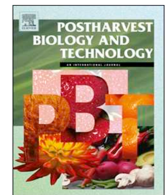
Postharvest Biology and Technology 153 (2019) 96–106

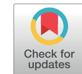
Contents lists available at ScienceDirect

Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio



Insights gained from metagenomic shotgun sequencing of apple fruit epiphytic microbiota



Dario Angeli[a,1], Abdoul Razack Sare[b,1], M. Haissam Jijakli[b], Ilaria Pertot[a,c], Sebastien Massart[b,*]

[a] Department of Sustainable Agro-Ecosystems and Bioresources, Research and Innovation Centre, Fondazione Edmund Mach, via Mach 1, 38010 San Michele all'Adige, Italy

[b] Laboratory of Integrated and Urban Phytopathology, Gembloux Agro-Bio Tech, University of Liège, Passage des déportés 2, 5030 Gembloux, Belgium

[c] Center Agriculture Food Environment (C3A), University of Trento, via Mach 1, 38010 San Michele all'Adige, Italy

ARTICLE INFO

Keywords:
Microbiota
High-throughput sequencing
Postharvest pathogens
Biocontrol
Apple
Phytobiome

ABSTRACT

The epiphytic plant microbial communities living at the surface of fruit have been the source of most current biocontrol agents (BCAs) and can influence fruit quality during storage. Despite this interest, their taxonomical and functional composition has been poorly studied so far. This paper describes the use of high-throughput sequencing (HTS) technologies to characterise the microbial phytobiome residing on apple surface at the taxonomic and functional levels through shotgun metagenome sequencing. Apples from the Pinova cultivar bearing no symptom of disease development were sampled in an orchard at harvest, and their epiphytic microbiota was isolated. After DNA extraction, 14.1 Gbases of raw sequences were generated by HTS. These sequences were annotated following two pipelines in parallel: (i) they were individually analysed by the MG-RAST server, and (ii) they were *de novo* assembled into contigs and the contigs were annotated by the IMG server. Our results showed a very high fungal and bacterial diversity, with a higher proportion of fungal sequences (79.0%) than bacterial sequences (13.8%). Among fungi, the phylum *Ascomycota* prevailed, while *Bacteroides* were dominant in the bacterial population. Among them, 24 species corresponded to known apple pathogens like *Aspergillus* spp., *Botrytis* spp., *Sclerotinia* spp., and *Penicillium* spp. for fungi, and *Erwinia* spp. and *Agrobacterium* spp. for bacteria. Moreover, several contigs were assigned to species of known BCA strains belonging to the following genera: *Filobasidiella* spp., *Talaromyces* spp, *Candida* spp., *Saccharomyces* spp., *Bacillus* spp., and *Enterobacter* spp. The functional analysis showed similar patterns of abundance and function in all samples, identified genes potentially involved in biocontrol properties, but also underlined the complexity of datum interpretation and the incompleteness of current databases.

1. Introduction

Plants host abundant and diverse microbial communities associated with specific functions that may influence plant health and productivity. These functions can be classified into five categories: i) improvement of nutrient acquisition and plant growth, ii) protection against abiotic stress, iii) induction of resistance against pathogens, iv) direct antagonism against pathogens, and v) interaction with other trophic levels, such as phytophagous arthropods (Massart et al., 2015). According to their ecological niche, microorganisms can reside within the tissues (endophytes) and/or on the plant surface (epiphytes). Conditions in the phyllosphere are challenging for most microorganisms because of solar radiation, wide fluctuations in temperature and humidity, and variable nutrient availability (Lindow and Brandl, 2003; Vorholt, 2012). Most of the microorganisms residing in the phyllosphere, including the carposphere, are deposited on plant surfaces by air movement or rain. Most of them cannot multiply, while a few can become epiphytic residents thanks to their ability to grow and survive on fruit skin (Hirano and Upper, 1983). The carposphere microbiota plays an important role in the development of plant diseases, including postharvest fruit diseases, and might also impact fruit maturity and ripening (Saminathan et al., 2018; Buchholz et al., 2018). Examples of diseases are blue mould caused by *Penicillium expansum,* grey mould caused by *Botrytis cinerea,* and bull's eye rot caused by *Neofabrea* spp. Many other genera, like *Fusarium* sp., *Alternaria* sp., *Nectria* sp., *Mucor* sp., *Sphaeropsis* sp., *Phytophthora* sp., *Cladosporium* sp., and *Phomopsis*

Abbreviations: HTS, high-throughput sequencing

* Corresponding author.
E-mail address: sebastien.massart@uliege.be (S. Massart).

[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.postharvbio.2019.03.020
Received 30 November 2018; Received in revised form 5 March 2019; Accepted 26 March 2019
0925-5214/ © 2019 Elsevier B.V. All rights reserved.

sp., include species that can cause diseases on apple fruit (Pelliccia et al., 2011). A large part of the biocontrol agents (BCAs) of postharvest pathogens were isolated from carposphere microbial communities. Some studies demonstrated a relationship between the microbial populations of plant surfaces and biological disease control (Massart et al., 2015; Abdelfattah et al., 2016; Sylla et al., 2013). In fact, microbial communities may contribute to disease control by interacting with host plants, pathogens, and BCAs. A better understanding of these interactions may provide novel opportunities to develop innovative biocontrol methods against plant pathogens (Massart et al., 2015).

Although considerable research has been led on plant phyllosphere populations for several decades (Andrews and Kenerley, 1980, 1981; Blakeman, 1981; Pennycook and Newhook, 1981), little information is available on the microbiota of apple fruit surfaces. Some studies investigated the diversity of *in vitro* culturable microorganisms and estimated the population size of culturable fungi and bacteria to be approximately 8×10^3 and 9.5×10^4 colony forming units (cfu) *per* cm^2, respectively (Teixidó et al., 1998, 1999; Chand-Goyal and Spotts, 1996).

The composition and impact of microbial communities have been poorly studied to date (Massart et al., 2015), mainly because of the lack of techniques to analyse the non-culturable part of the populations. Recent developments in High-Throughput Sequencing (HTS, also called Next Generation Sequencing – NGS) technologies and bioinformatic analyses have eased this bottleneck. Two approaches are available to study the microbiota composition through DNA analysis by HTS technologies. Amplicon sequencing-based protocols, also called metabarcoding, are by far the most popular ones for plant microbiome studies. A specific genomic region, for example a fragment of the 16S rRNA gene for bacteria and of the 18S rRNA gene or the Internal Transcribed Spacer (ITS) region for fungi, is amplified by PCR and sequenced at high throughput (thousands of sequences) to describe the microbial community at the taxonomic level in a sample. Metagenome sequencing is a second approach. The extracted genomic DNA is sheared randomly into small fragments and directly sequenced at very high throughput (typically millions of sequences). The generated sequences randomly cover the genomes of the microorganisms; they are further assembled together into contigs, and the contigs are annotated at the taxonomical and functional levels (Massart et al., 2015).

More recently, pioneering studies using metabarcoding have been carried out to characterise the apple epiphytic microbiota (leaf, flower, or fruit) taxonomically, revealing diverse and dynamic microbial communities. The epiphytic microbiota is influenced by the environment and by disease management practices, and can include more than 600 bacterial and fungal genera (Abdelfattah et al., 2016; Glenn et al., 2015; Yashiro et al., 2011; Vepštaitė-Monstavičė et al., 2018; Yashiro and McManus, 2012; Shade et al., 2013; Shen et al., 2018).

The taxonomical approach has been extensively used so far to characterise the plant microbiota. It is relatively easy to apply, but it only partially describes the taxonomic composition and does not provide any information on the genes present in the microbial communities. Even though information can be obtained from the taxonomic level, the identification of functional traits associated with the microbiota can allow for a better understanding of its influence. Rather than focus on microbial taxonomy, the recruitment of genes by a given plant for a specific function might be more stable because different species may bring the same genes and thus the same function (Lemanceau et al., 2017). Although the functional approach using shotgun metagenome sequencing is much more complex, it should therefore be prioritised for a better understanding of the microbiota ecology and of its positive or negative interactions with the plant (Violle et al., 2007). Some intermediate approaches have been proposed (Louca et al., 2016; Zarraonaindia et al., 2015), *e.g.*, after applying a taxonomical approach, the functions of the microbiota were extrapolated from the observed genera or species. Nevertheless, these approaches are biased by the fact that microorganisms are genetically and phenotypically very diverse even within a single species, especially concerning their active metabolic pathways and host-interaction properties which can significantly vary among strains (Dini-Andreote and van Elsas, 2013).

Metagenome analysis can therefore help to identify the genes present in the plant microbiome, allowing for gene- and pathway-based functional analysis. Such functional analysis could yield a more accurate description and a refined understanding of the role of microbial communities. This approach has already been applied to study rhizosphere or phyllosphere communities of soybean (Mendes et al., 2014; Delmotte et al., 2009), rice (Sessitsch et al., 2012; Knief et al., 2012), barley (Bulgarelli et al., 2015), arabidopsis (Delmotte et al., 2009), or lettuce (Cardinale et al., 2015; Kröber et al., 2014). To our knowledge, it has never been applied to the apple microbiota, including carposphere microbial communities. Therefore, the objective of this study is to characterise the bacterial and fungal communities residing on the surface of apples at the taxonomical and functional levels by using a shotgun metagenomics approach.

2. Materials and methods

2.1. Sampling material preparation

Sampling was performed in an organically managed orchard in Belgium on 9th October 2013. The coordinates of the orchard were 50° 46.199′ N and 5° 9.607′ E. The apple trees (cv. Pinova, 2 m height and 4 cm average diameter) were 2 years old and were in their first year of yield. The fungicide programme was based on 16 applications of sulfur/lime- and copper-based fungicides. The surveyed trees were not close to the orchard borders. Three random replicates (P1-P2-P3) corresponding to eight apples each collected from four neighbouring trees (on two different rows, two apples from each tree) were sampled. The fruit were immediately transported to the laboratory to isolate epiphytic microorganisms. Each replicate was split into two groups of four apples each, which were placed in washing bags containing 1 L of phosphate potassium buffer with Tween 20 (KH_2PO_4 [0.05 M], K_2HPO_4 [0.05 M], and 0.05% (wt/vol] Tween 80, pH 6.5) (Lahlali et al., 2008) and shaken on a rotary shaker (Thermoshake THO 500/1, Gerhardt GmbH CO. KG) at 2 x g for 20 min at room temperature. The washing suspensions from the two groups were pooled.

2.2. DNA extraction, library preparation, and sequencing

The washing solution was immediately filtered on 0.22-µm sterile filters. The filters were then washed with 2 mL of sterile water, and the suspension was centrifuged at 22,873 x g for 10 min (Eppendorf Centrifuge 5415R, VWR, Sigma Aldrich, USA). The supernatant was discarded, and the pellet was added into the lysing matrix tube. The FAST DNA SPIN kit for soil (MP Biomedicals) was used to extract DNA according to the manufacturer's instructions, with one modification: an initial lysing step with a Mini-BeadBeater-8 (Biospec Products, USA) at maximum speed for 40 s was added. Samples were incubated on ice for 2 min and shaken again for 40 s at maximum speed. The extracted DNA was subjected to whole genome amplification using the GenomePlex® Whole Genome Amplification Kit (Sigma-Aldrich, USA) according to the manufacturer's instructions. The library was prepared for sequencing using the Truseq DNA kit (Illumina), and the paired-end sequencing of 2 × 100 nucleotides (nt) was done on a Hiseq 2000 sequencer (Illumina) by DNAVision (Gosselies, Belgium).

2.3. Bioinformatic analysis

The sequences were demultiplexed using the standard Illumina pipeline. The remaining sequences were submitted to MG-RAST (https://www.mg-rast.org/) using standard parameters for quantitative metagenomics analysis. The taxonomical and functional annotation of the sequences was made using M5NR database (Minimal e-value 10^{-5},

minimum identity 60%, and minimum alignment length 15 bp). The tables were downloaded from MG-RAST, and figures computed in excel and in Krona (https://github.com/marbl/Krona/wiki). For the sake of clarity, only the most abundant taxonomic groups (> 0.5%) were included in the figures.

Each sequence was then quality controlled independently and *de novo* assembled into contigs using CLC Bio (Qiagen, Copenhagen) using standard parameters. Three sequencing projects were created with the contigs (P1, P2, and P3), and a combined-assembly Gold project in Integrated Microbial Genome and Metagenome Expert Review (IMG/MER) comparative data analysis system (Chen et al., 2017; Huntemann et al., 2016) was submitted for further qualitative analysis under the Gold analysis Project Id Ga0136170. The phylogenetic lineage contig candidates issued by the Usearch algorithm (Huntemann et al., 2016) were proposed on the IMG interface with a minimum identity of 30%. IMG retained 2 other levels of identity percentages, 60% and 90%, that were compared to our data.

The identification of biocontrol-related genes in our dataset was carried out as follows: first, a database composed of 159 genes potentially involved in biocontrol was created based on a scientific literature review (Daguerre et al., 2014; Lemanceau et al., 2017). Gene names and synonyms were searched in UniProt database (http://www.uniprot.org/). These genes and their presence or absence in the dataset were further analysed. In addition, the taxonomical assignment of the contigs including the identified genes was also screened to identify the potential species of origin.

In order to evaluate the specificity of the taxonomical classification proposed by IMG, we randomly selected three genes with very high identity (> 90%) from the five most abundant phyla (Actinobacteria, Ascomycota, Bacteroidetes, Basidiomycota, Firmicutes, and Proteobacteria). The gene sequences were compared with the nt and nr databases using BLASTN and BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi), using standard parameters and a threshold e-value < 10^{-7}. The first 100 hits were analysed for each gene, and the proportion of the hits with an identical taxonomy or an identical function was calculated.

3. Results

3.1. Metagenome sequencing

After demultiplexing, adaptor trimming and quality control, the total numbers of sequenced nucleotides were 4.7 Gb (46.3 M high-quality sequences of 2 × 100 nt average length), 2.4 Gb (23.7 M sequences), and 7.0 Gb (69.7 M sequences) for samples P1, P2, and P3, respectively. Their GC contents were 48 ± 7%, 46 ± 8%, and 46 ± 8% for P1, P2, and P3 respectively. The sequences were further submitted to the MG-RAST server (MG-RAST ID: mgm4688630.3, mgm4688631.3 and mgm4719666.3). The proportion of sequences that failed the quality control on MG-RAST represented 31% for P1, 29% for P2, and 38% for P3.

3.2. Taxonomic analysis at the phylum level using MG-RAST

The MG-RAST algorithms processed each 2 × 100 nt generated sequence individually. The detailed taxonomic annotation of the sequences is available online in the MG-RAST server and is summarised at the phylum level in Fig. 1. The percentages of reads annotated at the phylum level were 95.3%, 98.9%, and 98.5% for samples P1, P2, and P3, respectively. The three samples presented similar taxonomic profiles. The metagenomes included sequence reads annotated in 84, 82, and 85 phyla in P1, P2, and P3, respectively. The two most abundant phyla were Ascomycota and Basidiomycota, which represented together 86, 77, and 83% of the assigned reads of P1, P2, and P3,

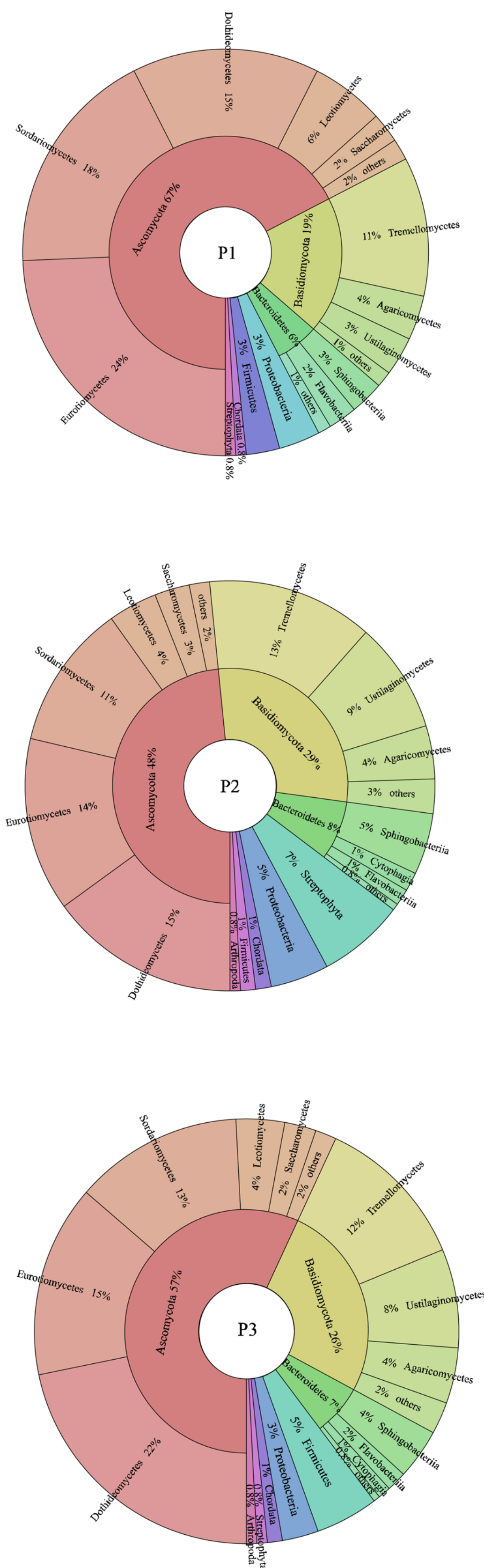


Fig. 1. Krona charts representation of the most abundant phyla and classes (relative proportion of taxa higher than 0.5%) in the 3 apple samples respectively (P1, P2 and P3).

respectively. The relative proportion of each phylum among samples ranged between 48 and 67% for Ascomycota, and between 19 and 29% for Basidiomycota. The most abundant bacterial phyla were Bacteroidetes, Proteobacteria, and Firmicutes, with cumulated proportions of 10, 14, and 14% in samples P1, P2, and P3, respectively. Their individual relative abundance varied among samples: it ranged between 6 and 8% for Bacteroidetes, 3 and 5% for Proteobacteria, and 1 and 5% for Firmicutes.

3.3. Taxonomic analysis at the genus and species levels using MG-RAST

All the genera with assigned sequences are available online in the MG-RAST server. The percentages of total sequences assigned at the genus level were almost 95.3, 94.7, and 98.5% for P1, P2, and P3, respectively. A total of 2223 bacterial species and 1536 fungal species were theoretically identified in at least one sample. The total numbers of bacterial candidate genera were 1863, 1741, and 1896 in P1, P2, and P3, respectively, and the total numbers of fungal candidate genera were 1134, 884, and 1223 in P1, P2, and P3, respectively.

The twenty most abundant microbial genera of each sample were highlighted (S.2); they represented a cumulative abundance ranging from 5.3 to 6.3 %. In total for the 3 samples, 25 different genera were included in these lists. Those low cumulative percentages suggest a high diversity of the microbiota residing on the apple surface. Sixteen highly abundant genera were present in all three samples, three were present in two of the 3 samples (*Puccinia*, *Schizosaccharomyces*, *Malassezia*), and six were present in one sample (*Ajellomyces, Nectria, Veillonella, Talaromyces, Mucilaginibacter,* and *Staphylococcus*).

The most abundant genera included well-known pathogen species of apple, as detailed in Fig. 2A. The most abundant apple pathogen genus was systematically *Aspergillus*, followed by *Botrytis*, *Sclerotinia, Penicillium*, *Nectria,* and *Colletotrichum*. Noticeably, several abundant fungal genera like *Ustilago*, *Magnaporthe*, *Gibberella*, *Pyrenophora,* and *Phaeosphaeria* included pathogenic species of cereal crops. Bacterial genera including pathogen species were present in much lower proportions:

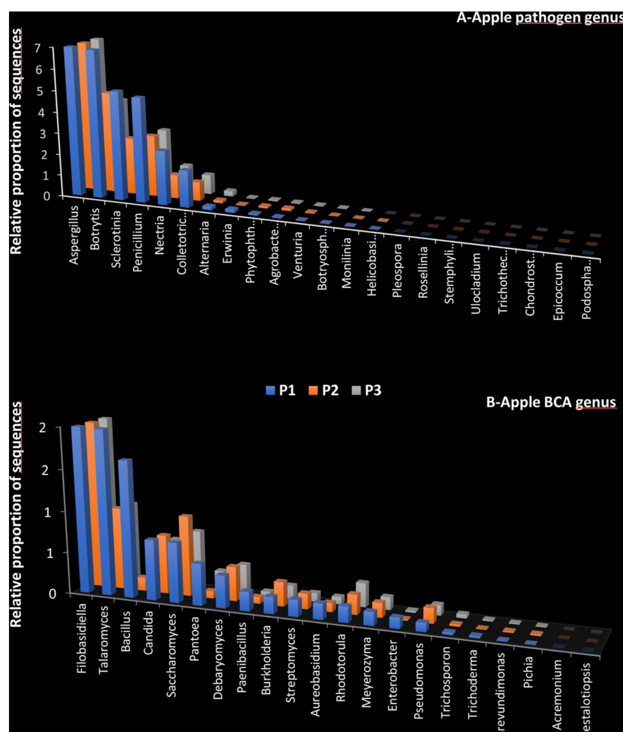


Fig. 2. Relative proportion of sequences belonging to genera including pathogens (A) or BCA (B) in each apple sample (normalized per 1000 reads for each sample). Each color represents a sample.

Table 1
Statistics of predicted features of sequences that passed the quality control on MG-RAST samples.

Sample	P1	P2	P3
Unknown (%)	13	11	7.5
Predicted unknown protein (%)	44.2	40	41.5
Predicted annotated protein (%)	42.5	43.8	43
Ribosomal DNA (%)	0.3	5.2	8

the most abundant were *Agrobacterium* and *Erwinia.* In total, 22 fungal and bacterial genera that included apple pathogens were detected. A genus that included a BCA species (*Filobasidiella*) was also present.

Considering the genera including beneficial fungi and bacteria identified on Pinova, a high relative proportion of epiphytic yeasts were identified (Fig. 2B). *Filobasidiella* was the most abundant genus, followed by *Talaromyces*, *Bacillus*, *Candida*, and *Saccharomyces.* In total, 21 genera including apple-beneficial microorganisms were detected.

At the species level, a total of 24 pathogenic species of apple including bacteria and fungi (S.1) were detected, with 21, 19, and 22 species in P1, P2, and P3, respectively. *B. cinerea*, *S. sclerotiorum*, *A. niger,* and *A. flavus* were the dominant fungal species across all samples, while *A. tumefaciens* and *E. amylovora* were the most abundant bacterial species. The numbers of bacteria, yeasts, and fungal species including BCA strains were 21, 22, and 24 in P1, P2, and P3, respectively. Among them, *Saccharomyces cerevisiae*, *Debaryomyces hansenii*, *Aureobasidium pullulans*, *Meyerozyma guilliermondii,* and *Rhodotorula glutinis* were the most abundant ones.

3.4. Functional analysis using MG-RAST

The functional annotation of the sequences is summarised in Table 1. Approximately half of the sequences that passed the MG-RAST quality control were not annotated or annotated as unknown proteins. The sequences annotated as known proteins or rDNA represented 42.8, 49, and 51% of the sequences in samples P1, P2, and P3, respectively. The relative proportions of functional subsystem categories were obtained for the three samples (Fig. 3). A total of 28 subsystem categories was identified, and the most dominant ones were “carbohydrates”, “amino acids and derivatives”, “clustering-based subsystems”, and “protein metabolism”.

3.5. Functional analysis using IMG: a global overview

The contig sequences obtained from the 3 samples were also pooled together, representing 320,126 contig sequences (min. = 200, average = 978.3, max. = 64,323, with 4.44% unmapped reads). An overview of the IMG statistics of the results is presented in Table 2.

Whatever the database used for annotation (see M&M section), only a small proportion of genes was functionally annotated (maximum 18% with pfam annotation) among all the protein-coding genes. As compared to the minimum identity threshold of 30% proposed by default by IMG, the total number of protein-coding genes assigned to a function dropped from 132,960 (26.23%) to 83,119 (16.40%) and 21,241 (4.20%) at 60% and 90% identity, respectively.

Table 3 shows the percentages of protein-coding genes assigned at the phylum level, with a 30% minimum identity percentage for each functional feature of COG, Pfam, KO, and Enzymes.

3.6. Functional analysis using IMG: genes and pathways involved in biocontrol

We further focused the functional analysis on the detection of 159 annotated genes potentially involved in the modes of action of BCA (S.3) and of genes involved in plant-pathogen interaction pathways.

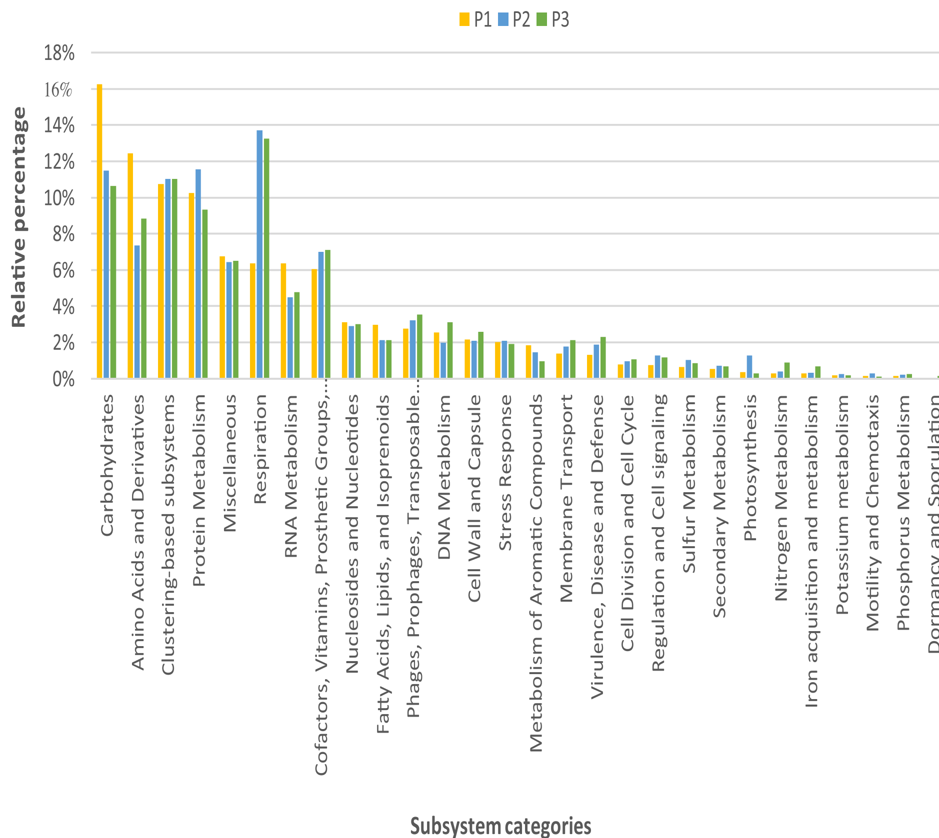


Fig. 3. Subsystem categories representation of the 3 apple samples. Only the most abundant subsystem categories (> 1%) have been shown. Each color represents a sample.

Table 2
Apple assembled metagenome statistics after IMG analysis.

	Number	Percentage
Number of contigs submitted	320126	
Number of contigs processed in IMG	319576	
Number of bases	313044027	
Genes identified	509073	100.0%
RNA genes	2140	0.4%
rRNA genes	347	
5S rRNA	106	
16S rRNA	50	
18S rRNA	45	
23S rRNA	73	
28S rRNA	73	
tRNA genes	1793	
Protein coding genes	506933	99.6%
Unassigned (> 30% identity)	373973	73.0%
with COG	70820	14.0%
with Pfam	90837	18.0%
with KO	74561	15.0%
with Enzyme	40912	8.0%
with MetaCyc	21822	4.0%
with KEGG	47081	9.0%

Sixty-five biocontrol genes were detected in the apple metagenome. No gene was found in relation to competition, although many genes identified within the microbiota were annotated within the carbohydrate metabolism and therefore potentially involved in competition for nutrients. A total of 31 genes linked to mycoparasitism, plant resistance, and secondary metabolite production were found to be carried by BCA species, mostly *F. neoformans*. Some of the genes were taxonomically assigned to pathogens or other likely commensal or mutualistic species.

Nine percent of the protein-coding genes were associated to KEGG numbers (Table 3), and we primarily focused on the plant-pathogen interaction pathway. In this pathway, fourteen different KEGG numbers represented by 168 genes (S.4) were identified in our dataset (Fig. 4).

3.7. Validation of the functional analysis using IMG

To explore functional assignment accuracy, annotated contigs were analysed in depth by BLASTX (Table 4). The results showed that the proportion of hits with the same function varied greatly, ranging from 12% (for a Phage baseplate assembly protein W) to 100% (for a DNA polymerase III subunit, Basal replication machinery, a Multisubunit NA+/H + antiporter, and an Fe-S cluster assembly scaffold protein

Table 3
Number of protein coding genes annotated per gene catalogue for the five most prevalent bacterial and fungal phyla.

Gene catalogue	Total (metagenome)	Firmicutes	Bacteroidetes	Proteobacteria	Ascomycota	Basidiomycota	Sum for the 5 phyla
with COG	70,820	3,314	6,720	3,210	24,302	19,200	56,746
with Pfam	90,837	3,258	7,034	3,169	32,676	24,115	70,252
with KO	74,561	2,695	4,958	2,603	27,282	26,434	63,972
with Enzyme	40,912	1,502	2,965	1,442	15,300	14,088	35,297

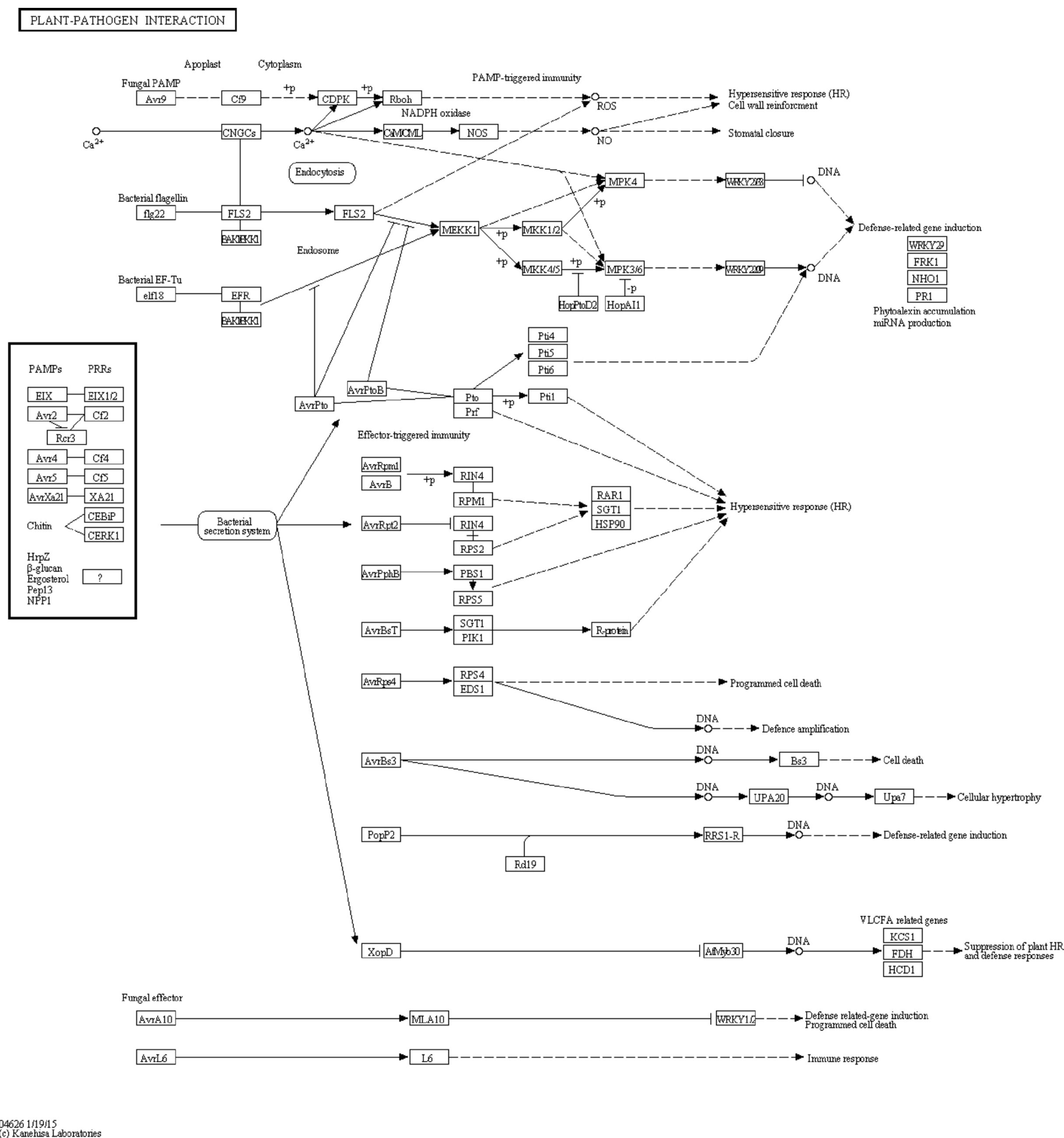


Fig. 4. Map of the KEGG Plant Pathogen interaction pathway. Identified genes in green represent genes identified in the metagenome of apple (cv. Pinova) and involved in this pathway. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

SufB). The average functional concordance was higher for bacteria (84%) than fungi (61%). A similar trend was observed at the taxonomic level: the assignment concordance was low for fungi (4%, ranging from 0 to 14% at the genus level) and higher for bacteria (50%, ranging from 0 to 100% at the genus level). The best hit (with the lowest e-value) was for the same species as in the IMG assignment for nearly all bacterial genes (11 out of 12), but was different for all the fungal genes. BLASTN analyses were also carried out. If a gene sequence was divergent from database sequences, the e-values obtained by BLASTN (comparing nucleic acids) were higher than by BLASTX (comparing amino acids). BLASTN results greatly differed from BLASTX results, with only 145 hits with an e-value lower than 10^{-7} (as compared to 1799 with BLASTX). This underlines the divergence between the actual retrieved sequences and the gene sequences stored in the databases.

4. Discussion

The microbial communities of harvested fruit play a key role in fruit conservation because they host and most probably establish complex trophic interactions with both postharvest plant pathogens and biocontrol agents. In addition, they could also influence fruit maturation and ripening (Buchholz et al., 2018; Ravanbakhsh et al., 2018). Better understanding their composition and role may therefore substantially improve the storage of harvested fruit. The present study describes the first use of metagenome shotgun sequencing to characterise the carposphere microbiota. It illustrates the insights gained at the taxonomical and functional levels, including the identification of important functions, of plant pathogens or biocontrol agents. In addition, it also suggests that further research should focus on handling the

Table 4
NCBI blastn and blastx of contigs anotated in IMG with a minimum of 90% identity for the most important phyla; Only the top 100 hits are considered. Number in brackets are the total number of species hit compt; "/" means that there is no strain information provided in NCBI.

Best Hits at 90% Identity or more	Gene ID	% identity	Consensus Sequence length	COG e-value	COG Percent Identity	Functional annotation (COG)	Taxonomic assignment (homolog genome)	NCBI Blast x	
								Nber hit (e-value < 10e-7) for the first 100 top hits	Nber of hit functional ok _ (=N total-hypothetic)
Ascomycota	Ga0136170_10000658	93.51	444	1.4e-72	79.88	Actin-related protein	Cochliobolus heterostrophus C5	100	65
Ascomycota	Ga0136170_10000324	100	132	4.6e-07	33.08	Ribosomal protein S8	Paracoccidioides brasiliensis Pb01	100	79
Ascomycota	Ga0136170_11752731	90	340	3.4e-05	12.06	NADPH-dependent curcumin reductase CurA	Pyrenophora tritici-repentis Pt-1C-BFP	100	44
Basidiomycota	Ga0136170_100025814	91.43	451	3.3e-08	8.63	Signal recognition particle GTPase	Cryptococcus neoformans var. neoformans JEC 21	100	61
Basidiomycota	Ga0136170_10012902	95.5	384	6.9e-22	31.91	Serine/threonine protein kinase	Malassezia globosa CBS 7966	100	59
Basidiomycota	Ga0136170_10323302	97.18	682	5.1e-33	10.26	DNA polymerase III subunits, Basal replication machinery	Ustilago maydis 521	100	58
Actinobacteria	Ga0136170_10426561	100	935	5.5e-89	17.96	DNA polymerase III subunits, Basal replication machinery	Propionibacterium acnes TypeIA2 P.acn17	100	100
Actinobacteria	Ga0136170_12856881	100	713	1.6e-15	16.14	Multisubunit NA+/ H + antiporter	Propionibacterium acnes SK182B-JCVI	100	100
Actinobacteria	Ga0136170_114742410	93.33	379	4.7e-03	11.87	Transposase (or an inactivated derivative)	Corynebacterium diphtheriae 31A	100	79
Bacteroidetes	Ga0136170_10058142	96.32	116	1.8e-25	87.07	Phage baseplate assembly protein W	Chryseobacterium sp. YR485	99	12
Bacteroidetes	Ga0136170_10137111	90.29	528	5.4e-60	28.62	Fatty acid biosyntheis, Deoxyxylulose pathway of terpenoid biosynthesis	Pedobacter caeni DSM 16990	100	99
Bacteroidetes	Ga0136170_11033701	97.48	412	7.6e-37	36.41	Fe-S cluster assembly scaffold protein SufB	Epilithonimonas tenax DSM 16811	100	100
Proteobacteria	Ga0136170_10129211	98.29	563	1.4e-22	16.99	Predicted acyl esterase	Erwinia billingiae OSU19-1	100	33
Proteobacteria	Ga0136170_11035071	99.16	381	3.5e-35	60.39	Lipid A biosynthesis	Pantoea agglomerans P10c	100	92
Proteobacteria	Ga0136170_11611921	99.26	207	1.6e-45	65.71	Cytochrome oxidase Cu insertion factor, SCO1/SenC/PrrC family	Neisseria macacae ATCC 33926	100	99

(*continued on next page*)

Table 4 (*continued*)

Best Hits at 90% Identity or more	Gene ID	% identity	Consensus Sequence length	COG e-value	COG Percent Identity	Functional annotation (COG)	Taxonomic assignment (homolog genome)	NCBI Blast x: Nber hit (e-value < 10e-7) for the first 100 top hits	NCBI Blast x: Nber of hit functional ok _ (=N total-hypothetic)
Firmicutes	Ga0136170_10087881	97.92	335	1.2e-28	46.74	HEAT repeat	Bacillus psychrosaccharolyticus ATCC 23296	100	96
Firmicutes	Ga0136170_11208853	100	301	2.3e-08	21.77	3',5'-cyclic AMP phosphodiesterase CpdA	Staphylococcus epidermidis 3d11	100	99
Firmicutes	Ga0136170_12287461	93.81	1163	1.8e-54	16.35	Chromosome segregation ATPase	Staphylococcus warneri 1DB1	100	96

Best Hits at 90% Identity or more	NCBI Blast x				NCBI Blast n				
	Nber of hit taxonomical ok at strain level (number of species ok)	Number of genus taxonomical Ok	Top hit (strain)	Top hit (species)	Nber hit (e-value < 10e-7) for the first 100 top hits	Nber of hit taxonomical ok at strain level (number of species ok)	Number of genus taxonomical Ok	Top hit (strain)	Top hit (species)
Ascomycota	0	0	no	no	15	0	0	no	no
Ascomycota	0	1	no	no	15	0	0	no	no
Ascomycota	1 (1)	2	no	no	1	0	0	no	no
Basidiomycota	1 (4)	14	no	no	1	0	0	no	no
Basidiomycota	1 (1)	4	no	no	37	0	0	no	no
Basidiomycota	1 (1)	3	no	no	0	0	0	no	no
Actinobacteria	1 (16)	43	/	yes	27	1 (20)	21	/	yes
Actinobacteria	0	1	no	no	11	0	0	no	no
Actinobacteria	1 (9)	95	/	yes	10	1 (3)	10	no	no
Bacteroidetes	0 (26)	26	/	yes	1	0 (1)	1	/	yes
Bacteroidetes	0 (1)	37	/	yes	No	0	0	no	no
Bacteroidetes	0	0	no	yes	2	0	0	no	no
Proteobacteria	0 (1)	1	/	yes	0	0	0	no	no
Proteobacteria	0 (7)	16	/	yes	3	0 (1)	2	/	yes
Proteobacteria	1 (1)	76	yes	yes	1	0	1	No	yes
Firmicutes	0 (1)	96	/	yes	0	0	0	no	no
Firmicutes	0 (25)	97	/	yes	10	0 (8)	10	/	yes
Firmicutes	0 (6)	100	/	yes	11	0 (1)	10	/	yes

hurdles that currently impede the use of this approach, *e.g.*, the incompleteness of databases, the influence of bioinformatics analysis and its parameters, as well as the lack of accuracy of the taxonomic and functional assignment of sequences in databases, mostly for fungi.

This study is focused on representative samples of the Pinova apple cultivar from an organic orchard in Belgium. Although its results cannot be generalised, they are in agreement with previous published data using amplicon sequencing (Abdelfattah et al., 2016; Vepštaitė-Monstavičė et al., 2018; Shen et al., 2018). They provide a global picture of the apple carposphere fungal metagenome of our samples, even if differences in microbial populations have been highlighted between different fruit parts (Abdelfattah et al., 2016). It is worth underlining that most current studies were carried out on apple fruit bought from the supermarket, while only two gave details on orchard sampling: random harvest (Shen et al., 2018) and random harvest followed by pooling to reach 300 g of fruit (Vepštaitė-Monstavičė et al., 2018). Besides the technological and bioinformatics improvement, developing sampling recommendations should also be a priority to ensure that the results from future independent studies can be compared.

A high fungal and bacterial diversity was observed in the three samples, as the 20 most abundant microbial genera *per* sample only represented 6.3% of the microbiota at most. This could be explained by the fact that we surveyed an organic orchard. Before harvesting, apples were treated with copper to control postharvest diseases, and the copper-based treatment is indeed associated with higher fungal diversity and abundance in apple (Granado et al., 2008). Concerning the fungal composition of the apple surface, members of the phylum *Ascomycota* were dominant in all samples (47–67% of the total sequences) followed by *Basidiomycota* (20–30%). This is in accordance with recent publications based on high-throughput sequencing of amplified ITS PCR products from the apple carposphere (Abdelfattah et al., 2016; Vepštaitė-Monstavičė et al., 2018; Shen et al., 2018). Differences were detected in the relative abundance of fungal genera: *Ascomycota* were mainly represented by the genera *Aspergillus* spp., *Botrytis* spp., *Sclerotinia* spp., and *Penicillium* spp., while *Filobasidiella* spp. was the most abundant *Basidiomycota* genus, as previously observed (Glenn et al., 2015). In agreement with recent microbiota studies carried out on apple (Vepštaitė-Monstavičė et al., 2018; Leff and Fierer, 2013), we found that the bacterial communities were dominated by the phyla *Bacteroides* (5–8% of the total sequences), *Proteobacteria* (3–4%), and *Firmicutes* (3–4%), which were mostly represented by the genera *Bacillus* spp., *Paenibacillus* spp., *Burkholderia* spp., *Streptomyces* spp., *Enterobacter* spp., and *Pseudomonas* spp.

A significant number of sequences appeared to be closely related to fungal apple-pathogenic genera like *Aspergillus* spp., *Botrytis* spp., *Sclerotinia* spp., and *Penicillium* spp., and apple-pathogenic bacteria like *Erwinia* spp. and *Agrobacterium* spp. However, not all the species from these genera are pathogenic. The analysis was also carried out at the species level: in total, 24 apple-pathogenic species including six bacteria and 18 fungi (all assigned at the species level in MG-RAST) were identified in the metagenome data (S.1). Interestingly, the potential presence of pathogens was not correlated with any disease symptom. These preliminary results are based on only 3 composite samples, but if they are confirmed at a larger scale and the taxonomic assignment bias can be solved (see below), they might indicate quite a widespread prevalence of many plant pathogens on healthy fruit. Genera including biocontrol strains were also identified. The most abundant one was *Filobasidiella* spp., a genus including several biocontrol strains, while other genera like *Talaromyces* spp, *Candida* spp., *Saccharomyces* spp., *Bacillus* spp., and *Enterobacter* spp. were also identified in lower proportions, and also observed in previous studies using amplicon sequencing (Abdelfattah et al., 2016; Vepštaitė-Monstavičė et al., 2018; Shen et al., 2018). Overall, the taxonomic results of our metagenome sequencing study are in accordance with the results of the published studies using amplicon-targeted approaches. Importantly, the relative abundance of both fungi and bacteria taken together can be obtained through metagenome sequencing, whereas they are studied separately by the amplicon sequencing approach.

In addition, the presence of genera including cereal crop pathogens might represent an important challenge to be deciphered. The various compartments of the phyllosphere are frequently colonised by microorganisms that reach the surface carried by the wind, dust, rain, and animals (insects, arachnids, birds, *etc.*). The presence of genera including cereal crop pathogens might be due to the presence of a recently harvested maize field located close to the orchard. A high abundance of the *Ustilago* and *Phaeosphaeria* genera has already been observed on the apple surface (Abdelfattah et al., 2016). If these results are confirmed at a larger scale, this will suggest that these genera might be part of the apple microbiota, and that they grow and multiply there with a potential role in the community structure. Such a hypothesis would need further analysis, for example through a targeted transcriptomic study of a few species whose genomes are sequenced or through a metatranscriptomic approach, *e.g.*, by high-throughput sequencing of the RNA extracted from the microbiota. Metatranscriptome analysis has indeed a major advantage over metagenome analysis because the relative proportion of identified genes depends on their expression level and not on the presence of a coding gene. It therefore reflects the microbial activity within the microbiota and not the sole presence of cells. This approach was very recently combined with amplicon sequencing to characterise the epiphytic microbiota of watermelon fruit (Saminathan et al., 2018). Nevertheless, it is also more complex to handle and might be biased: the protocols used to harvest microbiota from plant samples are usually quite long (20 min to 1 h at least), while microorganisms can react very quickly to environmental changes by significantly modifying their gene transcription and thereby impact the overall results of a metatranscriptome analysis.

Valuable taxonomic information was extracted from the data mined in the present study, and only a low proportion of sequences remained unassigned in MG-RAST. Nevertheless, an important bottleneck cannot be underestimated at this stage, *i.e.*, the incompleteness of databases. The lack of data in databases raises another challenge about the specificity of taxonomic assignment. For example, if a single species is sequenced as belonging to a genus or a family, sequencing reads from a close relative species whose genome is not sequenced will be mis-assigned to the sequenced species. Nevertheless, the potential assignment bias decreases at higher taxonomic levels like the genus and the family levels, which are the current taxonomic levels used for amplicon sequencing approaches. In addition, bacterial genome sequences are currently much more abundant than fungal sequences, so that the appropriate taxonomic assignment is easier. Therefore, particularly for fungi, if the plant-pathogenic species is the only sequenced species within a genus or a family, this might over-estimate the presence of plant pathogens, and the results should be interpreted with great care. Nevertheless, this lack of specificity is lower at higher taxonomical levels such as orders or phyla. As the nucleic acid databases and the number of sequenced species are growing exponentially, this challenge will be progressively addressed in the future, and its impact will be reduced. The development of genome databases will also improve the taxonomic classification of strains and species. However, this challenge will remain a key bottleneck because accurate taxonomic assignment at the species level of DNA sequences generated by high-throughput sequencing of the ITS region for fungal microbiome analysis is a challenging and as yet unsolved problem, even for the more popular approach of amplicon sequencing. Therefore, amplicon-based or shotgun-sequencing approaches should be selected with special care depending on the objective of the experiment and on the available resources (databases, algorithm computational power, and funds).

It has been suggested in the literature that the functional core microbiota could be more stable over time compared to the taxonomical core microbiota (Glenn et al., 2015). The three analysed samples presented higher similarities at the functional level than in their taxonomic profiles. Despite similarities among samples, the proportions of the

major phyla varied among samples, mostly as regards Actinomycetes (48–67%), Basidiomycetes (19–29%), and Firmicutes (1–5%). At the functional level, variability was much lower, with only the functional subsystem category of respiration presenting divergent proportions in the three samples (6, 13, and 14% in P1, P2, and P3, respectively). Diverse pathways related to biocontrol properties have been identified in the apple carposphere metagenome. Out of the 159 scanned biocontrol genes, 40% were detected. However, although several biocontrol genes were assigned to *Filobasidiella neoformans* (a species including BCA strains), other genes were assigned to known pathogens (S.3). This observation underlines a key element for such an assay: the functional interpretation of sequences must be done concomitantly with taxonomic assignment.

A large proportion of sequences were not properly annotated at the functional level. This observation underlines a current limitation of metagenomics sequencing for the phyllosphere or carposphere microbiota, and might result from three factors: i) the small size of the analysed sequences (100 nt for MG-RAST) that limited the probability of finding homologies, ii) the poor characterisation of fungal genes in the databases while fungi represented most of the generated sequences, and (iii) the very limited amount of annotated sequences from apple microorganisms in the databases. To investigate if the small size of the sequences used with MG-RAST had an impact on annotation, the assembled contigs were also annotated. These longer sequences could be theoretically assigned with better accuracy than shorter sequences. Nevertheless, annotation was not improved, indicating that the lack of properly annotated sequences in the database might be the main bottleneck. The carposphere is a particular ecological niche that might harbour many as yet non-sequenced microorganisms not included in the databases, even though the gene families currently considered as important for biocontrol properties or plant–pathogen interactions are better characterised (Massart et al., 2015). Even for the microorganism species sequenced in the databases, the strains present on the apple surface can be genetically distant from the sequenced strain.

The limitation regarding proper annotation is particularly important for fungi as compared to bacteria whose gene functions are better characterised. Many fungal sequences were homologous to hypothetical proteins or proteins with an unknown function. In addition, intronic and non-coding regions might be abundant in fungi while they are absent or less abundant in bacteria. It is therefore not surprising that the proportion of sequences unassigned at the functional level or assigned to a gene with an unknown function was close to 70% for both MG-RAST and IMG.

The validation of annotations is also a very important step. We used BLASTX and BLASTN comparisons on a small subset of contigs to evaluate the robustness of the taxonomical and functional assignments in IMG. The results highlighted numerous ambiguities at the taxonomical and functional levels, mostly for fungi. This underlines a limitation of the proposed approach and suggests alternatives for future experiments. A first alternative corresponds to the HTS technologies that generate longer sequencing reads (up to 10–20 kb), like the technologies provided by Pacific Bioscience or Oxford Nanopore Technologies. Another alternative is single-cell genome sequencing, which is currently emerging for metagenome studies (Gawad et al., 2016; Gladka et al., 2018; van den Bos et al., 2018). Single-cell sequencing will allow for specific assignment of the genes to a single organism or discover and characterise a new species, but it is still a costly technology and would need the parallel sequencing of a considerable quantity of individual cells. In summary, a combination of the approach used in this study with single-species-targeting genomics might provide a great progress.

By underlining key advances and taxonomic and functional information on the carposphere microbiome and by pinpointing the remaining challenges, the present work is paving the way for future studies addressing the functional characterisation of the carposphere microbiota to understand its ecology, physiology, evolution, and role in plant health and fruit conservation.

Acknowledgments

This research was financially supported by the INNOVA project, supported by the European Commission through the Seventh Framework Program under contract number 324416. The authors would like to thank the center of excellence in fruit research Proefcentrum Fruitteelt (Sint Truiden-Belgium) for technical support, and the F.R.S.-FNRS for the FRIA (Funds for the Research in Industry and Agriculture), PhD grant numbers 5103816F and 5200818F.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.postharvbio.2019.03.020.

References

Abdelfattah, A., Wisniewski, M., Droby, S., Schena, L., 2016. Spatial and compositional variation in the fungal communities of organic and conventionally grown apple fruit at the consumer point-of-purchase. Hortic. Res. 3 (August), 16047.
Andrews, J.H., Kenerley, C.M., 1980. Microbial populations associated with buds and young leaves of apple. Can. J. Bot. 58 (April (8)), 847–855.
Andrews, J., Kenerley, C., 1981. Direct observation and enumeration of microbes on plant surfaces by light microscopy. In: Blakeman, J.P. (Ed.), Microbial Ecology of the Phylloplane. Academic Press, London, pp. 3–14.
Blakeman, J., 1981. Microbial Ecology of the Phylloplane. Academic P. London.
Buchholz, F., Kostić, T., Sessitsch, A., Mitter, B., 2018. The potential of plant microbiota in reducing postharvest food loss. Microb. Biotechnol.(March).
Bulgarelli, D., et al., 2015. Structure and function of the bacterial root microbiota in wild and domesticated barley. Cell Host Microbe 17 (March (3)), 392–403.
Cardinale, M., Grube, M., Erlacher, A., Quehenberger, J., Berg, G., 2015. Bacterial networks and co-occurrence relationships in the lettuce root microbiota. Environ. Microbiol. 17 (1), 239–252.
Chand-Goyal, T., Spotts, R.A., 1996. Enumeration of bacterial and yeast colonists of apple fruits and identification of epiphytic yeasts on pear fruits in the Pacific Northwest United States. Microbiol. Res. 151 (December (4)), 427–432.
Chen, I.-M.A., et al., 2017. IMG/M: integrated genome and metagenome comparative data analysis system. Nucleic Acids Res. 45 (January (D1)), D507–D516.
Daguerre, Y., Siegel, K., Edel-Hermann, V., Steinberg, C., 2014. Fungal proteins and genes associated with biocontrol mechanisms of soil-borne pathogens: a review. Fungal Biol. Rev. 28 (December (4)), 97–125.
Delmotte, N., et al., 2009. Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. Proc. Natl. Acad. Sci. U. S. A. 106 (September (38)), 16428–16433.
Dini-Andreote, F., van Elsas, J.D., 2013. Back to the basics: the need for ecophysiological insights to enhance our understanding of microbial behaviour in the rhizosphere. Plant Soil 373 (December (1–2)), 1–15.
Gawad, C., Koh, W., Quake, S.R., 2016. Single-cell genome sequencing: current state of the science. Nat. Rev. Genet. 17 (March (3)), 175–188.
Gladka, M.M., et al., 2018. Single-cell sequencing of the healthy and diseased heart reveals Ckap4 as a new modulator of fibroblasts activation. Circulation(January) p. CIRCULATIONAHA.117.030742.
Glenn, D.M., Bassett, C., Dowd, S.E., 2015. Effect of pest management system on 'Empire' apple leaf phyllosphere populations. Sci. Hortic. (Amsterdam) 183, 58–65.
Granado, J., et al., 2008. Culturable Fungi of stored 'Golden delicious' apple fruits: a one-season comparison study of organic and integrated production systems in Switzerland. Microb. Ecol. 56 (November (4)), 720–732.
Hirano, S.S., Upper, C.D., 1983. Ecology and epidemiology of foliar bacterial plant pathogens. Annu. Rev. Phytopathol. 21 (September (1)), 243–270.
Huntemann, M., et al., 2016. The standard operating procedure of the DOE-JGI Metagenome Annotation Pipeline (MAP v.4). Stand. Genomic Sci. 11 (December (1)), 17.
Knief, C., et al., 2012. Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. ISME J. 6 (July (7)), 1378–1390.
Kröber, M.S., et al., 2014. Effect of the biocontrol strain Bacillus amyloliquefaciens FZB42 on the microbial community in the rhizosphere of lettuce under field conditions analyzed by whole metagenome sequencing. Front. Microbiol. 5.
Lahlali, R., Massart, S., De Clercq, D., Serrhini, M.N., Jijakli, M.H., 2008. Assessment of Pichia anomala (strain K) efficacy against blue mould of apples when applied pre- or post-harvest under laboratory conditions and in orchard trials - Springer. Eur. J. Plant Pathol. 123, 37–45.
Leff, J.W., Fierer, N., 2013. Bacterial communities associated with the surfaces of fresh fruits and vegetables. PLoS One 8 (3), 1–9.
Lemanceau, P., Blouin, M., Muller, D., Moënne-Loccoz, Y., 2017. Let the core microbiota be functional. Trends Plant Sci. 22 (July (7)), 583–595.
Lindow, S.E., Brandl, M.T., 2003. Microbiology of the phyllosphere. Appl. Environ. Microbiol. 69 (4), 1875–1883.

Louca, S., et al., 2016. High taxonomic variability despite stable functional structure across microbial communities. Nat. Ecol. Evol. 1 (December), 1–12.
Massart, S., Martinez-Medina, M., Jijakli, M.H., 2015. Biological control in the microbiome era: challenges and opportunities. Biol. Control 89 (June), 98–108.
Mendes, L.W., Kuramae, E.E., Navarrete, A.A., van Veen, J.A., Tsai, S.M., 2014. Taxonomical and functional microbial community selection in soybean rhizosphere. ISME J. 8 (August(8)), 1577–1587.
Pelliccia, C., Antonielli, L., Corte, L., Bagnetti, A., Fatichenti, F., Cardinali, G., 2011. Preliminary prospection of the yeast biodiversity on apple and pear surfaces from Northern Italy orchards. Ann. Microbiol. 61 (December (4)), 965–972.
Pennycook, S.R., Newhook, F.J., 1981. Seasonal changes in the apple phylloplane microflora. New Zeal. J. Bot. 19 (July (3)), 273–283.
Ravanbakhsh, M., Sasidharan, R., Voesenek, L.A.C.J., Kowalchuk, G.A., Jousset, A., 2018. Microbial modulation of plant ethylene signaling: ecological and evolutionary consequences. Microbiome 6 (December (1)), 52.
Saminathan, T., et al., 2018. Metagenomic and metatranscriptomic analyses of diverse watermelon cultivars reveal the role of fruit associated microbiome in carbohydrate metabolism and ripening of mature fruits. Front. Plant Sci. 9 (January), 4.
Sessitsch, A., et al., 2012. Functional characteristics of an endophyte community colonizing rice roots as revealed by metagenomic analysis. Mol. Plant Microbe Interact. 25 (January (1)), 28–36.
Shade, A., McManus, P.S., Handelsman, J., 2013. Unexpected diversity during community succession in the apple flower microbiome. MBio 4 (February (2)) pp. e00602-12-e00602-12.
Shen, Y., et al., 2018. Differentiated surface fungal communities at point of harvest on apple fruits from rural and peri-urban orchards. Sci. Rep. 8 (December (1)), 2165.
Sylla, J., Alsanius, B.W., Krüger, E., Reineke, A., Strohmeier, S., Wohanka, W., 2013. Leaf microbiota of strawberries as affected by biological control agents. Phytopathology 103 (10), 1001–1011.
Teixidó, N., Usall, J., Gutierrez, O., Viñas, I., 1998. Effect of the antagonist Candida sake on apple surface microflora during cold and ambient (shelf life) storage. Eur. J. Plant Pathol. 104 (4), 387–398.
Teixidó, N., Usall, J., Viñas, I., 1999. Efficacy of preharvest and postharvest Candida sake biocontrol treatments to prevent blue mould on apples during cold storage. Int. J. Food Microbiol. 50 (September (3)), 203–210.
van den Bos, H., Bakker, B., Spierings, D.C.J., Lansdorp, P.M., Foijer, F., 2018. Single-cell sequencing to quantify genomic integrity in cancer. Int. J. Biochem. Cell Biol. 94 (January), 146–150.
Vepštaitė-Monstavičė, I., et al., 2018. Distribution of apple and blackcurrant microbiota in Lithuania and the Czech Republic. Microbiol. Res. 206, 1–8.
Violle, C., et al., 2007. Let the concept of trait be functional!. Oikos 116 (May (5)), 882–892.
Vorholt, J.A., 2012. Microbial life in the phyllosphere. Nat. Rev. Microbiol. 10 (12), 828–840.
Yashiro, E., McManus, P.S., 2012. Effect of streptomycin treatment on bacterial community structure in the apple phyllosphere. PLoS One 7 (January (5)), e37131.
Yashiro, E., Spear, R.N., Mcmanus, P.S., 2011. Culture-dependent and culture-independent assessment of bacteria in the apple phyllosphere. J. Appl. Microbiol. 110 (5), 1284–1296.
Zarraonaindia, I., et al., 2015. The soil microbiome influences grapevine-associated microbiota. MBio 6 (March (2)) pp. e02527-14.