Highlighting the complexity of pathogenesis: the host microbiota impacts disease development on apple fruit and is a cornerstone for its biocontrol.

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Abstract

Background: Apple fruit is the most produced temperate fruit with a trade value estimated at 7.5 billion $ and is usually stored up to one year after harvest. Postharvest pathogens often compromise storage, responsible for up to 55% of fruit losses, depending on the country and fruit. They are also a source of mycotoxin contamination. A sustainable way to control that pathogen is using beneficial microorganisms called biocontrol agents (BCA). Despite promising efficacy in laboratory conditions, BCA's efficacy is variable and often reduced once applied at a large scale through either orchard or postharvest treatment. We hypothesized that the epiphytic microbiota plays a role in the variability of BCA efficiency (*Pichia anomala*, strain K) and postharvest disease development due to *Botrytis cinerea* on apples.

Results: A diverse set of 18 epiphytic microbial communities were harvested from apple carposphere and bio-banked. The analysis of their bacterial and fungal taxonomic composition and carbon metabolic footprint confirmed that contrasted microbiotas were harvested. Their impact on *B. cinerea* disease development was evaluated through a standardized *in vivo* bioassay. The reduction of *B. cinerea* rot development ranged from 20% to 80% when the microbiotas were applied alone. In addition, three microbiotas enhanced the biological control efficiency of strain K (up to +100%, whatever the tested microbiota concentrations) while others limited its action (down to -27%). A co-clustering analysis of biocontrol efficacy with carbon profiles or taxonomic composition was carried out. It identified promising molecules whose high metabolization was associated with high biocontrol by the microbiota and taxa with higher abundance in microbiota limiting *B. cinerea* rot development. Putative beneficial taxa were isolated from the most efficient microbiota. *In vivo* bioassays confirmed the efficacy of two molecules and two strains belonging to species never mentioned for their biocontrol properties against plant disease.

Conclusion: This study, the first of its kind, demonstrates that natural epiphytic microbiota significantly influences postharvest disease development in apples and the variability in biocontrol efficacy. Our approach identified promising molecules and taxa that enhance biocontrol, offering new insights for sustainable postharvest pathogen management.
Keywords: fruit; microbiota; postharvest; food security; biocontrol; prebiotics

Introduction

In plant pathology, the disease triangle (host-pathogen-environment) has traditionally been an instrumental paradigm in understanding pathogenesis considering 3 favourable conditions: a susceptible host, a virulent microorganism, and conducive environmental conditions (temperature, air humidity, surface wetness…). Nevertheless, it is becoming clear that studying the interactions between hosts, pathogens, and the physical environment is often insufficient. Plant health is also profoundly influenced by its epiphytic and endophytic microbial communities. These plant-associated microbiota interact with the plant and the pathogen while being influenced by the environment [1]. The contribution of plant-associated microbiota to disease development has led to the concept of pathobiome that integrates the plant-pathogen interactions within their biotic environment and the environmental conditions [2–4]. The impact of pathogen development on the plant-associated microbiota has been demonstrated in many studies, even on plant residues [5], with sometimes an effect depending on the organ [6]. These modifications of the plant microbiota have been exploited for targeted isolation of beneficial strains and, sometimes, the design of disease-resistant synthetic communities from roots [7,8] or leaves [9]. On the other hand, the impact of the residing microbiota on disease development has been much less studied. For example, an observational study suggested a crucial role of rhizosphere protists in plant health [10]. Despite being postulated, for example, in a recent review[11], the impact of resident fruit microbiota on disease development has never been systematically addressed, even though the trophic relationships with the resident microbiota could also impact a pathogen. On the other hand, in several publications, modifying the environment by applying carbon or nitrogen sources of mineral or organic origin reshaped the initial microbiota, impacting pathogen or disease development [12–15].

Once harvested, the food commodities can be stored for up to one year under the threat of postharvest pathogens, some relying on fruit wounds for disease development. The postharvest losses threaten food security and sustainability, with up to 55% food losses [16]. They remained stable during the past decades despite developing and applying chemical,

physical, and biological treatments. Apple is the most-produced temperate fruit, with an annual production of 93 million tons in 2021 (https://www.fao.org/faostat). Like any other eukaryotic organ, apple fruit hosts an epiphytic microbiota whose composition has already been characterized in contrasted agricultural conditions worldwide [17–26]. A very high diversity was observed, with very few taxa corresponding to the core microbiome compared to other plant species [25]. Importantly, genera of postharvest pathogens such as *Botrytis*, *Monilinia*, *Neofabraea*, and *Penicillium* have been frequently observed, and their detection frequency tended to rise during storage [24], even without disease development. So, understanding if this asymptomatic presence could be linked with the composition and impact of the epiphytic microbiota is particularly promising for developing new biocontrol solutions.

On the other hand, many biocontrol agents (BCA) were isolated from apple epiphytic microbiota and developed into microbial-based biopesticides that represent a promising alternative to overcome the limits of synthetic chemical pesticides. However, despite decades of research and the progress made to understand the BCA's ecology and mode of action, their efficacy is unstable and reduced compared to synthetic pesticides [27]. Besides the impact of abiotic conditions, once sprayed, beneficial microorganisms like BCA, either applied alone or in association, encounter a resident microbiota, where complex ecological relations occur. These interactions could affect directly or indirectly their reliability and efficacy in natural conditions, explaining the observed instability.

So, shifting from the disease triangle to the pathobiome paradigm is a promising axis to improve the control of diseases in terms of efficacy and reliability, also postharvest [1,28,29]. Studies have shown that applying BCA might further alter the composition of apple fruit [30] or flower [31] epiphytic microbiota or of the rhizosphere of plants such as cucumber [32] or rice [33]. Nevertheless, apart from hypotheses, little information is available on the impact of the epiphytic microbiota on the efficacy of beneficial microorganisms like BCAs. This information is essential for understanding the ecological fitness of BCA within the resident microbiota and how its composition might impact their efficiency.

Therefore, our study aimed to answer two key questions: (i) what is the impact of the resident epiphytic microbiota on postharvest disease development and the efficacy of a BCA, and (ii) how can this impact be leveraged for limiting postharvest losses due to pathogens? For this purpose, diverse apple carposphere microbiota were sampled, taxonomically and metabolically characterized, and tested *in situ* for their impact on *B. cinerea* disease development and on the efficacy of a well-characterized BCA, *P. anomala* strain K[34]. Then, hierarchical clustering analyses were applied to drive the selection of prebiotics (molecules

driving the plant microbiota toward a composition limiting the development of plant pathogens[29])whose efficacy was further evaluated *in vivo*. Candidate biocontrol strains were also isolated from beneficial microbiota and evaluated *in vivo*.

Material and methods

Sampling and plant materials

Apple fruits were harvested in September 2016 in Belgium from four different orchards, just before harvest. Orchards corresponded to two commercial orchards managed under organic farming (Site A - 50°33'47.5"N; 4°43'17.8"E) or under Integrated Pest Management (IPM), (Site B - 50°48'36.7"N; 4°57'08.8"E), one 35-years old conservation orchard never treated with pesticides from the Fruit Tree Genetic Resources Collection of Walloon Agronomic Centre of Gembloux (Site C – CRA-W - 50°33'47.5"N; 4°43'17.8"E) and a research orchard of CRA-W combining organic farming with reduced spraying of copper (Site D - 50°33'06.8"N; 4°39'11.0"E). Two *Malus sylvestris* accessions and 13 different cultivars of *M. domestica*, representing 18 samples in total, were sampled. More details are available in supplementary material 1.

Microbiota harvest and biobanking

A pool of four fruit was considered as a sample replicate and 5 independent replicates were extracted per microbiota. Each replicate was put into a sterile plastic bag with 1L of Phosphate Buffer Saline buffer (8 g/l NaCl, 200 mg/l of KCl, 1.44 mg/l of Na_2HPO_4, and 245 mg/l of KH_2PO_4, final solution pH=7.4). Then, fruits were bath sonicated (Ultrasonic cleaner, VWR USC100TH; 45 kHz) for 15 min and shaken at room temperature for 20 min at 120 rpm (2 x g). The microbiota washing process was repeated twice to improve the microbiota harvest as previously determined [23]. The biobanking, quantification, and preparation of microbiota have been carried out as described in Supplementary Material 2.

In vivo evaluation of disease control

The ability of microbial communities, isolated strains, and potential prebiotic molecules to control disease development by *Botrytis cinerea* Pers. was evaluated by standardised *in vivo* tests. . The microbial communities (normalized at 10^5 CFU/ml) were applied alone (n=10) or in combination with 10^5 CFU/ml of *P. anomala* strain K (n=18) on sterilized commercial 'Golden delicious' wounded apples following standardized protocol [35] described in Supplementary material 3.

Eight prebiotic candidates were also tested *in vivo* following the same protocol at 0.5% and 1% (w:v) concentration with three selected microbiota. Twelve strains isolated from microbiota limiting the development of *B. cinerea* were tested *in vivo* at 10^7 CFU/ml alone to evaluate their biocontrol efficacy.

Taxonomical characterisation of the microbiota

Laboratory protocol

Two biological replicates of each sample (18 microbiota x 2 replicates) were centrifuged for 10 min at 10,000 x g and the pellet was used as starting material for DNA extraction with FASTDNA spin kit (MP Biomedicals) according to manufacturer's instructions. The v3-v4 16 rRNA gene and tThe ITS1 region were targeted using respectively Bakt_341F/ Bakt_805R [36] and ITS1-F_KYO2F/ ITS2_KYO2R [37] modified primers with Illumina™ adapters. PCR mix and thermal cycle were identical to previously published protocols [23]. The amplicon presence was checked on 1% gel agarose (90 V, 40 min). Individual tags were added to the PCR products before the sequencing on Illumina MiSeq (2 x 300 nt) according to standard protocols.

Bioinformatics analyses

Demultiplexed paired-end FASTQ reads with trimmed adapters and primers were analysed using QIIME 2 (q2) version 2019-4 [38]. Quality filtering, denoising, merging, chimera removal, and Amplicon Sequence Variants (ASVs) feature tables were done with DADA2 [39] implemented in q2 with default setting for 16s rRNA. For fungi, forward and reverse read lengths were truncated at positions 232 and 182, respectively, to keep high-quality sequences. Sequences were aligned in q2-MAFFT algorithm [40]; then aligned sequences were used to build phylogenetic trees with FastTree 2 [41], which was further rooted (q2 midpoint-root). Rarefaction curves were computed in q2 diversity alpha-rarefaction with 20 steps at 19,200 and 5,789 maximum sequence depth for 16S rRNA gene and for ITS1 ASVs, respectively. ASVs were taxonomically assigned with vectorized search method VSEARCH [42] in the q2 feature classifier plugin. SILVA_132 and UNITE version 8, both at 99% of sequence similarity, were the taxonomic reference databases for bacteria and fungi, respectively. Cytoplasmic contamination sequences (chloroplast and mitochondria) were removed from the bacterial features table with the q2 script taxa filter table. The q2 diversity core metrics phylogenetic plug in was used to calculate alpha (Observed OTUs, Faith Phylogenetic Diversity (Faith PD), Shannon and Pielou's Evenness) and beta

(Bray Curtis, Weighted and Unweighted Unifrac distance metrics) diversity indices on rarefied feature tables.

Metabolic profile of the microbiota

The carbon metabolization profile of 17 microbiota (excepting Gb), of the BCA *P. anomala* strain K and of the pathogen *B. cinerea* strain V was assessed with commercial Biolog Ecoplates PM1 and PM2A microplate systems (Biolog, Hayward, CA, USA), containing 190 different carbon sources [43,44] and following the protocol detailed in supplementary material 4. Each plate contained a negative control corresponding to sterilized water. The repeatability of the method was first checked using two aliquots of the same biological replicate of one selected microbiota called GoGx on a PM2A microplate (Figure Sup. Mat 4). Optical Densities (O.D) at 492 nm wavelength was recorded twice at 10s interval (two technical replicates) with a 96 well plate Spectrophotometer Multiskan Ascent (Thermo Labsystems, Helsinki, Finland) at T0 and each 24h up to 7 days. The results of this test (unpresented results) suggested that 24 h was ere used for downstream analysis to allow fair comparison among the microbiota and because at this moment, we recorded the smallest variability between replicates during the repeatability tests (See detailed protocol in Supplementary material 4).

Statistical data analysis

Comparisons of efficacy in controlling disease development during bioassays were made by the Analysis of Variance (ANOVA) at 5% type I error rate ($\alpha = 0.05$) using RStudio version 3.5.1. Means of the normalized apple wound diameters were separated with the Tukey's HSD test with the Agricolae 1.3-0 [45] R package. Corrected carbon source O.D data were scaled, co-clustered and Spearman correlations visualized in heatmaps with dendrograms using the R package Heatmap [46].

The impact of bacterial or fungal alpha diversity indices on the biological efficacy was estimated with Pearson correlation (α=0.05). In addition, Spearman rank pairwise correlations between all the biological efficacies (alone and at the three tested concentrations with the BCA), the taxa identified and the carbon sources metabolised were computed with the Microbiome R package [47] and plotted on R as heatmaps to drive the identification of beneficial strains and prebiotics of biocontrol. Because Spearman correlation investigates monotonic association rank and does not require parametric distribution, no distribution hypothesis was further assumed. Hence no p-values were inferred for later exploration.

HTS-guided selection of beneficial strain and metabolism-based selection of prebiotics

Carbon sources or taxa whose metabolization or abundance showed a strong correlation with the biocontrol properties of the microbiota were evaluated *in vivo* to confirm their ability to support disease control as prebiotic or beneficial strains respectively.

The microbiota containing the highest abundance of targeted taxa were plated on PDA and R2A media, then each pure colony was purified twice and kept on the media used for isolation. Suspensions were prepared with sterile deionized water for Colony PCR. One millilitre of each suspension was heated during 30s in microwave (750 W) and used directly as DNA templates with ITS1-F [37] and ITS4 [48] primers, following the published protocols. Amplicons were sent to Macrogen (Amsterdam-Zuidoost, Netherland) for purification and dideoxy sequencing (Sanger). BLASTn, analysis against the NCBI ITS databases was carried out to determine the taxonomy of the isolates. The taxonomic assignments were further confirmed in the online BLAST tool UNITE database.

Results

Diverse microbiotas were harvested

The samples' fungal and bacterial taxonomic composition were analyzed from 3,628,894 and 3,605,618 paired-end reads respectively. The reads were clustered into 1,916 bacterial and 5,442 fungal ASV, respectively. Analysis of beta diversity plots by Bray Curtis distance metrics for both bacterial and fungal ASV (Supplementary Material 5) showed that samples were well scattered but tended to cluster by location (PERMANOVA pairwise comparison pseudo-F test q-value < 0.05) [which included several factors like surrounding environment (plants, soil), management practices, and cultivars]. Among the 18 sampled microbiota, Proteobacteria (57 – 94%) and Bacteroidetes (2 – 24 %) were the most abundant bacterial phyla. The two major fungal phyla were Ascomycota (27 – 71%) and Basidiomycota (9 – 63%). Ten and 19 identified genera were present in more than 80% of the samples for bacteria and fungi, respectively, including seven of the eight genera considered as the core microbiota of apple surface. The observed percentages showed a high variability of taxa abundance (Supplementary material 6.a & b) between samples, but similar abundance between replicates. This fulfiled our objective of harvesting contrasted microbial communities for further analyses.

In addition, the average colony firming units (cfu) concentration of all microbiota samples was 2 10^4 +/-1.49 10^4 CFU / cm² of apple surface with a minimum of 2 10^3 CFU/cm² (GoGx microbiota) and a maximum of 6.75 10^4 CFU/cm² (M. *sylvestris* O57 microbiota). These concentrations were used as a proxy to normalise biocontrol assay (Supplementary material 2) and were in accordance with previous isolation studies from apple fruit [30,49].

The microbiota significantly impacted disease development

The ten microbiota tested alone provided a significant control (df=9, F=7.549, p=2.12 10e-10) of the disease caused by *B. cinerea* (strain V) with significantly different efficacies, ranging between 27% and 82% (Figure 1). One microbiota (P) provided a disease control significantly higher than the biocontrol strain K applied at the same concentration (10^5CFU/ml) while seven microbiota (Rf, Po, Api, GoGx, GoTi, R48R0, Cer) presented an efficacy similar to the strain K. Two microbiota (JoTi and Ms) developed a lower protection than strain K.

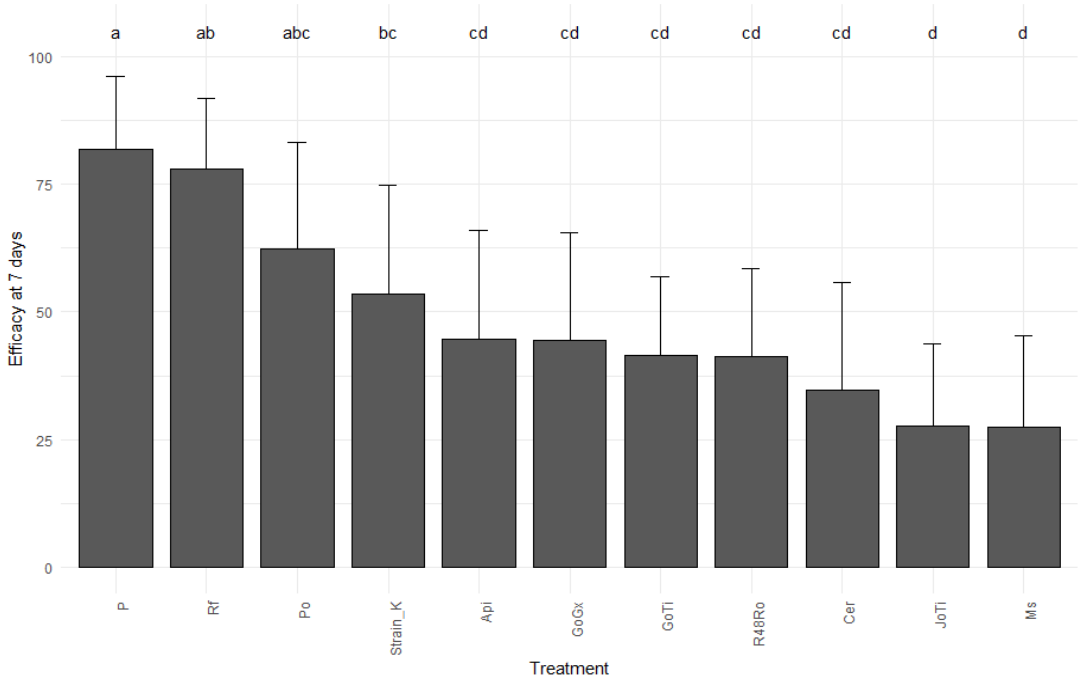


Figure 1. Efficacy of the apple microbiota (applied at 10^5 CFU/mL; n=10) against *B. cinerea* rot development on apple wounds. Isotonic water was used as a control. Letters above each bar represent Tukey statistical mean groups. Error bars on each bar represent standard deviations.

The microbiota modulated the efficacy of the biocontrol agent strain K

Overall, a highly significant impact of the microbiota on the strain K efficacy was observed (df=17, F=14.81, p value = 2 10e-16). The efficacy was improved by 100% for three

microbiota (JoGx, O57 and Plp) or decreased by 27% with Ms (Figure 2). Importantly, the microbiota Cer, JoTi and R48Ro whose efficacies alone were very low, significantly raised the protection against *B. cinerea* when applied with strain K.

The impact of the microbiota was also tested at two other concentrations: 10^4 and $5x10^4$ cfu/ml. An interaction effect (Supplementary Material 7) between the concentration and the microbiota samples was observed (F=2.369, df=34, p< 0.001) but concerned only some microbiota. For example, the 3 microbiotas presenting the highest efficacy increase (+100%) over 10^5 cfu/ml maintained their level of efficacy at the two other concentrations. For the other microbiotas, the efficacy either raised or dropped according to the applied concentration. Nevertheless, a high variability was observed(Figure 2), an often-observed trend in biocontrol as the variability of the bioassay is raising when the efficiency is dropping for a biocontrol agent [50].

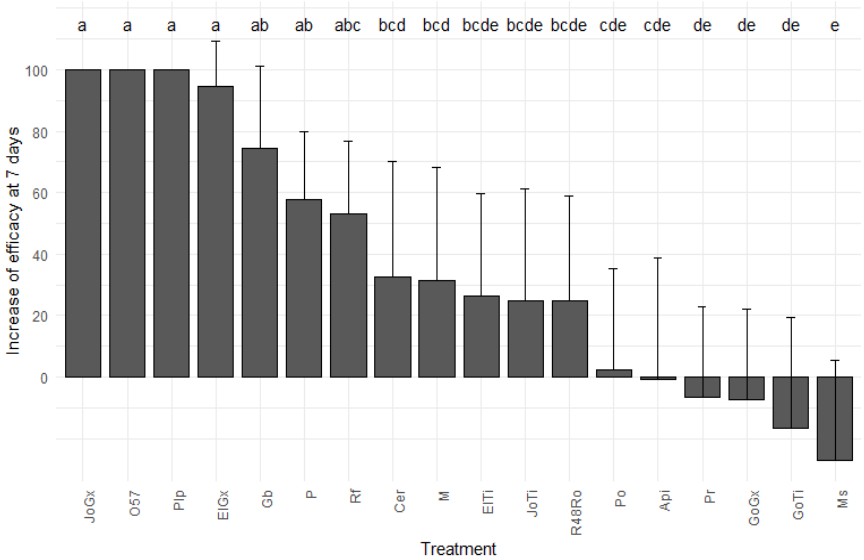


Figure 2. Impact (in percentages of efficacy modification) of the application of apple microbiota (n=18) on the biocontrol efficacy of strain K against *B. cinerea* V on apple wounds. Letters above each bar represent Tukey statistical mean groups. Error bars on each bar represent standard deviations. The strain K applied alone reached an efficacy of 52% and corresponded to 0% in this graph.

The microbiotas are contrasted carbon metaboliser

The repeatability of the test was suitable as the prior evaluation of two biological replicates of GoGx did not show any significant difference in their metabolization profile with the lowest difference observed after 24h (Supplementary material 4). The seventeen microbiotas (all but

Gb microbiota whose stock was empty) showed contrasted profiles of carbon source metabolization and were able to metabolize 186 carbon sources with high variability profile (Supplementary material 8). Among all the carbon sources tested, four (D-threonine, tricarballylic acid, L-threonine and acetamide) were not metabolised by any of the microbiota. Sixteen and eleven carbon sources were metabolised by only one and two microbiota, respectively. Nineteen carbon sources were metabolised by at least 12 microbiota (70 %). No carbon source was metabolised by all the microbiota tested, but the most metabolised were D-galactose and laminarin. In total, 116, 183 and 189 carbon sources were metabolised (D.0 > 0.1) by strain K, *B. cinerea* strain V and strain DSC, respectively. Arbutin, salicin, D-tagatose and D-fructose were highly metabolized (D.O.>0.5) by at least one strain of *B. cinerea* while the most metabolised carbon source was i-erythritol for the BCA.

The 17 microbiota and the 3 strains were clustered according to their metabolization pattern (Supplementary Material 8). The largest cluster included fifteen microbiota; and the strain V, the BCA and strain DSC clustered together. Two microbiota (Plp and M) were distant from the others. The 190 carbon sources were clustered into 5 groups. The clusters 1, 2 and 3 included molecules strongly metabolised by the BCA strain and pathogens but slightly metabolised by most microbiotas. In the cluster 5, some molecules such as L-threonine, oxalomalic acid, beta-hydroxy-butyric acid, and L-arabitol, were much more metabolised by the BCA strain compared to the pathogenic strains and the microbiotas.

The abundance of some taxa is correlated with disease protection, whatever the microbiota

First, bacterial or fungal alpha diversity indexes were not correlated with the efficacy of apple microbiota applied alone or with the BCA. On the other hand, Spearman correlations between biological efficacy (alone and at all concentrations) and taxa abundance identified clusters positively or negatively correlated with the biocontrol activity with or without the strain K. (Figure 3). Cluster 1 corresponded to taxa abundant in microbiota such as members of *Drechslera*, *Galactomyces*, *Chitinimonas* or *Acaulospora* that were associated with biocontrol when co-applied with strain K. Cluster 2 included taxa such as *Kondoa*, *Neofabrae* and *Hymenobacter*, whose abundance in microbiota was correlated with a high protection for the microbiota applied with strain K or alone. Cluster 3 corresponded to taxa, such as *Rhodococcus*, *Variovorax*, and *Polaromonas* that were correlated with high microbiota protection when applied alone but not when the strain K was co-applied, suggesting

antagonism. It would correspond to taxa supporting strain K activity. Other clusters were also of interest. The cluster 4 included taxa, belonging for example to *Ralstonia*, *Janibacter*, *Erwinia*, *Cutibacterium* and *Aeromicrobium* genera, that were more abundant when the microbiota strengthened the disease severity applied alone or with the strain K. Detailed table of these taxa with their correlation values are available in Supplementary Material 9a.

The metabolization of some carbon sources is correlated with disease protection

For the carbon source, the metabolization of Glycyl-L-Proline, D-Fructose-6-_Phosphate, D-Arabinose, L-Asparagine, alpha-D-Glucose, D,L Malic acid, L-Threonine, Glycolic_Acid, L-Fucose and D-Fructose was positively correlated with the efficacy of the microbiota applied alone (Figure 4). In addition, the metabolization of D-Saccharic_Acid, Acetic_Acid, beta-Methyl-D-_Glucuronic_Acid, Lactitol, gama-Amino_Butyric_Acid , L-valine and Inulin was correlated with the efficacy of strain K. Meanwhile, higher metabolisation of molecules such as Xylitol, 2-Deoxy_Adenosine, N-Acetyl-L-_Glutamic_Acid, L-Serine, Methyl_Pyruvate, i-Erythritol, L-Proline and D-Alanine was positively correlated with the protection of strain K or the microbiota. For another cluster of molecules (m-Inositol, Pectin, L-Isoleucine, Maltitol, Maltose, Butyric_Acid and L-Methionine), their higher metabolization was correlated with a lower control of *B. cinerea* by the strain K or the microbiota. Detailed table of these carbon sources correlations values are available in Supplementary Material 9b.

Figure 3. Clustered heatmap of correlations between biological efficacy and taxa abundance in the studied microbiota. Each color represents a correlation, and the strength of the correlation is showed at right side in the legend. Each column represents microbial assigned taxa and each line represents the efficacy of biocontrol assay at each concentration. Blue and red colours indicate positive and negative correlations, respectively.

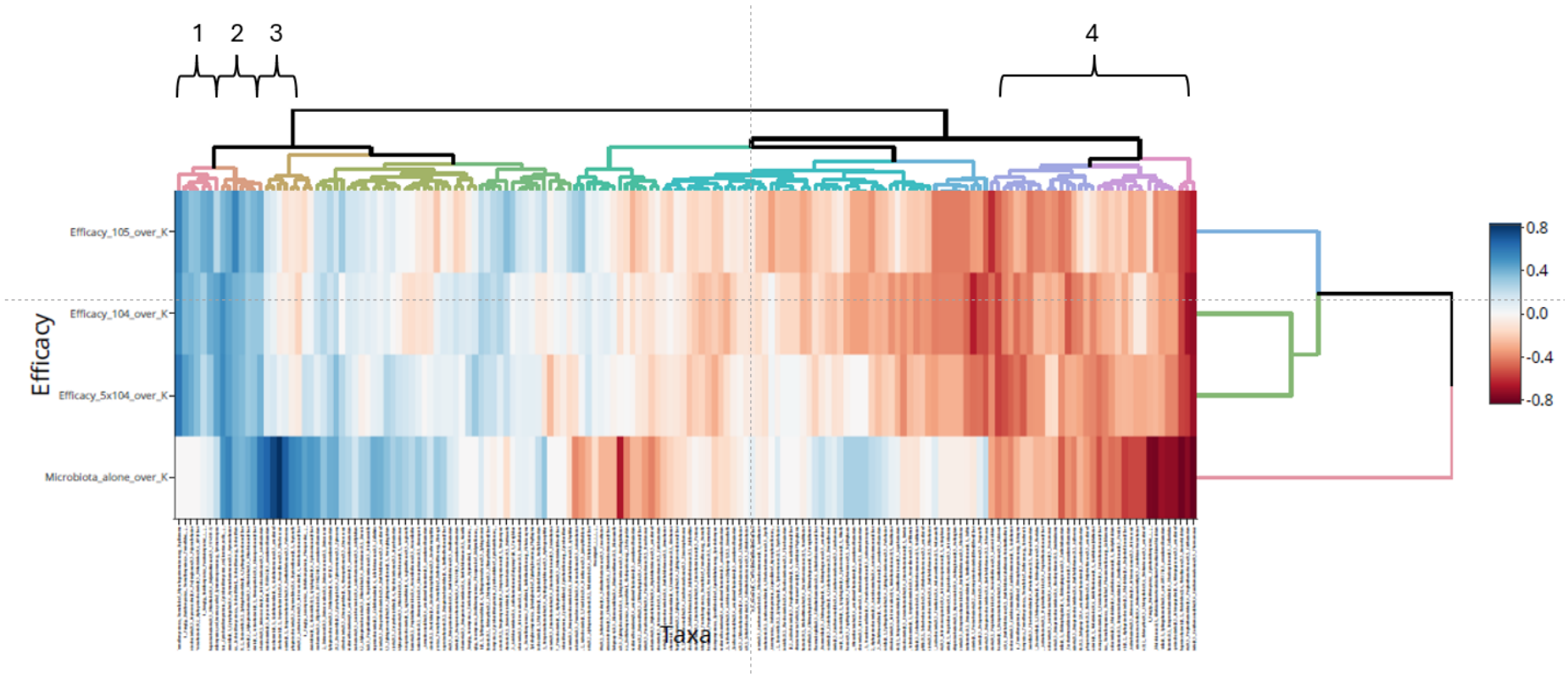
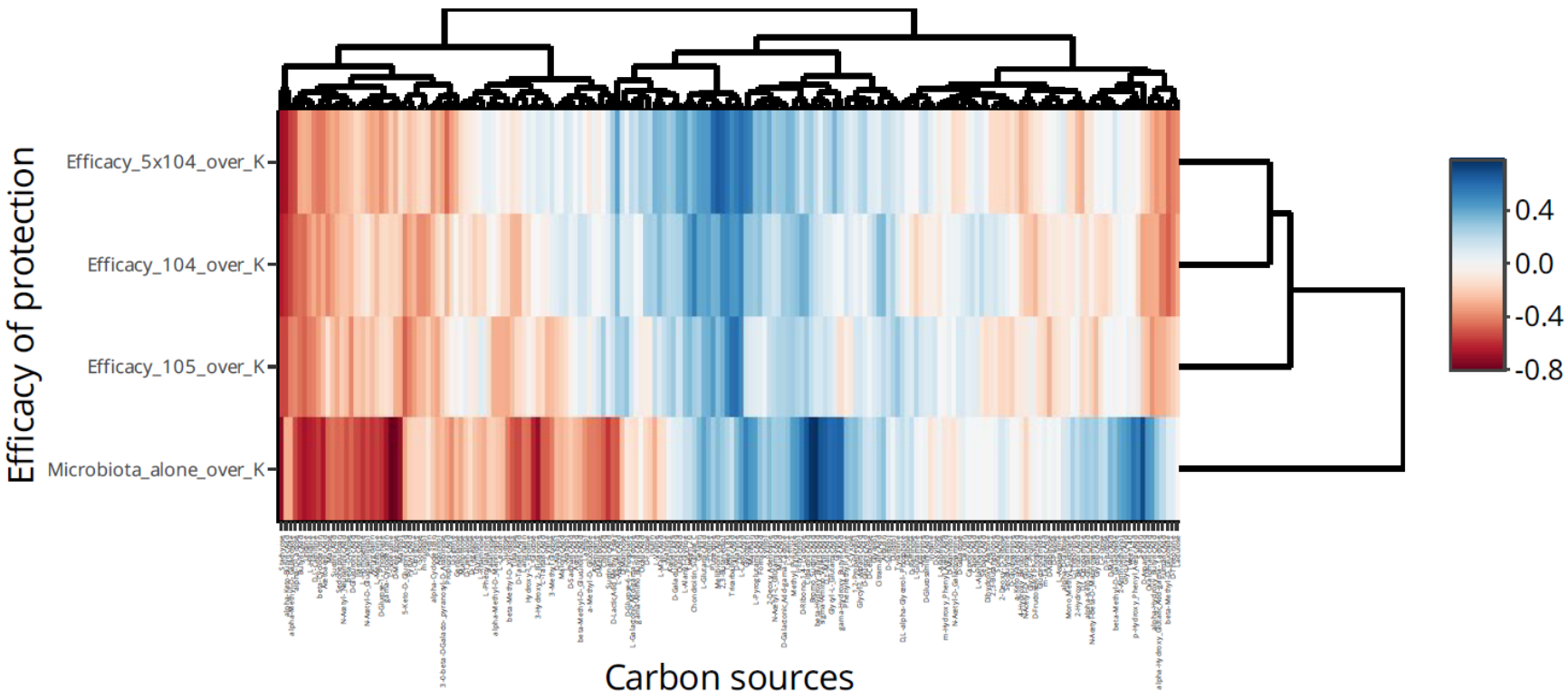


Figure 4. Clustered heatmaps of correlations between biological efficacy of microbiotas (applied alone or co-applied at 3 concentrations with strain K) and the efficacy of carbon source metabolization for the studied microbiota. Each column represents a carbon source and each line represents the efficacy of biocontrol assay at each concentration. Each colour represents a correlation, and the strength of the correlation is showed at right side in the legend. Blue and red colours indicate positive and negative correlations, respectively.



Selection and *in vivo* evaluation of prebiotics for disease control

Eight molecules were selected based on the correlation of their metabolization by the microbiota and the disease control brought by the microbiotas: L-alanine, Meso-erythritol, L-Threonine, Xylitol, GABA, 2-deoxy-D-Ribose, L-proline, and gluconic acid. In addition, their availability, cost and presence in edible food were the additional criteria. Their prebiotic potential was tested with strain K and with a microbiota showing middle to low protection (GoGx). The GoGx microbiota applied without molecule as control brought 30% (C.V. = 52%) of protection while the application of GoGx + strain K achieved a protection of 54 % (C.V.: 62%). Both values were similar to the obtained efficacies during the initial evaluation of GoGx efficiency. The impact of prebiotic molecules applied with GoGx on *B. cinerea* pathogenesis was variable with efficacy ranging from 91% to 4% compared to the control of isotonic water. L-alanine and Meso-erythritol were the most efficient to improve the disease control of GoGx + strain K with 3.6X and 4.9X wound diameter reduction, although with high variability (Table 1).

Treatment	Molecules	Lesion diameter (mm)	Standard deviation (mm)	% of protection	Tukey's statistical analyses
Microbiota + strain K +	L-alanine	1,6	2,5	91%	d
Microbiota + strain K +	Meso-erythritol	2,2	2,9	87%	d
Microbiota + strain K +	L-Threonine	6,7	4,7	60%	cd
Microbiota + strain K +	-	7,9	4,9	54%	bcd
Microbiota + strain K +	Xylitol	8,0	5,4	53%	bcd
Microbiota + strain K +	GABA	8,8	7,7	48%	bc
Microbiota + strain K +	2-deoxy-D-Ribose	10,8	7,6	36%	abc
Strain K	-	11,2	3,2	34%	abc
Microbiota	-	11,9	6,1	30%	abc
Microbiota + strain K +	L-proline	14,3	12,6	15%	ab
Microbiota + strain K +	Gluconic acid	16,3	10,7	4%	a
Isotonic water	-	16,9	5,6	n.a.	a

Table 1: Evaluation of the prebiotic properties of 8 molecules when applied (at 0.5%) with GoGx microbiota (and strain K) against *B. cinerea* through an *in vivo* bioassay on apple.

These two molecules and the xylitol (closest molecule compared to the control) were also tested *in vivo* (at 0.5%) with GoGx or with one of two other microbiotas (Api and GoTi, belonging to the same statistical group as GoGx in term of efficacy) and with strain K (figure 4). The strain K alone achieved a protection of 51 % (C.V. = 41%) compared to the control. Whatever the microbiota, the addition of L-alanine did not raise the disease protection compared to strain K applied alone, including when applied with GoGx, indicating a variable

effect throughout time. The application of meso-erythritol or xylitol to Api and GoGx microbiota significantly improved the protection compared to strain K alone but no difference was observed when added to GoTi.

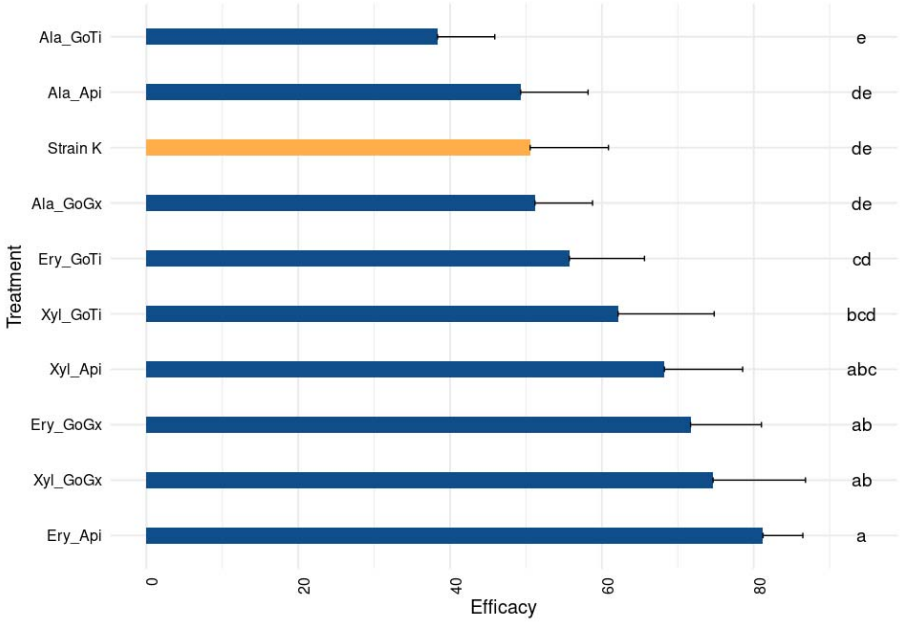


Figure 4. Evaluation of the prebiotic potential of L-Alanine (Ala), meso-erythritol (Ery) and xylitol (Xyl) applied at 0.5% with 3 microbiota (Api, GoGx, GoTi) and the strain K against disease development by B. *cinerea*. The control in orange corresponds to the strain K applied alone. Letters for each bar represent Tukey statistical mean groups. Error bars on each bar represent standard deviations.

Selection of beneficial fungi and *in vivo* evaluation of their efficacy

The three best microbiota (JoGx, O57 and Plp) were selected for their biocontrol properties and their potential higher relative abundance in fungal taxa identified as beneficial. After plating and isolation, 125 strains were isolated, purified, and identified by sequencing their ITS (Many isolates belonged to known BCA genera (*Aureobasidium, Debaryomyces, Metschnikowia, Rhodotorula,* and *Sporobolomyces*) but none of them belonged to the genera highlighted in the beneficial clusters 1, 2 and 3. On the other hand, two isolated strains belonged to species (*Farysia acheniorum* and *Kalmanozyma fusiformata*) included in genera never mentioned in biocontrol. Evaluation of beneficial properties was carried out by *in vivo* biocontrol assay on 12 isolates. *In vivo* assays showed that all the isolated strains provide a significant protection against *B. cinerea* rot (df=11, F=3.541, p= 9.89 10e-05) with an efficacy ranging from 93 to 61 % (Supplementary material 10). Two strains of *Metschnikowia*

pulcherrima and the strain of *Farysia acheniorum* controlled disease development by at least 90%.

Discussion

The impact of resident microbiota on disease development is variable and could rely on a complex network of interactions.

Microbial communities of apple fruit can present attractive, functional biocontrol traits and genes [51], which can be harnessed for the ecological management of postharvest pathogens. Except hypotheses casted in the literature, there has been no evaluation of the impact of epiphytic microbiota on fruit disease development. In this publication, we demonstrated the impact of a diverse set of microbiotas from apple carposphere on disease development and its biocontrol through a combination of standardized *in vivo* bioassays, taxonomic profiling by HTS technologies, and metabolic profiling of carbon sources. The complexity of the interactions network is highlighted by the contrasting results obtained.

First, the harvested microbiota included taxa usually detected on apple fruit and their composition was contrasted. Some genera including pathogenic species on apple were detected, such as *Aeromonas*, *Erwinia*, *Pseudomonas*, *Alternaria*, *Aspergillus*, and *Mucor*. In addition, genera including biocontrol agents, such as *Bacillus, Enterobacter, Pantoea, Rahnella, Aureobasidium, Candida, Metschnikowia, Pichia, Saccharomyces, Trichoderma, Acremonium, Pestalotiopsis, Rhodotorula, Sporobolomyces*, and *Cryptococcus* were identified. Overall, the HTS profile of amplicons from V3-V4 region of 16S rRNA and from ITS1 showed that the most abundant fungal and bacterial phyla and genera were similar to previous studies on apple fruit [17,19–23,25,51]. The alpha diversities metrics (Pilou-eveness, phylogenetic diversity, count observed OTUs and Shannon – see supplementary material 6) and the taxonomy profiles also presented contrasted pattern among the samples collected.

The contrasted microbiotas exhibited variable levels of protection against *B. cinerea*, ranging from 30% to 100%. In our bioassay, the protection, obtained under standardized *in vivo* conditions, primarily depended on the microbiota composition, its growth on the fruit, and its ecological interactions with the pathogen. The pathogen and the members of the microbiota will indeed develop a complex set of interactions from mutualism to competition.

Noteworthy, the pathogenesis of *B. cinerea* was never reinforced, whatever the microbiota. This phenomenon could be attributed to competition for available nutrients and a niche occupation by microbiota members, limiting the pathogen development. This competition for nutrients and/or space can explain why microbiota originating from Kefir grains (a completely different ecological niche) could also inhibit disease development due to *P. expansum* on apple [52]. Compared to the BCA strain K alone, whose efficacy was 50% at the same low concentration of application (10^5 cfu/ml), most of the apple microbiome samples were less effective, probably because in most of them, only a proportion of beneficial microorganisms was implicated (or developed their phenotype) in the biocontrol interactions.

In addition, the microbiotas and, in some cases, their concentration impacted the biocontrol efficacy of the strain K which either raised (up to double) or dropped (down to 27%). The biocontrol properties of the most protective microbiotas (JoGx, O57 and Plp) were not impacted by the 3 tested concentrations while other microbiota presented variable results depending on the concentration. The variable impact of the microbiota recalled the variability of BCA efficacy in real conditions and suggests that the BCA efficacy is strongly influenced by the variability of ecological relationships occurring with the resident microbiota in the processes of biocontrol phenotype expression. Interestingly, three microbiotas raised the efficacy of the BCA strain K while providing only a low protection when applied alone. They may have a synergistic interaction with strain K, allowing biocontrol when combined, and could be a valuable source of helper strains. Helper strains do not present biocontrol properties but support the efficacy of biocontrol strains [53]. On the other hand, the Ms microbiota was the more conductive for pathogenicity as it presented a low protection when applied alone and decreased the strain K efficacy.

The demonstration of the impact of resident microbiota on disease development and biocontrol strain efficacy open an innovative research area to understand this phenomenon. Omics technologies have been widely used to understand the mode of action of biocontrol agents in the past [54]. In this context, future research should deploy them to follow the evolution of the composition and metabolism of the microbiota over time when interacting *in situ* with a plant pathogen or a biocontrol agent. Interaction models [55]used to decipher in situ BCA-pathogen interaction could be adapted to the study of microbial communities within a proper ecological framework [1].

Which component of microbiota alpha diversity is important for pathogen control?

Our results represent the first interventional study on fruits where resident microbiota are harvested and further tested for biocontrol properties. A higher richness or evenness was not associated with the higher biocontrol efficacy of apple fruit microbiota against *B. cinerea*. Nevertheless, observational studies comparing healthy and diseased plant material suggested that high alpha bacterial diversity promoted disease control [56]. For example, high bacterial diversity was linked to tobacco wilt disease control in soil [57] and tobacco wildfire disease control in phyllosphere [58] . In other studies, both bacterial and fungal alpha diversity indices were significantly higher in healthy sugar beet (Kusstatscher, Cernava, *et al.* 2019), or healthy rhizosphere, rhizoplane and endospheric tomato plant free of *Fusarium oxysporum* sp. *lycopersici* infection [59]. A first explanation of this apparent contradiction is that our results are unique as they are based on bioassays with microbiota application, not on observations prior or after disease development. Also, we tested one standardised condition and evaluating the association of diversity with biocontrol under wider range of bioassay conditions might provide a more complete overview. Our results uncover the complexity of interactions: beyond the diversity of the microbiota, the crucial factor could be the active members of the network developing biocontrol properties and their ecological interactions. This complexity was also recently suggested by a large-scale observational study on the association between *Arabidopsis* leaf- and root-associated microbiota and disease development. The authors suggested that potential microbiota biomarkers controlling disease development were highly dynamic as a significant humped-back relationship was observed between the alpha diversity of microbiota and pathogenesis [60]. In another study based on bioassays, a simplified community of 4 strains had a similar effect on root-rot disease development of *Astragalus mongholicus* than a more complex community of 13 strains [7].

Microbiome-guided selection of beneficial microorganisms by HTS taxonomic profiling can be strengthened by functional characterization of microbiota but remains a challenge

An HTS-guided strategy can be applied to select beneficial microorganisms whose presence or abundance in microbial communities is associated with beneficial properties. This concept is recent and has only been applied on lettuce[8], grapevine[61], citrus[9] or *Coptis*

chinensis[62], *A. mongholicus* [7] and *A. thaliana* [60]. Theoretically, this selection drastically reduced the number of isolated strains to be tested as new beneficial microorganisms (including from taxa unsuspected to have a beneficial effect) compared to empirical methods (blind microorganism isolations and biocontrol testing).

Nevertheless, this strategy has biological and technical limitations. First, microorganisms present at very low proportions can play a fundamental role in the microbiota functions [63], but their low proportion, often accompanied by higher variability, will limit their probability of being highlighted and selected. In addition, if the comparison is carried out between microbiota of healthy and diseased tissue, it can introduce a bias as the microbiota modification on diseased tissue might be a consequence of disease development without influencing the first stages of disease initiation. Our approach of microbiota application before pathogen inoculation limited this bias although the taxonomic composition of the microbiota might have changed between the application on the wounds and the application of the pathogen 24 hours later, reinforcing the need of analysing the evolution over time. At technical level, a HTS test includes many steps, each of them being a potential source of bias, from primer selection to taxonomic assignation [64]. For example, the taxonomic accuracy of metabarcoding is usually not reaching the species level, although this challenge might be progressively solved by the advances in long-read sequencing technologies. The second technical challenge might be more complex to address. Indeed, strain isolation protocols targeting specific taxa might be missing as some taxa could be difficult to isolate due to the lack of information on their ecology or impossible to culture under the current knowledge. One way to tackle this challenge might rely on culturomics technics as currently explored in human gut studies [65].

During this study, we could not isolate strains of the genera identified in beneficial clusters, confirming the difficulty of HTS-guided selection. This challenge was compensated by another approach relying on the isolation of strains from the most beneficial microbiota. Most isolated taxa were already mentioned in the literature for their biocontrol properties but, one strain belonged to *F. acheniorum*, a species never cited as biocontrol agent on plants. This strain showed a biocontrol efficacy of 90% at 10^7 cfu/mL against *B. cinerea*. Another isolated microorganisms "new to biocontrol", such as *Kalmanozyma fusiformata*, also presented biocontrol properties. Both strains should be better characterized for their biocontrol properties and metabolisms to evaluate their compatibility with existing biocontrol strains, for

example, within synthetic communities. Our approach was *in fine* guided by biocontrol properties of the microbiota and showed interest for isolating a more diverse set of putative beneficial microorganisms.

Engineering the microbiota for disease control by targeted selection of prebiotics molecules

The functional catabolic diversity of microbiota can be evaluated by studying their ability to metabolize various sources of nutrients. Such functional characterization can lead to the identification of prebiotics of biocontrol [29]. These molecules are substrates that can modulate the microbial composition of the microbiota toward an improved control of disease development. For instance, a rare sugar (tagatose) has been tested as a prebiotic of biocontrol to control grapevine leaf downy and powdery mildew, causing a shift in the leaf microbiota [66]. The application of glutamic acid has also reshaped tomato rhizosphere by increasing the abundance of several taxa while, at the same time, disease symptoms were reduced [14]. Our study showed that the tested microbiota metabolized well molecules present on apple such as fructose, sucrose, glucose, rhamnose, xylose, galactose, arabinose, galacturonic acid, and several amino acids (serine, glycine, threonine, alanine, proline, valine).

Correlating the metabolic profile of microbiota with their biocontrol properties when applied alone or in the presence of strain K identified several clusters of potentially valuable molecules with high Spearman correlations with the biocontrol properties. Eight of these molecules were further tested in bioassays including a microbiota, the strain K and/or the pathogen. The results were variable (from -50% to +37% in efficacy) depending on the molecule. This observation suggested that the large-scale correlations carried out should be considered as a preselection and underlined the need to confirm in vivo the candidate molecules. The L-xylitol and Meso-erythritol increased the efficacy of the microbiota alone, and in the presence of strain K. . These two molecules are rare sugars at low concentrations in apple fruit. Rare sugars have been shown to impact pathogen development and biocontrol of BCA. For instance, the rare sugar D-tagatose inhibited the growth of pathogens like *Phytophthora spp*. [67] and increased the efficacy of BCA on many fruits and vegetables [68]. L-xylitol and meso-erythritol correspond to the most promising biocontrol molecules in this pathosystem and deserve further characterization of their mode of action. Indeed, meso-erythritol was not well metabolized by any of the 3 tested microbiota (Api, GoGx, GoTi) and

the two pathogenic strains of *Botrytis*, but was the highest molecule metabolized by the strain K. This can explain why this molecule had increased the biocontrol activity in the presence of the strain K. Without the strain K addition, this molecule may have promoted other beneficial microorganisms toward a biocontrol phenotype. Again, following over time the evolution of the composition and metabolism of the microbiota and the strain K in situ after their application with the molecule is of prime interest. On the other hand, another selected carbon source, L-alanine, was reported to increase the efficacy of BCAs on apple [69,70] and pear [71] but did not improve the efficiency of the microbiota in our bioassay, even in the presence of strain K that metabolized it very efficiently compared to the microbiotas and *B. cinerea*.

Overall, as suggested by our results, the targeted selection of molecule could be envisioned as part of the biocontrol strategy against plant pathogens, applied alone to modify the resident microbiota or applied with BCA applied alone or in synthetic communities to foster the expression of their biocontrol phenotype. Beyond our exploratory study, a broader screening of molecules, preferably recognized as GRAS, could be envisaged to improve the field efficacy of biocontrol agents applied alone or in synthetic communities.

Conclusion

This study demonstrated that apple fruit epiphytic microbiotas significantly influenced disease development by postharvest pathogens with or without the co-application of a beneficial strain. The tested microbiotas also showed contrasted taxonomic and carbon metabolization profiles, allowing the identification of promising molecules (that can be called biocontrol prebiotics) and microbial strains for disease control. Our results also show the importance of the pathobiome concept and underline that residing microbiota is an essential component to be considered when developing solutions to control postharvest diseases. A thorough understanding of this role could identify more biocontrol prebiotics or beneficial strains in the future. They could shift the microbial composition toward an ecological network favouring disease control or the efficacy of biocontrol organisms.

Declaration

Ethics approval and consent to participate

Not applicable

Availability of data and materials

Sequence data are available on the National Centre for Biotechnology Information (NCBI) under the Sequence Read Archive (SRA) accessions PRJNA663271 to PRJNA662931. Other data are provided within the manuscript or supplementary information files.

Competing interest

The authors declare that they have no competing interests

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Authors' contribution

A.R.S.: design of the experiments, sampling, laboratory and bioinformatics analyses, data interpretation, paper writing;
M.H.: design of the experiments, data interpretation, paper edition;
S.M.: design and supervision of the experiments, sampling, data interpretation, paper writing

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Supplementary materials

Supplementary material 1. Details on the sampled apple fruit for epiphytic microbiota harvest

Supplementary Material 2. Additional details on microbiota harvesting protocols and evaluation of culturable bacteria and fungi (including yeast).

Supplementary material 3. Detailed protocol on biocontrol in vivo bioassay

Supplementary material 4: Metabolic profiling of the microbiotas

Supplementary material 5: Beta-diversity plots for the harvested microbiotas

Supplementary material 6: Taxonomy composition and alpha diversity indexes of harvested microbiotas

Supplementary Material 7: Impact (in %) of 18 microbiotas applied at 3 concentrations on the biocontrol efficacy of strain K against *Botrytis cinerea*

Supplementary material 8. Co-clustering analysis of the metabolization of 190 carbon sources by 17 apple fruit microbiota

Supplementary material 9. A: Detailed table of Spearman correlation between taxa identified by metabarcoding and the efficacy of microbiotas and B: Detailed table of Spearman correlation between carbon metabolisation intensity and the efficacy of microbiotas