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(Article begins on next page)

Genetic and functional characterization of the bacterial community on fruit of three raspberry (*Rubus idaeus*) cultivars

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Abstract.

BACKGROUND: Raspberry breeding programs allowed the development of highly yielding cultivars, but often resulted in a reduction of plant plasticity and resistance to abiotic and biotic stresses. The epiphytic bacterial community on leaves and fruit may play a crucial role in overcoming these shortcomings, influencing host performance and health status.

OBJECTIVE: The bacterial community associated to two red-fleshed (Imara and Regina) and one white-fleshed (Anne) raspberry varieties was described. The bacterial community was functionally characterized to identify strains with plant growth promoting or plant protection traits.

METHODS: Microbial community was assessed using both culture-independent and -dependent methods. Strains were tested for production of acetoin, siderophores, indoleacetic acid and ammonia, ACC deaminase activity, biofilm formation, biological control of *Erwinia amylovora*, *Botrytis cinerea* or *Drosophila suzukii*.

RESULTS: The fruit bacterial community clearly differed between red- and white-fleshed raspberry. Thirteen isolates produced plant growth and resistance promoting substances, while twelve bacterial isolates were able to manipulate either auxin or ethylene metabolism. Five strains inhibited the growth of *Erwinia amylovora* and *Botrytis cinerea*, and one increased *Drosophila suzukii* mortality.

CONCLUSIONS: This study also offers new insights for the biotechnological exploitation of bacteria isolated from raspberry.

Keywords: *Rubus idaeus*, metagenome, plant growth promoting, biological control, *Erwinia amylovora*, *Drosophila suzukii*, *Botrytis cinerea*, IAA, acetoin, ACC deaminase, siderophore

1. Introduction

Plants offer different specialized niches to microbial communities, that create a continuum from the rhizosphere to the endosphere and the phyllosphere [1]. Plant-microbe interactions and plant-associated microbial

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communities have been widely studied in the rhizosphere, whereas the study of epiphytic communities has received growing attention only in the recent years [2]. Nonetheless, phyllosphere can be densely populated by microorganisms that can act as endophytes, when present within plant compartments or as epiphytes, if anchored on the surfaces of plants, for instance on the cuticle [2–4]. Bacteria form the dominant group, reaching a surprisingly dense population of approximately 10^4 – 10^5 bacteria mm^{-2} of leaf surface or up to 10^8 bacteria g^{-1} leaf material [5]. Some bacteria, described as plant growth promoting bacteria (PGPBs), play important roles in determining plant fitness [6]. PGPBs directly benefit host plants via hormone production (e.g., indoleacetic acid, cytokinin, and zeatin) and improved nutrition, or indirectly by acting as biocontrol agents, inducing systemic resistance and ethylene stress resistance [2, 6–9].

The phyllosphere of different plant species has recently been the subject of in-depth sequencing approaches, which generated comprehensive catalogues of microbial life [10–12]. These studies demonstrated that the phyllosphere is colonised by a diverse microbiota, which is plant species-specific. At higher phylogenetic ranks, leaf-associated bacterial communities consist of recurring taxa, whereas the composition may differ at the species level. In this context, high-throughput technologies have provided comprehensive datasets portraying microbial life in the phyllosphere by employing transcriptomics and metaproteogenomics, a combination of shotgun metagenomics and proteomics [13].

Raspberry (*Rubus idaeus*) is one of the most valuable horticultural crop with an annual production reaching, in 2016, 795,249 tons (FAO Stat). Raspberry is an ancient fruit that has been traced back to the Middle Age when the wild fruit were used for medicinal purposes [14]. Recently, several studies highlighted the benefits of raspberry to human health. In animal and human studies, the consumption of raspberries has been associated with a decreased risk of developing several chronic diseases, including cardiovascular disease, type 2 diabetes mellitus (T2DM) and several types of cancer [14–16]. Several constraints affect raspberry yield, quality and nutraceutical characteristics. Among them, pests and pathogen attacks are crucial threats for the sustainable production of high quality berries. Indeed, considerable pesticide inputs are needed in raspberry cultivation to control fungal (e.g. *Botrytis cinerea*, *Sphaerotheca macularis*) and bacterial (e.g. *Erwinia amylovora*, *Pseudomonas syringae*) diseases and pests (e.g. *Drosophila suzukii*).

Despite the importance of microorganisms in influencing plant health status, performances, productivity and fruit quality and storability, the study of the microbial community of raspberry phyllosphere has been neglected.

A more holistic understanding about bacteria inhabiting this niche in raspberries is needed to better understand the intermicrobial interactions within the plant microbiota and to better define the functional relevance of the microbial networks for plant fitness and health status. Therefore, in this study, culture-independent (NGS) and -dependent (bacteria isolation and typing) methods have been applied to characterize the microbiome of three raspberry cultivars (“Regina”, “Imara” and “Anne”). These cultivars have been chosen mostly due to their organoleptic and textural differences and by their contrasting secondary metabolite composition, being “Regina” and “Imara” red-fleshed, while “Anne” is white-fleshed.

Isolated strains were also subjected to a functional characterization either related with plant growth promotion (i.e. production of acetoin, siderophore, indoleacetic acid and ammonia, ACC deaminase activity assay and biofilm formation) or plant protection (i.e. biological control of *Erwinia amylovora*, *Botrytis cinerea* or *Drosophila suzukii*).

2. Materials and methods

2.1. Sample origin

Three raspberry cultivars were studied: “Anne” (white flesh), “Regina” and “Imara” (red flesh). Plants were grown in the experimental field of Fondazione Edmund Mach - Research and Innovation Centre at Pergine

(Trento), located in the north of Italy (Trentino Alto Adige region - 46.0744°N, 11.2334°E, 525 m a.s.l.). Raspberry plants were all grown in 7 L pots, under hail net. A fertigation system was applied to guarantee water supply with a commercial fertigation recipe for raspberry. Five plants for each accession were maintained following standard pruning. Berries were harvested manually at the full ripe stage, and brought to the laboratory within half an hour from picking time.

2.2. Metagenomic analysis

2.2.1. DNA extraction and sequencing

Ten g of raspberry fruit were washed with 90 mL of NaCl solution (8.5 g L⁻¹). To extract all bacteria associated with phyllosphere, washing was carried out for 15 min under gentle agitation (100 rpm) at 4°C temperature to avoid mechanical tissue damage and bacterial multiplication. The washing solutions were centrifuged at 20,000 × g for 20 min at 4°C. The pellet of each sample was immediately frozen and stored at -20°C before DNA extraction.

Genomic DNA was extracted and purified using NucleoSpin® soil kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to manufacturer's instructions. V3-V4 regions were amplified with 16S Amplicon PCR Forward = 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and Reverse = 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC primers according to Illumina protocols.

PCR products were purified using the Agentcourt® AMPure® XP Beads (Beckam Coulter Company, Brea, USA), checked for quality using a Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany) and quantified with Qubit® fluorometer (Thermo Fisher Scientific, Waltham, USA). The amplicons were coupled to dual indices and Illumina sequencing adaptors attaches using the Nextera XT Index Kit (Illumina), pooled in equal proportions and sequenced paired-end in an Illumina MiSeq (Illumina Inc., San Diego, USA) at IGA Technology Services (Udine, Italy). To prevent focusing and phasing problems due to the sequencing of "low diversity" libraries such as 16S amplicons, 30% PhiX genome was spiked in the pooled library.

2.2.2. Bioinformatic analysis

Trimomatic was used to remove low-quality reads using a sliding window of 50 bp length with an average phred score ≥ 20 [17]. Sequences shorter than 120 bases were discarded. The sequences were analyzed using the Mothur software package version 1.35.1 [18]. The paired-end reads were assembled and aligned to the SILVA 16S rRNA sequences database [19]. Sequences were de-noised to remove sequencing error with the command "pre.cluster" and chimeric sequences were removed using the VSEARCH algorithm [20] implemented in Mothur. Sequences were clustered into OTUs at 97% sequence identity using the nearest neighbor clustering methods. The sequences were classified using the references Ribosomal Database Project database (RDP) provided in Mothur. OTUs that were singletons and doubletons were removed. Alpha diversity (Pielou and Shannon indices) were calculated using the 'vegan' package v 2.0-5 of R (<http://cran.r-project.org>, <http://vegan.r-forge.r-project.org/>).

2.3. Culture dependent approach

2.3.1. Bacteria isolation

Berry fruits (10 g) were blended in a solution of NaCl (8.5 g L⁻¹) in a Stomacher Lab-Blender 400 (Steward Medical, London, UK). Samples were serially diluted and plated on Luria-Bertani (LB) medium (Fisher Scientific) which were incubated under microaerophilic conditions at 30°C for 48 h. Colonies were randomly collected from the plates at the highest dilution. The random colony selection from the highest dilution plates allowed us to collect the most frequent and predominant species present in each sample. All the isolates were

stored at -80°C in LB broth supplemented with glycerol (20% vol/vol) (Sigma-Aldrich Srl., Milan, Italy) or on LB agar at 4°C for short-term storage.

2.3.2. Strains identification

DNA was extracted using Bacterial DNA isolation kit (Sigma-Aldrich, Milan Italy) according to manufacturer's instructions. 16S rRNA gene amplification was performed as previously described [21], using Lac16S-for (5'-AATGAGAGTTTGATCCTGGCT-3') and Lac16Srev (5'-GAGGTGATCCAGCCGCAGGTT-3') primers. The amplified fragment was then purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences AB, Uppsala, Sweden), according to the manufacturer's instructions and after drying was delivered to BMR Genomics (Padova, Italy) for sequencing. The obtained sequences were compared to those available in the GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST>) and those of the Ribosomal Database Project (<http://rdp.cme.msu.edu/index.jsp>) to determine the closest known relative species based on 16S rRNA gene homology.

2.3.3. Strains typing

Fingerprints of genomic DNA were obtained amplifying repetitive bacterial DNA elements (rep-PCR). PCR amplification was carried out using (GTG)₅ primer as described by Gevers et al. [22]. Amplification was performed on a MyCycler (Bio-Rad) with an initial denaturation at 94°C for 5 min followed by 30 cycles consisting of 1 min at 94°C , 1 min at 40°C , 8 min at 72°C and a final extension of 15 min at 72°C . PCR products were separated on a 1.5% (w/v) agarose gel in $1 \times$ TAE buffer. 1 kb Plus DNA (Invitrogen) was used as a marker. After electrophoresis, the gels were stained and photographed under UV transillumination as reported above. The repeatability of rep-PCR fingerprints was determined by triplicate loading of independent triplicate reaction mixtures prepared with the same strain. Conversion, normalization, and further analysis of the rep-PCR patterns were carried out with Fingerprinting II InformatixTM software program. Similarities among profiles were calculated by clustering the Pearson's correlation matrix using the Unweighted Pair-Group Method with Average (UPGMA) algorithm.

2.4. Functional characterization

2.4.1. Acetoin production

The Voges-Proskauer test was carried out according to Blomqvist et al. [23]. One mL of 0.3% creatine, 3 mL of freshly prepared 5% (w/v) α -naphthol in absolute alcohol, and 0.6 mL of 40% (w/v) KOH were added to 5 mL of bacterial culture supernatant. The acetoin formed as a result of glucose metabolism is oxidized in alkaline conditions to diacetyl, which reacts with the guanine group of creatine and gives a red colour in presence of α -naphthol [24]. All analyses were performed in duplicate.

2.4.2. Siderophore production

Siderophore production was tested using Chrome azurol S agar medium according to Schwyn and Neilands [25]. Plates were inoculated using a final concentration of bacteria of 10^6 CFU mL⁻¹ and incubated at 30°C for 48–72 h. Development of yellow-orange halo around the growth was considered as positive for siderophore production. All analyses were performed in duplicate.

2.4.3. Indoleacetic acid (IAA) production

The test was performed according to Ahmad et al. [26]. Briefly, fully grown cultures were centrifuged at 3000 rpm for 10 min. The supernatant was mixed with two drops of orthophosphoric acid and 4 mL of the Salkowski reagent (50 mL, 35% (v/v) of perchloric acid, 1 mL 0.5 M FeCl₃ solution). Development of pink colour indicates IAA production. All analyses were performed in duplicate.

2.4.4. Ammonia production

Freshly grown cultures were inoculated in 10 mL peptone water in each tube and incubated for 24 h at 30°C. Development of brown to yellow colour after the addition of 0.5 mL of Nessler's reagent considered as positive for ammonia production [27]. All analyses were performed in duplicate.

2.4.5. ACC deaminase activity assay

Plant growth promoting rhizobacteria lower ethylene levels by deamination of 1-aminocyclopropane-1-carboxylic acid (ACC) the immediate precursor of ethylene in plants. The enzyme catalyzing this reaction, ACC deaminase, hydrolyzes ACC to 2-ketobutyrate and ammonia. ACC deaminase activity was determined according to Penrose and Glick [28] and expressed as the amount of 2-ketobutyrate produced per mg of protein per hour. The 2-ketobutyrate produced was evaluated by comparing the absorbance at 540 nm of the sample to a standard curve of 2-ketobutyrate ranging between 0.1 and 50 µM. All measurements were made in five replicate samples and averaged.

2.4.6. Biofilm formation

The colorimetric assay of biofilms with crystal violet staining was performed according to Burton et al. [29]. Briefly, strains were inoculated in a microtiter plate wells and incubated for 24 h at 30°C. Media and unattached cells were removed, while the microtiter plate wells were washed three times with 200 µL of PBS (0.1 M, pH 7.4). The wells were stained with crystal violet 0.1% (w/v) for 15 min. Wells were washed 3 times with PBS to remove the unbound crystal violet and then air-dried for 15 min. The crystal violet in each well was solubilized by adding 200 µL of ethanol. The plate was read at 630 nm using a microtiter plate reader (Multiskan Ascent, Labsystems, Helsinki, Finland). Analyses were performed in triplicate.

2.4.7. Cell surface hydrophobicity determination

Cell surface hydrophobicity (CSH) was determined as previously described [30]. Selected bacterial strains were grown over night in LB medium. At the end of incubation cells were harvested by centrifugation and resuspended in PBS to obtain a final OD_{600 nm} of 0.8 (A0). One mL of hexadecane was added to cell suspension and after a 10 min-incubation at room temperature, the two-phase system was mixed by vortexing for 5 min at 1400 rpm. After a 15 min incubation at room temperature the aqueous phase was removed and its OD_{600 nm} was measured (A1). The percentage of microbial adhesion to solvent was calculated as follow: $(1 - (A1/A0)) \times 100$. Analyses were performed in triplicate.

2.4.8. Antagonistic activity against *Erwinia amylovora* and *Botrytis cinerea* in vitro

The effect of strains on *B. cinerea* development was tested as previously described [31]. The antibacterial activity against *Erwinia amylovora* was assessed according to Spinelli et al. [32] with some modifications. Antagonistic activity was assessed on LB diluted 1:2 and agar overlays were prepared by mixing 500 mL of melted agar and 20 mL of bacterial suspension at 10^8 CFU mL⁻¹. Antagonist colonies were transferred to the surface of the overlay agar plates with a loop and the plates were incubated at 26°C for 48 h. Antagonism was detected as the presence of a halo of inhibition. Analyses were performed in triplicate.

2.4.9. Insecticidal activity against *Drosophila suzukii*

Three bacterial-cell concentrations (2×10^6 , 2×10^4 , and 2×10^2 cells mL⁻¹, suspended in sterile 10 mM MgSO₄ solution) of *Er. aphidicola* were tested against adults of *D. suzukii* (2–3 weeks old). After mixing 1:1 (v/v) with a 20 mL/L agar solution maintained at 50°C, the bacterial suspension or supernatant were spread on the walls of a 50 mL Falcon tube. Five to ten insects were included in the tube, together with about 1 g fresh-cut cherry slices, and closed with a cotton tissue to allow air exchange. Controls were treated with sterile MgSO₄ solution

and LB medium. Each treatment was replicated 3 times. The tubes were incubated as previously described, and mortality was recorded every two days until 9 DAA.

2.5. Statistical analysis

Bioassay data were analyzed by one-way analysis of variance (ANOVA) followed by means separation with Fisher's least significant difference (LSD), using the appropriate models for a completely randomized design (laboratory bioassays) (SAS Institute, 2010). Percentages of adult mortality were transformed to arcsine [$\sqrt{\% \text{mortality}}$] before analysis to stabilize variance and reported means were back-transformed to percentages for presentation. Data were expressed as mean $\pm$ standard error (S.E.). Differences were considered significant at $p < 0.05$ and $p < 0.01$ level. Abbott's formula [33] was used to correct for control mortality (efficacy).

3. Results and discussion

3.1. Determination of raspberry microbiota through culture-dependent and -independent approaches

Sequencing of the bacterial 16S rRNA was performed to characterize the microbial communities. At phylum level, fruit from "Regina" and "Imara" (red flesh) were dominated by Proteobacteria, followed by Actinobacteria and Firmicutes. In "Anne" (white flesh), Firmicutes and Proteobacteria were almost equally abundant, while Actinobacteria were detected in a lower concentration (Fig. 1A). These three phyllosphere-associated generalists phyla are the most abundant in the phyllosphere of several plant species, suggesting substantial overlap in the key community members across host species [4, 7].

The three tested raspberry cultivars differed in terms of main genera present. "Anne" was characterized by the dominance of *Bacillus* followed by *Tatumella* and *Ochrobactrum*, while *Sphingomonas* was the main genus in "Imara" and "Regina" (Fig. 1B). *Bacillus* species are natural inhabitants of the phyllosphere [34] and have been shown to suppress plant diseases caused by microorganisms such as *Pythium torulosum* [35], *Pseudomonas syringae* [36], and *Erwinia amylovora* [37]. *Tatumella* genus is closely related to its sister taxon *Pantoea* [38, 39], which is commonly associated with plants including strains excreting antagonistic activities against bacterial plant pathogens [40]. Bacteria belonging to the genus *Tatumella* have also been found to be frequently associated with *D. suzukii* [41], one of the main raspberry pests. *Tatumella* was found to be the dominant bacterial genus in *D. suzukii*, constituting between 31% to 99% of total microbiota associated to *D. suzukii* adults and larvae feeding on undamaged cherry fruit [41]. Moreover, this genus was found to be characteristic of *D. suzukii* while being absent, or at minimal levels, in other species of *Drosophila* [41, 42].

Some *Ochrobactrum* species (e.g. *O. tritici*, *O. lupine* and *O. grignonense*) are considered as valuable candidates for biodegradation of phenol-contaminated soils [43]. Moreover, members of this genus are able to produce IAA, solubilize inorganic phosphate/zinc and increase N uptake by plants [44].

The genus *Sphingomonas* include a number of plant-protective species, able to suppress disease symptoms and decrease pathogen growth [45]. Innerebner et al. [46] showed that the inoculation with a *Sphingomonas* sp. strain reduced the population size of the plant pathogens *Pseudomonas syringae* pv. tomato DC3000 and *Xanthomonas campestris* pv. *campestris* LMG 568 on *Arabidopsis* leaves.

Shannon and Pielou biodiversity indexes were also calculated. The Shannon index (H) is the most widely used index based on species richness, and it is sensitive to changes in rare species. The Pielou index (J) is an index of evenness, and ranges from 0 to 1, with 1 representing perfect evenness and 0 complete dominance. A high biodiversity was observed in "Regina" (H = 2.63), followed by "Anne" (H = 0.86) and "Imara" (H = 0.68). Conversely, "Imara" and "Anne" showed the lowest J index (J = 0.19 and 0.31, respectively) being its microbiome dominated by one single bacterial genus, while "Regina" presented the highest index (J = 0.63), being *Sphingomonas*, the abundant genus, barely 40% of the whole microbiome.

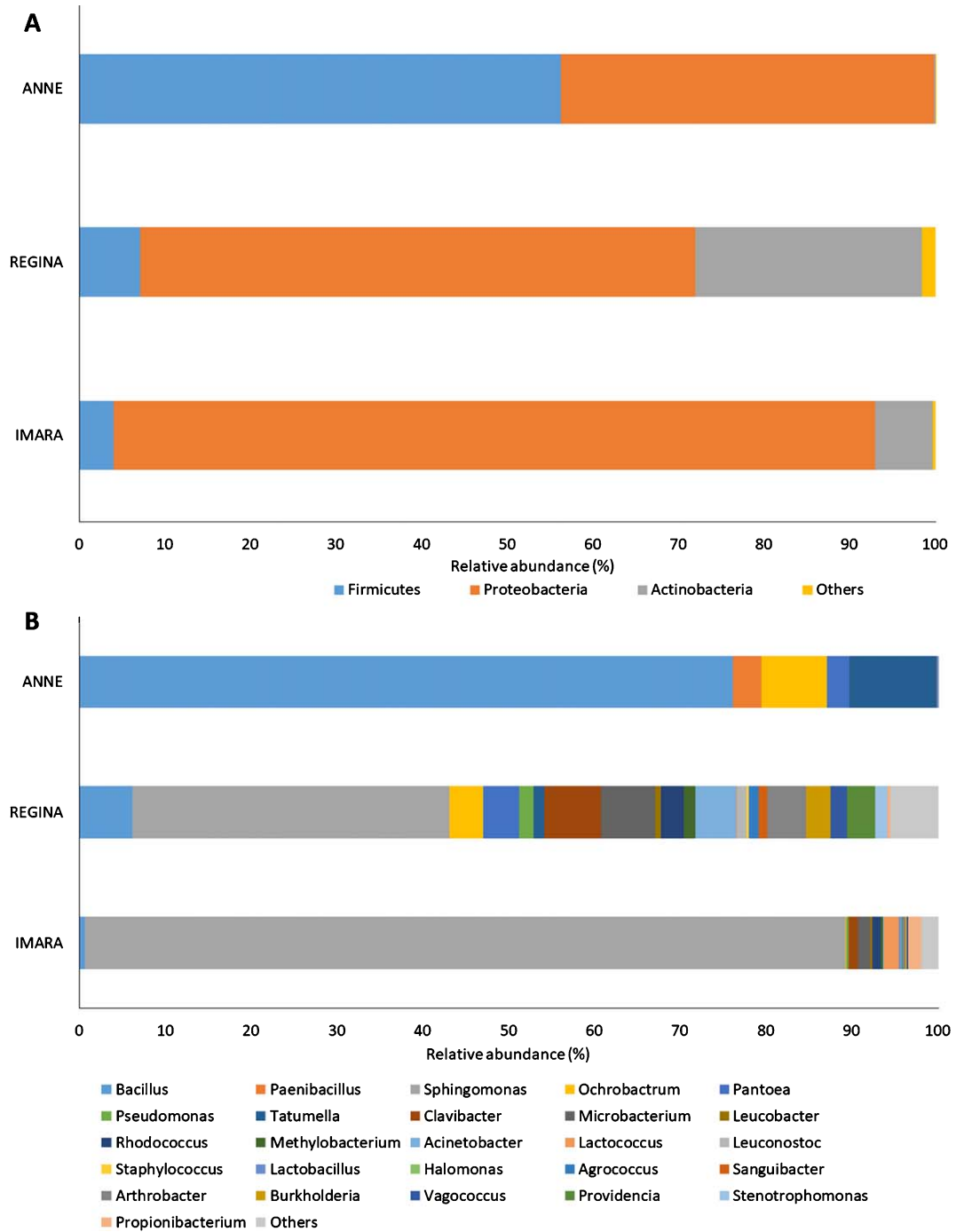


Fig. 1. Barplots showing the bacterial community composition at phylum (A) and genus (B) level based on 16S rRNA sequencing.

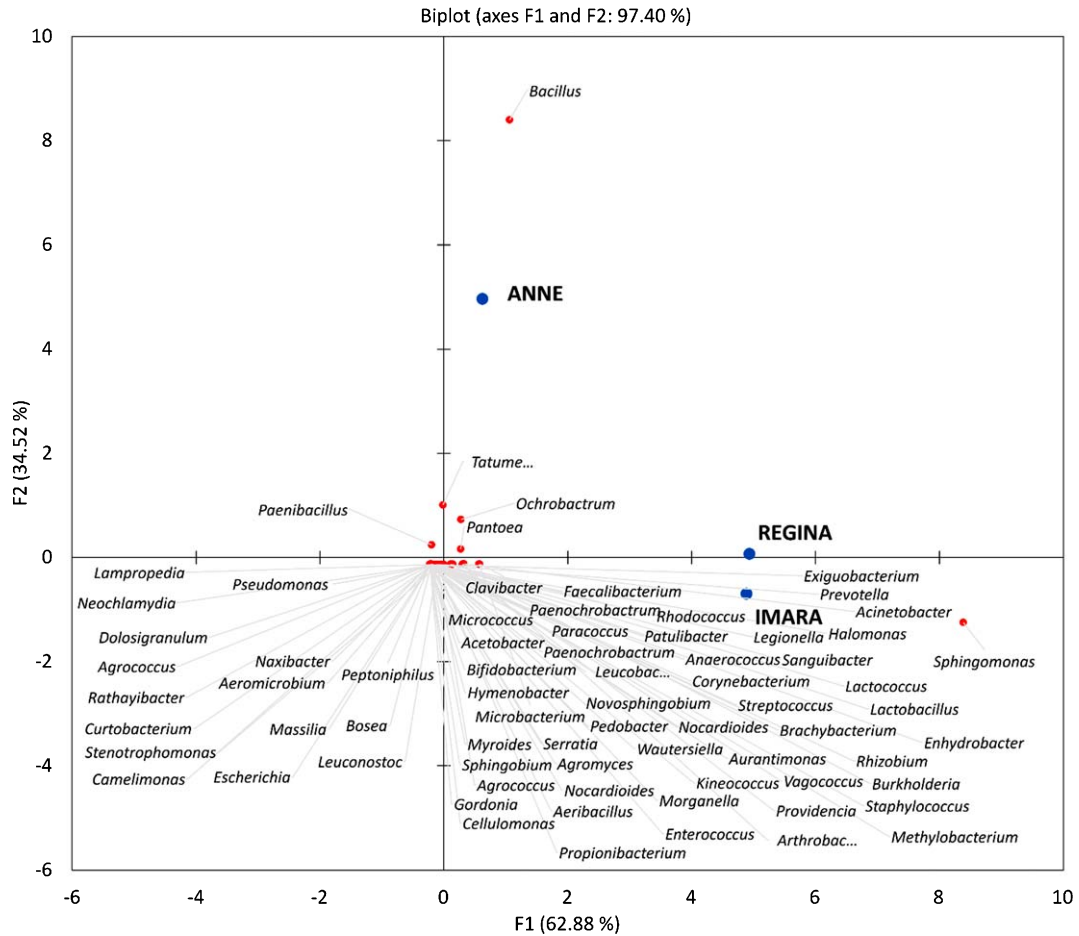


Fig. 2. Bi-plot ordination diagram of principal component analysis describing the main genera present in the analyzed raspberry cultivars and biodiversity indexes.

PCA analysis was performed, considering the genera detected, to visualize differences among samples (Fig. 2). The first two PCA components (F1: 62.88%; F2: 34.52 %) explained 97.40% of the total variance between the raspberry cultivars, based on microbial composition. “Anne” was mostly related to *Bacillus*. “Regina” was characterised by the presence of *Tatumella*, *Ochrobactrum* and *Pantoea*. Finally, “Imara” was characterised by *Sphingomonas*, *Clavibacter*, *Microbacterium*, *Rhodococcus*, *Arthrobacter*, *Burkholderia*, *Vagococcus*, *Providencia* and *Acinetobacter*.

Bacterial count was approximately $4.6 \log \text{CFU g}^{-1}$ raspberry in all cultivars. A total of 43 colonies were randomly collected from the highest countable dilution to increase the probability to pick up strains belonging to the dominant species. All bacterial isolates were identified by sequence analysis of 16S rRNA and the obtained sequences showed a similarity level $\geq 99\%$ (data not shown). In agreement with metagenomic data, twenty-two detected species/genera were differently distributed among the three cultivars (Fig. 3A). *Bacillus* spp. and *Penibacillus* spp. were the dominant species in “Anne”, *Sphingomonas* spp. and *Pseudomonas* spp. in “Imara”, and *Methylobacterium* spp., *Ochrobactrum* spp. and *Erwinia* spp. in “Regina”. In agreement with culture-independent analyses, the highest biodiversity was observed for “Regina”. Several species/genera, such

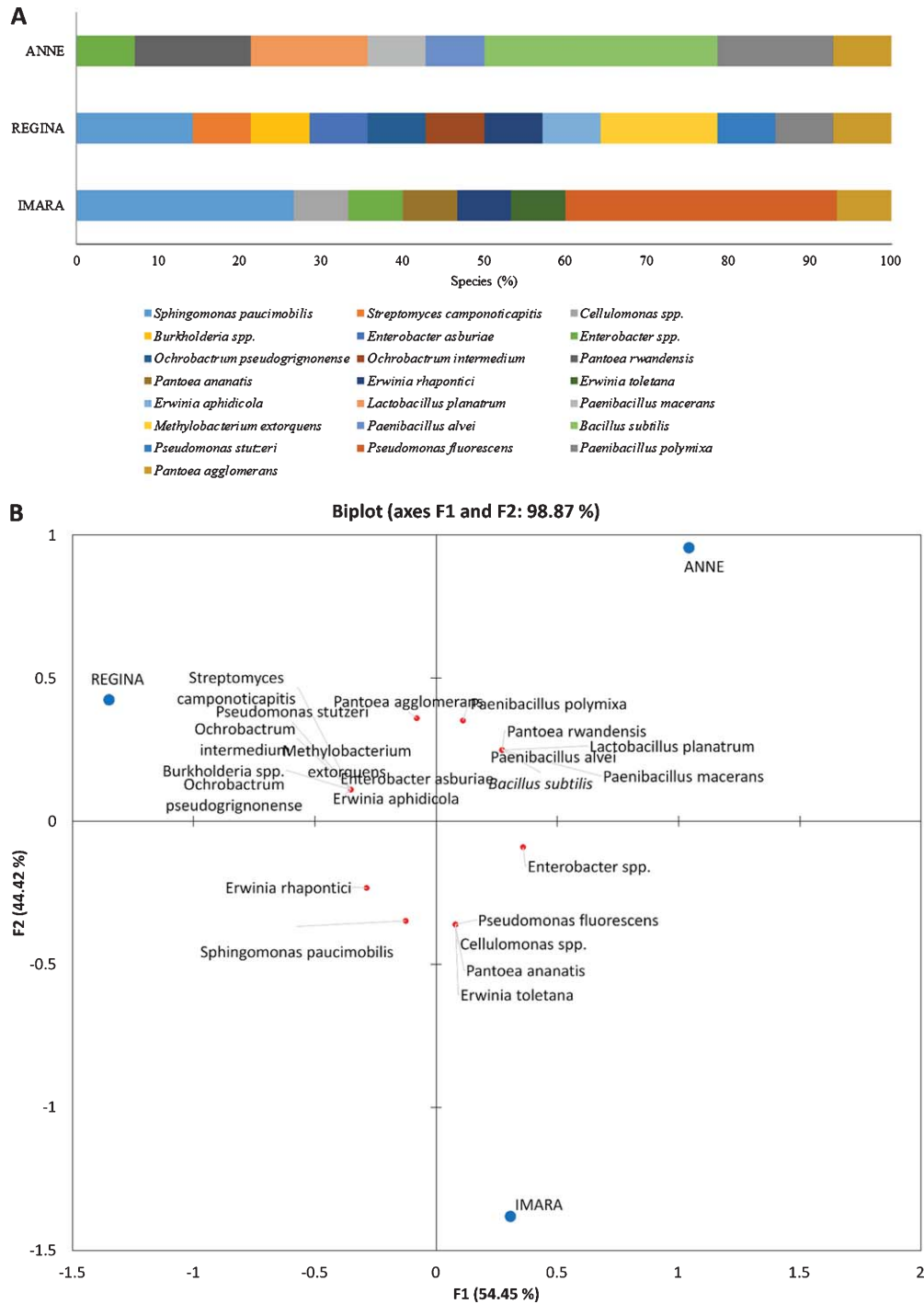


Fig. 3. (A) Percentage of bacterial species detected in the analyzed samples after sequencing of V3 and V4 regions of 16S rRNA. (B) Bi-plot ordination diagram showing species abundance (%) in the 3 cultivars.

as *Burkholderia* spp., *Methylobacterium extorquens*, *Pseudomonas stutzeri*, *Ochrobactrum pseudogrignonense*, *Ochrobactrum intermedium*, *Streptomyces camponoticapitis*, *Erwinia rhapontici*, *Erwinia aphidicola* and *Enterobacter asburiae*, were specifically associated to this cultivar (Fig. 3B). *Pantoea agglomerans* was the only isolate shared by all cultivars. The two red-fleshed cultivars shared 13% of isolates. PCA analysis was carried out to visualize the main differences between cultivars. The first two PCA components (F1: 54.45%; F2: 44.42%) explained 98.87% of the total variance (Fig. 3B). “Imara” hosted four unique bacterial species: *Cellulomonas* sp., *Erwinia toletana*, *Pantoea ananatis* and *Pseudomonas fluorescens*. Finally, “Anne” shared 9% of species with “Regina” or “Imara”, while it harboured cultivar-specific species such as *Pantoea rwandensis*, *Lactobacillus plantarum*, *Bacillus subtilis*, *Paennibacillus macerans* and *Paenibacillus alvei* (Fig. 3A and B).

Culture-independent and -dependent methods provided analogous results concerning both species abundance and diversity, however some discrepancies among the two methods were also observed. In particular, no strain belonging to the genus *Tatumella* was isolated. Differences among the methods are probably imputable to a different selectivity of the growing medium and to the competition on plate that may mask the presence of slow growing or less competitive species.

Some of the detected species/genera are associated to interesting plant growth-promoting traits. Many *Paenibacillus* species can directly promote the crop growth via biological nitrogen fixation, phosphate solubilization, production of the phytohormone indole-3-acetic acid (IAA), and release of siderophores that enable iron acquisition [47]. Production of siderophores on the phyllosphere has also been associated with the ability of plant symbiotic species to inhibit pathogenic ones [48]. Several strains of *Paenibacillus* can also protect against insect herbivores and phytopathogens, including bacteria, fungi, nematodes, and viruses. This is accomplished by the production of antimicrobials and insecticides, and by triggering a hypersensitive defensive response of the plant, known as induced systemic resistance (ISR) [49]. *Paenibacillus macerans* is used in commercial biofertilizers, while *Paenibacillus polymyxa* strain KNUC265 protects against the bacterial pathogens *Xanthomonas axonopodis* and *Erwinia carotovora* in pepper and tobacco, respectively, using bacterial volatiles and diffusible metabolites as elicitors [49].

Raspberry fruit harboured a detectable population of other possible PGPBs which were scattered among the three cultivars. *Burkholderia* spp. can colonize the rhizosphere and tissues of some important crops, such as wheat [50], corn [51], grapes [52] and sugarcane [53]. Members of this genus have direct and positive effects on plant growth through phosphate solubilization and the production of hormones [54]. In addition, indirect beneficial effects consist of the production of antagonistic compounds for pathogens, such as hydrolytic enzymes and antibiotics [55].

Methylobacterium spp. was already found in the tissue of sugarcane, rice [56], sunflower, corn, and soybean [57]. These bacteria, using methanol as carbon source, promote the plant growth through several mechanisms, such as the production of auxins and cytokinins [57], the regulation of ethylene levels caused by stress in plants through the production of ACC deaminase [56], and synthesis of cellulase and pectin [56]. *Methylobacterium* is also known for its positive influence on strawberry aroma, being able to stimulate the emission of two furanones, namely 2,5-dimethyl-4-hydroxy-2H-furanone and 2,5-dimethyl-4-methoxy-2H-furanone which are key drivers of perceived strawberry flavour [58]. Bacteria from the genus *Pseudomonas* have been widely applied to produce organic compounds and to solubilize phosphates, and, thus, have high potential as PGPBs or biological control agents [59]. Several studies have found *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas aeruginosa*, and other *Pseudomonas* spp. promoting plant growth through the production of phytohormones and siderophores [60]. Some of the isolated species, such as *Pantoea agglomerans* and *Pseudomonas fluorescens*, are antagonists of *Erwinia amylovora* and they are commercially exploited for the biological control of fire blight [61]. Finally, this is the first report of the isolation of the insect pathogen *Erwinia aphidicola* from berry plants. This bacterium, described only on *Phaseolus vulgaris*, *Pisum sativum*, and *Capsicum annuum*, is associated with aphids [62–64].

Further characterization of the 43 isolates was performed by (GTG)₅-rep PCR fingerprinting. The repeatability of the assay was 95% as measured by the correlation index between replicates of the same strain. Considering an

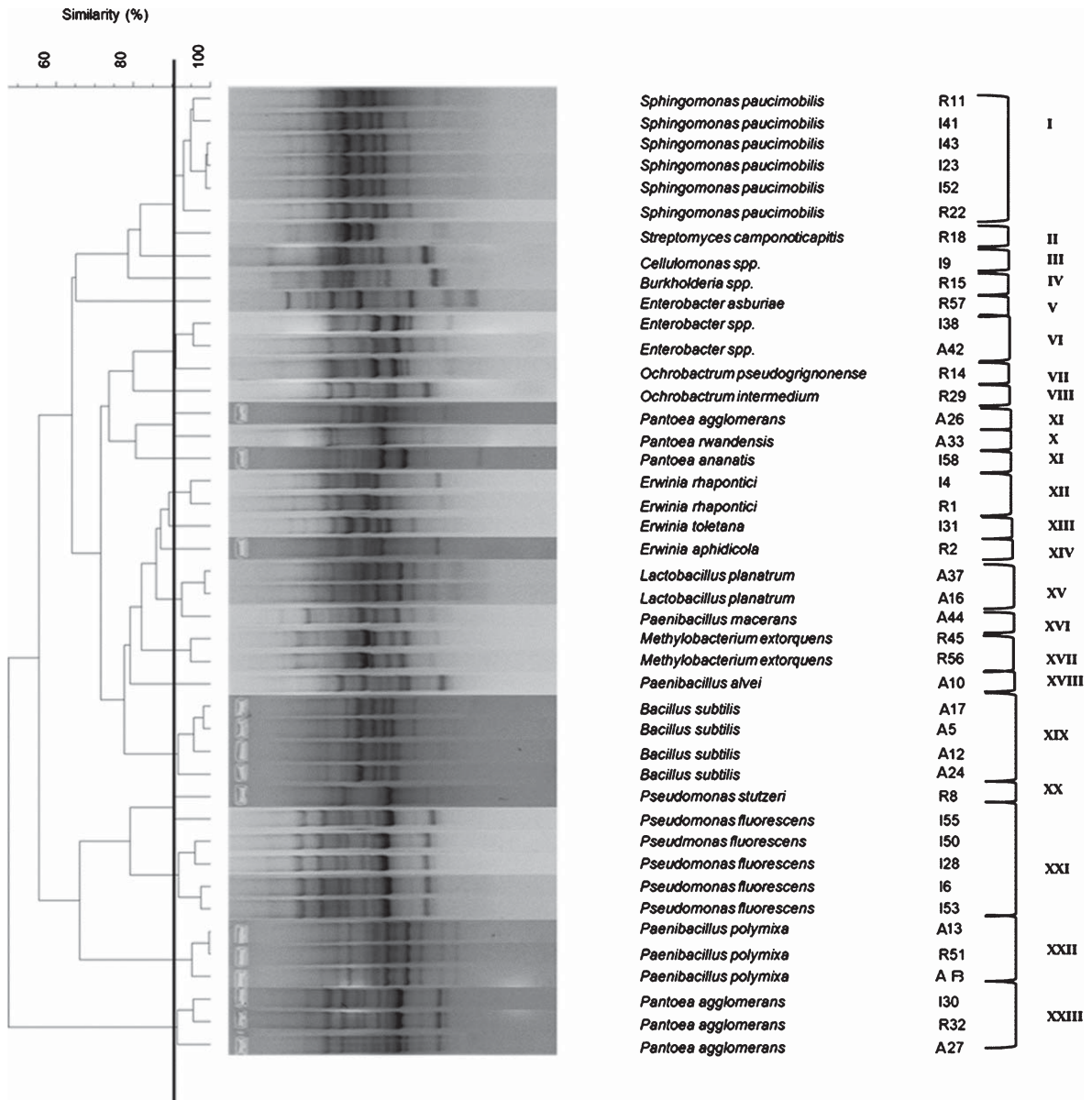


Fig. 4. Dendrogram generated after bioinformatics analysis with Fingerprinting II Informatic of rep-PCR profiles. Strains in bold represent those selected for functional characterization. R, F and I refer to isolation source: Regina, Anne or Imara, respectively.

arbitrary threshold of 90%, 23 clusters were identified, and 11 consisted of a single strain. Generally, rep-PCR resulted in a coherent classification at the species level (Fig. 4).

3.2. Functional characterization of strains

Based on fingerprinting results 25 strains were selected for further phenotypic characterization. Most of the tested strains (13 out of 25) were able to produce acetoin, a molecule playing a key role in elicitation of systemic

Table 1
Functional traits of representative strains randomly chosen among the different biotypes obtained by rep-PCR

Species	Strain	Acetoin production	IAA production	ACC deaminase activity ($\mu\text{M KB mg}^{-1} \text{ h}^{-1}$)	Syderophores	Ammonia production	<i>Er. amylovora</i> inhibition	<i>B. cinerea</i> inhibition
<i>Er. aphidicola</i>	R2	+	–	–	+	–	–	–
<i>Ps. stutzeri</i>	R8	+	–	–	+	–	–	–
<i>S. paucimobilis</i>	R11	–	–	–	+	–	–	–
<i>O. pseudogrignonense</i>	R14	+	–	–	±	–	+	–
<i>Burkholderia</i> spp.	R15	–	–	–	+	–	–	–
<i>S. camponoticapitis</i>	R18	–	–	–	±	–	–	–
<i>O. intermedium</i>	R29	+	+	–	+	–	–	+
<i>Pa. agglomerans</i>	R32	+	+	5.6 ± 1.2	+	+	–	–
<i>M. extorquens</i>	R45	+	–	27.8 ± 3.8	±	+	–	–
<i>P. polymixa</i>	R51	–	–	–	±	–	–	–
<i>E. asburiae</i>	R57	+	–	9.1 ± 0.9	++	+	+	+
<i>Er. rhapontici</i>	I4	+	–	–	+	–	–	–
<i>Cellulomonas</i> spp.	I9	+	–	–	+	–	–	–
<i>Ps. fluorescens</i>	I28	–	–	7.1 ± 1.8	++	+	–	–
<i>Er. toletana</i>	I31	+	–	7.9 ± 1.9	±	+	–	–
<i>Enterobacter</i> spp.	I38	+	–	–	+	–	–	–
<i>S. paucimobilis</i>	I41	–	–	–	+	–	–	–
<i>Ps. fluorescens</i>	I55	–	–	6.3 ± 0.7	++	+	–	–
<i>Pa. ananatis</i>	I58	–	–	11.5 ± 2.2	++	+	–	–
<i>B. subtilis</i>	A5	+	+	–	+	–	–	–
<i>P. alvei</i>	A10	+	–	–	±	–	–	–
<i>L. plantarum</i>	A16	–	–	–	+	–	–	–
<i>Pa. rwadensis</i>	A26	–	–	–	+	–	–	–
<i>Pa. rwadensis</i>	A33	–	–	6.9 ± 1.1	–	+	–	–
<i>P. macerans</i>	A44	–	–	17.4 ± 1.3	±	+	–	–

resistance in plant [65] (Table 1). In some bacterial species, such as *Pa. agglomerans*, *Enterobacter* sp., *B. subtilis*, *B. amyloliquefaciens*, *P. polymyxa*, *Erwinia* spp., acetoin can be readily reduced to 2,3-butendiol which is responsible for both plant growth and resistance promotion against pathogens and abiotic stresses [66–69].

The ability to produce indole-3-acetic acid (IAA) was detected in only three strains: R32 (*Pa. agglomerans*), R29 (*O. intermedium*) and F5 (*B. subtilis*) (Table 1). The ability of rhizobacteria to produce auxins has been associated with plant growth promotion, especially root initiation and elongation. Significant amounts of *in vitro* IAA production by *Bacillus* spp. and *Pa. agglomerans* have been documented by other authors [70–72]. The production of IAA by *O. intermedium* has been here reported for the first time. However, this trait has been described in *O. anthropi* TRS-2, isolated from tea rhizosphere by Chakraborty et al. [73]. The other strains did not show this feature even if aminotransferases associated with IAA production have been reported in a variety of plant-associated bacteria, including *Enterobacter* and *Pseudomonas* spp. [74–76], suggesting the strain-dependence of this trait.

Plant growth can also be stimulated by PGPRs that produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase. In this study, the ACC deaminase activity was determined through the evaluation of ammonia and 2-ketobutyrate. Nine strains showed this feature with values of 2-ketobutyrate production ranging from

5.6 μM 2-ketobutyrate $\text{mg}^{-1} \text{h}^{-1}$ (R32) to 27.8 μM 2-ketobutyrate $\text{mg}^{-1} \text{h}^{-1}$ (R45) (Table 1). The highest activity detected for *M. extorquens* is in agreement with other studies showing the presence of this trait in several species belonging to this genus [77–79]. Almost all strains produced siderophores (Table 1). This activity was particularly evident for *Pa. ananatis*, *E. asburiae*, and *Ps. fluorescens*.

Ethylene is an important signalling molecule regulating plant responses to pathogen attack or abiotic stress [80]. Ethylene is a crucial modulator of fruit ripening process and is involved either directly or indirectly in the regulation of metabolites that determine the fruit quality [81, 82]. Ethylene is synthesized from S-adenosyl-methionine (SAM) via 1-aminocyclopropane-1-carboxylic acid (ACC) through the catalytic activity of ACC synthase (ACS). The subsequent oxidation of ACC to ethylene is catalyzed by ACC oxidase (ACO) [83]. ACC deaminase cleaves ACC, the immediate precursor of ethylene, to produce 2-ketobutyrate and ammonia.

Ethylene production greatly varies among raspberry cultivars and, in some of them, its concentrations increase 75-fold during ripening [84, 85]. As the fruit pigments change from green to red there is a progressive softening, loss of skin strength and a breakdown of cell walls in the mesocarp. An increase in cellulase (endo-1,4- β -D-glucanase) in both drupelets and receptacles accompanies these changes [84–86]. During ripening, cell wall breakdown and cellular enlargement in the mesocarp cause the softening of drupelets. As a result, ripe raspberry fruit are extremely delicate and very susceptible to damage during harvesting and marketing [87]. Interestingly, in other berries, such as strawberry, the activity of endo-1,4- β -D-glucanase is dependent on reduced levels of IAA, more than on endogenous ethylene [88, 89]. IAA is also involved in other aspects of fruit development since it stimulates fruit set and fruit growth by pericarp cell division and elongation. Finally, IAA also play a significant and independent role during the ripening of climacteric and non-climacteric fruits. For example, an important cross-talk between auxin and ethylene was demonstrated in peach fruit during ripening [90]. In grape berry, elevated concentrations of ethylene, prior to the initiation of ripening, lead to an increased production of IAA, suggesting a complex involvement in berry ripening [91].

This body of evidence suggests that bacterial strains able to influence ethylene and/or IAA production by plant tissues may influence fruit set, development and ripening. In this view, bacteria able to produce IAA and subtract ethylene molecular precursors by ACC deaminase activity may increase fruit set and yield. Moreover, the lower ethylene production may reduce fruit abscission and softening and thus, harvest losses. Finally, the reduction of ethylene may also allow the fruit to remain for a longer period on the cane where they can accumulate greater amount of photoassimilates, resulting in higher fruit sugar content and quality.

Iron is an essential growth element both for microorganisms and plants. Phyllosphere is an oligotrophic niche where the scarce bioavailability of iron fosters microbial competition for this limiting factor [2]. Under iron-limiting conditions bacteria produce siderophores to competitively acquire ferric ion [52 and reference therein]. Bacterial siderophores can deprive of this essential element those pathogenic bacteria or fungi producing siderophores with lower affinity [92]. Siderophore production can contribute to the biological control of pathogens in the phyllosphere [2], rhizosphere [92, 93] and even on fruit during postharvest [94]. Some siderophores produced by *Pseudomonas*, such as pseudobactin 358, may elicit plant induced resistance (IRS) [95, 96], while others, such as pyochelin, have been suggested to act as antifungal compounds [97].

All the bacterial species isolated in this work on raspberry fruit, except for *Pa. rwadensis*, showed the ability to produce siderophores. However, the degree of siderophore production differed from species to species, being *Ps. fluorescens*, *E. asburiae* and *Pa. ananatis* the highest producing isolates. The ability to form siderophores is well known in *Pseudomonas* genus. Fluorescent pseudomonads produce and excrete, under iron-limiting conditions, yellow-green fluorescent siderophores designated pyoverdines [98]. *E. asburiae* ability to form siderophores is probably related to the presence of many genes for two siderophores, aerobactin (Enterobactin_01228–01236) and enterobactin (Enterobactin_03893–03912) [99]. *Pa. ananatis* is widely recognized as an ubiquitous phytopathogenic bacterium capable of infecting diverse hosts including maize, rice and pineapple as well as eucalyptus [199]. A scan of the genome revealed that it has genes associated with iron acquisition and siderophore biosynthesis, including *alcaA*, *lucA* and siderophore-interacting protein/WP_014606889.1 [101, 102].

Interestingly, *E. asburiae* (R57), one of the isolates with the highest siderophores production, was the only strain showing an inhibition activity against both *Erwinia amylovora* and *B. cinerea*. Isolates R14 and R29, belonging to *O. pseudogrignonense* and *O. intermedium*, were able to inhibit *Erwinia amylovora* or mycelial growth of *B. cinerea*, respectively. However, other strong siderophore-producing isolates, such as *Ps. fluorescens* I28 or *Pa. ananatis* I58, had no inhibitory effect against any of the tested plant pathogens. These results suggest that even though siderophores may contribute to inhibiting raspberry bacterial and fungal pathogen, their production is not sufficient, *per se*, in explaining pathogen inhibition. *E. asburiae* may inhibit *B. cinerea* infection also by ACC deaminase activity. In fact, despite the production of ethylene by *B. cinerea* via the 2-keto-4-methylthiobutyric acid (KMBA) pathway, reduction in plant ethylene delays ripening making fruit more resistant to the pathogen [103].

The presence of *E. asburiae* R57 and its possible effect on raspberry quality and storability needs further investigations. In fact, R57 shows both *B. cinerea* inhibition and ACC deaminase activity suggesting that it could be usefully applied in pre-harvest to reduce fruit losses and increase quality.

Nine isolates from raspberry fruit can produce ammonia (Table 1). Three of these species, namely *Pa. agglomerans*, *Ps. fluorescens* and *M. extorquens*, include symbiotic epiphytes commonly associate to crop plants. Moreover, *Pa. agglomerans* and *E. asburiae* were the ones showing also several other PGP activities such as acetoin, ACC deaminase and siderophore production which may exert synergic effects of plant growth and health.

Several bacteria belonging to different phylogenetic groups share the ability to reduce atmospheric N₂ to ammonium via the enzyme nitrogenase. Several of these species, including diazotrophic bacteria, can colonize the phyllosphere of wild and cultivated plants [104 and reference therein]. Nitrogen is an essential nutrient for plants, and its deficiency severely affect raspberry plants by causing stunted growth, chlorosis, reduction of photosynthesis and, ultimately, reducing yield and fruit quality. In raspberry, a significant uptake of N occurs via leaves [105]. Nutrient uptake by leaves provides essential elements to the plant when soil conditions restrict root uptake, or during periods of rapid growth, when requirements may exceed root supply [106]. The agronomic practice of foliar fertilization allows a faster and more efficient absorption and translocation of nitrogen than soil application. In raspberry, up to 50% of the applied nitrogen is absorbed within 32 h and transported throughout the plant within 7 days [105]. Therefore, foliar fertilization represents an important tool for more precise tailoring of nutritional inputs to plant needs. Nonetheless, a single foliar application provides an insufficient nutrient supply [105]. In this view, the colonization of leaves with an abundant population of ammonia-producing bacteria might provide a natural and constant nitrogen fertilization, thus maximizing the positive effects on fruit production and quality.

Strains were also tested for their ability to form biofilms and for their cell surface hydrophobicity. Biofilm formation provides several selective advantages for epiphytic bacteria, such as protection from environmental stresses, competitors and antimicrobial molecules, activation of enzymatic processes that require high cellular density, and acquisition of new genes via horizontal gene transfer [107]. The ability of bacterial pathogens to form protective biofilm may substantially reduce the efficacy of pesticide application, or even increase virulence [107, 108]. Similarly, in PGPB-based products, the ability to form biofilm by single strains or synthetic communities may enhance their effectiveness and reliability by reducing the susceptibility of the growth promotion effect to environmental and cultural stresses [109]. Therefore, biofilm formation on plant tissues represents a hotspot for microbial interactions that locally shape microbial assemblages [1].

Results of this study revealed that biofilm formation was variable among the tested strains (Fig. 5A). The highest OD_{600 nm} values were observed for R57 – *Enterobacter asburiae* – (2.16 ± 0.39), F33 – *Pantoea ananatis* – (1.80 ± 0.11) and I58 – *Pantoea rwadensis* – (2.17 ± 0.11) strains, while the lowest for I9 – *Cellulomonas* spp. – (0.84 ± 0.28) and R15 – *Burkholderia* spp. – (0.81 ± 0.19). The other tested strains showed an OD_{600 nm} higher than 1, ranging from 1.05 ± 0.22 (R14 – *O. pseudogrignonense*) to 1.55 ± 0.24 (I28 – *Pseudomonas* spp.). The low ability of *Cellulomonas* and *Burkholderia* genera to form biofilms has been shown also by other authors [110, 111]. Yung et al. [110] showed a certain variability among *Cellulomonas* species in biofilm forming ability, mostly in response to N limitation. The *Burkholderia* ability to form biofilms is strain-specific and depends on

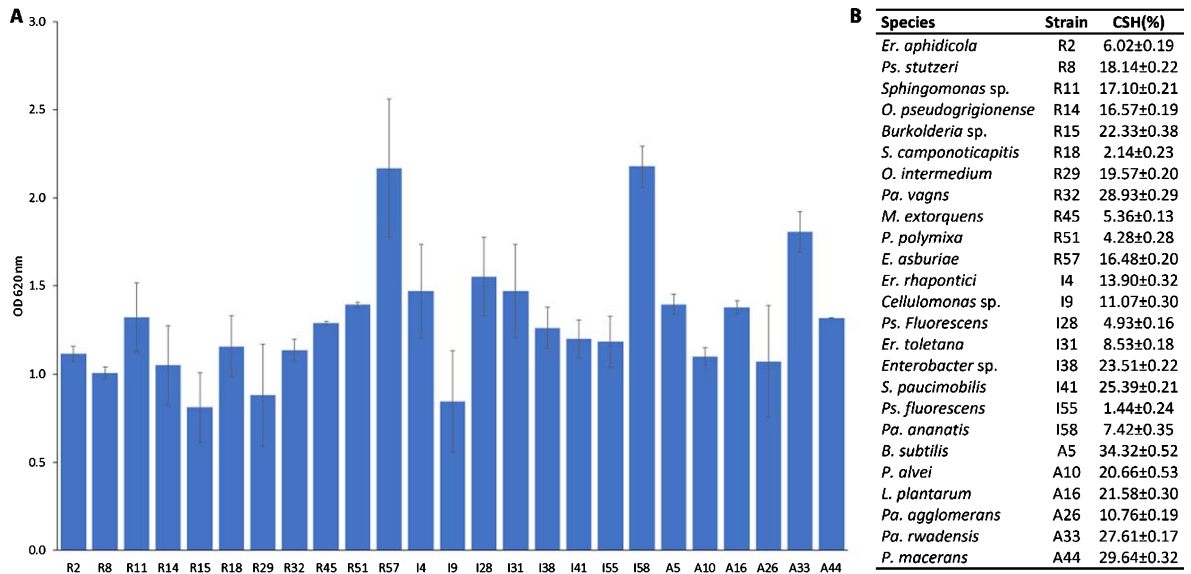


Fig. 5. (A) Biofilm formation, expressed as OD at 595 nm, of tested strains. Vertical bars indicate standard errors. (B) Hydrophobicity of tested strains based on the affinity to hexadecane.

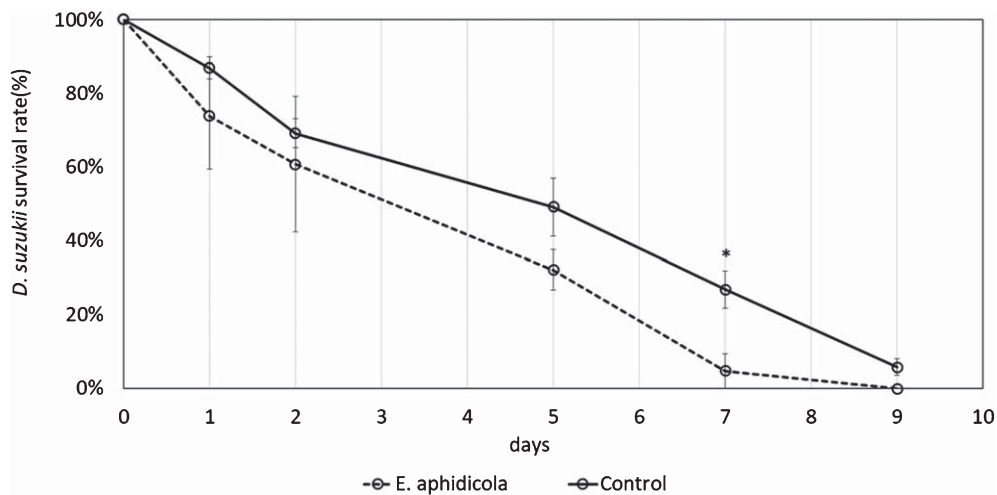


Fig. 6. Effect of *Erwinia aphidicola* application on the survival of *Drosophila suzukii*. Each point is the average of three replicates. Standard error is reported. Values, in each day, marked with an asterisk are significantly different.

the conditions tested [111]. The ability of microorganisms belonging to *Enterobacteriaceae* family to adhere to polystyrene plates is not surprising, since *Enterobacteriaceae* are well known for their ability to form biofilms; e.g. *Enterobacter cloacae* GS1, a plant growth-promoting bacterium, colonizes rice roots as microcolonies and forms biofilm-like structures on the root surface [112].

In order to investigate the relationship between CSH and biofilm-forming ability, the aptitude of tested strains to adhere to hexadecane was determined. Strains showed a certain variability with CSH values varying over a wide

range, from $4.93 \pm 0.30\%$ (I9) to $34.32 \pm 0.21\%$ (F5) (Fig. 5B). With a few exceptions, strains showing biofilm-forming capacity on polystyrene plates had the highest CSH values. The link between adhesion properties and hydrophobicity often lead to controversial results. *Halomonas venusta* strains showed differences in their adhesion onto polystyrene, even differences in their Lewis acid-base characteristics and hydrophobicity were not substantial [113]; on the other hand, Kouidhi et al. [114] reported a correlation between cells surface hydrophobicity of *Staphylococcus aureus* and their adhesion potencies to various surface. Our data suggested that under our experimental conditions, hydrophobicity alone is not responsible for adhesion and cannot be used as predictor of biofilm formation.

Finally, preliminary experiments were performed to test possible pathogenic effect of bacterial isolates against *D. suzukii*, the main raspberry pest. The experiments were performed with *Er. aphidicola*, since this species, initially isolated from pea plants, has been demonstrated to be pathogenic for insects [62, 115]. These isolates did not result pathogenic toward the host plant and treatments of *D. suzukii* adults with a bacterial suspension (10^6 CFU mL⁻¹) sharply decreased the survival rate of this insect (Fig. 6). This is the first report on the pathogenic effect of *Er. aphidicola* on *Drosophila suzukii*. However, further experiments are needed to understand the mode of action and pathogenicity of *Er. aphidicola* and to verify its possible use on raspberry as a protective agent against *Drosophila suzukii*.

4. Conclusions

This study provides both taxonomic and functional information regarding raspberry (*Rubus idaeus* L.) microbiome. Taxonomic analysis revealed the presence of a continuum of microorganisms throughout the plant and communities with functional profiles shaped by specific niche characteristics. However, the three tested cultivars were clearly differentiated by the bacterial community hosted on their fruit with “Anne”, the white flesh variety, having the most distinctive community. The detected differences are probably related to the influence of genotype on microbial composition. Several studies demonstrated the impact of genotype in structuring microbial community [116–119]. These results suggested a certain degree of co-evolution or coinheritance between a genotype and its associated microbiota. However, more detailed and comprehensive studies will need to be conducted in raspberry to support this idea. Moreover, clarifying the interactions between plant genotypes and the phyllosphere microbial community is a crucial prerequisite for managing the phyllosphere microbiota, for example, in terms of disease resistance, plant health and fruit productivity and quality. Finally, this study represents a preliminary, but promising example of the biotechnological potential of bacteria isolated from raspberry. Further studies on the performance of these isolates, or synthetic community, on the growth and resistance of plant will uncover the mechanism and the potential of these PGPR exhibiting multiple PGP traits.

Author contribution

GP and ID performed bacteria isolation and microbiology experiments. GP and AC wrote the first draft. LO performed the metagenomic analysis. BF and LG provided the plant material and performed fruit quality analysis. FS conceived and supervised the experiments and wrote the final draft of the experiment. All authors contribute to the discussion of the results, revised and approved the manuscript.

Conflict of Interest

The authors have no conflict of interest to report.

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