant differences in the yield of lactate, acetate and mannitol from D-fructose metabolism (Table S7), likely

indicating that mannitol dehydrogenase is more active in *C. intestini* (which produces less lactate, but more mannitol and acetate). Furthermore, the present data suggested that invertase/sucrase was less active in *C. praedatoris*, as sucrose was not fully metabolised. Once sucrose is converted to D-glucose and D-fructose, D-glucose can be fermented to lactate and ethanol or, in lower amounts, acetate, whereas D-fructose is again utilised as an electron acceptor and reduced to mannitol. Genome analyses furthermore indicated that both species can produce polysaccharides from sucrose degradation (Table S6). Dextran, levan and inulin may be involved in biofilm formation, which is important for adhesion and colonisation of the gut tissue. Furthermore, the latter two are known as prebiotics that stimulate the growth of gut beneficial bacteria in mammals (Dogsa et al., 2013; Schmid et al., 2019; Velazquez-Hernandez et al., 2011).

The small size of the *Convivina* genomes (1.5–1.6 Mbp) is a hallmark of gene reduction involved in carbohydrate transport and metabolism (category G) in LAB, as was recently shown in other small genome *Lactobacillaceae* species (Maeno et al., 2021). Using the formula for correlation between genome size and number of genes involved in category G ($y = 95.452x − 52.387$) (Maeno et al., 2021), *C. intestini* and *C. praedatoris* should have approx. 100 and 93 category G genes, respectively. The observation of only 77 and 83 genes, respectively, in both type strains confirmed a reduction in category G genes, especially in *C. intestini*. Although gene reduction is not as high as in fructophilic LAB, it likely highlights adaptation and specialisation to carbohydrate-rich habitats, which is in accordance with their isolation sources. The present and an earlier study (Cini et al., 2020) demonstrated that both *C. praedatoris* and *C. intestini* occur in European *V. velutina* populations, whereas *C. intestini* was earlier isolated from gut samples of *B. terrestris* bumble bees (Praet et al., 2015). Considering its high abundance in samples in the present study, it is unlikely that *C. intestini* is merely a prey bacterium transiting the hornet gut. The strong reduction in carbohydrate metabolism genes in *C. intestini* limits this organism to carbohydrate-rich habitats, which is provided in the gut of both hosts. It seems plausible to speculate that *Convivina* bacteria are present in more carbohydrate-rich habitats linked to social insects, or specifically the clade Aculeata, but that this relatively recently described LAB genus (Praet et al., 2015) has not been identified or misidentified in earlier studies.

CONCLUSION

Our study provided a comprehensive description of the cultivable bacterial community of the crop, midgut and hindgut compartments, as well as the ovaries of the invasive hornet *V. velutina*. While these results were obtained through the analysis of samples from a single nest of Asian hornets in Belgium, they were largely congruent with 16S rRNA amplicon sequencing data reported in Italy (Cini et al., 2020) and China (Zhang et al., 2022). The results obtained suggested that the Asian hornet bacterial community was dominated by *Convivina*, *Fructobacillus*, *Lactococcus*, *Sphingomonas*, *Spiroplasma* and *Lactiplantibacillus*. We reported and discussed the functional genomic potential of two *Convivina* species from the hornet gut: *C. intestini*, which was previously isolated from a bumble bee, and the novel species *C. praedatoris*. Comparative functional genomics analyses of both species indicated specialisation towards amino acid and carbohydrate metabolism, respectively.

Description of *Convivina praedatoris* sp. nov.

Convivina praedatoris (L. gen. n. *praedatoris*, from a predator, referring to the isolation source of the reported strains).

Gram-positive, rod-shaped cells, 1.5–10 μm long and about 0.8 μm wide. Rods can be short or elongated and can occur in chains of 2 or 3 cells. Aerobic growth on MRS occurs at 15–37°C and anaerobic growth occurs at the tested temperatures (28°C and 37°C). Grows anaerobically in MRS broth at pH 5 to 8 and NaCl concentrations of 0 to 6%; after prolonged incubation up to 7 days weak growth is obtained at 7% NaCl. Catalase and oxidase negative. Growth on MRS supplemented with 2% fructose occurs in both aerobic and anaerobic conditions. The type strain grows with D-glucose, D-fructose, sucrose, D-maltose and D-trehalose as single carbon source, but not with D-ribose, D-cellobiose, D-fucose, D-melibiose, starch or chitin. Lactic acid, acetic acid and ethanol are produced as end products from glucose, fructose and sucrose metabolism. Mannitol is produced during growth in fructose and sucrose, with higher mannitol levels produced during fructose metabolism.

The type strain LMG 32447[T] (=R-78119[T], CECT 30512[T]) was isolated in 2019 from the hindgut of the Asian hornet *V. velutina* sampled in Essene, Belgium. The accession numbers of the 16S rRNA and partial *phe*S gene sequences of *C. praedatoris* LMG 32447[T] are OW028325 and OW028324, respectively. The whole-genome sequence of strain LMG 32447[T] has a size of 1.5 Mbp and a G + C content of 40.18 mol %. This whole-genome sequence is publicly available under accession number CAKOEU010000001 – CAKOEU010000022.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

All data generated during the present study are archived at the European Nucleotide Archive. 16S rRNA gene amplicon sequencing data are accessible through Bioproject PRJEB51475. Annotated whole-genome sequences and *C. praedatoris* LMG 32447^T 16S rRNA and *phe*S gene sequences through Bioproject accession number PRJEB47662.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Data S1. Supporting Information