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Apple Blossoms from a Swiss Orchard with Low-Input Plant Protection Regime Reveal High Abundance of Potential Fire Blight Antagonists

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ABSTRACT

Erwinia amylovora causes fire blight, a serious disease of Rosaceae plants, including apple and pear. A predominant path of bacterial infection is entry through nectartodes after multiplication on the stigma. Depending on the inhibitory abilities of the native blossom microbiota, it may control the outbreak of fire blight and, therefore, may bar potential plant protection with reduced input of synthetic chemicals. Blossoms of five apple varieties in a low-input orchard, which had no fire blight history despite disease outbreaks in close proximity, were analyzed to assess bacterial and fungal communities. Metabarcoding indicated low microbial diversity and the presence of a few dominant operational taxonomic units (OTUs), including known fire blight antagonists such as *Metschnikowia pulcherrima* and *Aureobasidium pullulans.* The most dominant bacterial taxon (bOTU_01) was classified as *Erwinia* spp. To resolve sequences of species within bOTU_01, we used analyses of sequence variants and DNA signatures (i.e., nucleotide polymorphisms that

are indicative for different species or species groups). These analyses revealed that >94.5% of the sequences of bOTU_01 derived from *E. tasmaniensis*, a potential *E. amylovora* antagonist. The latter was represented by up to 0.006% of the sequences. Cultivation-based analyses confirmed the prevalence of *E. tasmaniensis*. The high abundance of native potential *E. amylovora* antagonists likely indicates that this special set of native apple blossom microbiota counteracted the establishment of *E. amylovora* in this low-input orchard. This may allow for a new approach to assess possible components of synthetic apple blossom communities to mitigate fire blight infections.

Keywords: antagonists, apple blossoms, bacteriology, biological control, disease control and pest management, *Erwinia amylovora*, *Erwinia tasmaniensis*, fire blight, low-input orchard, metabarcoding, microbiome, sub OTU-level analysis

The genus *Erwinia* (family *Enterobacteriaceae*) includes phytopathogenic and nonphytopathogenic bacterial species, some of which are associated with economically significant pome fruit trees. *Erwinia amylovora,* the causative agent of the devastating disease "fire blight", is the most important pathogen of this genus. It infects Rosaceae plants, including apple, pear, and quince. *E. pyrifoliae* causes fire blight on Asian (Nashi) pear and is considered to be

geographically restricted to Korea and Japan (Rhim et al. 1999; Shrestha et al. 2003). Epiphytic species within the genus include *E. billingiae* and *E. tasmaniensis*, which also reveal potential for use as antagonists in *E. amylovora* biocontrol (Jakovljevic et al. 2006; Mergaert et al. 1999).

The fire blight pathogen multiplies on the stigma after being transported to a blossom by bees or other pollinating insects and can be washed down the style into the floral nectary by water, subsequently infecting the underlying tissue (Stockwell et al. 1998). Therefore, most fire blight management strategies have focused on the reduction of inoculum in the orchard and treatments to prevent blossom infections (Norelli et al. 2003). Currently, the most effective fire blight control is the application of antibiotics such as streptomycin (Sundin et al. 2009). Due to the emergence and spread of streptomycin resistance (Chiou and Jones 1995) and increasing public concerns about using antibiotics in agriculture, all field applications of streptomycin in Switzerland were banned in 2016.

Therefore, there is a need for alternatives to control fire blight, and biocontrol may offer such alternatives. For decades, antagonists of the fire blight pathogen have been isolated using classical cultivation and subsequent in vitro and in vivo analyses for assessing the antagonistic activity of a selected strain Mikiciński et al. (2020). In general, growth and subsequent efficacy of a biocontrol agent may vary and is dependent on the location and the conditions that may differ (Johnson and Stockwell 1998).

With the availability of new DNA-sequencing technologies and the processing of large data sets, the possibility has been provided to characterize entire microbial communities (Aleklett et al. 2014) rather than isolating and characterizing individual strains. Because of the recognition of its ecological and economical importance, plant-associated microbiota research has significantly increased over the past 10 years. Biocontrol strategies based on the microbiota and use of the microbial diversity in an environment might overcome inconsistencies experienced with applying individual organisms in biocontrol (Berg et al. 2017). Microbiota studies may assist the development of synthetic communities or they can support the development of strategies to promote already existing beneficial microflora by allowing the fostering of biocontrol agents that already exist in the community of a growing area. This might improve efficacy and consistency of the applied biocontrol agent. Initial studies performed on apple blossoms in Wisconsin and Connecticut, United States revealed specific differences in bacterial communities. Either they were dominated by members of the phyla *Deinococcus–Thermus* and TM7 (Shade et al. 2013) or the genus *Pseudomonas* and family *Enterobacteriaceae* (Steven et al. 2018).

To characterize a microbiota with antagonistic potential against the fire blight pathogen, an orchard with a low-input plant protection regime was selected. No fire blight incident was reported in this orchard, although the disease occurred in the immediate vicinity. The two main research goals were to (i) characterize bacterial and fungal communities in apple blossoms from varieties Ariwa, Boskoop, Idared, Milwa/Diwa, and Golden Delicious and (ii) infer explanations for the absence of fire blight infection in this orchard. Therefore, we collected apple blossoms from the stage at which they are most susceptible to *E. amylovora* infections and assessed bacterial and fungal communities using metabarcoding. Thus, the presented study provides the information needed for hypothesis-driven studies in fire blight protection on synthetic communities with antagonistic potential.

MATERIALS AND METHODS

Field site. Blossom samples were collected during full bloom in 2017 in an apple orchard at the Agroscope Research Station in Waedenswil, Switzerland (GPS: 47°13′18.1″ N, 8°40′38.9″ E). The orchard (800 m^2 in size), with eight rows of trees and a planting distance of 4 m between rows and 1.5 m within rows, was established between 1998 and 2007 and consists of five apple varieties (i.e., Ariwa, Boskoop, Idared, Milwa/Diwa, and Golden Delicious). Golden Delicious trees were located at the borders of the orchard for pollination, while the trees of the other varieties were planted in rows. Varieties Idared and Golden Delicious have been tested in two consecutive years in *E. amylovora* shoot infection. Idared was classified as moderate and highly susceptible and Golden Delicious as moderate susceptible in both years (Kostick et al. 2019). The orchard had a low-input managing regime to study naturally occurring insect and mite populations. Records from 2010 to 2017 show that fertilizers and herbicides were used every year but no insecticides and other pesticides until 2015. In 2015, Myco-sin (containing 75% sulfuric acid clay) was applied three times at 8 kg/ha for fire blight control. In 2016, LMA (at 20 kg/ha, potassium

alum for fire blight control) was applied, as well as the fungicides Slick (1×), Flint (1×), and Captan (2×). Before blossom sampling in 2017, there were no pesticide treatments. This low-input orchard has no history of fire blight infection, although fire blight was present for several years in a conventional orchard in the immediate vicinity (approximately 100 m) (Supplementary Fig. S1), which was eradicated in November 2016.

Blossom sample collection. To characterize the microbiota of apple blossoms, eight replicate samples consisting of 25 lateral blossoms each were collected randomly from two trees of the five varieties Ariwa, Boskoop, Idared, Milwa/Diwa, and Golden Delicious. Samples were taken at full bloom in 2017 (i.e., when 80 to 100% of the lateral blossoms were open), which represents the window of infection for the fire blight disease. Blossoms sampled met two criteria: first, petals were open and intact and, second, anthers were yellow to dark yellow but not brownish. The blossom petals were carefully removed by hand wearing gloves while the remaining blossom material was collected in plastic bags (i.e., 25 blossoms/bag). Material was kept on ice during sampling and transportation to the laboratory. Sample processing was performed the same day.

Sample processing and DNA extraction. For DNA extraction, 25 ml of 1× buffer (K$_2$HPO$_4$ at 2.5 g liter^{-1} and KH$_2$PO$_4$ at 1.2 g liter^{-1}) was added to each plastic bag containing 25 blossoms from one sample. Bags were shaken briefly and sonicated for 1 min (37 kHz, sweep mode, Elmasonic S 30 H). Blossom extracts were transferred to sterile 15-ml centrifuge tubes and stored at –20°C until DNA extraction. After thawing, extracts were centrifuged (2,205 × g) for 30 min at 4°C and the pellet was resuspended in 2 ml of the supernatant, of which 0.5-ml aliquots were transferred to sterile microcentrifuge tubes and centrifuged for 10 min at 9,900 × g. DNA was extracted from the resulting pellets using the BioSprint 96 DNA Plant Kit (Qiagen) according to the manufacturer's protocol. Shortly, each pellet was resuspended in 300 μl of RLT Lysis buffer and incubated for 30 min at 65°C and 1,000 rpm in a thermomixer. After centrifugation for 5 min at 5,000 × g, 200 μl of each supernatant was transferred to 96 deep-well plates containing isopropanol and MagAttract Suspension G (magnetic silica beads). DNA was eluted in 100 μl of sterile MilliQ water and stored at –20°C until further processing.

PCR reaction and sequencing. The fragment of the bacterial small subunit of the ribosomal RNA (rRNA) gene (16S rRNA) containing the variable regions V5 and V6 was amplified according to Shade et al. (2013) using the primer pair 799F (5′-AACMG-GATTAGATACCCKG-3′)/1115R (5′-AGGGTTGCGCTCGTTG-3′) (Chelius and Triplett 2001; Reysenbach and Pace 1995). The fungal ribosomal internal transcribed spacer (ITS)2 was amplified using the primer pair ITS3 (5′-CAHCGATGAAGAACGYRG-3′)/ITS4 (5′-TCCTSCGCTTATTGATATGC-3′) (Tedersoo et al. 2014). Primers were extended with CS1 or CS2 adapters to allow multiplexing with the Fluidigm Access Array System (Fluidigm, South San Francisco, CA, U.S.A.). PCR mixtures consisted of 1× GoTaq Flexi buffer colorless (Promega Corp.), 2.5 mM MgCl$_2$, 0.2 mM dNTPs, 0.2 μM of each primer, GoTaq G2 (Hotstart Polymerase) at 1.25 U/reaction, and 4 μl of isolated DNA template per reaction in a 25-μl volume. PCR conditions were 2 min at 95°C; 35 cycles of 40 s at 94°C, 40 s at 58°C, and 1 min at 72°C; and a final amplification step of 10 min at 72°C. The approximate length of amplification products was 335 bp for bacteria and 400 bp for fungi. Integrity and quality of the PCR products were analyzed on 1% agarose gels. PCR was replicated four times and quality-controlled replications were pooled prior to sequencing on an Illumina MiSeq platform (Génome Québec Innovation Center at the McGill University, Canada). Raw sequences were deposited in the

NCBI Sequence Read Archive database with the accession number PRJNA609888.

Sequence processing and taxonomic classification. Sequences were processed and clustered into operational taxonomic units (OTUs) using a customized pipeline adapted from Frey et al. (2016) and Mayerhofer et al. (2017), which was mainly based on USEARCH v9 (Edgar 2010). Briefly, quality control of the sequences included, first, the removal of remaining PhiX sequences, merging of paired-end reads, and removal of primer sequences (allowing for one mismatch) using cutadapt (Martin 2011). Then, all sequences with a maximum expected error of at least 1 were discarded. Finally, sequences were clustered into operational taxonomic units (OTUs) with 97% sequence identity along with de novo chimera removal, and targets were verified using Metaxa2 for prokaryotic 16S (Bengtsson-Palme et al. 2015) and ITSx for eukaryotic ITS2 sequences (Bengtsson-Palme et al. 2013). Qualifying prokaryotic sequences were taxonomically assigned based on the SILVA database v123 (Quast et al. 2013) using the naïve Bayesian classifier implemented in MOTHUR v.1.36.1 (Schloss et al. 2009). The same classifier was used to compare eukaryotic sequences to a custom-made, GenBank-derived database in order to distinguish fungal from other eukaryotic sequences (Frey et al. 2016). Fungal sequences were taxonomically assigned using the same classifier but based on the UNITE database v7.2 (Nilsson et al. 2019). A bootstrap analysis was used to estimate the robustness of the taxonomic assignments on each taxonomic level. Assignments with a bootstrap value <80% were considered as unclassified. Only sequences classified as bacteria or fungi were kept for further analyses.

Estimating microbial diversity and statistical analysis. Statistical analyses were done in R version 3.2.2 (R Core Team 2016). Sequencing depth was assessed by rarefaction analysis and Good's coverage. The α diversity was assessed as OTU richness (i.e., the number of OTUs per sample) as well as Simpson evenness and inverse Simpson indices. The inverse Simpson index indicates the effective number of OTUs in a sample, which is the number of equally abundant OTUs that correspond to the detected diversity. To correct for the different sequencing depths among samples, communities were subsampled with 10,000 iterations to the minimal sequence number. Average values for OTU richness, Simpson evenness, and inverse Simpson indices were calculated using 'summary.single' in MOTHUR v 1.36.1. Similarly, Bray-Curtis dissimilarities used for pairwise community comparisons were obtained as average values of 10,000 subsamplings using 'dist.shared' in MOTHUR v 1.36.1. The α-diversity data were tested for normal distribution and similarity of variance among groups using Shapiro and Levene tests. Subsequently, analysis of variance (ANOVA) and pairwise Tukey's honestly significant difference were used to test for differences of α diversities among apple varieties. For comparisons of microbial community structures, permutational analysis of variance (PERMANOVA) was calculated using 'adonis' of the R package vegan (Oksanen et al. 2018), and the test assumption of homogeneous multivariate dispersions among groups was assessed using 'betadisper' of the same R package. *P* values of multiple tests were corrected based on the Benjamini-Hochberg *P* value adjustment method.

Sub-OTU-level classification of bacterial OTU_01 sequences. DADA2 version 1.16 (Callahan et al. 2016) was used to define sequence variants (SVs). Default parameters of DADA2 were applied with three exceptions: (i) sequences without primers were discarded, (ii) a more stringent quality control was applied with a maximum expected error of 1 instead of 2, and (iii) eukaryotic sequences were trimmed using cutadapt (Martin 2011). The last 50 bp of forward and the last 100 bp of reverse reads were removed

due to their generally low quality. Their inclusion would have led to lower quality of the entire sequences, not allowing them to pass subsequent quality control. The first two adaptations of the DADA2 pipeline were chosen to use the same conditions for sequence quality control prior to SV definition as for OTU clustering, and the third was necessary because DADA2 separately filters forward and reverse reads. The α and β diversities based on SVs were obtained as described for OTUs. To identify SVs that were associated with bacterial OTU (bOTU)_01, we mapped SVs to the bOTU_01 centroid sequence using USEARCH v9 (usearch_global) and selected SVs with a minimum sequence identity of 97% to the bOTU_01 centroid sequence.

A classification key was developed to taxonomically assign the SVs mapped to bOTU_01 as well as all sequences that clustered within bOTU_01. This classification key was based on specific nucleotide polymorphisms, and allowed us to classify bacterial species or species groups based on these signatures. First, a list of genera potentially included in bOTU_01 was generated using a BLAST search (NCBI) which yielded all genera that had an identity of at least 96% to the bOTU_01 centroid sequence. Second, all species of these genera and all species within the family *Erwiniaceae* were retrieved from the List of Prokaryotic Names with Standing in Nomenclature (LPSN) (Parte 2018). This generated a list with 196 species of 23 genera (Supplementary Table S1). Sequences of their type strains were then compared with the sequences within bOTU_01 for exact matches. Only 14 species of five genera revealed a perfect match with sequences within bOTU_01. These were aligned (Supplementary Fig. S2) and DNA sequence signatures that differentiated species or species groups were identified. The classification algorithm was implemented in Python version 3.5. Unique sequences included in a bOTU_01 were determined using -fastx_uniques in USEARCH v9 and aligned using Mafft v7.310 (Katoh and Standley 2013) with default parameters. The aligned sequences were then classified according to the established DNA sequence signatures.

Cultivation of Boskoop blossom isolates. Four days after sampling blossoms for the metabarconding approach, an additional eight blossom samples (each containing 25 blossoms) were collected from Boskoop trees of the low-input orchard. Petals and stems were removed and blossoms were transferred into a 50-ml Falcon tube. To each sample, 25 ml of 1× buffer (as described above) was added. Samples were shaken for 1 h at 500 rpm and vortexed for an additional 30 s. From the supernatant of each sample, a 1-ml aliquot was transferred to a fresh 2-ml reaction tube, glycerol (40% stock solution) was added to a final concentration of 20%, and the mixture was stored at −80°C. DNA extraction, amplification, sequence processing, and taxonomic classification for these additional samples were performed as described above. For cultivation studies, 100 μl of a 10^{-3} dilution from each sample was plated on King's B (KB) (King et al. 1954), lysogeny broth (LB) (Roth), and tryptic soy broth (TSB) (Oxoid) agar and incubated for 4 days at 26°C.

Identification of Boskoop blossom isolates. The cultivated colonies of a KB plate were assigned to groups according to their morphology. Groups represented by only one colony were not further analyzed. For matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) identification, all colonies were tested when a group consisted of two to three colonies. If groups consisted of 4 to 10 members, at least three colonies were analyzed; if there were 10 or >10 members, five colonies were analyzed. If MALDI-TOF did not yield an identification (cut-off score < 2) of the selected members of a morphology group, the 16S rRNA gene or ITS2 was sequenced from randomly selected individual colonies of this subgroup. MALDI-TOF was performed according to

Gekenidis et al. (2014) using a MicroFlex biotyper, and the MALDI Biotyper software (database version 4.0.0.1; Bruker Daltonics). Briefly, cell material from a colony was smeared onto a MALDI target and covered with 1 µl of matrix solution (α-cyano-4-hydroxycinnamic acid at 10 mg ml^{-1}) dissolved in acetonitrile-water-trifluoroacetic acid (50:47.5:2.5 [vol/vol/vol]). The MALDI target was processed using the instrument's standard settings for bacteriological classification.

For 16S rRNA or ITS2 amplification, a crude DNA extract was prepared by suspending colonies in 100 µl of KAWA buffer (Kawasaki 1990), followed by vortexing, incubation at 85°C for 20 min, and centrifugation at 12,000 × g for 1 min. The supernatant was used as PCR template. The ITS2 PCR was performed as described above. For 16S rRNA gene amplification, the universal primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rP2 (5′-ACGGCTACCTTGTTACGACTT-3′) (Weisburg et al. 1991) and the Hotstar Taq DNA polymerase (Qiagen) were used. Amplification conditions were 15 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 56°C, and 1.5 min at 72°C; followed by a final extension of 10 min at 72°C. DNA sequencing was performed by Microsynth AG (Balgach, Switzerland) and sequences were identified by aligning type strain sequences retrieved from LPSN and close matches of BLAST searches (NCBI).

RESULTS

Apple varieties and their blossom associated microbiota. Amplicon sequencing of the V5 and V6 regions of the bacterial 16S rRNA and the fungal ITS2 resulted in a total of 1.5 million reads for bacteria and 1.2 million reads for fungi. The reads were assigned to 588 bacterial and 746 fungal OTUs using a 97% sequence identity cut-off. A Good's coverage of 100% revealed extensive sequencing depths (Table 1).

Microbial communities in the apple blossoms of the varieties Ariwa, Boskoop, Idared, Milwa/Diwa, and Golden Delicious (see Material and Methods, field site) were determined using α- and β-diversity metrics. The α diversity was assessed as the number of OTUs in a sample (i.e., the OTU richness), the distribution of OTU abundances (i.e., Simpson evenness), and the combination thereof (i.e., the inverse Simpson index). Among varieties, significant differences for bacterial communities were detected for the indices Simpson evenness (ANOVA, $F = 3.8$, $P = 0.017$) (Supplementary Table S2) and inverse Simpson ($F = 6.4$, $P = 0.002$) (Supplementary Table S2). Pairwise comparisons revealed that these differences of bacterial α diversities were caused by the variety Boskoop, which had an increased inverse Simpson index as compared with the other varieties (Fig. 1A). The β diversity was determined using pairwise community comparisons based on Bray-Curtis dissimilarity. Similarly to α diversity, the bacterial β diversity (Supplementary Fig. S3) was significantly different among varieties (PERM-ANOVA, Pseudo-$F = 7.7$, $P = 0.0002$) (Supplementary Table S3). Again, this difference was driven by the bacterial communities of Boskoop blossoms (PERMANOVA, $P < 0.016$) (Supplementary Table S3).

Fungal communities did not significantly differ among varieties in α diversities (ANOVA, $P = 0.2$) (Supplementary Table S2; Fig. 1B) but did in terms of β diversity (PERMANOVA, Pseudo-$F = 2.2$, $P = 0.0001$) (Supplementary Table S3). Pairwise comparisons revealed significant differences of fungal communities between Golden Delicious and the varieties Ariwa, Idared, and Milwa/Diwa (Supplementary Table S3). Furthermore, fungal communities of Milwa/Diwa and Ariwa blossoms significantly differed in their β diversity.

Dominant bacterial and fungal taxa. The 10 most abundant bOTUs and fungal OTUs (fOTUs) accounted, on average, for 97.6 and 71.6%, respectively, of the relative abundance per sample (Supplementary Fig. S4). Thus, the remaining 578 bOTUs and 736 fOTUs represented only 2.4 and 28.4%, respectively, of the mean relative abundance per sample (Fig. 2A and B). Bacterial communities of all varieties were dominated by bOTU_01 that was assigned to *Erwinia* sp. (bootstrap value of 96%) (Table 2). On average, bOTU_01 had a mean relative abundance of 81.6%, with the variety Boskoop showing a significantly lower abundance (67.3%; ANOVA, $F = 8.5$, $P < 0.0001$) (Supplementary Fig. S5).

TABLE 1
List of samples, operational taxonomic unit (OTU) numbers, number of sequences per sample, sequencing depth, and the ubiquitous bacterial and fungal OTUs (bOTU and fOTU, respectively)

Parameters[a]	Bacteria	Fungi
Number of samples	40	40
Number of OTUs	588	746
Number of sequences	1,555,631	1,188,229
Mean number of sequences per sample ± STD	38,891 ± 6,397.2	29,706 ± 11,653.9
Mean number of OTUs per sample ± STD	86 ± 37.1	100 ± 33.8
Mean Good's coverage per sample ± STD	1.0 ± 0.00	1.0 ± 0.00
Mean sequence lengths (nucleotides) ± STD	300 ± 3.0	318 ± 34.2
Number of OTUs that occur in all samples (ubiquitous OTUs)	4	6
Ubiquitous OTUs and their taxonomic assignment	bOTU_01 *Erwinia* sp.	fOTU_01 *Metschnikowia pulcherrima*
	bOTU_02 *Rosenbergiella* sp.	fOTU_02 *Podosphaera leucotricha*
	bOTU_03 *Pseudomonas* sp.	fOTU_03 *Aureobasidium pullulans*
	bOTU_16 Oxalobacteraceae	fOTU_04 Pleosporales
		fOTU_09 Capnodiales
		fOTU_13 Tremellales

[a] STD = standard deviation.

The bOTU_01 was followed by bOTU_02 (*Rosenbergiella* sp., bootstrap value of 83%) (Table 2) that covered, on average, 12.7% of the mean relative abundance. With 28.4%, the blossoms of Boskoop showed a significantly (ANOVA, $F = 13.4$, $P < 0.0001$) (Supplementary Fig. S5) increased abundance of bOTU_02 while this bOTU did not exceed a mean relative abundance of 8.8% in the other varieties. Similarly, bOTU_03 (*Pseudomonas* sp., 100%) covered, on average, 1.8% of bacterial sequences. All other bOTUs covered, on average, <0.5% of bacterial sequences (Fig. 2A).

The most dominant component of fungal communities was fOTU_01, which was assigned to the species *Metschnikowia pulcherrima* (bootstrap value of 99%) (Table 3). Its mean relative abundance per variety ranged from 19.9 to 37.6%, with an average of 28.2% (Fig. 2B). In Golden Delicious blossoms, fOTU_01 had a mean relative abundance of 19.9%, which was similar to that of fOTU_04 (Pleosporales, bootstrap value of 97%) with 20.0% (Supplementary Fig. S6). The fOTU_02 had an identical sequence with *Podosphaera leucotricha* (bootstrap value of 100%), which is the causal agent of apple powdery mildew, and a mean relative abundance of 9.3 and 11.1 in Boskoop and Idared, respectively. In Milwa/Diwa blossoms, fOTU_02 had a mean relative abundance of 21.8% and, therefore, was the second most dominant fungal taxon in the blossoms of this apple variety. The third most abundant fOTU was fOTU_03, which was assigned to *Aureobasidium pullulans* (bootstrap value of 100%), with an average relative abundance of 8.9%.

In summary, the community compositions in the analyzed apple blossoms of all five varieties were dominated by only a few bacterial and fungal OTUs (Supplementary Fig. S4). This was particularly true for the bacterial community and bOTU_01 that, on average, had an abundance of 81.6%.

Species classifications of bOTU_01 sequences. Sequence clustering into OTUs was based on a sequence identity threshold of 97%. The bOTU_01 consisted of 189,871 different sequences and was classified as *Erwinia* sp., with a bootstrap value of 96%. This OTU was of particular interest in this study because it most likely contained the fire-blight-causing agent *E. amylovora* and, therefore, more in-depth analyses were applied for this OTU by assessing unique sequences and SVs (using DADA2) within bOTU_01. Before this analysis, the congruence of SVs and OTUs was assessed at the entire community level, which yielded highly correlated α ($\rho_{Bacteria} \geq 0.87$, $\rho_{Fungi} \geq 0.60$) and β ($\rho_{Bacteria} = 0.90$, $\rho_{Fungi} = 0.89$) diversities, with the same patterns of similarities among varieties (Supplementary Tables S2 and S3; Supplementary Fig. S3). Then, SVs falling within bOTU_01 were determined, which yielded six SVs. To classify these SVs, as well as all sequences clustered within the OTU, we developed, a classification key based on DNA sequence signatures (i.e., specific nucleotide polymorphisms that are indicative for different species or groups of species) (see Material and Methods). The assignment of bOTU_01 sequences according to this classification key resulted in 14 species of five genera (i.e., *E. amylovora*, *E. tasmaniensis*, *E. endophytica*, *E. billingiae*, *E. rhapontici*, *E. persicina*, *E. aphidicola*, *E. iniecta*, *Siccibacter colletis*, *Phytobacter ursingii*, *Pantoea rwandensis*, *P. agglomerans*, *Buttiauxella warmboldiae*, and *B. izardii*). The marker sequence did not allow discriminating *P. agglomerans* from *B. izardii*, as well as *P. rwandensis* from *B. warmboldiae* (Supplementary Table S1). Furthermore, *E. billingiae*, *E. aphidicola*, *E. rhapontici*, *E. persicina*, and *E. iniecta* could not be distinguished. Therefore, these were grouped and are referred to as *Erwinia* group BARPI (Fig. 3). Based on the developed classification key (Fig. 3), two SVs were classified to *E. tasmaniensis*
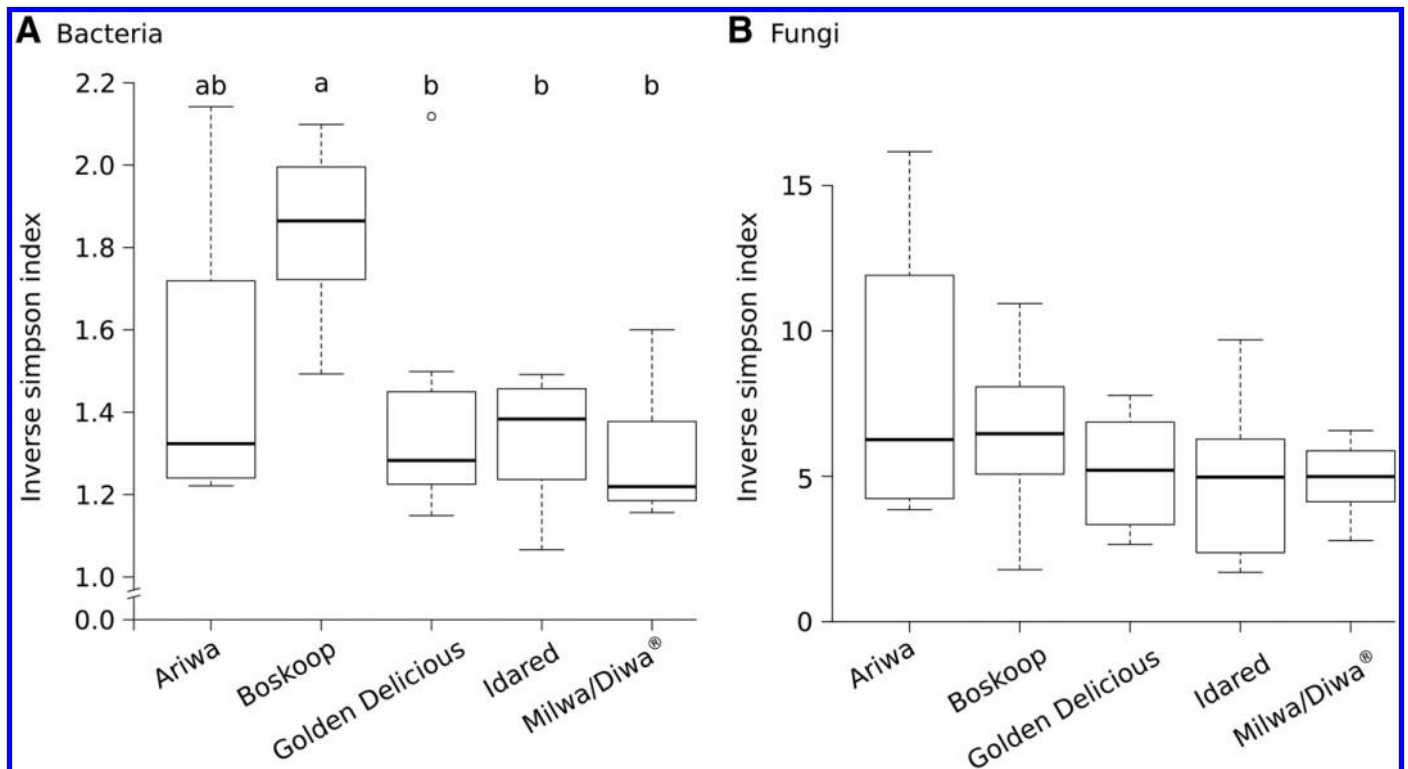

Fig. 1. A, Bacterial and **B,** fungal α diversity on apple blossoms sampled from varieties Ariwa, Boskoop, Golden Delicious, Idared, and Milwa/Diwa. Different letters indicate significant differences according to pairwise group comparisons. Analysis of variance: $F = 6.4$, df $= 4$, $P = 0.002$ (A) and $F = 1.9$, df $= 4$, $P = 0.236$ (B).

(bSV_01 and bSV_04); one each to *Erwinia* group BARPI (bSV_5), *E. amylovora* (bSV_127), and *P. agglomerans* (bSV_22); and one remained unclassified (bSV_15). The *E. tasmaniensis* SVs 1 and 4 were most abundant and covered 96.0 and 2.5%, respectively, of the abundance of bOTU_01, while the SV classified as *E. amylovora* covered 0.004%. Highly similar results were obtained when classifying all 1,263,244 sequences within bOTU_01 according to the DNA signatures. The majority of 94.5% sequences (*n* = 1,194,207) were classified to *E. tasmaniensis* based on the DNA signatures and the most abundant sequence was identical to the one of type strain *E. tasmaniensis* Et1/99 isolated from apple blossoms in Tasmania. The *Erwinia* group BARPI included 15,767 sequences, representing 1.2% of bOTU_01. Only 75 sequences, representing 0.006%, of bOTU_01 were classified as *E. amylovora*

and 559 sequences (0.044%) were assigned to *P. agglomerans*. In total, 52,420 sequences of bOTU_01 (4.1%) could not be attributed to a species or species group based on the developed classification key. In addition, few sequences (0.017%) were classified to *E. endophytica, S. colletis, Phytobacter ursingii,* or *Pantoea rwandensis,* which were not detected using the SV approach.

Cultivation based verification of the Boskoop blossom microbiota. To verify the data obtained with metabarcoding, isolates of eight Boskoop samples were cultivated on KB agar (Supplementary Fig. S7), yielding 239 colonies of five distinct morphology types (Table 4). The dominant morphology type A comprised 169 colonies. Using MALDI-TOF, all 39 tested colonies of type A were identified as *E. tasmaniensis* (scores 2.1 to 2.4). In addition, the 16S rRNA gene sequence from a subset of MALDI-
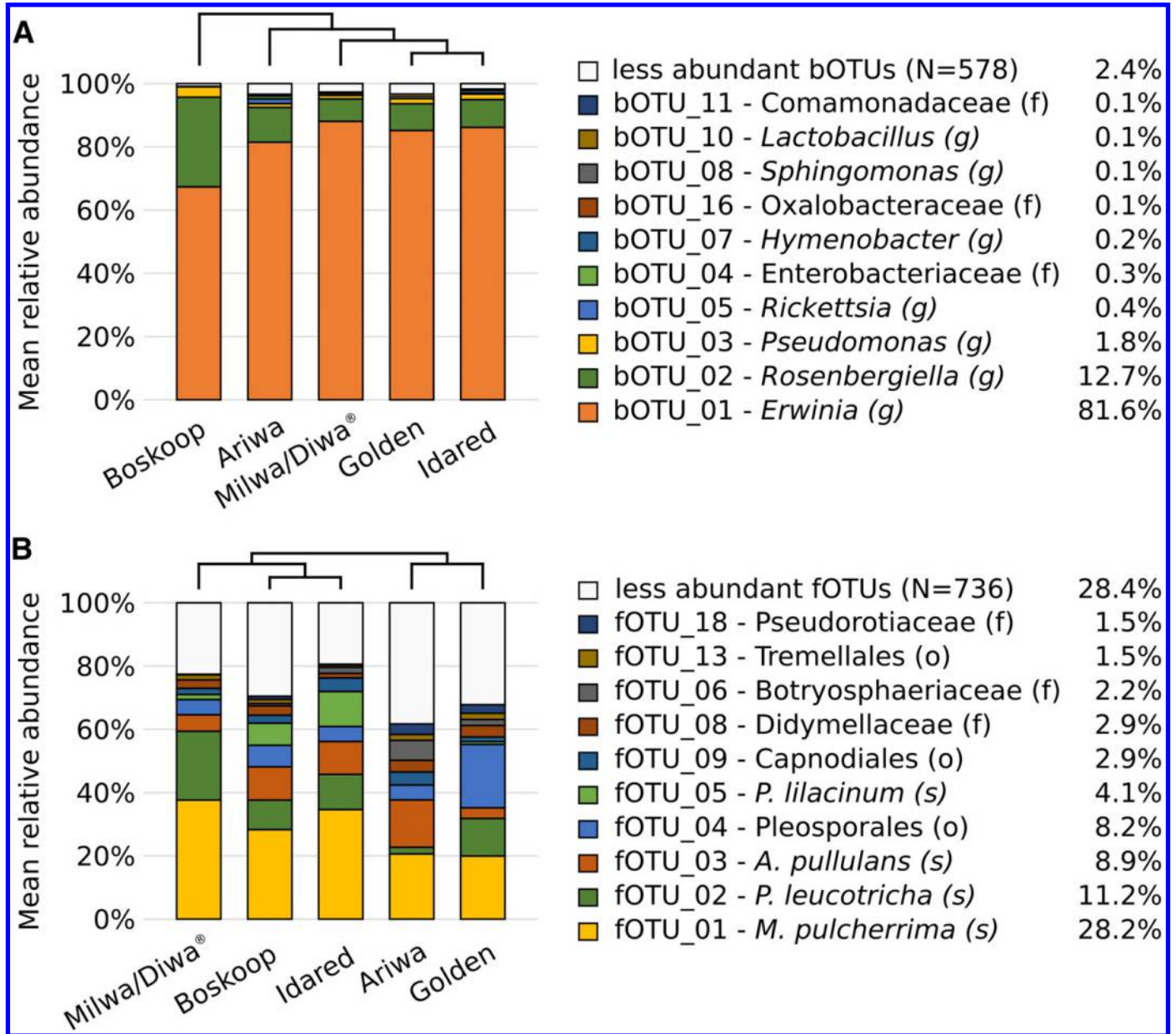
Fig. 2. Compositions of **A,** bacterial, and **B,** fungal communities on blossoms of five apple varieties, each represented by eight samples. The 10 operational taxonomic units (OTUs) with highest mean relative abundances are colored and assigned to the species (s) or the lowest possible level (s = species, g = genus, f = family, and o = order); bOTU and fOTU = bacterial and fungal OTUs, respectively. Dendrograms are based on cluster analyses of mean relative abundances using Ward's clustering criterion. Fungi include *Purpureocillium lilacinum, Aureobasidium pullulans, Podosphaera leucotricha,* and *Metschnikowia pulcherrima.*

TOF-identified *E. tasmaniensis* isolates had the highest identity (>99%) to *E. tasmaniensis* ET1/99 (NR_074869.1) and perfectly matched to the signatures in the developed classification key (Fig. 3). The second most abundant colony morphology (B, 39 colonies) was identified as *Rosenbergiella* sp. based on the 16S rRNA gene sequence. Colony morphology C (18 colonies) (Table 4) was detected in four samples and identified as *M. pulcherrima* (C) based on the ITS2 sequence (CP034461.1, MN913467.1).

DISCUSSION

Fire blight was first reported in 1780 in North America, from where it spread to other parts of the world (e.g., Europe and the Mediterranean region and, recently, East Asia) (Myung et al. 2016; Park et al. 2016; van der Zwet and Bonn 1999). In addition to the cultivation of less susceptible apple varieties, the treatment of blossoms with microbial antagonists that suppress establishment and growth of *E. amylovora* on floral tissue is an appealing alternative to the application of chemical pesticides such as antibiotics. However, the efficacy of a biocontrol agent varies greatly depending on location and year (Johnson and Stockwell 1998). With the availability of new DNA technologies, it is possible to analyze microbial communities in a cultivation-independent manner aimed at understanding their ecological functions and providing opportunities in disease control (Berg et al. 2017; Poudel et al. 2016).

The study presented here revealed that the microbiota of apple blossoms is dominated by only a few bacterial and fungal species and that the apple variety, with the exception of Boskoop, had little effect on the community structure (Figs. 1 and 2; Supplementary Fig. S3). In large agreement with our results, Steven et al. (2018) have reported no effect of the apple varieties Braeburn, McIntosh, and Sunrise on the bacterial community structures.

The dominating taxon in this study, bOTU_01, was assigned to the genus *Erwinia* (Fig. 2). Nevertheless, the studied orchard had no fire blight record and showed no symptoms. Therefore, we analyzed the 1,263,244 sequences of bOTU_01 in greater detail. The majority ($n = 1,194,207$) matched the partial 16S rRNA gene sequence

TABLE 2
The 10 bacterial operational taxonomic units (bOTUs) with highest mean of relative abundance (MRA)[a]

bOTU	MRA (%)	Marker length (bp)	Taxonomic assignment, bootstrap value	Defined *Erwinia amylovora* antagonists (reference)
bOTU_01	81.6	300	*Erwinia* (g), 96	*E. tasmaniensis* (Jakovljevic et al. 2006)
bOTU_02	12.7	300	*Rosenbergiella* (g), 83	
bOTU_03	1.8	300	*Pseudomonas* (g), 100	*Pseudomonas fluorescens* A506* (Wilson and Lindow 1993)
	…	…	…	*P. fluorescens* EPS62e (Pujol et al. 2005)
	…	…	…	*P. graminis* 49M (Mikiciński et al. 2016)
	…	…	…	*P. rhizosphaerae* JAN (Paternoster et al. 2010)
bOTU_05	0.4	302	*Rickettsia* (g), 100	…
bOTU_04	0.3	300	Enterobacteriaceae (f), 100	…
bOTU_07	0.2	295	*Hymenobacter* (g), 100	…
bOTU_16	0.1	301	Oxalobacteraceae (f), 100	…
bOTU_08	0.1	302	*Sphingomonas* (g), 100	…
bOTU_10	0.1	301	*Lactobacillus* (g), 100	*Lactobacillus plantarum*, PM411, TC54, TC92 (Roselló et al. 2013)
bOTU_11	0.1	301	Comamonadaceae (f), 100	…

[a] Abbreviations: g = genus and f = family; * indicates commercialized.

TABLE 3
The 10 fungal OTUs (fOTUs) with highest mean of relative abundance (MRA)[a]

fOTU	MRA (%)	Marker length (bp)	Taxonomic assignment, bootstrap value	Defined *E. amylovora* antagonists (reference)
fOTU_01	28.2	215	*Metschnikowia pulcherrima* (s), 99	*M. pulcherrima* (Seibold et al. 2004)
fOTU_02	11.2	289	*Podosphaera leucotricha* (s), 100	…
fOTU_03	8.9	303	*Aureobasidium pullulans* (s), 100	*A. pullulans** (Kunz 2004)
fOTU_04	8.2	299	Pleosporales (o), 97	…
fOTU_05	4.1	309	*Purpureocillium lilacinum* (s), 100	…
fOTU_09	2.9	297	Capnodiales (o), 100	…
fOTU_08	2.9	303	Didymellaceae (f), 100	…
fOTU_06	2.2	307	Botryosphaeriaceae (f), 100	…
fOTU_13	1.5	274	Tremellales (o), 100	…
fOTU_18	1.5	294	Pseudorotiaceae (f), 100	…

[a] Abbreviations: s = species, g = genus, f = family, and o = order; * indicates commercialized.

Fig. 3 classification key

188,189,190	35,39,49	210,221,222,223	209	12	Taxon	OTU Sequences	OTU in %	SVs Sequences	SVs in %
GGC	CTA	GCTT	C	C	*Erwinia tasmaniensis*	1,194,207	94.535%	947,572	98.545%
		TGGA	C	C	*Erwinia amylovora*	75	0.006%	38	0.004%
	TGC			C	*Erwinia* group BARPI*	15,767	1.248%	12,276	1.277%
		GCTT	Y	T	*Erwinia endophytica*	49	0.004%	0	0.000%
	TTA	RYTT	T	C	*Siccibacter colletis*	93	0.007%	0	0.000%
GGT	TGC	GCTT	C	C	*Phytobacter ursingii*	28	0.002%	0	0.000%
ACT			C	C	*Pantoea rwandensis***	46	0.004%	0	0.000%
	TTA	GCTT	T	C	*Pantoea agglomerans****	559	0.044%	581	0.060%
					Unclassified	52,420	4.150%	1,096	0.114%
					Sequences in bOTU_01	1,263,244	100.000%	961,563	100.000%

Fig. 3. Classification key used to assign sequences of bacterial operational taxonomic unit (bOTU)_01 as well as sequence variants (SVs) within bOTU_01 to different bacterial species. The bOTU_01 contains 1,263,244 sequences that were classified to seven species and one species group (*Erwinia* BARPI) based on DNA sequence signatures as defined in the methods. The *Erwinia* group BARPI (*) includes five species (i.e., *Erwinia billingiae*, *E. aphidicola*, *E. rhapontici*, *E. persicina*, and *E. iniecta*). *Pantoea rwandensis* (**) and *Buttiauxella warmboldiae*, as well as *P. agglomerans* (***) and *B. izardii*, cannot be discriminated based on the marker sequences (Supplementary Table S3). Ambiguous nucleotides are indicated following the code of the International Union of Pure and Applied Chemistry (IUPAC) (i.e., R corresponding to A or G and Y to C or T). Positions of DNA sequence signatures correspond to the positions within the alignment provided in the supplements (Supplementary Fig. S2).

TABLE 4
All colony morphologies of isolates obtained from Boskoop blossoms with their identification and abundance[a]

Type	Photo[b]	Morphology[c]	Taxon[d]	N[e]	CFU (n) Mean	Min	Max	Total	SVs (%) Mean	Min	Max
A		White-beige, circular, smooth, ca. 0.4 cm	*Erwinia tasmaniensis*[f,g]	39[f], 5[g]	21	11	33	169	55.0	47.5	60.6
B		Orange brown, circular, smooth, ca. 0.3 cm	*Rosenbergiella* sp.[g]	6[g]	5	0	21	39	33.0	29.2	39.2
C		White, circular, smooth, ca. 0.1–0.2 cm	*Metschnikowia pulcherrima*[g]	6[g]	2	0	7	18	18.8[h]	12.7[h]	24.3[h]
D		Fluorescent, circular, smooth, ca. 0.5–0.7 cm	*Pseudomonas* sp.[f]	5[f]	1	0	6	8	3.5[i]	2.0[i]	7.6[i]
E		Yellow, circular, smooth, ca. 0.5 cm	*Pantoea agglomerans*[f]	4[f]	1	0	5	5	0.1	0.0	0.5

[a] Isolates were grown on King's B plates (n = 8). Identity was determined with either matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) or DNA-marker sequencing. Sequence variants (SVs) were obtained using DADA2. Min = minimum and Max = maximum.
[b] Black bar equals 0.5 cm.
[c] Colonies of each morphology type could be assigned to a single taxon.
[d] Taxonomic assignment.
[e] Number of colonies identified.
[f] Species assignment by MALDI-TOF (scores: 2.0 to 2.4).
[g] Species assignment based on 16S rRNA gene or ITS2 sequence.
[h] Five SVs perfectly matching to isolate sequences.
[i] Eight SVs assigned to the genus *Pseudomonas* without a species assignment.

of type strain of *E. tasmaniensis* Et1/99 (Fig. 3), which we also obtained when using an algorithm to build SVs. Interestingly, two SVs were classified as *E. tasmaniensis*. Although the most abundant SV was identical to the *E. tasmaniensis* type strain, the other one had a single substitution of the first base pair. This substitution was observed in all samples and was present in neither the reference sequences nor the 100 best BLAST results, indicating a systematic but nonbiological origin of this SV. More species within bOTU_01 were identified based on the DNA signatures when using all sequences within bOTU_01 as compared with the SV approach. All of these species (i.e., *E. endophytica, S. colletis, P. ursingii,* and *P. rwandensis*) had low abundances (≤0.007% of bOTU_01 sequences), which may explain that these were either erroneous sequences introduced during metabarcoding or extremely rare species. A rarely detected species in both approaches was *E. amylovora*, which indicated its presence in the studied orchard despite the absence of fire blight symptoms.

The dominant presence of *E. tasmaniensis* and presence of *Rosenbergiella* sp. as well as *M. pulcherrima* in the low-input orchard was confirmed in a cultivation-based approach. Sequencing of the 16S rRNA gene as well as the protein-based analysis using MALDI-TOF identified the dominant colony morphology on KB plates as *E. tasmaniensis*. Neither the cultivation on TSB and LB plates (Supplementary Fig. S8) nor an extended incubation for 10 days at room temperature displaced *E. tasmaniensis* as the dominant CFU on the plates. *E. tasmaniensis* has been first described in 2006. The type strain Et1/99 but also strain Et2/99 have been isolated as the dominant bacteria present in apple and pear flowers in Australia (Tasmania and Knoxfield, Victoria). The inoculation of immature pear or apple seedlings with these isolates has not resulted in necrotic symptoms. Thus, this species has been considered not pathogenic for the common fire blight hosts apple and pear (Geider et al. 2006). Indeed, despite the high accumulation of *E. tasmaniensis* in the investigated Swiss orchard, no visible disease symptoms on blossoms or tree shoots were observed. An *E. tasmaniensis* isolate from Germany has been included in the study of Jakovljevic et al. (2008) and two more German isolates and two from South Africa in the report of Wensing et al. (2011) but the occurrence as dominant species in apple blossoms has only been reported from Australia. Further studies will have to show how widespread this *Erwinia* sp. is in the apple-growing regions of Switzerland and Europe. The same applies to the largely unknown genus *Rosenbergiella* (family *Enterobacteriaceae*), to which the second most dominant bacterial bOTU_02 of the apple blossoms was assigned. *Rosenbergiella nectarea* has been isolated from blossom nectar of *Amygdalus communis* (almond) and *Citrus paradisi* (grapefruit) in northern Israel and was first described in 2013 (Halpern et al. 2013). Only 559 sequences could be assigned to *P. agglomerans*, which is known to be an effective colonizer of apple blossoms (Pusey and Curry 2004). Sequences of bOTU_01 and bOTU_02 were assigned to the family *Enterobacteriaceae* (class: *Gammaproteobacteria*, phylum: *Proteobacteria*). The third most dominant bOTU, bOTU_03, was assigned to *Pseudomonas* (class: *Gammaproteobacteria*, phylum: *Proteobacteria*) with a relative abundance of 1.8% only. Steven et al. (2018) have detected a bOTU assigned to *Pseudomonas* as the dominant taxon on stigmata and a negative correlation between *Pseudomonas* and *Enterobacteriaceae*, implying a niche exclusionary relationship between these taxa. Very different dominating bacterial taxa (i.e., members of *Deinococcus–Thermus* (Class: *Deinococci*, Phylum: *Deinococcus–Thermus*) and TM7 have been detected in Gala apple blossoms of an orchard in Wisconsin (Shade et al. 2013). Nonetheless, the PCR applied by Shade et al. (2013) were also used in our survey, excluding a primer bias as cause for the differences,

although this cannot exclude possible bias due to DNA preparation (e.g., partially lysed spore-forming bacteria or loss of DNA during preparation). However, the consistency of metagenome- and cultivation-based results corroborated the dominance of *E. tasmaniensis* in the apple blossoms. Further surveys covering larger geographic areas and more apple varieties are necessary to explore the differences in the dominant members of the apple blossom microbiomes and to identify contributing factors.

bOTU_05, with a mean relative abundance of only 0.4% (Fig. 2A), was assigned to the genus *Rickettsia* (Table 2), which includes obligate intracellular parasites or symbionts of many arthropods (Perlman et al. 2006). Most likely, *Rickettsia* sequences are detected because small arthropods or their traces were sampled along with the apple blossoms. In addition, bOTU17, with a relative mean abundance of 0.03% (and thus not listed in the results), is assigned to *Buchnera aphidicola*, the primary endosymbiont of aphids (Baumann et al. 1995).

The analysis of the fungal community in the same apple blossoms showed a higher diversity compared with the bacterial microbiota. The 736 less-abundant fOTUs had, on average, a relative abundance of 28.4% (Fig. 2B). Within the 10 fOTUs with highest mean relative abundance, fOTU_02 (*P. leucotricha*, the causative agent of apple powdery mildew) was the second most abundant fOTU, most probably because no plant protection products had been applied in the orchard before sampling. fOTU_01 and fOTU_03, assigned to *M. pulcherrima* and *A. pullulans*, respectively, covered 30% of all fungal sequences. Strains of *M. pulcherrima* have mainly been known for their potential against postharvest pathogens (Piano et al. 1997) but are also capable of inhibiting the growth of *E. amylovora* (Duffy et al. 2006; Seibold et al. 2004, 2006).Two strains of *A. pullulans* have been reported as active ingredients of a biocontrol product against fire blight (Kunz 2004). Thus, in the investigated low-input apple orchard, potential *E. amylovora* fungal antagonists dominate the communities in apple blossoms of all five varieties. This may explain why *E. amylovora* was never able to establish a disease-causing population in the studied orchard, though phenotypically present in the immediate vicinity (Supplementary Fig. S1).

In the studied orchard, an *A. pullulans*-based product could be favorable for fire blight control, because this would mimic an already present native fire blight antagonist that successfully established in the blossom under the given environmental conditions. Previous studies on bacterial fire blight antagonist have focused on genus *Pseudomonas* and members of the genus *Pantoea* (family *Enterobacteriaceae*), with commercially available strains *Pseudomonas fluorescens* A506 (Wilson and Lindow 1993), *Pantoea agglomerans* C9-1 (Ishimaru 1988), *P. agglomerans* P10c (Vanneste et al. 2006; Smits et al. 2015), and *P. agglomerans* E325 (Braun-Kiewnick et al. 2011; Pusey et al. 2011). In addition, *Bacillus subtilis* QST 713 (Aldwinckle et al. 2002) is also available as a commercial product. However, none of these bacterial biocontrol strains corresponded to a genus that dominated the native bacterial community in the blossoms analyzed in the present study. In view of the niche exclusion relationship between members of *Enterobacteriaceae* and various taxa, the use of *Pseudomonas-* or *Bacillus*-based products might even have an adverse impact on fire blight control.

High-throughput sequencing technologies allow for cultivation-independent investigations at a high resolution and sensitivity. The detection of *E. amylovora* sequences in the studied orchard was only possible with a sequencing depth achieved by high-throughput sequencing. Furthermore, analyses of SVs were necessary to differentiate sequences of phytopathogenic and nonphytopathogenic *Erwinia* spp., because they were clustered within the same OTU at 97% sequence identity. Specific DNA sequence signatures can be

used to differentiate and accurately assign metabarcodes at a low taxonomic level. Metabarcoding enables the assessment of microbial communities, including bacteria and fungi, on sample sites with a unique character. This yields information needed for the assembly of synthetic communities that best match or complement naturally occurring antagonists in apple blossoms of a defined area and its given environmental conditions.

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