Joana Vanessa Gomes Pereira

The impact of pathogenic bacteria on plant microbiome – *Pseudomonas syringae pv. actinidiae*, a case study

Dissertação para a obtenção de grau de Mestre em Biodiversidade e Biotecnologia Vegetal
sob orientação científica da Professora Doutora Joana Cardoso da Costa e do Doutor Igor Clemente Tiago
e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Agosto 2017

· U C ·

Universidade de Coimbra

The impact of pathogenic bacteria on plant microbiome – *Pseudomonas syringae* pv. *actinidiae*, a case study

Joana Vanessa Gomes Pereira

Dissertação para a obtenção de grau de Mestre em Biodiversidade e Biotecnologia Vegetal sob orientação científica da Professora Doutora
Joana Cardoso da Costa e do Doutor Igor Clemente Tiago e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

This work was supported by Project PDR2020 "I9K - InovKiwi - Desenvolvimento de estratégias que visem a sustentabilidade da fileira do kiwi através da criação de um produto de valor acrescentado"

Universidade de Coimbra | 2017

Acknowledgments

Gostaria de manifestar vivamente a minha profunda gratidão e os meus sinceros agradecimentos a quem me possibilitou esta experiência ao longo deste ano letivo. Obrigada a todos as pessoas que contribuíram para a realização deste trabalho, etapa final de mais um período de aprendizagem.

Aos meus orientadores Professora Doutora Joana Costa e Doutor Igor Tiago por fizeram com que este trabalho fosse possível, por contribuírem para o meu aprimoramento profissional, e principalmente, em meu crescimento pessoal. A vós vos agradeço o vosso apoio nesta árdua tarefa, pois sem vocês não era possível ter atingido os objetivos. Muito obrigada!

À Catarina e à Eva pela disponibilidade e apoio que me deram no laboratório!! À Daniela e ao Sandro, os meus parceiros de trabalho durantes estes meses e que estiveram sempre lá quando foi necessário. Muito obrigada!!!

À empresa 'Delícias do Tojal' pela cedência das amostras biológicas para a realização deste estudo. Muito Obrigada!

A todos os restantes elementos do laboratório de Microbiologia do Departamento de Ciências de Vida da Universidade de Coimbra.

Aos meus amigos e cúmplices, por todo o apoio e confiança em mim, demonstrados; e pela motivação que me deram em especial às minhas companheiras (Liliana, Cristiana, Ronice, Paula). Obrigada malta! :)

Por último, tendo consciência que sozinha nada disto teria sido possível, dirijo um agradecimento à minha família, em especial aos meus pais e à Inês, por colocarem sempre os meus interesses em primeiro lugar e por todos os sacrifícios que fizeram para conseguir alcançar os meus objetivos de estudante. O meu profundo agradecimento. Adoro-vos e a vocês vos dedico este trabalho.

Summary

Resumo

Nos últimos anos, a produção de kiwi diminuiu devido aos surtos de *Pseudomonas syringae* pv. *actinidiae* (Psa), o agente causador do cancro bacteriano em plantas do género *Actinidia* tornando-se uma doença pandémica que ameaça a sustentabilidade da indústria do kiwi nos principais países produtores, incluindo Portugal.

Para entender o impacto da Psa na comunidade microbiana de *Actinidia deliciosa*, foi caracterizado o microbiota das folhas de pomares saudáveis e com doença. Pretendeu-se eliminar fatores que poderiam alterar o microbiota entre as amostras. Desse modo, foram recolhidas folhas de *A. deliciosa* de pomares vizinhos, removendo a influência de condições abióticas na estrutura do microbiota. Além disso, em ambos os pomares praticava-se agricultura biológica, sem aplicação de substâncias químicas, evitando a seleção artificial de populações de bactérias com base na sua resistência a pesticidas. Com esta abordagem, as alterações identificadas no microbiota entre plantas saudáveis e doentes permitirão avaliar o real impacto da doença e identificar grupos de microrganismos com potencial aplicação na modelação da doença e no estado de saúde da planta.

A diferença na susceptibilidade à Psa entre as plantas femininas e masculinas de *A. deliciosa* é bem conhecida. Desse modo, também foi nosso objetivo determinar se a estrutura do microbiota das plantas femininas e masculinas pode desempenhar um papel nessa característica, abrindo o caminho para a descoberta de novos agentes de prevenção / controle contra Psa.

Duas metodologias foram aplicadas para determinar a composição estrutural do microbiota, métodos dependentes e independentes de cultivo, já que pretendemos não só construir uma coleção de cultura de microrganismos que poderá ser usada em estudos futuros, mas também obter uma "imagem instantânea" e detalhada da estrutura do microbiota. A combinação destas duas metodologias permitiu uma análise detalhada da diversidade estrutural do microbiota associada ao estado fitossanitário de *A. deliciosa*.

Em ambas as abordagens foram identificados três filos predominantes, tanto em pomares saudáveis como doentes, nomeadamente *Proteobacteria*, *Actinobacteria* e *Firmicutes*, mas com abundâncias relativas distintas entre as amostras. Além disso, observou-se que plantas masculinas tendem a apresentar maior diversidade do que

plantas femininas (valores mais pronunciados nos resultados obtidos por métodos independentes de cultivo), sendo essa diferença mais óbvia em plantas doentes.

Em geral, a Psa induziu uma alteração dramática na estrutura do microbiota das folhas, traduzida numa maior diversidade apesar do domínio da ordem *Pseudomonadales*. Em ambas as abordagens (métodos dependentes de cultivo e métodos independentes de cultivo), a existência de populações bacterianas potencialmente fitopatogénicas foi observada em amostras de pomares doentes, isto é, populações pertencentes aos géneros *Pseudomonas*, *Serratia*, *Pantoea e Erwinia*, entre outras. Os resultados globais fornecem evidências de que, embora a Psa seja a causa do cancro bacteriano nas plantas de kiwi, a presença de outras populações patogénicas também pode contribuir para a doença e declínio das plantas afetadas.

Este estudo permitiu um avanço na compreensão da estrutura e composição das comunidades bacterianas em *A. deliciosa* e determinou ainda o impacto da Psa no microbiota.

Palavras-chaves: Kiwifruit; *Pseudomonas syringae* pv. *actinidiae; Actinidia deliciosa;* métodos dependentes de cultivo; métodos independentes de cultivo

Abstract

In last years the kiwifruit production decreased due to outbreaks of *Pseudomonas syringae* pv. *actinidiae* (Psa), the causative agent of the bacterial canker of kiwifruit, becoming a pandemic disease threatening the sustainability of the kiwi industry in major producing countries, including Portugal.

In order to understand the impact of Psa in the microbial community of *Actinidia deliciosa*, the microbiota of leaves was characterized from healthy and disease orchards. We intended to eliminate possible factors that could alter the microbiota between samples. In accordance, the studied *A. deliciosa* leaves were collected from plants in neighbor orchards removing the influence of abiotic conditions in the microbiota structure. Moreover, both orchards were organic, with no applied chemicals avoiding, once more, the artificial selection of bacterial populations based on their resistant to pesticides. With this approach, the identified alteration on the microbiota between heath and disease plants will allow assessing the impact of the disease and identify groups of microorganisms with a potential role on the modulation of the disease or on the plant health status to be used in the near future.

Difference on the susceptibility to Psa between *A. deliciosa* females and males plants is well known. In accordance, it was also our goal determine if the microbiota structure of females and males may play a role on this feature, paving the way to the discovery of new preventing/controlling agents against Psa.

Two methodologies were applied to determine the microbiota structural composition, namely culture–dependent and culture-independent methods since we intended, not only to construct a culture collection of microorganisms that could be used in future studies, but to get a detailed snapshot of the "total" microbiota. The combination of these two methodologies allowed a detailed analysis of the microbiota structural diversity associated with the phytossanitary status of *A. deliciosa*.

In both approaches, three predominant phyla were identified in both healthy and diseased orchards, *Proteobacteria*, *Actinobacteria* and *Firmicutes*, but with distinct relative abundance among sample. Additionally, it was observed that male plants tend to have higher diversity than female plants (more pronounced on culture-independent methods results), being this difference more obvious on diseased plants.

Overall, Psa induced a dramatic alteration on the structure of leaves microbiota in both approaches, translated in increased diversity despite the dominance from *Pseudomonadales* order. In both approaches (culture -dependent and -independent methods) the existence of putative phytopathogenic bacterial populations was observed in diseased orchards samples, i.e. populations belonging to genera *Pseudomonas*, *Serratia*, *Pantoea* and *Erwinia,* among others. The overall results provide evidence that, although Psa is the cause of kiwifruit bacterial canker, the presence of other pathogenic populations may also contribute for the disease and aid on the deteriorating condition of the diseased plant.

This study has enabled a breakthrough in understanding the structure and composition of bacterial communities in *Actinidia deliciosa* and determined the impact of Psa in the plant leaves microbiota.

Keywords: Kiwifruit; *Pseudomonas syringae* pv. *actinidiae; Actinidia deliciosa;* culture dependent methods; culture independent methods

Symbols and abbreviations

ABM2	Alkaliphilic buffered medium 2
CFU	Colony forming units
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
Dmg	Margalef Diversity
EDTA	Ethylenediamine tetracetic acid
EPPO	European and Mediterranean Plant Protection Organization
H'	Shannon- Weaver index
J'	Pielou's evenness index
NGS	Next Generation Sequencing
pb	Base pair
PBS	Potassium phosphate buffer solution
PCR	Polymerase Chain Reaction
Psa	*Pseudomonas syringae* pv. *actinidiae*
RAPD	Random Amplified Polymorphic DNA
rpm	Revolutions per minute
rRNA	ribosomal Ribonucleic acid
S	Species
TAE	Tris-acetate-EDTA buffer
Taq	Thermus aquaticus
UV	Ultraviolet
v/v	Volume/volume
w/v	Weight/volume
mM	Milimolar

ml	Mililiter
µl	Microliter
ʎ	Simpson diversity
ºC	Celsius Degree
%	Percentage
g	Grams

List of figures

List of tables

1 Introduction

1.1. The *Actinidia* culture

The genus *Actinidia* (Fig.1) was described for the first time in the XII century by Chongxiu Bencao (Ferguson & Huang, 2007). It is commonly named as kiwifruit and in China is known as "mihoutao" meaning "macaque monkey peach". *Actinidia* belongs to the division *Magnoliophyta*, class *Magnoliopsida*, order *Ericales*, family *Actinidiaceae* and comprises at least 76 species (Ferguson & Huang, 2007). It is a dioecious climbing vines plant (Ferguson, 2013).

The fruit is an edible berry with hundreds of small and dark seeds embedded in the green flesh. Moreover, it has a hairy and light brown skin. Most *Actinidia* species are native from eastern Asia, with a remarkably wide geographic distribution in the southern provinces of China (Li *et al.*, 2009). The diversity origin of this genus is likely centered between the Yangzi (Chang Jiang) and Pearl (Zhu Jiang) rivers. The typical warm and moist environments typical of the above-mentioned regions are the most suitable for *Actinidia* species growth. The diverse soil conditions and variable microclimates influenced by mountain ranges probably contributed to the increased rates of species diversity (Ferguson, 2013).

Figure 1. Images from *Actinidia* plant structures. Adult plant (A), flower (B), immature kiwifruit (C) and mature kiwifruit (D) (this sudy).

The first country in the world to develop a commercial orchard of *A. deliciosa* "Hayward" (green kiwifruit) was New Zealand in 1930. The huge success leads to the rapid exportation of plants to Italy, China, Chile, France, Greece, Japan and the United States. Since then new cultivars are available namely the kiwi berry (*A. arguta*), and

other variants of the species *A. chinensis* "Hort16A", ZESPRI" (gold kiwifruit) (Ferguson & Huang, 2007; Stonehouse *et al.*, 2013).

1.2. Socio-economic importance of *Actinidia* culture

In last years the production of kiwifruit is expanding because of the consumer demands based on its good taste, nutrition and benefits for health, namely a high content of vitamin C, E, K, potassium and fiber among others (Stonehouse *et al.*, 2013).

Actinidia is an important commercial crop in several countries. In 2014, China was the most important producer worldwide with a production of about 1 183 856 ton[3], followed by Italy (373 372 ton[3]), New Zealand (301 515 ton[3]), Chile (161 403 ton[3]) and Greece (75 762 ton[3]) (FOASTAT, 2016). Portugal was the 11th world kiwifruit producer, with 12 thousand tones exported, representing an income of 13 million euros (FOASTAT, 2016), and it was the 4th largest producer of kiwifruit in the European Union, with a total annual production in 2015 of approximately 28 331 tones, with a total area of 2 350 ha (INE, 2015).

Portugal has an excellent soil and climatic conditions for kiwifruit production and since 1990 this culture began to gain some commercial value due to the low-cost production. Kiwifruit production is mainly located in the coastal part of the North and Central Regions of Portugal, namely in the sub-regions Entre Douro e Minho and Beira Litoral and in the central region of Portugal (Bairrada). In 2015, Portugal imported 10 934t of kiwifruit with cost of 12 466 000 € and exported 14 970t with an income of 15 560 000 € (INE, 2015; European Comission, 2014).

In March 2010, the first focus of kiwi bacterial canker caused by *Pseudomonas syringae* pv. *actinidiae* (Psa) was reported in Portugal, on the region of Entre o Douro and Minho (Santa Maria da Feira and Valência) (Balestra *et al.*, 2010). In 2011, new cases were reported on the North of Portugal (Amarante, Arouca, Vila Verde, Castelo de Paiva among others) and on the following years the disease spread to the center of Portugal (Oliveira do Bairro, Anadia, Cantanhede, Águeda, Soure, Leiria among others) (Pinto *et al.*, 2014; DGAV, 2013). The bacterial canker of kiwifruit is a quarantine disease responsible for a negative impact in the economy of producing countries. Until 2018, losses of 310 to 410 million € in New Zealand are expected due to Psa (Khandan *et al.*, 2013).

1.3. *Pseudomonas syringae* pv. *actinidiae* (Psa) on kiwifruit

Currently, the gram-negative bacterium Psa is the major threat for kiwifruit crop worldwide as the causal agent of the "kiwifruit bacterial canker" (Fig. 2). This is a severe disease that promotes plant mortality due to the its high level of aggressiveness, reflected in important economic losses in kiwifruit production (Cameron & Sarojini, 2014).

The first reported on this emerging disease was in in Japan, on cultivar *Actinidia deliciosa* (Hayward) in 1983 (Serizawa *et al.*, 1989). After this first outbreak, the disease was rapidly spread to the Hayward cultivar in Korea in 1988 (Koh *et al*., 1994), in Italy in 1992 (Scortichini, 1994) and in China in the same year (Wang *et al*., 1992). In last years, Psa has been detected in all major kiwifruit producers countries where *A. deliciosa* and *A. chinensis* are cultivated, such as New Zealand (Everett *et al.*, 2011), Portugal (Balestra *et al.*, 2010), France (Vanneste *et al.*, 2011), Spain (Balestra *et al*., 2011; Abelleira *et al.*, 2011), Italy (Balestra *et al*., 2009) and Turkey (Bastas, 2012). Meanwhile, different populations of this pathogen (Psa1, Psa 2, Psa 3 and Psa 5) have been identified in all major areas of kiwifruit cultivation through genotypic and phenotypic of Psa strains (Ferrante & Scortichini, 2015; Fujikawa & Sawada, 2016).

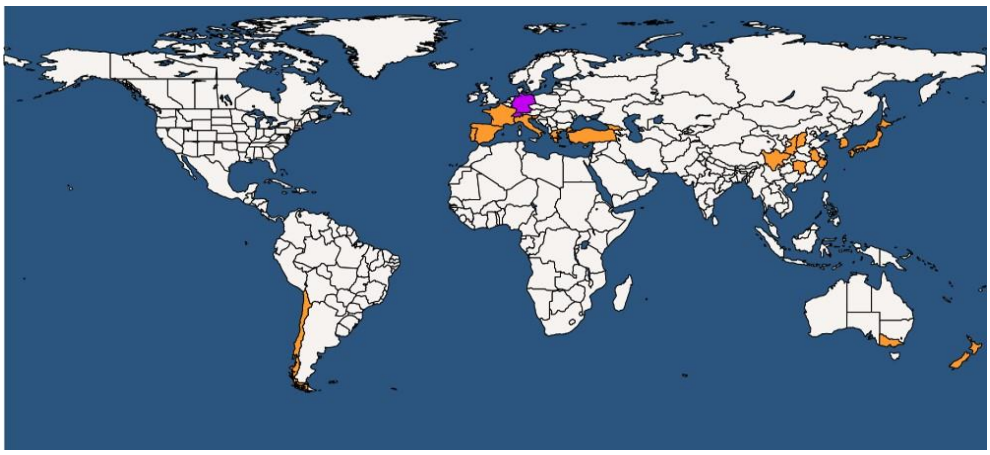

Figure 2 Current distribution of kiwifruit bacterial canker in world. Orange: presence of Psa; Purple: presence of Psa transiently, under eradication (EPPO, 2017a).

Psa is able to colonize and persist in several *Actinidia* spp., namely *A. deliciosa* (green kiwifruit), *A. chinensis* (yellow kiwifruit), *A. arguta* (baby kiwifruit) and *A. kolomita* (beauty kiwi) (EPPO, 2014)

Psa can colonize the above ground organs of kiwifruit plants being the major entry sites natural openings, mainly lenticels in stems, and stomata in leaves (Fig. 3)

(Bae *et al.*, 2015). Another possible colonization path are wounds caused by agriculture operations (e.g. pruning without equipment disinfection), intensive and strong precipitation and wind (DGAV, 2013). The transmission can occur during the entire year, but it is favored during spring and autumn when climatic conditions are more suitable for Psa multiplication (mild temperatures, abundant rainfall and high humidity). Psa optimal multiplication temperature is 25ºC, nevertheless its remains active between 10ºC and 30ºC but its dispersion and infection is limited (European Commission, 2014; Garcia, 2015).

Psa spread between orchards is mostly due to a combination of factors, namely agricultural practices that favors the presence of Psa inoculums in the orchards, which can be easily spread through infected tools, heavy rainfalls, strong winds and pollinators. Furthermore, the detection of new disease focus is mostly related with the commerce of infected plant material (EPPO, 2017b).

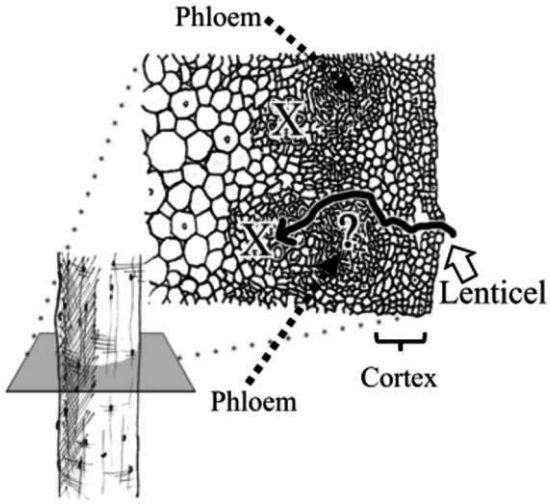

Figure 3 Possible path of infection in kiwifruit stem by Psa. Black lined arrows indicate possible infection paths. X xylem. Image adapted from (Bae *et al.*, 2015).

The most common symptoms in *Actinidia* caused by Psa infection (Fig. 4) include dark brown necrotic angular spots surrounded by yellow chlorotic halos on leaves and cankers with white to reddish (oxidation) exudate production on trunk and branches (Serizawa *et al.*, 1989). The wilting of flower accompanied by flower and bud drop is also observed as well as fruit specks and scabs (Marcelletti *et al.*, 2011).

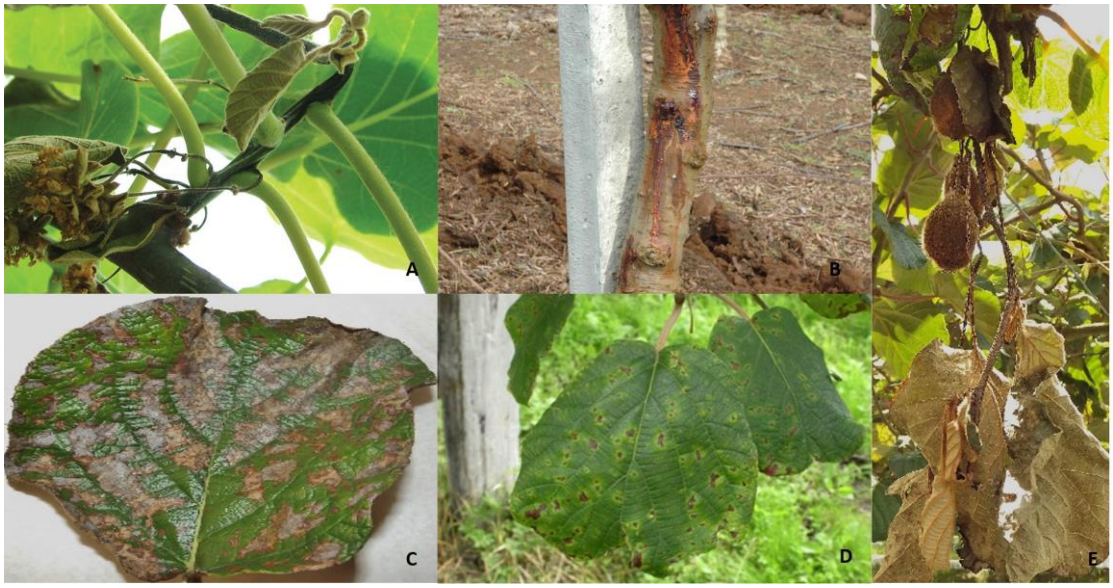

Figure 4 Characteristic symptoms of Psa infection in *Actinidia* spp. (A) Floral buds with brown necrosis; (B) Areas under the bark with a reddish exudate; (C) and (D) Leaf with brown necrosis surrounded by a yellow halo; (E) Wilt and death of branches. Adapted from (Moura *et al.*, 2015).

For kiwifruit cultivation, it is much more easy to understand the disease origin because its domestication is recent (1930s), unlike most crop plants which have been domesticated thousands of years ago (Lindow, 2015; McCann *et al.*, 2013). The problem of plant domestication is that the exhaustive and continuous anthropogenic selection of plants from wild ancestors, leads to the progressive homogenization of plant genotypes, thus corrupting the natural genetic variability present in non-domesticated ancestors (Bulgarelli *et al.*, 2013). Consequently, the intensive cultivation of clonal plants with low genetic diversity has led to the emergence of several problems, such as the decrease on efficiency of nutrient use, increase of pests and diseases impact and inability to overcome abiotic stress (Strange & Scott, 2005; Wulff *et al.*, 2011; Morris *et al.*, 2008; Gopal & Gupta, 2016). As the production of kiwi on a global scale is recent, and consequently the introduction of Psa, it is possible to follow the evolution of this disease in *Actinidia* from the 1st outbreak to the global pandemic, and in the future develop more efficient and targeted measures to prevent, mitigate and control Psa (McCann *et al.*, 2013).

1.4. Measures to prevent, mitigate and control Psa

To control the spread of the bacterial canker disease, the kiwifruit industry is committed in developing strategies that minimize both damage and spread. These strategies include good orchard hygiene practices, breeding resistant varieties, scheduled spraying of bactericidal compounds, use of elicitors that activate the plant's immune system and the use of biological control options, as well as reliable detection methods (Vanneste, 2013). Currently, chemicals treatments for control of plant pathogenic bacteria are based on the use of antibiotics (mostly streptomycin) or heavy metals (mostly copper). Some countries in Asia, and New Zealand allowed the use of such products; on the contrary, only copper derived products are legally used in Europe. Nevertheless, these products used in excess have high phytotoxicity, leaving residue in the fruit and leading to the appearance of bacterial resistance. All these treatments are only use in the prevention or as mitigators since there is not any curative treatment known against Psa (Cameron & Sarojini, 2014).

In order to prevent the entry or spread of dangerous plant diseases and pests the European and Mediterranean Plant Protection Organization (EPPO) (organization responsible for cooperation and harmonization in plant protection within the European and Mediterranean region), added an alert to the list A2 for Psa (EPPO, 2012). This alert list contains all organism recommended for regulation and defined as quarantine organisms. Psa belongs the A2 list because it is present in the EPPO region but is not widely distributed and it is officially under control. In addition, Psa is a quarantine organism because it has the potential to cause important economic losses to the EPPO region, a region with important kiwifruit producers (e.g: Italy, Portugal) (EPPO, 2012).

In 2012, the European Union Commission, intending to control the dispersal of the disease, approved the execution nº2013/756/UE that details the measures of emergency to block the introduction and dispersal of Psa. For this effect, propagation material or live pollen can only be moved into and within the EU territory accompanied by a phytosanitary passport to the diploma compliance (Pinto *et al.*, 2014).

1.5. Phyllosphere microbiota

Plants are attractive host for microbes offering distinct environments namely, the phyllosphere (above ground) and rhizosphere (below the ground) (Fig. 5) (Berlec, 2012). Moreover, plants may be divided in other different habitats, providing abiotic and biotic conditions for the development of microbial life; such as the endorhiza (internal environment of root system), the anthosphere (flower), the spermosphere (seed), the carposphere (fruit), the endosphere (inner tissues) and ectosphere (outer surfaces) (Berg *et al.*, 2014). This study only focus the phyllosphere and more concretely leaves.

The phyllosphere is a microbial habitat defined by the surface and interior of the above-ground organs of vascular plants, mainly dominated by leaves (Müller & Ruppel, 2014; Bulgarelli, *et al* 2013; Vorholt, 2012). Buds, flowers and trunks represent a less representative fraction of the plant structure (Lindow & Brandl, 2003). The study of the microbiota associated with this habitat is of special interest due to its large and exposed surface area and to its connection with the air microbiome (Berg *et al.*, 2014). The phyllosphere is considered one of the largest microbial habitats on earth, with a total area of leaf surface estimated of $1x10^9$ km^2, populated by a large and diverse community of bacteria, archaea, yeast, fungi and algae. Bacterial are the most common microorganism in this habitat with a typical cell density of 10^6-10^7 cells/cm^2 of leaf tissue (Lindow & Brandl, 2003; Rastogi *et al.*, 2013; Vorholt, 2012; Whipps *et al.*, 2008).

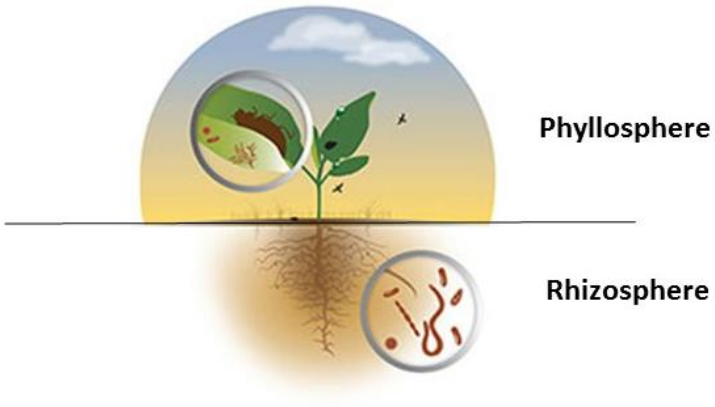

Figure 5 Different environments in plants colonized by microbiota Adapted from (Phytobiomes, 2017)

The microbial communities can colonize different parts of the plants. The surface colonizers of plant's tissues are considered epiphytes, whereas microbes residing inside of plant tissues (the endosphere), whether in leaves, roots or stems, are named endophytes. Numerous studies have revealed that series of microbial traits are beneficial for host plant, so the colonization of plant by this microorganism can promote plant growth through the production of hormones, and even, protect plants against pathogens through the production of antibiotic compounds, or by competing for nutrients resources and space (Berg, 2009). Additionally, some microorganisms stimulate plant immune system (Pieterse *et al*., 2012) to suppress plant pathogen proliferation and ultimately leading to plant protection (Vorholt, 2012). They may also promote tolerance to abiotic stress (e.g. drought) and influence crop yield and quality by nutrient mobilization and transport (Berg *et al*., 2014).

As microbial community around plants have an essential role in plant health and development, it is relevant to understand the processes that are responsible for determining the composition of the microbiota from phyllosphere (Bodenhausen *et al*., 2014).

The phyllosphere composition can be shaped by a multiplicity of factors, such as temperature, humidity, ultraviolet radiation, pollution, growing season and nutrient availability. In addition, precipitation and wind can also contribute to the temporal variability of the phyllosphere microbiota (Turner *et al.*, 2014). In this context, the host plant genotype and innate immune system also play a major role in the microbial community dynamic (Copeland *et al.,* 2015; Vorholt, 2012). Host genotype can be the primary factor driving microbiota composition, while in other cases the geographical location can directly influence the community composition (Vorholt, 2012). In fact, geographical location has been identified as a main driver of microbiota diversity in a lettuce field (Rastogi *et al.*, 2012). Anthropogenic factors, such as pesticides, management agricultural practices, such as organic vs conventional farming, application of antibiotics and nitrogen fertilization have strong effects on the community composition (Bringel & Couée, 2015; Rastogi *et al*., 2013).

The common microbial communities between plants constitute the core microbiota and plays a key role in the ecosystem function within a habitat. Any change in the core microbiome composition cause debilitative or destructive diseases on plants and this impact is manifested in plant trait expression (Rout, 2014; Gopal *et al.*, 2013). For example, pathogen outbreaks are associated with shifts in the entire community of

the core microbiota (Berg *et al.*, 2014). The microbial members within the plant microbiome cause differences in expression of plant phenotypes, due to their influence on plant functional traits (Gopal *et al.*, 2013; Rout, 2014; Berg, 2009). For these reasons, the characterization of the community structure and phylogenetic diversity found in a plant species is crucial since it could provide information leading to more suitable, sustainable and efficient agriculture practices (Copeland *et al.*, 2015; Schlaeppi & Bulgarelli, 2015).

1.6. Importance of plant microbiota in sustainable agriculture

With the predictable growth of human population, it will be required an increase on current agricultural production to satisfy the demand for food. The decline of arable land (limiting nutrient resources), global warming leading to constant change in climatic conditions, and the rise of diseases has been affecting severely agriculture crop yield. Therefore, to ensure global food supply for a growing population, the cropping system should be optimized and enhanced to maximize production in the limited fertile land while minimizing the resources required (Parnell *et al.*, 2016; Imam *et al.*, 2016; Kroll *et al.*, 2017).

One way to foster a sustainable agriculture is through the integration of benefic microorganism to plants that will increase crop yield through enhance nutrient update by plants (inoculants), decrease crop loss due to pests and diseases (biocontrol), improve plant growth and increase abiotic stress tolerance. Thus, this is a possible organic alternative to the use of chemical pesticides, harmful to humans and environment, making a profit out of plant microbiota (van Overbeek, 2017). Currently, there are agriculture companies that are coating seeds with beneficial microbes or adding them to the soil to increase the production of crops, such as soybean and corn, while reducing the need for fertilizers and pesticides (Busby *et al.*, 2017; Imam *et al.*, 2016; Müller *et al.*, 2016). Moreover, strategies that involve managing the plant microbiota will have an even greater impact in production yield in regions of the world with low fertilization and water availability, as well as those mostly affected by diseases (Sessitsch & Mitter, 2015).

1.7. Leaves microbiota characterization

The characterization of the microbiome present in leaves can be achieved by culture -dependent and/or -independent methods based on the phylogenetic assignment of the *Bacteria* populations obtain from the 16S rRNA gene sequence.

1.7.1. 16S RNA gene

16S small ribosomal subunit gene (16S rRNA) is a molecular marker gene used in microbial ecology to determinate the phylogenetic classification of detected taxa, and to characterize bacterial community diversity in the environment (Willey *et al.*, 2014; Woese, 1988; Mizrahi-Man *et al*., 2013). This gene is suitable for taxonomic studies because it has several aspects that make it an optimal phylogenetic marker. First, 16S rRNA gene is universal among all prokaryotic organisms, allowing the phylogenetic analysis between distinct taxa (Mizrahi-Man *et al.*, 2013; Willey *et al.*, 2014). Second, the gene is functionally conserved, resulting in low rates of mutation (clock-like mutation) throughout prokaryotic evolution, suggesting that any changes in random sequence are a more accurate measure of time (evolution). Furthermore, it is weakly affected by horizontal gene transfer. Finally, the 16S rRNA gene (~1 500 bp) has highly variable regions and other regions that are extremely conserved which act as good primers target (Fig. 6). Primers designed to target these conserved regions allow the amplification of the highly variable regions. The degree of dissimilarity between those regions allows to discrimination between closely related microbes (Mizrahi-Man *et al.*, 2013; Willey *et al.*, 2014).

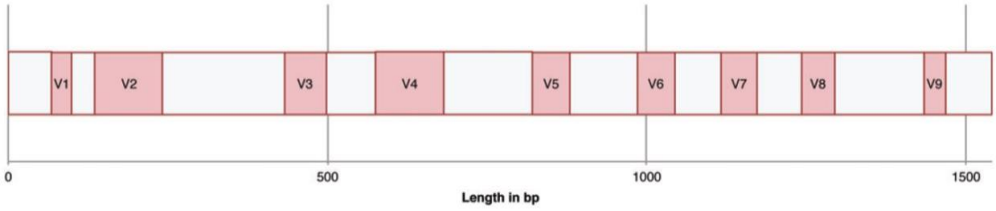

Figure 6 Structural representation of the 16S rRNA gene from Escherichia coli. The nine hypervariable regions (V1-V9) are flanked by nine highly conserved regions (white) (Cox *et al*., 2013) which can be used to identify microorganism. Adapted from (Clarridge & Alerts, 2004).

1.7.2. Culture-dependent methods

Culture-dependent methods, as the name suggests, imply isolation in culture the microorganisms present in a given sample. The use of this methodology in studies aiming the characterization of the phyllosphere microbiota is rather important since it allows building culture collections of microorganism that Microbiologists can use to perform a formal description and evaluate their potential role in the ecosystem. In particular, questions regarding specific characteristics or activities of a given microorganism that could be used for multiple applications can only be answered when researchers work directly with the organism (Costa & Tiago, n.d.; Rastogi *et al.,* 2011).

The microorganism isolation process is time-consuming and demanding in order to allow recovering as much as possible of the cultivable diversity present. One should try to sub-culture, if not all, most of the recovered colonies that are obtained in each different incubation condition. This increases drastically the number of isolates to screen and identify, thus the work needed to perform the task. During the screening process, molecular fingerprint techniques can be applied, such as Random Amplification Polymerase Deoxynucleic acid (RAPD) – Polymerase Chain Reaction (PCR). These techniques allow decreasing the number of isolates that need to be identified, by grouping the overall isolates and choosing one or two representatives of each group to do the phylogenetic identification. The more exhaustive the work is, the closest it is to the true microbial diversity of the environment in define conditions (Costa & Tiago, n.d; Rastogi *et al.*,, 2011).

In order to identify the isolates recovered from a microbial community it is require the PCR-amplification of the 16S rRNA gene of each strain (Rosselli *et al.*, 2016) and to determine its sequence by the Sanger sequencing method. This type of sequencing can comprise the full length of the 16S rRNA gene, allowing a more accurate phylogenetic classification of the isolate, and also providing data for the calculation of the abundance and diversity of bacterial populations in each sample (Bikel *et al.*, 2015).

However, culture-dependent methods have some inherent bias. In laboratory conditions, the recovery of microbial diversity by standard culture technique is limited. The main limitations are the difficulty in mimicking natural environments in terms of nutrients (e.g. composition and concentration) and culture conditions (e.g. pH, salinity, temperature) which leads to the recovery of only a fraction of the total population.

These recovered microorganisms rarely represent the most abundant population in the environment since these methods select those that grow best in the available growth conditions (Al-Awadhi *et al.,* 2013; Turner *et al.,* 2014). It is estimated that the recovered populations on laboratory conditions constitute less than 2% of all microbial diversity present in the environment (Rinke *et al*., 2013; Wu *et al*., 2009; Blaser *et al.*, 2016; Solden *et al.*, 2016).

1.7.3. Culture-independent methods

New molecular approaches (culture-independent methods) have been developed in order to circumvent the diversity-related bias of the previous described method. These molecular biology methods revealed several microbial communities that had never been cultured before, recently named as "microbial dark matter" (Birtel *et al.,* 2015; Blaser *et al.,* 2016). Thus, culture-independent methods allowed to achieve higher resolution on determining richness and diversity of microbiota compared to culture-dependent methods (Yashiro *et al.,* 2011). Currently, there are some common platforms of Next Generation Sequencing (NGS) namely, Illumina, 454-Pyrosequencing (discontinued platform), IonTorrent, and more recently PacBio (Goodwin *et al.*, 2016).

To analyze the microbiota by 16S rRNA profiling, the methodology involves environmental DNA extraction and amplification of specific regions by PCR, followed by sequencing and phylogenetic analyses (Bikel *et al.*, 2015). However, no technique is perfect and all have their inherent bias. The mainly biases are linked with the type of sampling, protocols for DNA extraction, set of primers and sequencing (Vorholt, 2012). Environmental samples are complex in their chemical composition and may have inhibitory substances that if not properly removed during extraction steps can negatively affect downstream processes, e.g. amplification and sequencing of the 16S rRNA gene. Moreover, the design of a true universal primer set is difficult and may artificialy select groups of microrganism with erronious results (Fig. 7) (Ibarbalz *et al*., 2014; Myer *et al.,* 2016).

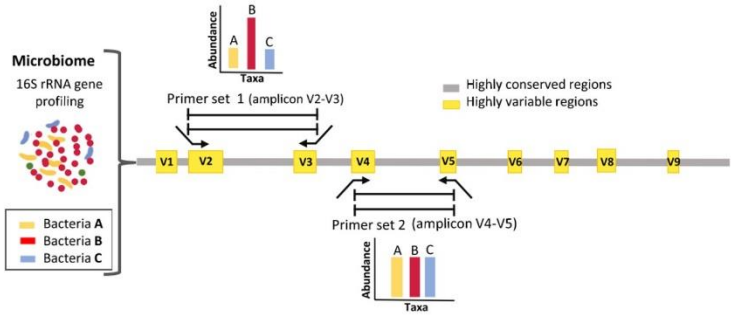

Figure 7 Importance of primer choice for the amplification of the hypervariable regions of the 16S rRNA gene. The figure illustrates that different sets of primers can amplify different kind of bacteria resulting in different abundance relative of hypothetical bacteria A, B and C. Adapted from (Bikel *et al*., 2015).

1.8. Goals

Actinidia deliciosa is a culture that has been seriously affected by the kiwifruit bacterial canker disease, responsible for huge economic losses in the major producing countries. Therefore, our goal was to determine the microbiota of leaves from healthy *A. deliciosa* and compare it with the microbiota obtained from *A. deliciosa* infected with *Pseudomonas syringae* pv. *actinidiae* (Psa), the causal agent of kiwifruit canker. With this approach, the identified alteration on the microbiota between heath and disease plants will allow assessing the impact of the disease and identify groups of microorganisms with a potential role on the modulation of the disease or on the plant health status to be used in the near future.

Moreover, there is a reported difference on the susceptibility to Psa between *A. deliciosa* females and males. In accordance, it is also our goal to determine if the microbiota structure of females and males may play a role on this feature, paving the way to the discovery of new preventing/controlling agents against Psa.

We intended to eliminate possible factors that could alter the microbiota between samples. In accordance, the studied *A. deliciosa* leaves were collected from plants in neighbor orchards removing the influence of abiotic conditions in the microbiota structure. Moreover, both orchards were organic, with no applied chemicals avoiding, once more, the artificial selection of bacterial populations based on their resistant to pesticides.

In order to fulfill our goals, the microbiota composition was determined by culture–dependent and culture-independent methods since we intended not only to construct a culture collection of microorganisms that could be used in future studies but to get a

detailed snapshot of the “total” microbiota. Namely, (1) identify and compare facultative aerobic heterotrophic microbial populations isolated from leaves of healthy plants (female and male) and disease plants (female and male); (2) determine and compare, by NGS the microbiota of disease plant (female and male) and healthy plants (female and male).

The combination of these two methodologies allowed a detailed analysis of the microbiota structural diversity associated with the phytossanitary status of *A. deliciosa*.

2 Materials and methods

2.1.Sampling

In June 2016, *Actinidia deliciosa* leaves were harvest from two organic kiwi orchards from "Delícias do Tojal", located in Vila Verde (N 41 º 68.930008'; W -8 º 4079839'), a region in the Northwest of Portugal (Fig. 8). The detection of Psa in both orchards was previously determined as a contracted service (FitoLab, IPN) according with EPPO standards. The presence of Psa was confirmed in the orchard with clear symptoms of bacterial canker disease – Diseased (D) while no Psa was detected in the neighbor orchard - Healthy (H).

From each kiwi orchard, two composite samples were collected, one comprising leaves from five female plants (F) and the second leaves from five male plants (M). From each plant, 10 leaves were handpicked. Samples were placed in individual plastic bags, labeled and transported to the laboratory in an icebox. The leaves were processed on the same day of the collection for the isolation of epiphytic and endophytic microorganisms and for total DNA extraction.

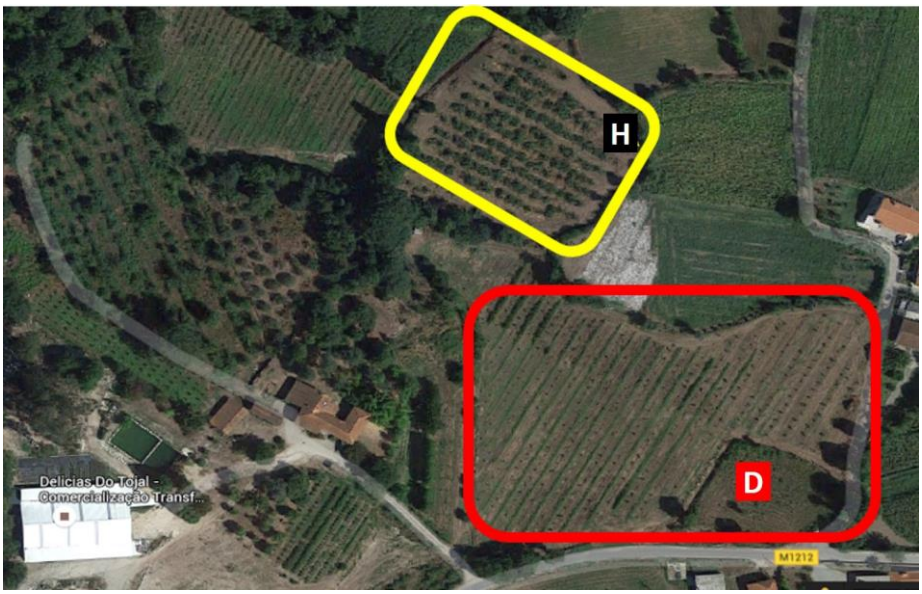

Figure 8 Geographic location of the studied orchards: H (healthy orchards – no Psa detected) and D (diseased orchards –Psa presence confirmed).

2.2.Processing of samples

From each orchard approximately ~150 g of female and male leaves were cut into small pieces and grind in a blender with 600 ml of ultra-pure autoclaved water (Milli-Q water filtered through 0.1 µm filter, autoclaved at 121 ºC for 20 minutes) to obtain a homogenized solution (Fig.9A).

Figure 9 Processing of leave samples. (A) Grinding of the samples to obtain a homogenate. (B) Filtration of the homogenate for the elimination of vegetable debris. (C) Storage of the sample for further analysis.

After this procedure for each sample, one part of the homogenized solution was filtered through sterile gauze cloth as the image demonstrates (Fig. 9B), to obtain a liquid solution without vegetable debris for isolation of epiphytic and endophytic microorganisms by culture-dependent methods.

Another part of homogenate solution (without filtration) was transferred directly to bottles and kept in the deep freezer at -80ºC (Fig. 9C) for posterior DNA extraction for culture-independent methods.

2.3. Culture-dependent methods

Several incubation conditions were used during isolation procedures, in an effort to maximize the diversity of recovered facultative aerobic heterotrophic bacterial populations: two solid culture medium (ABM2 – "Alkaliphilic Buffered Medium 2" and Difco™ R2A), six dilutions (10^0 to 10^{-6}), three pH (5.5, 7 and 8.5) and three temperatures (20ºC, 25ºC and 37ºC), were used.

2.3.1. Media

Table I Solid ABM2 medium composition

Composition	Quantity
Yeast extract	5 g
Tryptone	5 g
α-Ketoglutarate	1 g
Organic Buffer*	40 mM
Macronutrients solution 10x concentrated**	100 ml
Micronutrients solution 100x concentrated ***	10 ml
Cycloheximide	50 mg/ml
Agar	15 g
Demineralized water until	1000 ml

*According to the desirable pH value. Discriminated buffers in the table I.I in supplementary data.
**Macronutrients solution composition in the table I.II in supplementary data.
***Micronutrients solution composition in the table I.III in supplementary data.

Yeast extract, tryptone, α-ketoglutarate and the organic buffer were weighted and dissolved in 300 ml of demineralized water plus the micronutrients and macronutrients solutions previously measured. The volume of the solution was adjusted with demineralized water until 1000 ml. The solution was homogenized with a magnetic stirrer. The required pH's were obtained by adjusting the pH value of each medium with hydrochloric acid solution (final media pH 5.5) or potassium hydroxide tablets (final media pH's 7 and 8.5). Media were transferred into shot-bottles with agar and autoclaved at 121ºC for 20 min. After sterilized, media were cooled in water bath until 50ºC and plated into plastic Petri dishes. Media were preserved at room temperature.

Table II Solid R2A medium (Difco ™) composition.

Composition	Quantity
Yeast Extract	0.5 g
Proteose Peptone No.3	0.5 g
Casamino acids	0.5 g
Dextrose	0.5 g
Soluble Starch	0.5 g
Sodium Pyruvate	0.3 g
Dipotassium Phosphate	0.3 g
Magnesium Sulfate	0.05 g
Cycloheximide	50 mg/ml
Organic Buffer*	40 mM
Demineralized water until	1000 ml

* According to the desirable pH value. Discriminated buffers in table I.I in supplementary data.

The organic buffer was dissolved in 1000 ml of demineralized water and homogenized with a magnetic stirrer. The requested pH's were obtained by adjusting the pH value of each medium with hydrochloric acid solution (final media pH 5.5) or potassium hydroxide tablets (final media pH's 7 and 8.5). Additionally, all solutes were hydrated with the previous solution and autoclaved at 121ºC for 15 min. After sterilized, media were cooled on a water bath to 50ºC and plated into plastic Petri dishes. Media were preserved at the room temperature.

2.3.2. Incubation conditions and media inoculation procedures

In this study, several incubation conditions were used for each medium as shown in Figure 10.

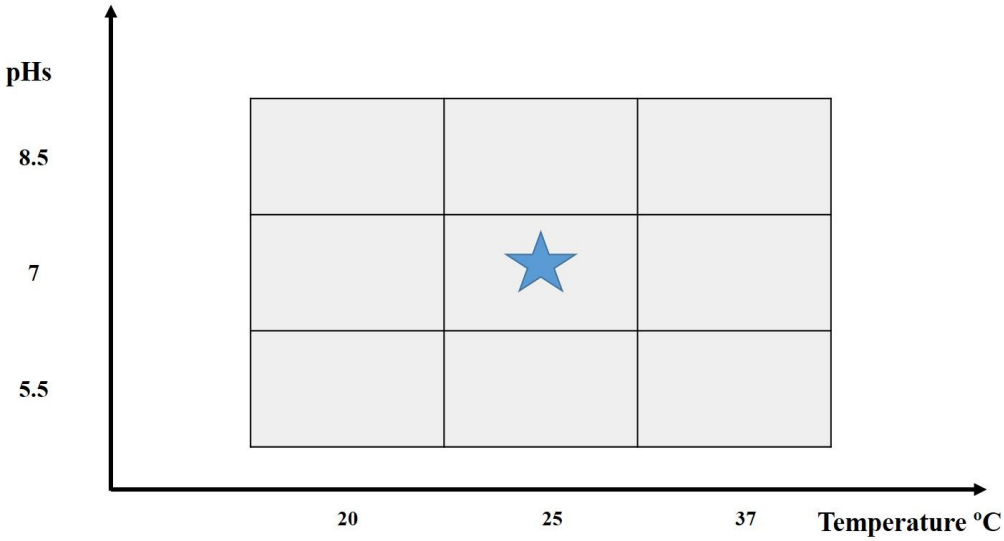

Figure 10 Incubation conditions used during isolation process. Mostly used incubation conditions for bacterial isolation are marked with a star. Each pH (5.5, 7, and 8.5) was tested for each temperature (20ºC, 25ºC and 37ºC).

The serial dilution technique was applied to produce cell suspension to be used during inoculation procedures. This technique allows obtaining cell suspensions in several concentrations, from high to lower cell concentrations, so that the inoculation results in the growth of isolated colonies. Under sterile conditions, 1ml of each sample was added to 9 ml of sterile potassium phosphate buffer solution (PBS), described in table III, mixed by vortex and further serially diluted (1:10) in the same buffer (Fig.11).

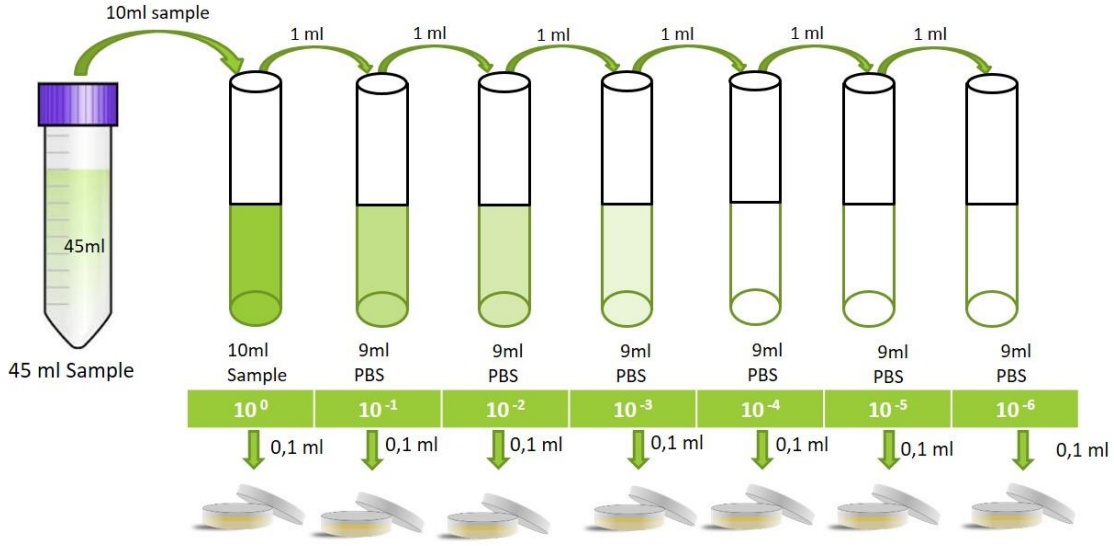

Figure 11 Experimental procedure of serial dilution technique and inoculation.

Table III PBS 10 mM composition.

Reagents	Weight
Sodium chloride	8.0g
Potassium chloride	0.2g
Sodium Dihydrogen Phosphate Dodecahydrate	2.9g
Potassium phosphate	0.2g
Distilled water	Up to 1000 mL

All reagents were weighted and dissolved in 1000 ml of distilled water. The pH was adjusted with hydrochloric acid solution at 7.2. Lastly, the solution was sterilized by autoclaving at 121°C for 20 minutes.

For all different media, different dilution levels were used for inoculation, according to table IV. In this study, the conditions highlighted in green were used.

Table IV Conditions used in both media according to temperature and pH values. The green conditions were used.

		Serial dilution						
Temperature ºC	**pH**	**1**	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**
20	5.5							
	7							
	8.5							
25	5.5							
	7							
	8.5							
37	5.5							
	7							
	8.5							

Solid media were inoculated with 100 µl of selected serial dilution bacterial suspension by spread plate method (Fig. 12) under sterile conditions.

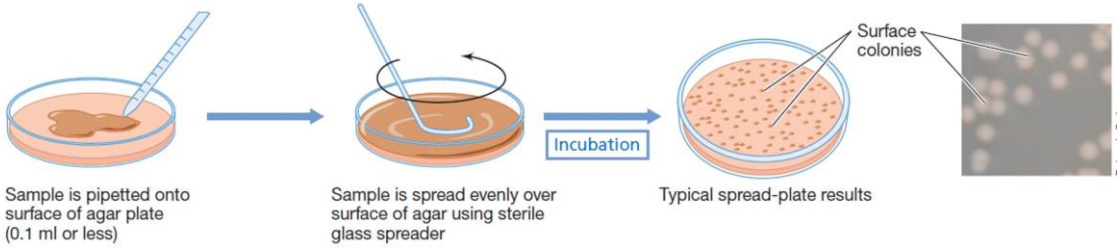

Figure 12 Explanatory picture of the spread-plate method (Madugan *et al.*, 2013).

After inoculation, plates were incubated for 5 days at defined temperatures (20ºC, 25ºC, and 37ºC) in inverted position to prevent condensation drops of the cap from falling on the surface of the agar. Visible colonies were enumerated to determine the number of colony forming unit (CFU) bacteria recovered on each condition. Isolation procedure encompassed random colonies selection. Each selected colony was summarily characterized according with morphologic characteristics (namely, pigmentation, shape, margin, size and texture) and subcultured in the same medium and temperature for purification purposes. For the process of subculturing, each selected colony was picked up with a sterile loop and inoculated into fresh solid medium; this was performed as often as necessary until pure cultures were obtained.

2.3.3. Preservation of pure culture isolates

Pure cultures of the isolates were cryopreserved in cryogenic-tubes with preservation medium (isolation medium supplemented with 15% (w/v) glycerol with pH value adjusted to isolation values). Cell mass was obtained with a loop (10 µl) by removing cell mass of the pure culture from the medium surface, and resuspended into the freezing tube containing the preservation medium. The suspension was homogenized by vortex and stored in a deep freeze at -80ºC.

Preservation medium was obtained by mixing two solutions 1:1 (v/v): liquid ABM2 medium 2x concentrated and glycerol 30%. ABM2 liquid was prepared to the double concentration for pH= 5.5, 7 and 8.5 (1x composition described at Table I). Glycerol 100% was weight (30 g) and diluted with 70 ml of Milli-Q filtered water (through 0.1 µm filter) for a glycerol solution with a final concentration of 30%. Both solutions were sterilized by autoclaving at 121ºC for 20 min. Solutions were cooled at room temperature, and preservation medium was prepared by mixing the two solutions, and then distributed by 2 ml cryogenic-tubes and store at -20ºC, until further use.

2.3.4. Total DNA extraction

DNA from each bacterial isolate was extracted by boiling, adapting the method of Wiedmann-al-ahmad *et al.*, 1994. One colony of each isolate was suspended in 50 µl of lysis buffer (Table V) and boiled in a water bath for 10 min, followed by freeze step at -20ºC for 5 min, to ensure the complete lysis of the cells. The suspension was defrosted and centrifuged at 13 200 rpm during 5 min in a benchtop centrifuge (Eppendorf 5415D) for deposit the cells debris. Supernatant was transferred into sterile Eppendorf 1,5 ml and stored at - 20ºC.

Table V Lysis buffer composition.

Reagents	Volumes
Tween 20 2%	500 µl
10x NH_4^+ Bioline	100 µl
H_2O Mili-Q sterile (sterilized by autoclaving (121ºC, 20 min)	400 µl

Lysis Buffer it was filtered through a membrane filter with a 0,2 µm pore diameter to a new Eppendorf.

2.3.5. Random Amplified Polymorphic DNA (RAPD)

Genomic diversity among the isolates was assessed through a DNA-fingerprint technique - Random Amplified Polymorphic DNA – RAPD). RAPD uses products from PCR amplification with a single primer with an arbitrary nucleotide sequence which anneals randomly at multiples sites on the genomic DNA and doesn't require any knowledge of DNA sequence of the target bacteria (Williams *et al.*, 1990)

In this study the amplification reactions were performed with a total volume of 30 µl. The best conditions to perform RAPD runs in thermocycler were obtained after testing several conditions as described below (Table VI). Concentration of primer, template and number of cycles were changed over the various attempts.

Table VI Concentration of reagents used for preparation of RAPD mix the various performed test.

Reagents	Standard	2nd Condition	3 rd Condition	4th Condition
H_2O Mili-Q sterile*	----------	----------	DNA was diluted 1:5. The concentrations to preparation of mix were the same standard condition.	New primer batch-OPA-03 was tested. The concentrations to preparation of mix were the same standard condition with exception in the concentration of primer. It was increased to 0.66 µM.
Buffer 10x	1x	1x		
$MgCl_2$ 50 mM	4 mM	4 mM		
dNTPs 10 mM**	2 mM	2 mM		
Primer OPA3 (1:10)***	0.33 µM	0.2 µM		
Taq DNA polymerase (5U/ µl)	0.05 U/ µl (Nzytech)	0.05 U/ µl (Bioline)		
Templet DNA	2 µl	2 µl		
PCR conditions	**Standard**	**2nd Condition**	**3 rd Condition**	**4th Condition**
Elongation	94 ºC for 1 min	Equal to Standard condition	Equal to Standard condition	Equal to Standard condition except the number of cycles that it was increase for 35
Annealing	45ºC for 1 min			
Extension	72ºC for 2 min			
Cycles	30			

*It was sterilized by autoclaving at 121ºC during 20 min.

**Deoxynucleotide (dNTP) solution mix described in table VII.

*** Stock solution was obtained by hydrating primer OPA3 in ultra-pure autoclaved water (Milli-Q filtered through 0.1 µm filter, autoclaved at 121 ºC for 20 minutes). Aliquot were carried out with a final concentration of 10 µM diluting 10 µl of stock solution (100 µM) in 90 µl ultra-pure autoclaved water (Milli-Q filtered through 0.1 µm filter, autoclaved at 121 ºC for 20 minutes).

.

Table VII Deoxynucleotide (dNTP's) solution mix).

Reagents	Final Concentration
dATP 100mM (Nzytech)	10 mM
dCTP 100mM (Nzytech)	10 mM
dGTP 100mM (Nzytech)	10 mM
dTTP 100mM (Nzytech)	10 mM
H_2O Mili-Q sterile *	-----

*Sterilized by autoclaving at 121ºC for 20 min.

At the end, the optimized conditions were as follows: 2 µl template DNA, 0.66 µM primer OPA-03 (5' AGT CAG CCA C 3'), 2 mM d'NTPs, 4 mM $MgCl_2$, 0.05 U/µl *Taq* DNA polymerase (Nzytech) with the respective buffer (1x) and sterile Milli Q water. The MyCycler Thermal Cycler (BIO-RAD) was used to run RAPD-PCR with the following temperature protocol: initial denaturation (94ºC for 5 min) followed by 35 cycles of denaturation (1min at 94ºC), annealing (1min at 45ºC), extension (1:30 min at 72ºC) and a single final extension step (7 min extension 72ºC).

2.3.5.1. Analysis of the amplification products

After the RAPD – PCR, it was added 4 µl of the loading buffer solution to the PCR products. The mixture was loaded into a well of a 2% agarose gel (TableVIII) stained with 7 µl of the ethidium bromide and electrophered in Tris-acetate - EDTA (TAE) electrophoresis buffer during 50 min at 75V. For comparison and standardization, in each lane the first well was loaded with a molecular weight marker (NZYDNA Ladder III, 200 to 10000 bp, Nzytech). The amplifications patterns were photographed under UV illumination (Image Analyzer Doc Tm RX +, Biorad, EUA).

Table VIII Composition of agarose gel at 2%.

Reagents	Weight
Agarose Nzytech	3 g
TAE solution (1x)*	150 ml
Ethidium Bromide	7 µl

* TAE solution (1x) was prepared with 40 ml TAE (50x) diluted in 2000 ml distilled water. TAE(50x) preparation described in table IX.I in supplementary data.

2.3.5.2. Analysis of DNA profiles

DNA profiles of isolates obtained by RAPD-PCR were grouped by visual inspection according with similarities between fragments observed by electrophoresis analysis. Isolates were grouped according to the generated profile determined by (1) number and similarity of each bands, and (2) to the migration of the bands when compared to each other and to the molecular weight marker. The intensity of the fluorescence of each band was not a factor used in the comparison between the RAPD profiles.

2.3.6. Amplification of 16S rRNA gene by Polymerase Chain Reaction (PCR)

The 16S rRNA gene was amplified only from representative isolates from each established RAPD group. The gene was amplified with bacterial-specific primers 27F (5' - GAG TTT GAT CCT GGC TCA G - 3') and 1525R (5'- AGA AAG GAG GTG ATC CAG CC - 3') (Rainey *et al.*, 1996).

In this study, the amplification reactions were performed with a total volume of 30 µl. The best conditions to perform the PCR runs were obtained after testing several conditions as described below (Table IX).

Table IX Concentrations of reagents used for preparation of mix in the various tests performed to amplify the DNA.

Reagents for the preparation of the mix	**Standard**	**2nd Condition**	**3rd Condition**
H_2O Mili-Q*	----------	----------	It was used the concentrations of 2nd condition..
Buffer 10x	1x	1x	
$MgCl_2$ 50 mM	4 mM	1.67 mM	
dNTPs 10 mM**	2 mM	2 mM	
Primer FW (1:1)***	0.41 µM	0.17 µM	
Primer RV (1:1)***	0.41 µM	0.17 µM	
Taq DNA polymerase (5U/ µl) Nzytech	0.05 U/µl	0.03 U/µl	
Templet DNA	2 µl	2 µl	
PCR conditions	**Standard**	**2nd Condition**	**3rd Condition**
Elongation	95 ºC for 1 min	Equal to Standard condition	95 ºC for 1 min
Annealing	55ºC for 1 min		53ºC for 1 min
Extension	72ºC for 2 min		72ºC for 2 min
Cycles	30		30

* Sterilized by autoclaving (121ºC, 20 min).

**Deoxynucleotide (d'NTP) mix was performed in the same manner as described in table VII.

*** Primer 27F and 1525R were suspended in sterile ultra-pure water (filtration and autoclaving (121ºC during 20 min). Aliquots were performed with a final concentration of 50 µM diluting 100 µM of stock solution in 50 µl ultra-pure water, sterile by filtration and autoclaving (121ºC during 20 min).

At the end, the conditions optimized for the amplification of DNA were as followed: 2 µl template DNA, 0.3 mM primer (forward and reverse), 2 mM dNTPs, 1.6 mM $MgCl_2$, 0.03 U/µl Taq DNA polymerase (Nzytech) with your respective buffer (1x) and sterile mili Q water. MyCycler Thermal Cycler (BIO-RAD) was used to amplify DNA with the following temperature protocol: initial denaturation (95ºC for 5 min)

followed by 30 cycles of denaturation (1min at 95ºC), annealing (1min at 55ºC), extension (2 min at 72ºC) and a single final extension step (10 min extension 72ºC).

2.3.6.1. Analysis of the amplification products

Amplicons were visualized according with the methodology described in section 2.3.5.1. The size of the amplicon was determined through comparison with a molecular marker.

2.3.6.2. PCR products purification

Amplicons were purified directly from TAE agarose gel using the NZYGelpure kit. All steps were carried out according to manufacture protocols. Briefly, 1500 b.p. DNA bands were cut and placed into a 2 ml Eppendorf tube containing a binding buffer; agarose was melted by placing the suspension at 50 °C for 15 min. The homogenized solution was loaded into a column with silica gel-based membrane and centrifuged for 1 min for a selectively adherence of the DNA fragments. All impurities (soluble agarose, nucleotides etc.) that did not bind to the membrane were removed through washing steps (twice) with a washing buffer and centrifuged at maximum speed for 1min. The flow-through was always discarded. At the end, the membrane was dried by an additional centrifugation step (1min). The filter was then transferred to a sterile Eppendorf 1.5 ml and DNA fragments were eluted with an elution buffer. DNA solution was stored at -20ºC.

2.4. Partial DNA sequencing of 16S rRNA

The PCR products were sequenced by Sanger's platform, as a contracted service. In our lab, multiwell plates were prepared with 5 µl of the template DNA and 5 µl of the primer 519R (CAG CMG CCG CGG TAA TWC) at final concentration of 5 µM (Turner, *et al*., 1999), and shipped to the sequencing facilities.

2.4.1. Phylogenetic analysis

16S rRNA partial gene sequence quality was checked with Sequence Scanner program. Good quality sequences were edited in BioEdit sequence editor for reverse complement (Hall, 1999). All sequences were clustered into groups of related sequences - operational taxonomic units (OTUs), with similarity cutoff of 98% using CD-HIT Suit Biological Sequence Clustering and Comparison (Huang *et al.*, 2010). One representative sequence of each OTU was used to retrieve the five closest relative sequences from the SILVA database (https://www.arb-silva.de/projects/living-tree/) and to obtain the taxonomic assign of each OTU. Phylogenetic analyzes were performed using ARB software package (Ludwig *et al.*, 2004). Phylogenetic trees were constructed using neighbor-joining (Saitou & Nei, 1987) with Jukes–Cantor correction (Jukes & Cantor, 1969) and bootstrap analysis (Felsenstein, 1985) of 1000 resamplings of the dataset.

To determine if the strains identified as *Pseudomonas* sp. were Psa, they were individually tested, as a contracted service (FitoLab, IPN), according with the EPPO standards.

2.5. Culture-independent technique

2.5.1. Harvesting and processing of samples

Samples collected in section 2.2 were used for extraction of total genomic DNA.

2.5.2. Total DNA extraction from *Actinidia* leaves

Extraction of total genomic DNA was carried out with The PowerMax Soil DNA Isolation Kit (MoBio Laboratories, USA). All steps were carried out according to manufacture protocol with an additional cleaning step.

Briefly, approximately 10 g of leaves from each sample were added to the PowerBead Tubes and homogenized. An anionic detergent solution was added, and the homogenized solution was vortexed for 10 min for cell lysis. After centrifugation, the supernatant was transferred to a clean sterile tube avoiding the pellet. Precipitation

solution was added, to precipitate non-DNA organic and inorganic material (such as humic acids, cell debris, and proteins) that constitute contaminating matter that reduce DNA purity and inhibit downstream DNA application. This step involved incubation at 4ºC for 10 min and centrifugation at room temperature at 2500 g for 4 min. Supernatant was transferred to a clean sterile tube avoiding any pellet. This precipitation step was repeated with a different precipitation solution to ensure complete remove of contaminating matter from the DNA. The supernatant was washed once more with an additional step (that does not exist on the manufacturer protocol) to remove any remaining contaminants. To the supernatant an equal volume of Chloroform:Isoamyl Alcohol solution (24:1; v/v) (SIGMA) was added, the solution was putted on ice during 10 min and centrifuged at 2500 g for 15 min. The upper phase was collected to a clean sterile tube and 30 ml of bidding solution were added. The solution was loaded onto a Spin Filter and centrifuged at 2500 g for 2 min at room temperature. In this step, the DNA is selectively bound to the silica membrane of the spin filter, while contaminants pass through the filter membrane leaving only DNA bound to the membrane. The same procedure was repeated until all volume of the solution was used. Then a final cleaning step was performed with an ethanol wash solution, by centrifuging at 2500 g for 3 min to clean the DNA bound to the silica filter membrane and remove contaminants. After discarding the flow through, the samples were centrifuged (2500 g for 5min at room temperature) again to removes residual ethanol wash solution (since ethanol can interfere with downstream DNA application). Spin Filters were placed in a Collection Tube, and sterile elution buffer was added to the center of the filter membrane to ensure a total release of the DNA from the silica Spin Filter membrane. Centrifugation was performed at 2500 g for 3 minutes at room temperature, the Spin Filter was discarded, and the DNA solution was stored at -20ºC.

2.5.3. 16S rRNA gene amplification from total DNA samples

In order to verify the integrity of genomic DNA from samples the 16S rRNA gene was amplified by PCR with universal pairs of primers specific for domain *Bacteria* before sending to sequencing facility. For domain *Bacteria* primers 27F (5'GAG TTT GAT CCT GGC TCA G- 3') and 1525R (5'- AGA AAG GAG GTC ATC CAG CC- 3') (Rainey *et al.,* 1996) were used. PCR amplification were performed in a total volume of 30 µl, with 0.05 U/ µl of Taq-DNA polymerase (Nzytech, Portugal), 0.41 µM each

primer, 2 mM dNTP's, 4Mm $MgCl_2$ and buffer 1x. It was used a MyCycler Thermal Cycler (BIO-RAD) to amplify the 16S rRNA gene using the temperature protocol: initial denaturation at 95ºC for 5 min followed by 30 cycles of: denaturation (1min at 95ºC), annealing (1min at 55ºC), extension (2 min at 72ºC) and a single final extension step (10 min at 72ºC). The present of PCR products and respective sizes were confirmed by 1% agarose gel electrophoresis as described in section 2.3.5.1.

2.5.4. DNA sequencing and data analysis

The structural diversity of the bacterial communities was determined by sequencing hypervariable regions from 16S rRNA gene in the Illumina's MiSeq platform.

For domain Bacteria primers MS357F (CCT ACG GGA GGC AGC AG) and 926 R (CCG TCA ATT YMT TTR AGT TT) covering regions V3-V5 were used. Raw data were analyzed by using mothur software package (http://www.mothur.org; Schloss *et al.*, 2009). Briefly, sequences were subjected to conservative quality control measures, namely initial quality trimming and assembly of contig reads sequences. Through the analyses workflow, all sequence reads with low quality and ambiguous bases, chimeras were removed from the data sets. After this, sequences were aligned, clustered into operation taxonomic units (OTU's) at a level of sequence similarity of 97% similarity and phylogenetically classified using the ARB-Silva taxonomic database (https://www.arb-silva.de/projects/living-tree/)

2.6. Diversity indexes and statistical analysis

The characterization of the microbiota assessed based on culture dependent and independent methods from within and between healthy and diseased orchards was supported by the measurement of two levels of inventory diversity: alpha and beta diversity.

2.6.1. Alpha diversity

The alpha diversity analysis (Hill, 1973) was individually performed for each orchard in order to compare the microbiota diversity between female and male samples. Margalef index (Dmg) was calculated following the formula: $Dmg = \frac{S-1}{\log(N)}$, being S the richness (total number genera) and N the total number of isolates. The Shannon

index (H') was determined from genera richness and their relative proportion following the formula: H'= -∑*pi*.ln(*pi*), being *pi* the specific richness of each genus. This index accounts for both abundance and evenness of the genus present. Pielou's evenness index (J') measured the ration between the observed diversity (H') and the maximal possible diversity, which accesses how equitable were the strains/sequences distributed by the genus (Pielou, 1966). The Simpson diversity index (1-D) determine the probability of two isolates/sequences belong to distinct genus. It was determined by the complement of Simpson alternative index ($D = \sum(pi)^2$). This index was sensitive to changes in genus abundance, being an indicative of dominance (Simpson, 1960).

2.6.2. Beta diversity

Beta diversity measures the similarity of the microbiota structure between the healthy and diseased orchards used in this study. A Jaccard index (Jaccard, 1912) was determined by the quotient between the intersection and the union of the pairwise compared genus among two orchards.

2.6.3. Principal component analysis and Venn diagram

A principal component analysis (PCA) – inter-species correlation and a Venn diagram based on the identified genus data were performed in order to understand the microbiota structure between orchards by using the software package CANOCO (Leps & Smilauer, 2003) and mothur (Schloss *et al*., 2009), respectively.

3 Results and Discussion

3.1. The impact of Psa on plant microbiome diversity based on culture - dependent methods

3.1.1. Bacteria quantification

The isolation of bacteria from the four samples matching different variables, namely, female-health (FH), male-health (MH), female-disease (FD) and male-disease (MD), was performed through the isolation procedures described in section 2.3.2. Selected colonies were subcultured until pure cultures were obtained (Fig.13). With this purification process, 1.258 isolates were cryopreserved.

Figure 13 Example of pure cultures in R2A used for cryopreservation.

The quantification of the recovered microorganisms from each condition is presented in Tables X to Table XIII. The isolation conditions described in the grey regions of the abovementioned tables were not tested because no growth was expected in higher dilutions, 10^{-4} to 10^{-6}, and in temperatures ranging between 20ºC and 25ºC. On the contrary, a higher bacterial growth was expected at 37ºC and in the lower dilutions 10^{0} to 10^{-2}, and for these reasons they were not also tested. A color code was used for comprehensive purposes, namely green regions corresponded to the maximal number of recovered bacteria while red regions represented the minimal number of recovered bacteria (Tables X to Table XIII).

A total of 291 isolates were recovered from the sample female health. The highest bacterial recovery was achieved with the medium R2A at 20ºC (total number of

recovered bacteria 108) and at 25ºC (total number of recovered bacteria 86), while on medium ABM2 for same conditions the recovery was lower (37 and 50 were the total number of recovered bacteria at 20ºC and 25ºC, respectively) (Table X).

Relatively to the medium R2A, the best conditions of isolation were at 20ºC with a pH=5.5 and 8.5 with a bacterial recovery of 29 and 26 isolates, respectively. These maximum bacterial recoveries were reached without dilution. At 25ºC, the best conditions were pH=5.5, also without dilution, with the growth of 28 isolates, and pH=7 with a tenfold dilution allowing the recovery of 29 isolates (Table X). At 37ºC the bacterial recovery was minimal due to our isolation strategy that did not include the isolation from the concentrated dilutions. The numbers recovered from the plated dilutions were in accordance with the numbers observed for the other tested temperatures (Table X).

Table X Number of bacterial recovered per isolation condition from the sample female health

			Female Health								
			Dilutions							Total Isolates	
Media	T°C	pH	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	pH	T°C
ABM2	20	5.5	1	3	0	0				4	37
		7	7	10	1	0				18	
		8.5	13	2	0	0				15	
	25	5.5	3	3	0	0				6	50
		7	13	7	1	0	0	0	0	21	
		8.5	16	4	1	2				23	
	37	5.5			3	0	0	0	0	3	4
		7			0	0	0	0	1	1	
		8.5			0	0	0	0	0	0	
R2A	20	5.5	29	1	5	0				35	108
		7	16	9	0	0				25	
		8.5	26	18	4	0				48	
	25	5.5	28	8	1	0				37	86
		7	0	29	0	0	0	0	0	29	
		8.5	5	14	0	1				20	
	37	5.5			2	0	0	0	0	2	6
		7			3	0	0	0	0	3	
		8.5			1	0	0	0	0	1	
										Total	291

30	25	20	15	10	5	0

A total of 384 isolates were recovered from the sample male health. The highest bacterial recovery was achieved with medium R2A at 20ºC (with a total number of

recovered bacteria of 130) and at 25ºC (with a total number of recovered bacteria of 109. On ABM2 medium and for the same conditions the recovery was lower (62 and 54 were the total number of recovered bacteria at 20ºC and 25ºC, respectively) (Table XI). The best isolation conditions were observed for the R2A medium at 20ºC, as above mentioned, at pH=5.5 and 8.5 with a bacterial recovery of 23 and 33 isolates, respectively. At 37ºC the bacterial recovery was minimal due our isolation strategy that did not include the isolation from less diluted samples. The numbers recovered from the plated dilutions were in accordance with the numbers observed for the other tested temperatures (Table XI). The ABM2 medium was less efficient since less bacteria were isolated. The same trend was also observed for the sample female health (Table XI).

Table XI Number of bacterial recovered per isolation condition from the sample male health.

			Male Health								
			Dilutions							Total Isolates	
Media	TºC	pH	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	pH	TºC
ABM2	20	5.5	10	10	7	0				27	62
		7	0	0	10	2				12	
		8.5	7	1	14	1				23	
	25	5.5	9	4	0	0				13	54
		7	7	1	7	4	0	3	0	22	
		8.5	9	6	0	4				19	
	37	5.5			0	0	0	0	0	0	8
		7			0	0	0	7	0	7	
		8.5			0	0	1	0	0	1	
R2A	20	5.5	17	23	10	4				54	130
		7	0	7	14	0				21	
		8.5	33	13	9	0				55	
	25	5.5	51	9	0	0				60	109
		7	1	17	2	1	0	1	0	22	
		8.5	9	7	7	4				27	
	37	5.5			0	1	0	0	0	1	21
		7			4	0	0	2	0	6	
		8.5			6	0	0	8	0	14	
										Total	384

A total of 270 isolates were recovered from the sample female disease. As previously observed in the other samples, bacteria recovery was higher in R2A medium without dilution or with the lower dilution (Table XII). A total number 68 isolates were

recovered at 20ºC, while 61 were recovered at 25ºC in R2A, while on medium ABM2 for same conditions the recovered numbers were lower (27 and 39 were the total numbers of recovered bacteria at 20ºC and 25ºC, respectively) (Table XII).

In R2A medium, at 20ºC, the maximum recovery was achieved at pH=7 with a 10 fold dilution with 25 isolates. At 25ºC, a higher bacteria growth was achieved at pH=5.5 with a 10 fold dilution with a total of 19 isolates. Contrarily to what was previously found, a higher number of bacteria was isolated at 37ºC despite our sampling strategy, with a total number of 47 isolates. This number was clearly higher than the numbers recovered from samples of healthy plants, namely 6 and 21 isolates from female and male, respectively (Table X and XII).

No substantial differences were observed between the total number of isolates in health and disease female samples, 291 and 270, respectively. Interestingly, the isolation conditions that gave the highest recovery numbers were distinct between female samples, since almost no isolates were recovered at 37ºC from health female samples, while at this temperature, almost 17% of the total isolates were recovered from disease female (Table XII).

Table XII Number of bacterial recovered per isolation condition from the sample female disease.

			Female Disease								
			Dilutions							Total Isolates	
Media	T°C	pH	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	pH	T°C
ABM2	20	5.5	0	0	0	0				0	27
		7	10	6	0	0				16	
		8.5	3	0	8	0				11	
	25	5.5	1	3	5	0				9	39
		7	2	5	3	1	2	4	0	17	
		8.5	3	4	4	2				13	
	37	5.5			10	0	0	0	0	10	28
		7			8	0	1	0	0	9	
		8.5			9	0	0	0	0	9	
R2A	20	5.5	16	0	0	0				16	68
		7	0	25	2	7				34	
		8.5	7	0	11	0				18	
	25	5.5	10	19	8	2				39	61
		7	7	6	4	0	0	0	0	17	
		8.5	1	2	0	2				5	
	37	5.5			11	0	0	0	0	11	47
		7			12	1	1	0	0	14	
		8.5			21	0	0	0	1	22	
										Total	270

A total of 313 isolates were recovered from the sample male disease. In accordance with the previous results, bacterial recovery was higher in R2A medium. A total number 84 isolates were recovered at 20ºC, while 73 were recovered at 25ºC in R2A, while for medium ABM2 in the same conditions 78 and 43were the total numbers of recovered bacteria, respectively) (Table XIII).

Similar numbers of bacteria were recovered at 20ºC with pH 8.5 and at 25ºC with pH=5.5. Similar to what was observed for female and male health samples, the bacteria recovery at 37ºC was minimal due our isolation strategy that did not include the isolation from less diluted samples.

A decrease in the total number of isolates was observed in the disease male sample when compared with the male health samples, 313 and 384, respectively. Nevertheless, the total number of recovered bacteria from male samples was always higher than those from female samples.

Table XIII Number of bacterial recovered per isolation condition from the sample male disease**.**

			Male Disease								
			Dilutions							Total Isolates	
Media	T°C	pH	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	pH	T°C
ABM2	20	5.5	0	5	8	4				17	78
		7	0	9	7	11				27	
		8.5	21	7	4	2				34	
	25	5.5	2	2	4	0				8	43
		7	7	4	4	1	0	0	0	16	
		8.5	6	13	0	0				19	
	37	5.5			5	0	0	0	0	5	16
		7			5	3	0	0	0	8	
		8.5			3	0	0	0	0	3	
R2A	20	5.5	8	9	2	5				24	84
		7	6	10	3	0				19	
		8.5	20	8	11	2				41	
	25	5.5	0	30	0	0				30	73
		7	8	11	5	0	0	0	0	24	
		8.5	4	6	6	3				19	
	37	5.5			4	0	0	0	0	4	19
		7			4	5	0	0	0	9	
		8.5			3	3	0	0	0	6	
										Total	313

30	25	20	15	10	5	0

In sum, the isolation conditions that allowed recovering higher numbers of bacteria were achieved with R2A medium in the temperature range between 20ºC - 25ºC for all samples. The experimental procedure in future experiments, and given the obtained results from plants with the disease, should include results from all the dilutions at 37ºC.

3.1.2. Bacterial fingerprinting

DNA was extracted from the 1258 isolates (section 2.3.4) and a fingerprint analysis was performed by RAPD (section 2.3.5), as previously described (Williams *et al.*,1990), aiming to identify identical strains. Nevertheless, only 473 (38%) isolates generated a suitable RAPD profile despite several attempts (section 2.3.5).

This methodology allowed clustering isolates into groups and select representative strains to be identified. Figure 14 shows an example of the obtained RAPD profiles from several isolates. Isolates KWT_291, KWT_121 and KWT_136 presented an equal RAPD profile whereby considered the same strain and were clustered into group A (Fig. 14). From this group, strain KWT_121 was selected as a representative and further used for identification purposes.

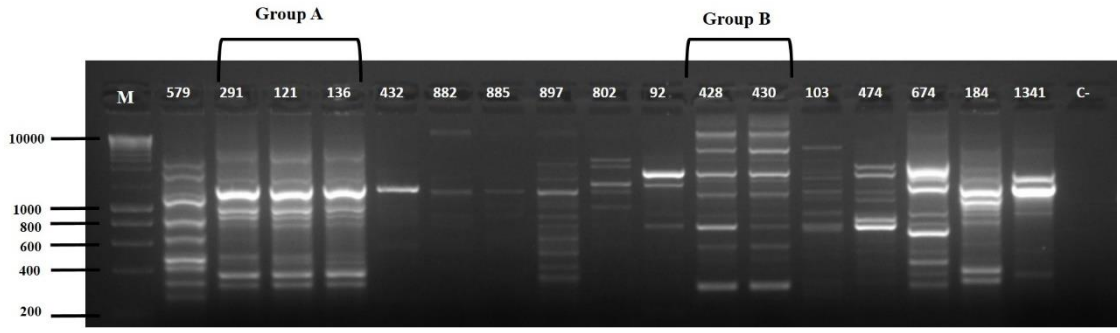

Figure 14 RAPD profiles obtained from strains isolated from plant samples. M: Ladder; Numbers match strains references; C-: negative control.

This approach was applied to all isolated strains that generated a suitable RAPD profile. Those isolates were grouped into 106 groups, of which 23 comprised a single strain (Table XIV).

RAPD-PCR is a methodology extremely sensitive to detect DNA variations and establish phylogenetic relationship between organisms. The lower reproducibility of DNA profiles may have been affected by the quality or quantity of DNA (Rastogi *et al.*, 2011).

Table XIV RAPD profiles, number of isolates and origin. FH: Female Heath; MH: Male Health; FD: Female Disease; MD: Male Disease.

RAPD Profile	Number of Isolates				RAPD Profile	Number of Isolates			
	FH	MH	FD	MD		FH	MH	FD	MD
1			10	5	**61**	1			
2			14		**62**	2			
3			16		**63**	2	4		
4			4		**65**	2			
5	1			34	**66**	2			
6			15		**67**	2			
7			7	2	**68**	3			
8			4		**69**	2			
9				8	**70**	7			
10			3		**71**	2	7		
11				2	**72**		10		
12	3				**73**		7		
13			3	1	**74**		6		
14			4		**75**		4		
16			5		**76**		4		
17			4		**77**		5		
18		1	6	15	**78**		5		
19			2		**79**		4		
20	1		1		**80**		1		
22			2		**82**		3		
23			16	31	**83**		2		
24			4		**84**		3		
25			2	6	**85**		2		
26			3	7	**86**		2		
27			2		**87**		2		
28			2		**88**		2		
29	2				**89**		3		
30			3		**90**		4		
31			2		**91**		3		
32			2		**94**		4		
33	2				**95**		1		
34			2		**97**		2		
35			2	1	**98**		2		
36			2		**99**		4		
37				2	**100**			1	
38				2	**101**			1	
39				3	**102**		1		
40				3	**103**	1			
41				2	**104**			1	
43	1			1	**105**			1	
44				5	**106**			1	
45				2	**107**			1	
46			1	2	**108**			1	
47				3	**109**			1	
50	3				**110**		1		
51	14				**111**			1	
52	3				**112**			1	
53	8				**113**			1	
54	3				**114**			1	
56	3				**115**	1			
57	4				**116**				1
58		1			**117**		1		
59	3				**118**		1		

The total number of isolates that gave a suitable RAPD profile varied among orchards. Namely, in the health orchard, only 27% of all isolates gave a suitable RAPD profile, 78 isolates from a total of 291 (27%) in the female sample and 102 isolates from a total of 384 (27%) in the male sample. The percentage of isolates efficiently typed was much higher in the diseased orchard, 155 isolates from a total of 270 (57%) in the female sample and 138 isolates from a total of 313 (44%) in the male sample (Table XIV). This technical limitation constitutes a bias since it narrows the potential diversity found in both orchards since only a limited fraction of all isolates were considered for the following analysis. Future studies will focus on the phylogenetic diversity encoded by the uncharacterized strains.

Figure 15 shows the distribution of the identified RAPD profiles according to samples. Populations were remarkably distinct between samples (Fig. 15 and 16). In the health orchards a total of 56 different RAPD profiles were identified, 26 in female health samples and 32 in male health sample. Only 2 profiles were common to both samples, namely RAPD 63 and 71. In the diseased orchards a total of 55 different RAPD profiles were identified, 41 in female disease sample and 22 in male disease sample. Eight profiles were common to both samples, namely RAPD 7, 13, 18, 23, 25, 26, 35 and 46 (Table XIV).

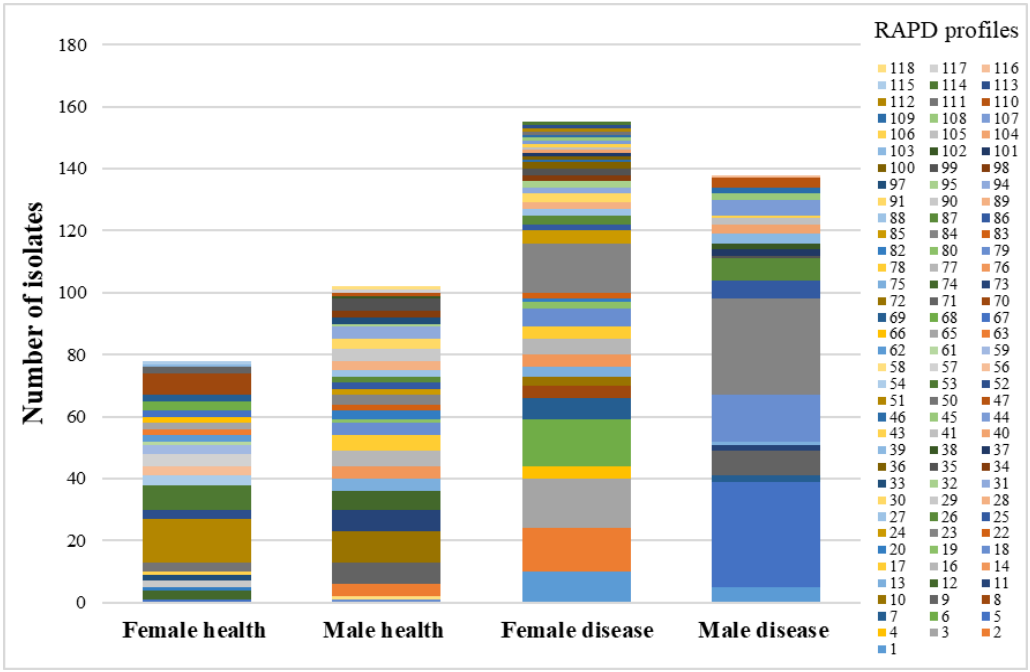

Figure 15 Distribution and proportion of RAPD profiles identified in samples from female and male plants from health and diseased orchards.

Differences in diversity profile between orchards were also notorious. Indeed, only 4 RAPD profiles were common to both orchards, but none was shared between all samples. RAPD profiles 5 and 43 were similar between female health and male disease samples. Both female samples had in common RAPD profile 20. The profile 18 was abundant amongst the isolates from the diseased orchard and one strain was recovered from the male health sample (Table XIV).

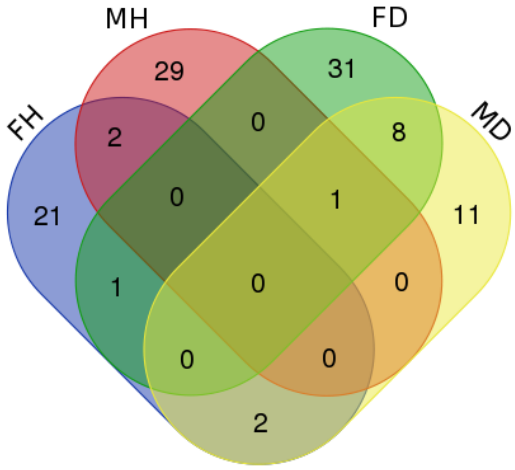

Figure 16 Venn diagram showing the unique and shared RAPD profiles from leaves of *Actinidia deliciosa*. FH: female-health; MH: male-health; FD: female disease and MD: male disease.

Our results suggest that the populations colonizing female and male plants from the same orchard are indeed very distinct (Fig. 15 and 16). Once the abiotic conditions are the same between plants, the most probable factor modulating this diversity is the plant gender.

Nevertheless, differences were notorious between males and females of both orchards. Since the abiotic conditions are the same between orchards, the most probable factor affecting the diversity between the two orchards is the presence of Psa.

3.1.3. Phylogenetic classification of the isolated strains

The partial sequence of the 16S rRNA gene was determined for all the 218 representative strains selected from the previous determined RAPD groups in order to infer the population diversity. The selection of representative isolates for identification was made according with total number of profiles obtained in each RAPD group. Namely, RAPD groups with 3 isolates one strains was selected while in RAPD groups with 4 or more isolates, two strains were selected. Through the application of these criteria into the health orchard isolates, 40 (51%) strains representing 78 isolates from the female sample were selected and 53 (52%) strains representing 102 isolates from male sample were selected. From the diseased orchard, 70 (45%) strains representing 155 isolates from the female sample were selected and 55 (40%) strains representing 138 isolates from male sample were selected.

From this analysis we were able to identify 49 operational taxonomic units (OTU's) (Table XV and Fig. 33 in supplementary data), with the majority belonging to phylum *Proteobacteria* but populations belonging to the phyla *Bacteroidetes, Firmicutes* and *Actinobacteria* were also identified (Fig. 17). Looking to the genus diversity between orchards, we can observe that the main populations identified in the orchard health were *Curtobacterium, Frigobacterium, Pseudoclavibacter, Methylobacterium, Shingomonas, Enterobacteriaceae* unclassified *and Escherichia-Shigella*. In the orchard disease, the Psa led to an increase of *Pseudomonas, Serratia, Pantoea, Erwinia, Raoultella and Pectobacterium* populations and reduction of previous populations. This shift observed for the populations identity and quantity may be due to the presence of Psa, that somehow altered the bacterial structural diversity existing in the leaves of the diseased plants.

3.1.3.1. Phyla

Phyla *Actinobacteria* and *Bacteroidetes* present values lower in disease female plants than in health female plants. In contrast, an exponential increase of phylum *Proteobacteria* was determined in disease female plants. Regarding the male plants, phyla *Actinobacteria* and *Firmicutes* present values lower in disease plants than health plant, whereas the phylum *Proteobacteria* increased in plant disease.

Table XV Identification and total number of the isolated strains from the studied samples. Each OTU was determined from a representative sequence with a 98% level of similarity. Total number of isolates from each cluster from female health, male health, female disease and male disease samples.

Domain	Phylum	Class	Order	Family	Genus	OTU's	Female - Health	Male - Health	Female - Disease	Male - Disease
Bacteria	*Actinobacteria*	*Actinobacteria*	*Micrococcales*	*Dermacoccaceae*	*Dermacoccus*	35	0,00	0,98	0,00	0,00
				Intrasporangiaceae	*Terrabacter*	48	1,28	0,00	0,00	0,00
				Microbacteriaceae	*Curtobacterium*	19	0,00	4,90	0,00	0,00
						25	2,56	4,90	0,00	0,72
						21	3,85	10,78	0,65	1,45
					Frigoribacterium	26	3,85	8,82	0,00	0,72
						29	3,85	4,90	0,00	2,90
					Microbacterium	27	0,00	1,96	0,00	0,00
						31	0,00	2,94	0,00	0,00
						24	0,00	0,00	0,65	0,00
						22	0,00	0,00	0,00	1,45
						33	5,13	1,96	0,00	2,17
						41	0,00	0,00	0,00	0,72
					Pseudoclavibacter	28	7,69	1,96	0,00	0,72
				Micrococcaceae	*Kocuria*	32	0,00	0,00	0,00	0,72
				Promicromonosporaceae	*Cellulosimicrobium*	30	0,00	0,00	0,00	0,72
			Geodermatophilales	*Geodermatophilaceae*	*Geodermatophilus*	42	0,00	0,98	0,00	0,00
	Bacteroidetes	*Sphingobacteria*	*Sphingobacteriales*	*Chitinophagaceae*	*Chitinophaga*	46	3,85	0,00	0,00	0,00
	Firmicutes	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	18	0,00	0,00	0,65	0,00
				Staphylococcaceae	*Staphylococcus*	11	0,00	0,00	0,65	0,00
						20	0,00	0,00	0,65	0,00
						0	0,00	1,96	0,00	0,00
						2	0,00	4,90	0,65	0,00
			Lactobacillales	*Enterococcaceae*	*Enterococcus*	1	2,56	0,00	0,00	0,00
				Streptococcaceae	*Streptococcus*	16	0,00	0,00	0,00	0,72

Table XV (continuation) Identification and total number of the isolated strains from the studied samples. Each OTU was determined from a representative sequence with a 98% level of similarity. Total number of isolates from each cluster from female-health, male-health, female-disease and male-disease samples.

Domain	Phylum	Class	Order	Family	Genus	OTU's	Female - Health	Male - Health	Female - Disease	Male - Disease
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Caulobacterales*	*Caulobacteraceae*	*Brevundimonas*	44	0,00	0,00	0,00	2,17
			Rhizobiales	*Aurantimonadaceae*	*Aurantimonadaceae unclassified*	8	0,00	1,96	0,00	0,00
				Methylobacteriaceae	*Methylobacterium*	40	1,28	8,82	0,00	0,00
			Sphingomonadales	*Sphingomonadaceae*	*Sphingomonas*	43	6,41	0,00	0,00	0,00
						45	0,00	0,98	0,00	1,45
						39	1,28	3,92	0,00	0,72
						38	0,00	16,67	0,65	0,72
		Gammaproteobacteria	*Enterobacteriales*	*Enterobacteriaceae*	*Pectobacterium*	23	0,00	0,00	17,42	0,00
					Enterobacteriaceae unclassified	47	0,00	0,00	0,65	0,00
						12	26,92	0,00	0,00	0,00
					Erwinia	15	0,00	1,96	2,58	0,00
						3	0,00	4,90	1,94	2,90
					Escherichia-Shigella	6	14,10	1,96	0,00	0,00
					Hafnia-Obesumbacterium	9	0,00	0,00	3,23	0,00
					Pantoea	10	2,56	5,88	24,52	15,22
						37	5,13	0,00	9,68	0,72
						34	0,00	0,00	1,94	0,00
					Raoultella	36	0,00	0,00	1,94	0,00
					Serratia	5	0,00	0,00	9,03	6,52
						17	0,00	0,00	1,29	0,00
			Pseudomonadales	*Pseudomonadaceae*	*Pseudomonas*	4	1,28	0,00	17,42	52,17
						7	2,56	0,00	1,94	0,72
						13	0,00	0,00	0,65	1,45
						14	3,85	0,98	1,29	2,17

The majority of the isolates recovered from samples of diseased plants belonged to the phylum *Proteobacteria*, namely 96.1% from female and 87.0% from male samples. The relative abundance of this phylum was lower amongst isolates from health plants, namely, 65.4% of the total isolates from female and 48.0% from male samples. In spite of the observed differences on the relative abundance, this was the most representative phylum among the bacterial isolates from *Actinidia deliciosa* leafs (Fig. 17).

The second most representative phylum was *Actinobacteria*, with contrary relevance between health and disease plants; namely, 28.2% of the isolates from female-health samples, 45.1% from male health, 1.3% from female disease and 12.3% from male-disease sample (Fig. 17).

In accordance, the phylum *Firmicutes* was also identified in all sample but it was less abundant and the relative distribution was not homogeneous among diseased and healthy plants. This phylum accounted for 2.6% of female health isolates, 6.9% of male health strains, 2.6% of female disease and 0.7% of male disease samples (Fig. 17).

The phylum *Bacteroidetes* was only identified in strains recovered from female-health sample, corresponding to 3.6% of total isolates (Fig. 17).

In sum, the relative abundance of each phylum had a similar distribution in samples from the same orchard (female and male), but meaningful differences were observed between healthy and diseased orchards (Fig.17). These results suggest that the presence of Psa has influence on the identity of the principal phylogenetic groups, favoring the dominance of *Proteobacteria* over *Actinobacteria* populations.

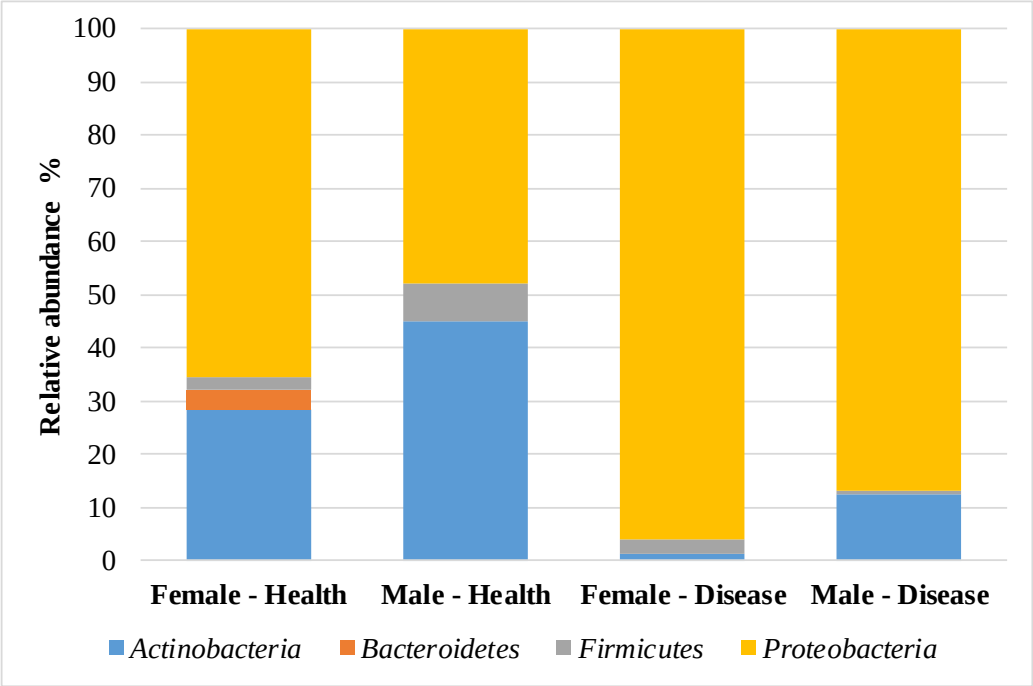

Figure 17 Phylogenetic characterization at phylum level of bacteria from *A. deliciosa* leaf samples obtained by culture-dependent methodology.

3.1.3.2. Order

The most abundant phylum in all samples, *Proteobacteria*, was divided into two orders, *Enterobacteriales* and *Pseudomonadales* (Table XV), with a very distinct relative abundance amongst samples (Fig. 18). The second most representative phylum *Actinobacteria* comprised two orders, *Micrococcales* and *Geodermatophilales*. The phylum *Firmicutes* encompassed orders *Bacillales* and *Lactobacillales*, while phylum *Bacteroidetes*, restricted to the female health sample, was represented by order *Shingobacteriales* (Fig. 18).

The isolated bacterial community from sample female health was mostly composed of isolates belonging to orders *Enterobacteriales* (48.7%), *Micrococcales* (28.2%), *Shingomonadales* (7.7%) and *Pseudomonadales* (7.7%). The orders with lower relative abundance were *Shingobacteriales* (3.8%), *Lactobacillales* (2.6%) and *Rhizobiales* (1.3%). In this sample no isolates from the orders *Caulobacterales*, *Bacillales* and *Geodermatophilales* were identified (Fig. 18).

Regarding the diversity of the isolates recovered from female disease sample the most abundant orders were *Enterobacteriales* (74.2%) and *Pseudomonadales* (21.3%). In addition, several other orders were also identified but with a significant lower relative abundance, namely *Micrococcales* (1.3%), *Shingomonadales* (0.7%) and *Bacillales*

(2.5%). In this sample it was not identified isolates belonging to orders *Caulobacterales, Rhizobiales, Shingobacteriales, Geodermatophilales* or *Lactobacillales* (Fig. 18).

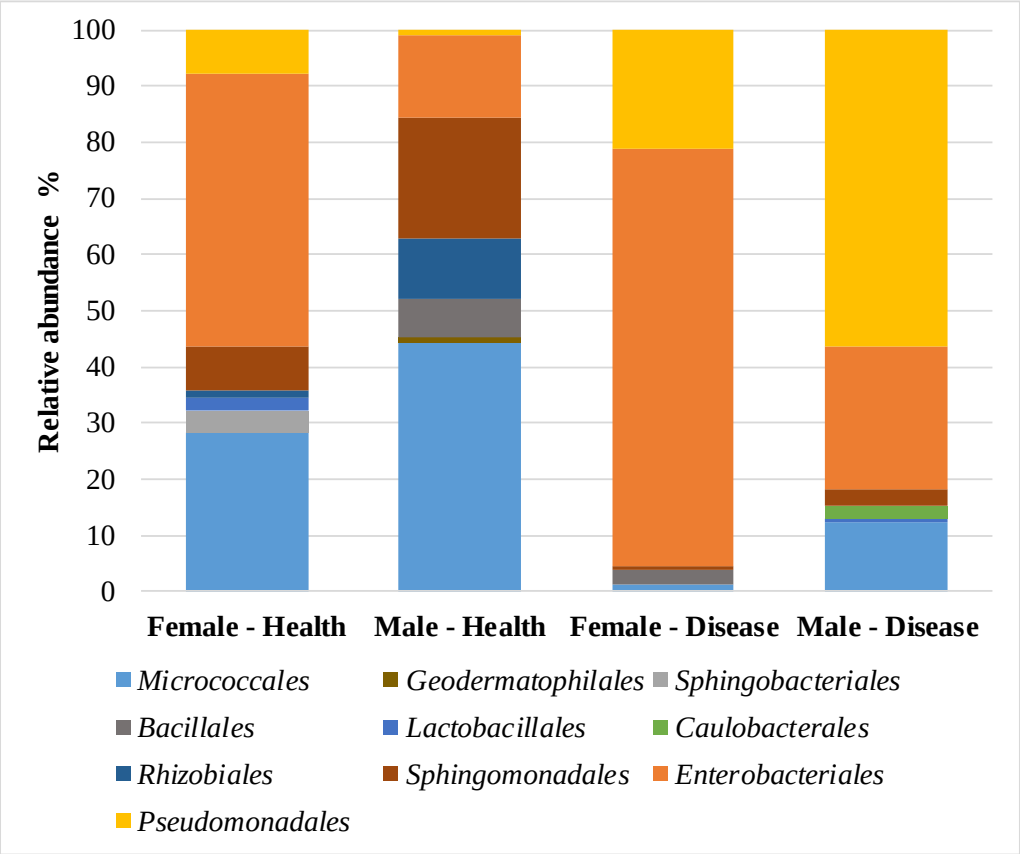

Figure 18 Phylogenetic characterization at order level of bacteria from *A. deliciosa* leaf samples obtained by culture-dependent methodology.

According with the obtained results we could argue that the presence of Psa induced a significant increase in the *Enterobacteriales* and *Pseudomonadales* orders when compared to the healthy orchards, namely from 48.7% to 74.2% and from 7.7% to 21.3%, respectively. This evidence was accompanied by a decreased in the relative abundance of orders *Micrococcales*, from 28.2% to 1.3%, and *Shingomonadales*, from 7.7% to 0.7%, most probably due to the presence of Psa. In addition, some minority orders were distinct between health and diseased plants. Indeed, the diversity of orders recovered from health female plants was higher than that observed for diseased female plants (Fig. 18).

The isolated bacterial community from sample male health was mostly identified as belonging to orders *Micrococcales* (44.1%), *Shingomonadales* (21.6%) and *Enterobacteriales* (14.7%). The remaining identified orders had lower relative abundance, namely *Rhizobiales* (10.8%), *Bacillales* (6.8%), *Geodermatophilales* (1.0%) and *Pseudomonadales* (1.0%). No isolates belonging to orders *Shingobacteriales*, *Lactobacillales* and *Caulobacterales* were recovered (Fig. 18)

Regarding the diversity from the isolates recovered from male disease sample the most abundant orders were *Pseudomonadales* (56.4%), *Enterobacteriales* (25.4%) and *Micrococcales* (12.3%). In addition, several other orders were also identified but with a significant lower relative abundance, namely *Shingomonadales* (2.9%), *Caulobacterales* (2.2%) and *Lactobacillales* (0.7%). In this sample no isolates from the orders *Rhizobiales, Shingobacteriales, Geodermatophilales* and *Bacillales* were identified (Fig. 18).

From the obtained results we could argue that the presence of Psa induced a significant decrease in the *Micrococcales* and *Shingomonadales* orders, namely from 44.1% to 12.32% and from 21.6% to 2.9%, respectively. This trend was followed by a tremendous increase in the relative abundance of *Pseudomonadales* from 1.0% to 56.4%, in the diseased plants, followed to a lesser extent by an increase in *Enterobacteriales* order, from 14.7% to 25.4%, most probably due to the presence of Psa. Changes in composition between male samples was also registered since orders *Rhizobiales Bacillales Geodermatophilales* were only found in healthy plants while orders *Caulobacterales* and *Lactobacillales* were exclusive of diseased plants (Fig. 18).

Another important comparison should be made between female and male plants from the same orchard.

Overall, orders *Enterobacteriales, Micrococcales, Shingomonadales, Pseudomonadales* and *Rhizobiales* were present in both healthy plants despite important differences in their relative abundance. A higher relative abundance of *Enterobacteriales* and a lower relative abundance of *Micrococcales* was observed in female plants when compared to male plants. In addition, the abundance of *Shingomonadales* was higher on males than female's plants (Fig.18). Nevertheless, some orders were restricted to one of the plant gender namely, *Shingobacteriales* and *Lactobacillales* were only identified in healthy female plants while *Bacillales* and

Geodermatophilales were limited to male samples. Importantly, order *Caulobacterales* was only identified in male diseased plants (Fig. 18).

Relatively to the diseased orchard the most abundant orders were *Enterobacteriales* and *Pseudomonadales* despite the plant gender, but with a heterogeneous distribution between females and males, since this dominance was most notorious on female plants (Fig. 18). Diseased female plants had a higher relative abundance of *Enterobacteriales* and a lower relative abundance of *Pseudomonadales* while on males the opposite was observed. Orders *Micrococcales* and *Shingomonadales* were also found in both genders but with a lower relative abundance. Nevertheless, some orders were restricted to one of the plant gender, namely orders *Caulobacterales* and *Lactobacillales* were only identified in male diseased plants while order *Shingobacteriales* was only identified in healthy female plants while order *Bacillales* was limited to female samples (Fig. 18). Importantly, orders *Rhizobiales*, *Shingobacteriales* and *Geodermatophilales* were only identified on healthy orchard (Fig. 18 and Table XV).

3.1.3.3. Genera

A total of 49 genera (Table XV) were recovered and were found to be evenly distribute by the samples with 19 genera identified in sample female-health, 23 in male-health, 22 in female disease and 23 in male-disease.

Only 3 genera were common to all samples, namely *Curtobacterium* (OTU 21), *Pantoea* (OTU 10) and *Pseudomonas* (OTU 14) (Fig. 19 and 20). Indeed, some genera were only identified in just one sample. Namely, five unique genera were identified on female health samples *Terrabacter* (OTU 48), *Chitinophaga* (OTU 46), *Enterococcus* (OTU 1), *Shingomonas* (OTU 43) and *Enterobacteriaceae unclassified* (OTU 12), while seven unique genera were identified in male health sample (*Dermacoccus* (OTU 35), *Curtobacterium* (OTU 19), *Microbacterium* (OTU 27 and 31), *Geodermatophilus* (OTU 42), *Staphylococcus* (OTU 0) and *Aurantimonadaceae* unclassified (OTU 8)).

Ten unique genera were identified in female disease sample (*Microbacterium* (OTU 24), *Staphylococcus* (OTU 11 and 20), *Bacillus* (OTU 18), *Pectobacterium* (OTU 23), *Enterobacteriaceae* unclassified (OTU 47), *Hafnia-Obesumbacterium* (OTU 9), *Pantoea* (OTU 34), *Raoultella* (OTU 36) and *Serratia* (OTU 17)), whereas six unique

genera were identified in male disease sample (*Microbacterium* (OTU 22 and 41) *Kocuria* (OTU 32), *Cellulosimicrobium* (OTU 30), *Streptococcus* (OTU 16) and *Brevundimonas* (OTU 44)).

Plants from the same orchard shared several genera between them. For instance, female and male samples from the healthy orchard had eleven common genera, two of which were not present in samples from diseased plants (*Methylobacterium* (OTU 40) and *Escherichia-Shigella* (OTU 6)). Regarding the diseased orchard, female and male plants shared ten genera, of which two were not present in samples from healthy plants (*Pseudomonas* (OTU 5) and *Serratia* (OTU 13) (Fig. 19 and 20). These results suggest that the plant gender has some influence on the diversity of the isolated microbial community given the high number specific bacterial genera of plant genera. Moreover, the microbial community isolated from plants of one orchard is more similar than when compared to the microbial community isolated from plants of the other orchard.

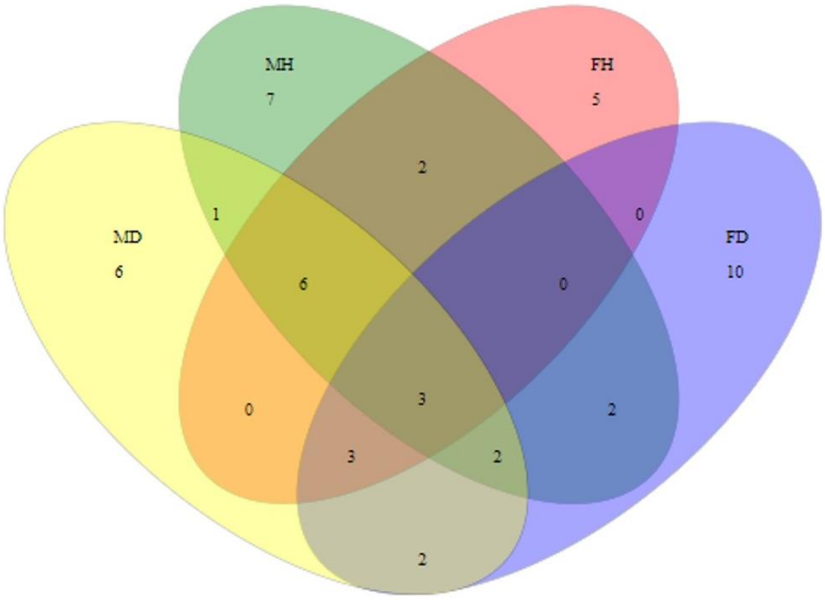

Figure 19 Venn diagram showing the number of shared genera between *A. deliciosa* leaf samples identified by cultivation-based approaches. FH: female-health sample; MH: male-health sample; FD: female-disease sample and MD: male disease sample.

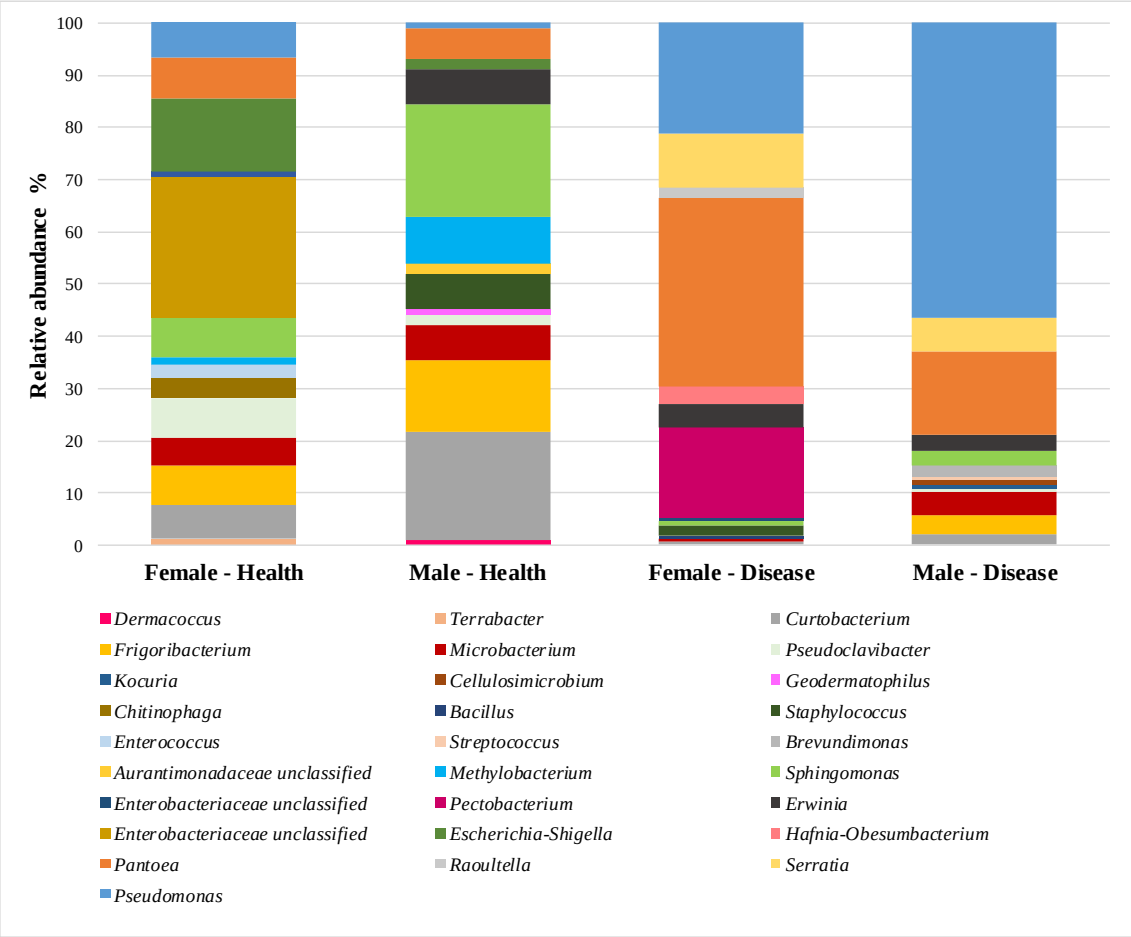

Figure 20 Phylogenetic characterization at genus level of bacteria from *A. deliciosa* leaf samples obtained by culture-dependent methodology.

3.1.4. Principal Component Analysis (PCA)

The PCA analysis performed on results obtained for the culture dependent methods was carried out with two focuses: distance between sample (PCA-inter-sample) and inter species correlation (PCA-inter-species).

3.1.4.1. PCA-Inter-sample

From Fig.21 we can observe that all samples were quite distinct in terms of the most representative populations, thus supporting our previous results that showed a bacterial diversity heterogeneity between samples.

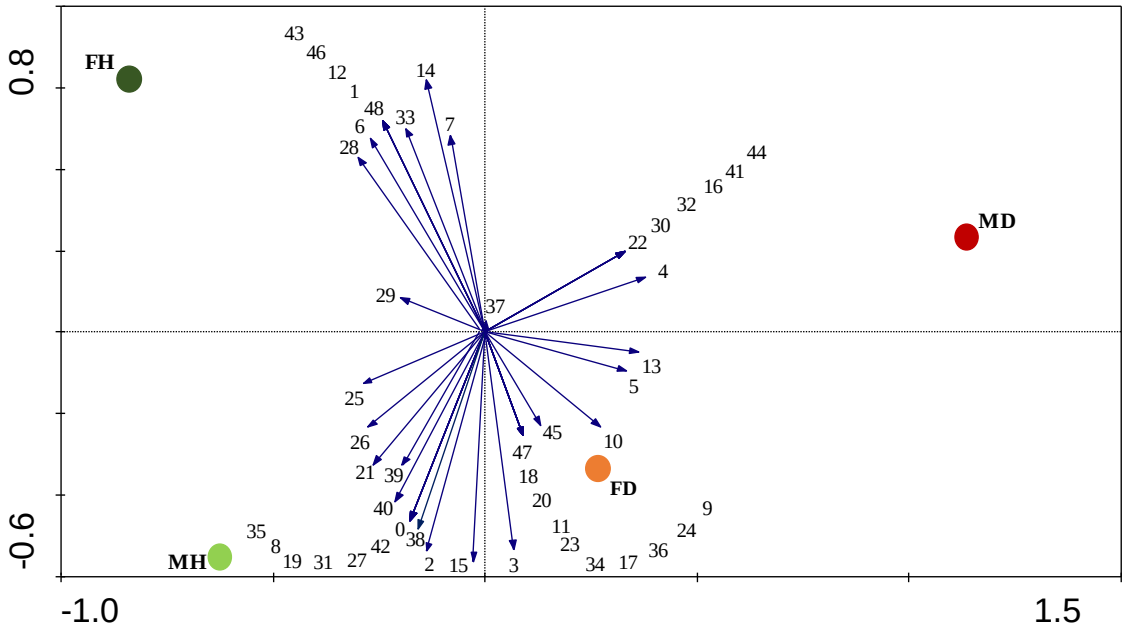

Figure 21 Principal component analysis (PCA) of OTUs profiles correlated from each sample (inter-sample). FH: female health, MH: male health; FD: female disease and MD: male disease.

3.1.4.2. PCA-Inter-species

In this analyses, we aim to determine if one or a group of bacterial populations can be related to a specific condition, in this case health or disease. From this PCA (Fig.22) we can observe a clear differentiation between the bacterial populations related to healthy orchards and disease orchards.

From the 49 different OTUs determined, only five OTUs, namely OTUs 4 (*Pseudomonas*), 5 (*Serratia*), 10 (*Pantoea*), 23 (*Pectobacterium*) and 37 (*Pantoea*) were mostly related (had higher relative abundance) with the two disease samples (female-disease and male-disease). So we can relate these five phylogenetic groups to the disease condition. On the other hand, it seems that in healthy orchards there is a gender differentiation concerning to population relatedness. While genus *Shingomonas* (OTU 39 and 38) and *Erwinia* (OTU 15 and 3) were relevant in male health samples, genus *Escherichia-Shingella* (OTU 6), *Enterobacteriaceae*-unclassified (OTU 12) and *Pseudomonas* (OTU 7 and 14) were mostly related to female health.

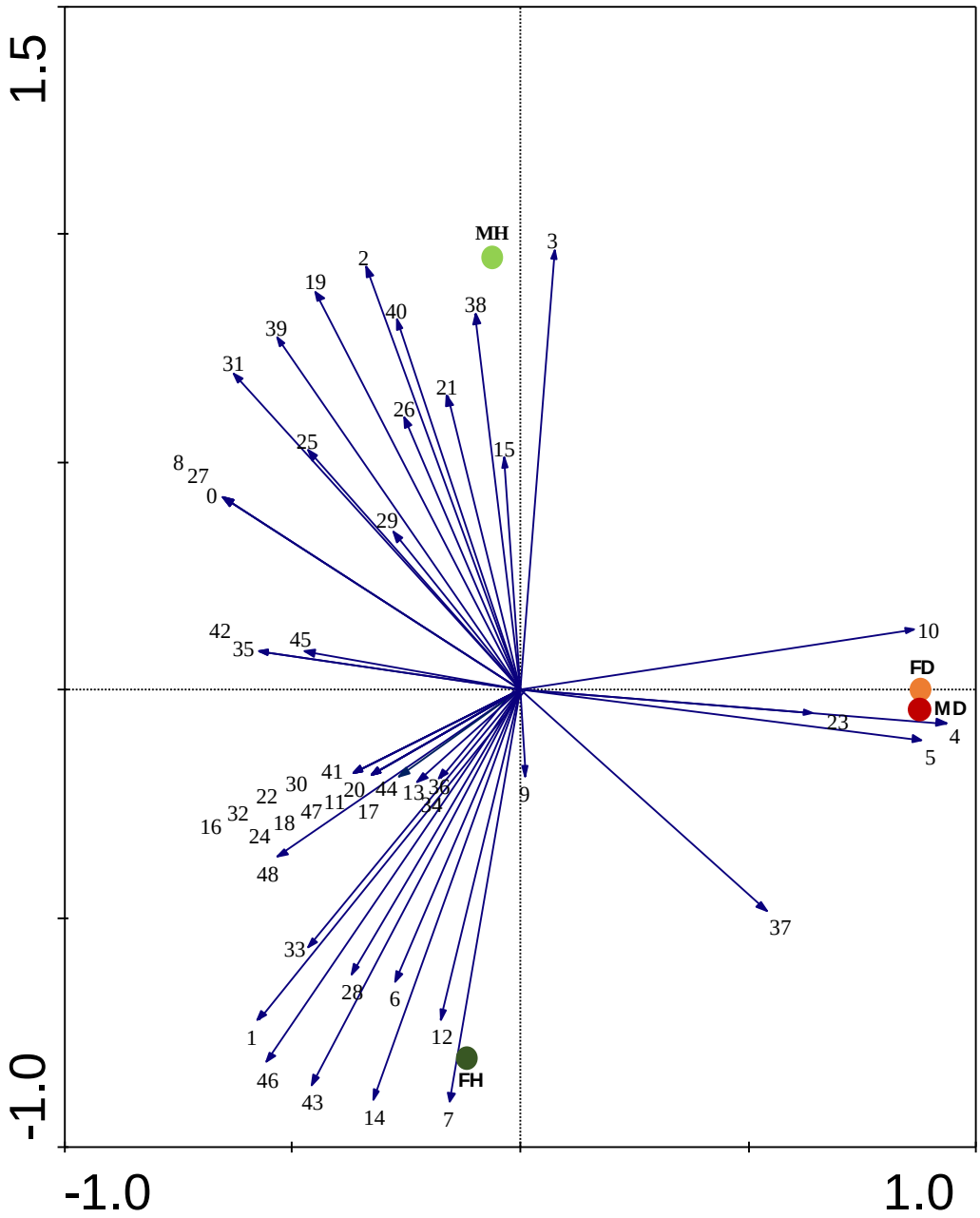

Figure 22 Principal component analysis (PCA) from OTU's recovered by culturing and identification of OTU's most present in each sample (inter-species). FH: female health; MH: male health; FD: female disease and MD: male disease.

3.1.5. Biodiversity index

To characterize the diversity of isolates recovered from each sample (FH, MH, FD and MD) α-diversity and β-diversity indexes were calculated. α-diversity indexes were used to measure the diversity within samples while β-diversity measured the diversity between orchards.

3.1.5.1. Diversity within orchards

Several parameters were used to estimate the diversity within orchards, namely Richness - total number of species (S), Margalef's diversity index (d), Shannon-Weaver (H'), Pielou's evenness index (J') and Simpson's diversity (λ).

Margalef's diversity index (Dmg) estimates the diversity of a community based on the total number of different species (S) as a function of the total number of individuals (N). The higher the value the greater the diversity. On the healthy orchard, Margalef's diversity index was Dmg= 4.13 and Dmg= 4.76 calculated from female and male sample, respectively. On the diseased orchard similar values were obtained from female and male sample, namely Dmg= 4.16 and Dmg= 4.46, respectively. In both orchards male samples presented higher index than female samples. These results suggested that the presence of Psa did not induced changes on the number of taxons. Nevertheless, a slight decrease was observed in the diseased male samples (Table XVI).

Shannon-weaver index (H') relates the specific richness of the isolates with their relative proportion. The higher the value of the index, grater the diversity of the sample. Similar Shannon-Weaver index values were obtained in the healthy orchards for both female and male samples (H'=2.53 and H'=2.82, respectively) (Table XVI). Regarding the diseased orchard, a decrease in this index was observed for female and male samples (H'=2.31 and H'=1.89, respectively). This trend was more pronounced in male samples. These results suggested that the presence of Psa led to a decreased in the microbiota diversity.

Pielou's evenness index (J') measures the distribution of one specie relatively to the other species present in the community. This index varies between 0 and 1. Values close 1 indicate that there is a balance on the distribution of genera abundance by the community. A value close to 0 indicates that the abundance of the genera in the community is not balanced whereby the community is dominated by a small number of

genera. On the healthy orchard the evenness of female and male sample was close to 1, J'=0.86 and J'=0.90, respectively. On the diseased orchard the evenness of female and male sample was lower, J'=0.75 and J'=0.61, respectively, with a more pronounced effect in males samples (Table XVI). These results suggested that Psa induced the dominance of some taxonomic groups and in fact an increased in the orders *Enterobacteriales* and *Pseudomonadales* was observed in the diseased orchard (Fig. 18).

Simpson's diversity (λ) or dominance index (1-D) reflects the probability of two randomly chosen individuals belong to the same species and varies between 0 (no diversity) and 1 (high diversity). The higher the value of the index the higher is the probability of two individuals being of the same species. On the healthy orchard the Simpson's diversity was λ=0.88 and λ=0.92 for female and male samples, respectively. On the diseased orchard the Simpson's diversity index was lower varying between λ =0.85 and λ =0.70 for female and male sample, respectively. These results confirm our previous assumption that the presence of Psa may induce a decrease in diversity.

In sum, the presence of Psa was translated in the dominance of two taxa, despite maintaining the same richness as the healthy orchard. Since these results account for only 38% of the total isolates further studies are need to strength our evidences.

Table XVI Alpha diversity indexes determined for each orchard and for each gender. Dmg: Margalef index; H': Shannon index; J': Pielou's evenness index; 1-D: Simpson diversity index.

	(H')	(J')	(1-D)	(Dmg)
Female Health	2.53	0.86	0.88	4.13
Male Health	2.82	0.90	0.92	4.86
Female Disease	2.31	0.75	0.85	4.16
Male Disease	1.89	0.61	0.70	4.46

3.1.5.2. Diversity between orchards

A Beta diversity analysis was performed to compare the observed diversity between the healthy and the diseased orchards. This analysis measured changes in the diversity of species (in this case of genera) from one environment (in this case the presence of Psa) to another (absence of Psa). The Jaccard similarity index was applied to compared the diversity of each orchard and determine which genera were shared and which were distinct. The higher the obtained percentage, the more similar two

populations are. Based on the obtained results, it was possible to infer that the populations were quite distinct, sharing only 25.75 % of their diversity. These results reinforced our previous assumptions that indeed Psa induced a dramatic alteration on the structure of the cultivated microbiota.

In accordance, female plants from healthy and diseased orchards were quite dissimilar, only sharing 9.4% of their diversity. Similarly, low similarity values were obtained from male plants since they only shared 17.7 % of their diversity. Based on this result we can conclude that the diversity of the cultivated microbiota from male plants was less affected by the presence of Psa than the cultivated microbiota from female plants.

3.1.6. Analysis of microbial richness

Rarefaction curves were constructed for each of the 4 samples (FH, MH, FD and MH) to determine if the richness determined for each sample was representative of the bacterial diversity existing in each sample.

Through the analysis of the rarefaction curves of each sample (Fig.23) we observe that none reaches a saturation point, although samples female health and male health show a slight tendency for saturation. Thus, we can presume that the bacterial diversity determined for each sample does not cover the totality of the existing bacterial diversity in each sample. This is probably due to the low numbers of isolates that were examined. From a total of 1258 isolates only 473 were positively typed by RAPD, clustered and phylogenetically classified. This was due to constrains to obtain a proper RAPD profile and to temporal limitations.

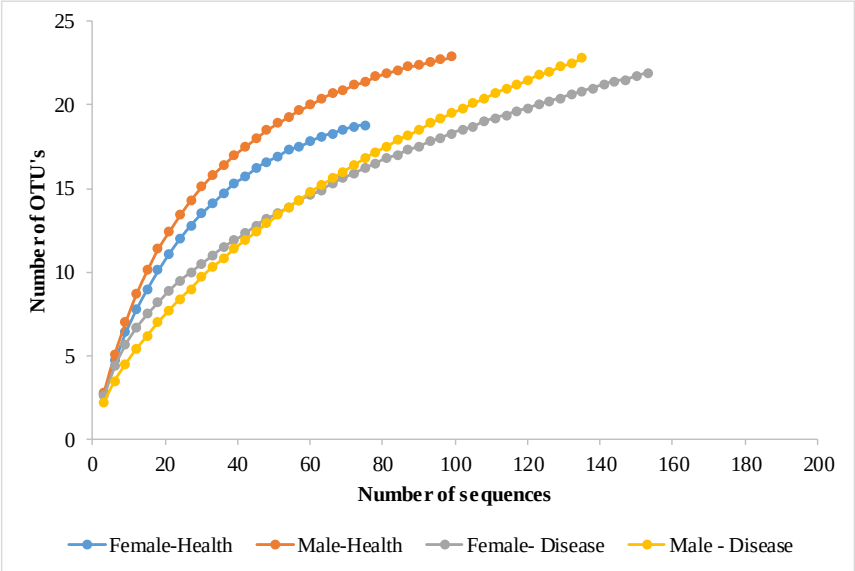

Figure 23 Rarefaction curve of observed OTU's richness for each sample and using an OTU's threshold of ≥98% identity.

3.2. Culture-independent methods

The composition of the microbiome from the phyllosphere of *A. deliciosa* was studied through the analysis of amplicons from the hypervariable regions V3-V5 of the 16S rRNA bacterial gene. The amplicon library was sequenced in Illumina Miseq platform and the analysis of raw data was performed in mothur software.

3.2.1. Quantification of DNA

To verify the quality of the genomic DNA extracted from leaves samples, quantification by spectrophotometry was done (Nanodrop 3300, Thermo scientific, USA). The gDNA is accepted as pure for the ratio A260/280 with values ranging from 1.8 - 2.0. The values measured for concentration and absorbance of gDNA are presented in table XVII.

Table XVII Quantification of DNA by spectrophtometry. The racio A 260/280 is an indicator of quality. DNA content in ng/µl.

Samples	[DNA] ng/µl	A260/280
Female health	4.9	1.93
Male health	4.5	2.01
Female disease	5.7	1.88
Male disease	4.8	1.82

The concentration and quality of the DNA samples was satisfactory to proceed with the metagenomic workflow. The DNA samples were tested for the amplification of the bacterial 16S rRNA gene. In Fig. 24, an electrophoresis image of the tested DNA samples is shown. One amplicon with 1500 bp was observed for each sample, that corresponds to expected size of the gene.

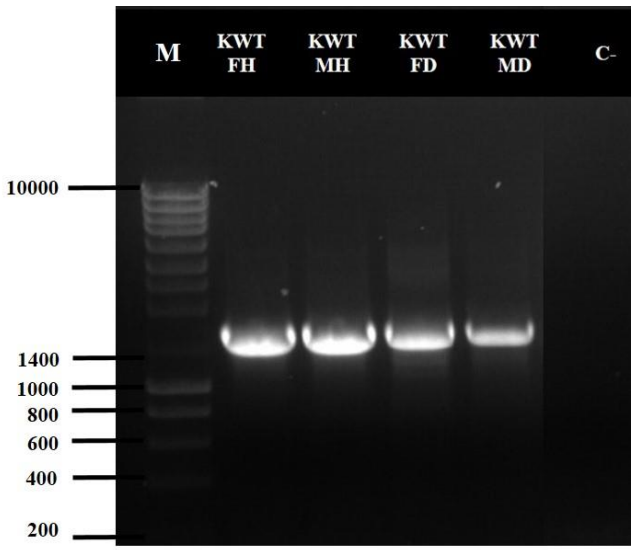

Figure 24 Agarose gel (1%) obtained after amplification by PCR the 16S rRNA gene. C-: negative control; M: ladder III Nzytech. FH: female health; MH: male health; FD: female disease; MD: male disease.

3.2.2. Phylogenetic analyses of Illumina sequences

After the analyses of the raw data, a total of 6670 high quality sequences were obtained (37 sequences for female health, 38 sequences for male health, 3803 sequences for female disease, 2793 sequences for male disease) that were clustered into 43 OTU's, and their relative abundances were calculated and are presented in Table XVIII. The low sequence numbers obtained for all samples (and even lower for samples female health and male health) was due to the high number of sequences that were classified as chloroplasts that were removed from the downstream analyses.

Table XVIII Identification and total number of the isolated strains from the studied samples. Each OTU was determined from a representative sequence with a 97% level of similarity. Total number of isolates from each cluster from female health, male health, female disease and male disease samples.

Domain	Phylum	Class	Order	Family	Genus	OTU's	Female - Health	Male - Health	Female - Disease	Male - Disease
Bacteria	*Bacteria_unclassified*	*Bacteria_unclassified*	*Bacteria_unclassified*	*Bacteria_unclassified*	*Bacteria_unclassified*	1	*0,00*	2,63	0,11	0,11
	Bacteroidetes	*Cytophagia*	*Cytophagales*	*Cytophagaceae*	*Cytophagaceae_ unclassified*	2	*0,00*	0,00	0,00	0,07
					Hymenobacter	3	*67,57*	52,63	1,08	20,77
					Spirosoma	4	*0,00*	0,00	0,00	0,21
				*Cytophagales_* unclassified	*Cytophagales_ unclassified*	5	*0,00*	0,00	0,00	0,21
		Flavobacteria	*Flavobacteriales*	*Flavobacteriaceae*	*Epilithonimonas*	6	*0,00*	0,00	0,00	0,07
	Cyanobacteria	*Cyanobacteria_ unclassified*	*Cyanobacteria_ unclassified*	*Cyanobacteria_ unclassified*	*Cyanobacteria_ unclassified*	7	*2,70*	5,26	0,29	0,43
	Deinococcus-Thermus	*Deinococci*	*Deinococcales*	*Deinococcaceae*	*Deinococcus*	8	*0,00*	5,26	0,00	0,00
	Firmicutes	*Bacilli*	*Bacillales*	*Paenibacillaceae*	*Paenibacillaceae_ unclassified*	9	*0,00*	0,00	0,00	0,11
			Lactobacillales	*Lactobacillaceae*	*Lactobacillus*	10	*0,00*	0,00	0,00	0,07
	Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*1174-901-12*	*1174-901-12_ge*	11	*0,00*	0,00	0,00	0,11
				Aurantimonadaceae	*Aurantimonadaceae_ unclassified*	12	*0,00*	0,00	0,00	0,11
					Aureimonas	13	*0,00*	0,00	0,00	0,32
				Methylobacteriaceae	*Methylobacteriaceae_ unclassified*	14	*0,00*	0,00	0,00	0,07
					Methylobacterium	15	*0,00*	0,00	0,08	2,11
				Rhizobiaceae	*Rhizobium*	16	*0,00*	0,00	0,00	0,04
				Rhizobiales_unclassified	*Rhizobiales_unclassified*	17	*0,00*	0,00	0,05	0,39
			Rhodospirillales	*Acetobacteraceae*	*Acetobacteraceae_ unclassified*	18	*0,00*	0,00	0,00	0,11
					Acidiphilium	19	*0,00*	0,00	0,00	0,07

Table XVIII Identification and total number of the isolated strains from the studied samples. Each OTU was determined from a representative sequence with a 97% level of similarity. Total number of isolates from each cluster from female health, male health, female disease and male disease samples

Domain	Phylum	Class	Order	Family	Genus	Cluster	Female - Health	Male - Health	Female - Disease	Male - Disease
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Sphingomonadales*	*MN_122.2a*	*MN_122.2a_ge*	20	*0,00*	0,00	0,00	0,04
				Sphingomonadaceae	*Sphingomonadaceae_ unclassified*	21	*21,62*	10,53	0,42	2,65
					Sphingomonas	22	*5,41*	15,79	0,71	7,02
					Zymomonas	23	*0,00*	0,00	0,00	0,04
				Sphingomonadales_ unclassified	*Sphingomonadales_ unclassified*	24	*2,70*	7,89	0,50	3,44
				uncultured	*uncultured_ge*	25	*0,00*	0,00	0,00	0,07
		Betaproteobacteria	*Burkholderiales*	*Burkholderiales_ unclassified*	*Burkholderiales_ unclassified*	26	0,00	0,00	0,00	0,04
				Comamonadaceae	*Comamonadaceae_ unclassified*	27	0,00	0,00	0,00	1,86
					Variovorax	28	0,00	0,00	0,00	0,32
					Xylophilus	29	0,00	0,00	0,00	0,54
				Oxalobacteraceae	*Duganella*	30	0,00	0,00	0,00	0,07
					Massilia	31	0,00	0,00	0,03	0,50
					Oxalobacteraceae_ unclassified	32	0,00	0,00	0,13	0,68
					Rugamonas	33	0,00	0,00	0,00	0,14
		Deltaproteobacteria	*Deltaproteobacteria_ unclassified*	*Deltaproteobacteria_ unclassified*	*Deltaproteobacteria_ unclassified*	34	0,00	0,00	0,00	0,36
			Myxococcales	*P3OB-42*	*P3OB-42_ge*	35	0,00	0,00	0,00	0,11
		Gammaproteobacteria	*Enterobacteriales*	*Enterobacteriaceae*	*Enterobacteriaceae_ unclassified*	36	0,00	0,00	0,03	0,21
					Erwinia	37	0,00	0,00	0,00	0,04
					Rahnella	38	0,00	0,00	0,03	0,00
					Sodalis	39	0,00	0,00	0,00	0,18
			Gammaproteobacteria_ unclassified	*Gammaproteobacteria_ unclassified*	*Gammaproteobacteria_ unclassified*	40	0,00	0,00	0,03	0,00
			Pseudomonadales	*Pseudomonadaceae*	*Pseudomonadaceae_ge*	41	0,00	0,00	0,26	0,14
					Pseudomonadaceae_ unclassified	42	0,00	0,00	40,13	24,18
					Pseudomonas	43	0,00	0,00	56,14	31,98

3.2.3. Phyla

Phylum *Proteobacteria* was the most representative phylogenetic group in diseased plants, corresponding to 98.53% in female plants and 77.94% in male plants. This phylum was also detected in healthy plants although in much lower percentage; 29.73% on female plants and 34.21% on male plants. Despite the observed differences on the relative abundance between healthy and diseased plants, this was the most representative phylum among bacterial sequences recovered from *A. deliciosa* leafs (Fig. 25). These findings were in accordance with our previous results based on culture-dependent methods (section 3.1.3.1).

The second most representative phylum was *Bacteroidetes*, with contrary relevance in healthy and diseased plants; namely 67.57% from female healthy plants, 52.63 % from male healthy plants, 1.08 % from female diseased plants and 21.35 % from male diseased plants (Fig.25).

The phylum "unclassified *Cyanobacteria*" was identified in all samples despite the low relative abundance. In the healthy orchard, 2.70% and 5.26% of the sequences were classified within this phylum in female plants and in male plants, respectively. The percentage of "unclassified *Cyanobacteria*" was lower in the diseased orchard, corresponding to 0.29% and 0.43% of the total sequences from female and male plants, respectively.

The phylum *Deinococcus-Thermus* was only identified in healthy male plants (5.26%), while *Firmicutes* were only present in male diseased plants (0.18%).

Lastly, "unclassified *Bacteria*" were recovered from all samples with low values of relative abundance. Within male plants, this phylum was detected in 2.6% of the sequences from heathy plants while accounted for 0.11% in diseased plants. Relatively to female plants, this group was only detected in diseased plants (0.11%) (Fig.25).

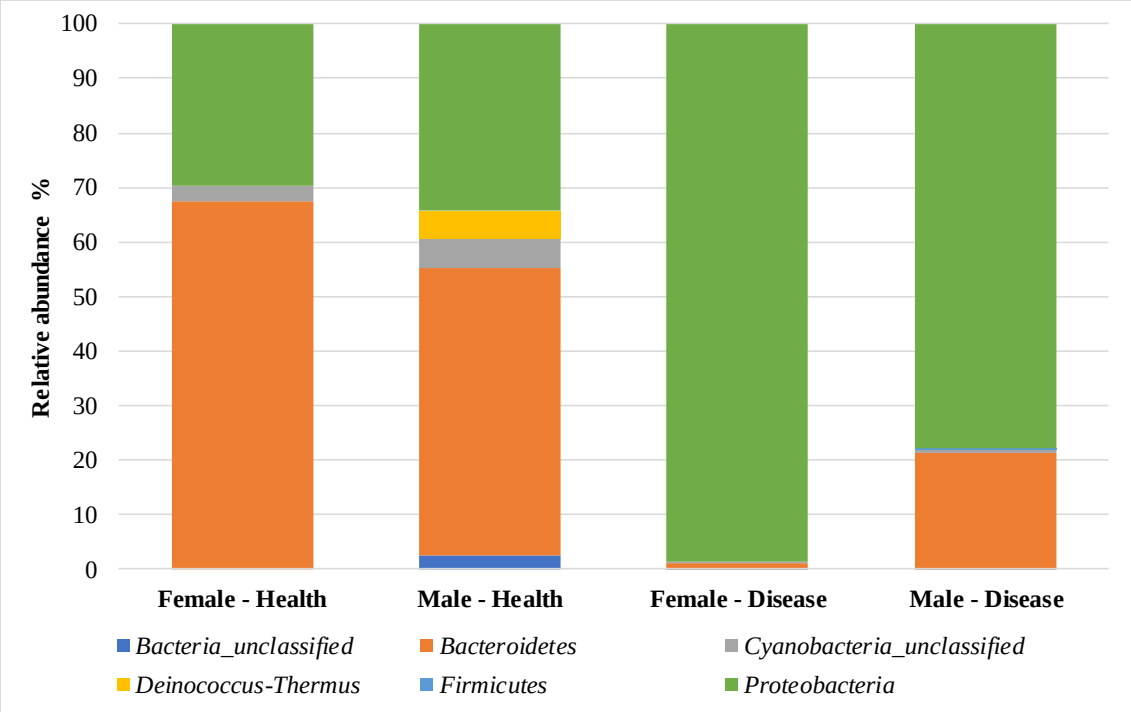

Figure 25 Phylogenetic characterization at phylum level of bacteria from *A. deliciosa* leaf samples obtained by culture-independent methodology.

Moreover, *Bacteroidetes* were dominant in healthy female plants becoming vestigial in diseased female plants. Concomitantly, a significant increase in *Proteobacteria* phylum in diseased female plants was observed when compared with healthy ones. The low amount of "unclassified *Cyanobacteria*" detected in healthy female plants became vestigial in diseased female plants. Phyla *Deinococcus-Thermus* and *Firmicutes* were not detected in female plant samples.

In male plants, similar changes were observed between healthy and diseased plants. Namely, the decrease in the abundance of phylum *Bacteroidetes* in diseased plants was accompanied by a significant increase in phylum *Proteobacteria.* Interestingly, phyla *Deinococcus-Thermus* and *Firmicutes* present in healthy plants were not recovered from diseased male plants.

In sum, the relative abundance of each phylum had, in general, a similar distribution between samples from the same orchard (health and disease). On the contrary, clear and significant differences on the microbiota composition were detected between orchards. Namely, the dominance of the phylum *Bacteroidetes* in the healthy

orchard, in clear contrast with the dominance of the phylum *Proteobacteria* in the diseased orchard (Fig.25).

3.2.4. Order

Within phylum *Proteobacteria* four orders were identified, *Rhizobiales*, *Shingomonadales*, *Burkholderiales* and *Pseudomonadales* (Table XVIII), with a very distinct relative abundance amongst samples (Fig. 26).

The second most representative phylum, *Bacteroidetes*, comprised two orders, *Cytophagales* and *Flavobacteriales*. Phylum *Firmicutes* encompassed orders *Bacillales* and *Lactobacillales* while phylum *Deinococcus-Thermus* was represented by order *Deinococcales*. Moreover, two unclassified orders were identified among phyla "unclassified *Bacteria"* and "unclassified *Cyanobacteria"* (Fig. 26).

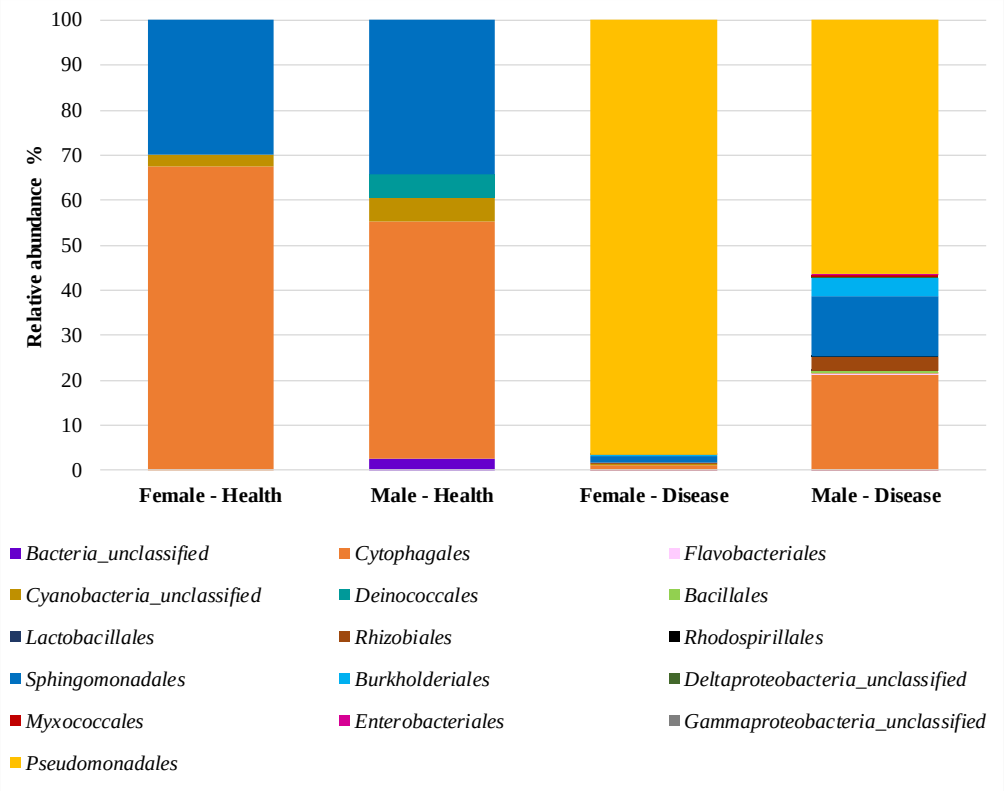

Figure 26 Phylogenetic characterization at order level of bacteria from *A. deliciosa* leaf samples obtained by culture-independent methodology.

The healthy orchard encompassed sequences classified within three orders, common to both female and male samples, namely *Cytophagales, Shingobacteriales* and *Cyanobacteria*-unclassified. *Cytophagales* was the most abundant order in both genders followed by the order *Shingomonadales* with similar values. *Cyanobacteria*-unclassified had a slightly higher relative abundance in male than in female plants. Two additional orders were only detected in healthy male plants - *Deinococcales* and *Bacteria*-unclassified (Fig.26).

Regarding the diseased orchard an obvious dominance of the order *Pseudomonadales* was determined. This trend was most notorious among female plants. In male plants, besides the later mentioned order, *Cytophagales* had also high relative abundance (Fig.26). Orders *Cytophagales* and *Shingomonadales* were also equality important in the composition of diseased male plants. Several other orders with relative less abundance were also present in the diseased orchard, namely, *Bacteria*-unclassified, *Cyanobacteria, Rhizobiales, Burkholderiales* and *Enterobacteriales* (Fig.26). Additionally, orders *Flavobacteriales, Bacillales, Lactobacillales, Rhodospirillales, Deltaproteobacteria*-unclassified and *Myxococcales* were only identified in diseased male plants while order *Gammaproteobacteria*-unclassified was limited to female plants (Fig.26 and table XVIII).

As previously observed at phyla level, significant differences on the microbiota composition were detected between orchards. These findings were corroborated by the analysis at order level since orders *Cytophagales* (67.57%) and *Shingomonadales* (29.73%) were the most abundant in healthy female plants while a single and dominant order *Pseudomonadales* (96.53%) was identified among diseased plants (Fig.26).

Based on the obtained results we could infer that the presence of Psa lead to a decrease in the relative abundance of orders *Cytophagales* and *Shingomonadales,* from 67.57 to 1.08% and from 29.73% to 1.63%, respectively. In addition, the reduction on the abundance of these orders was accompanied by an enormous increase in the *Pseudomonadales* abundance (96.53%) (Fig.26).

Like in healthy female plants, *Cytophagales* (52.63%) and *Shingomonadales* (34.21%) were the most abundant orders in healthy male plants. Nevertheless, two distinct orders were found in healthy male samples that were not identified in female samples, namely the *Deinococcales* and "*Bacteria*-unclassified*"*.

Interestingly, the characterized microbiome from diseased male plants was mainly composed of *Pseudomonadales* (56.30%) followed by *Cytophagales* (21.28%). These changes were accompanied by the identification of orders that were not present in healthy male plants, namely *Burkholderiales* (4.15%), *Rhizobiales* (3.15%), *Rhodospirillales* (1.79%), *Enterobacteriales* and *Cyanobacteria* (0.43%), *Deltaproteobacteria*-unclassified (0.36%), *Myxococcales* and *Bacillales* (0.11%), *Lactobacillales* and *Flavobacteriales* (0.07%). On the contrary, *Deinococcales* was not detected in diseased male plants.

In sum, our results suggested that the presence of Psa induced a significant alteration on the microbial population structure in both female and male plants. These findings were supported by the reduction of relative abundance of orders *Cytophagales* and *Shingomonadales* followed by a tremendous increase in the relative abundance of order *Pseudomonadales.*

3.2.5. Genera

A total of 43 OTUs (similarity value of 97%) (Table XVIII) were identified and phylogenetically classified (from a total of 6670 sequences of bacterial 16S rRNA gene). These OTUs were not evenly distributed by samples with 5 identified in samples female-health, 7 in male-health, 16 in female disease and 40 in male-disease. Only 5 OTUs were common to all samples (Fig. 27 and 28, Table XVIII), namely 21 (*Sphingomonadaceae*-unclassified), *24* (*Sphingomonadales*-unclassified), *22* (*Sphingomonas*), *3* (*Hymenobacter*) and 7 (*Cyanobacteria*-unclassified).

From the healthy orchard samples, only the male sample presented a unique OTU, namely 8 (*Deinococcus*). From the disease orchard, female sample had two unique OTUs, 40 (*Gammaproteobacteria*-unclassified) and 38 (*Rahnella*) and male sample encompassed twenty-six unique OTUs: 13 (*Aureimonas*), *20* (*MN_122.2a_ge*), *28* (*Variovorax*), *16* (*Rhizobium*), *39* (*Sodalis*), *25* (uncultured*_ge), 18* (*Acetobacteraceae*-unclassified), *2* (*Cytophagaceae*-unclassified), *33* (*Rugamonas*), *12* (*Aurantimonadaceae*-unclassified)*; 35* (*P3OB-42_ge*)*; 26* (*Burkholderiales*-unclassified), *30* (*Duganella*), *29* (*Xylophilus*), *14* (*Methylobacteriaceae*-unclassified), *27* (*Comamonadaceae*-unclassified), *37* (*Erwinia*), *11* (*1174-901-12_ge*), *9* (*Paenibacillaceae*-unclassified)*; 4* (*Spirosoma*)*; 10* (*Lactobacillus*)*; 34*

(*Deltaproteobacteria*-unclassified)*; 23* (*Zymomonas*)*; 6* (*Epilithonimonas*)*; 5* (*Cytophagales*-unclassified) and 19 (*Acidiphilium*).

The sample male health, male disease and female disease share 1 OUT: S (*Bacteria*-unclassified). The two samples from orchard disease shared 8 OTUs namely 36 (*Enterobacteriaceae*-unclassified), *41* (*Pseudomonadaceae_ge*), *15* (*Methylobacterium*), *43* (*Pseudomonas*), *42* (*Pseudomonadaceae*-unclassified), *17* (*Rhizobiales*-unclassified), *32* (*Oxalobacteraceae*-unclassified) and *31* (*Massilia*).

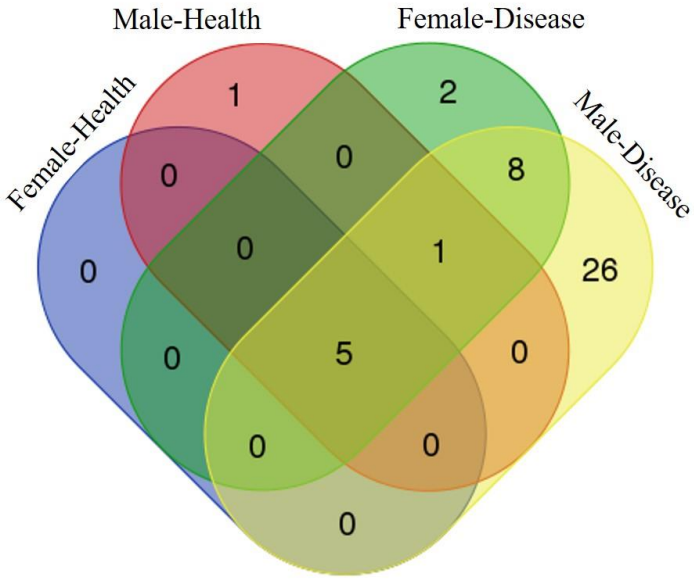

Figure 27 Venn diagram showing the number of shared genera between *A. deliciosa* leaf samples identified by cultivation-based approaches. FH: female-health sample; MH: male-health sample; FD: female-disease sample and MD: male disease sample.).

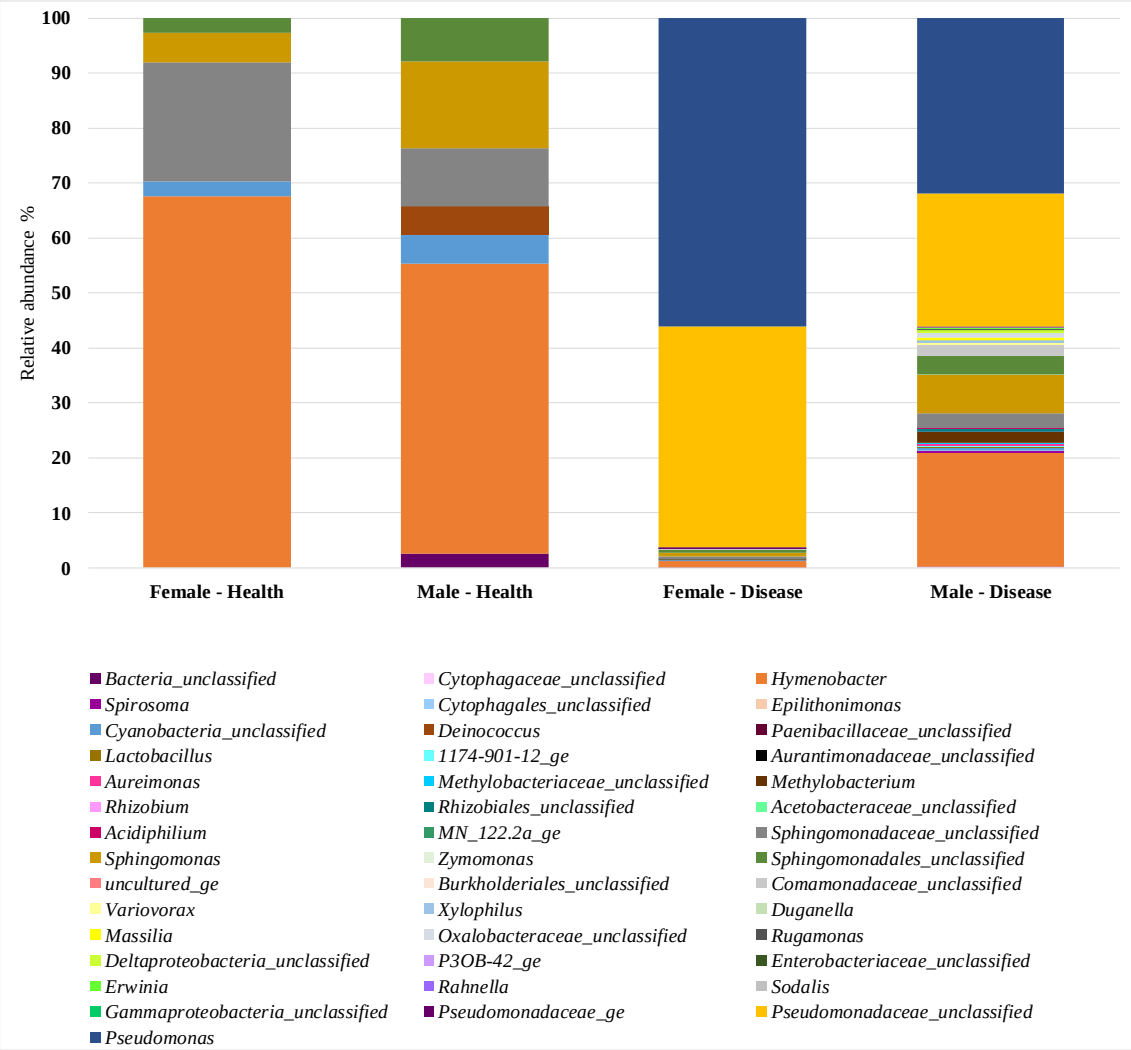

Figure 28 Phylogenetic characterization at genus level of bacteria from *A. deliciosa* leaf samples obtained by culture-independent methodology.

3.2.6. PCA Analysis

The PCA analysis performed on results obtained for the culture dependent methods was carried out with two focuses: distance between sample (PCA-inter-sample) and inter species correlation (PCA-inter-species).

3.2.6.1. PCA-Inter-sample

Based on results from Fig.29 we can conclude that samples are heterogeneous regarding the structural bacteria composition of each sample. Nevertheless, a clear distinction between healthy orchards and disease orchards is observed, providing

evidence of the existence of specific structural bacterial diversity for healthy plants and for disease plants.

Figure 29 Principal component analysis (PCA) from OTU's by uncultured methods**.** Clusters profiles correlated with samples (Inter-samples). FH: female health; MH: male health; FD: female disease and MD: male disease.

3.2.6.2. Interspecies

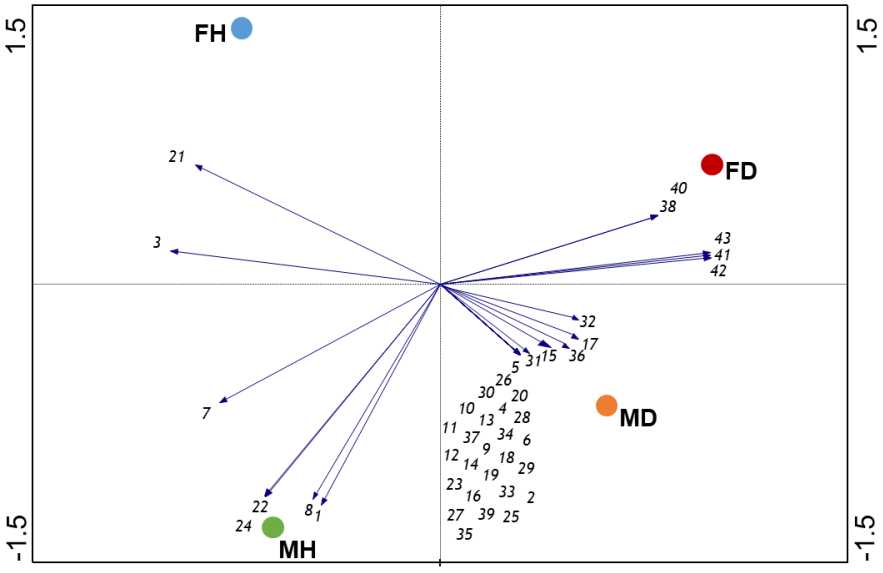

Figure 30 Principal component analysis (PCA) from OTU's by uncultured methods**.** Clusters profiles correlated with samples (Inter-species). FH: female health; MH: male health; FD: female disease and MD: male disease.

By analyzing the inter species correlation PCA result (Fig.30), we can identify the populations responsible for the difference between samples. For instance, we can

identify the existence of three OTUs, namely OTU41 (*Pseudomonadaceae-ge*), OUT42 (*Pseudomonadaceae*-unclassified) and OTU43 (*Pseudomonas*) that are accountable for the differentiation between healthy and disease orchards, and most probably are responsible for the disease condition.

3.2.7. Biodiversity index

The same parameters of diversity used in culture dependent methods (section 3.1.5) were applied to the culture independent methods.

3.2.7.1. Diversity within orchards

Lower values for the Margalef's diversity index (Dmg) were obtained for female health, male health and female disease samples, with Dmg=1.12, 1.65 and 1.82, respectively; when compared with the values obtained from the male diseased sample, 4.79 (Table XIX). In both orchards, male's samples had higher bacterial diversity than female's samples.

Shannon-Weaver index (H') was calculated from healthy female and male samples, H'= 0.95 and H'= 1.47 respectively; and from diseased female and male samples, H'= 0.89 and H'= 1.94, respectively. Comparing the female samples a decrease in diversity was found in the disease plants. On the other hand, among male samples an increase in diversity was detected in the disease plants. Nevertheless, the lower H' values obtained for both orchards support the existence of dominate genera.

In the healthy orchard the Pielou's evenness index values for female and male samples were J'=0.59 and J'= 0.76, respectively. Regarding the diseased orchard, the values for female and male samples were J'=0.32 and J'= 0.53, respectively. Based on these results, the diseased orchard presented lower J' values than the healthy orchard suggesting the presence of one or a few dominant genera, most obvious in diseased female plants (Fig.25). Thus, according with the Shannon-Weaver index and Pielou's Evenness, the lower diversity found in the diseased orchard was associated with the existence of dominance genera. This decrease in the microbial diversity may have been due to the presence of Psa, translated in a significant increase in the abundance of the *Pseudomonadales* order. Additionally, the presence of Psa may have also contributed to

the support of other orders detected in male diseased plants, namely *Cytophagales*, *Shingomonadales* and *Rhizobiales* (Fig. 26).

Finally, the Simpson diversity index (λ) or dominance index (1-D) was used since it measures the probability of two isolates belong to distinct genera, varying between 0 (no diversity - dominance) and 1 (high diversity). On the healthy orchard the Simpson's diversity varied between λ=0.49 and λ =0.68, for female and male samples, respectively. On the diseased orchard the Simpson's diversity index was higher, varying between λ =0.53 and λ =0.79 for female and male sample, respectively.

In sum, these results suggested an increase in diversity in the diseased orchard, more pronounced among male plants. This finding maybe explained by the presence of Psa that allowed the colonization of plants by bacterial genera that were not found in the healthy orchards. This microbiota diversity found in the disease orchard may play a role on the severity degree and extent of the kiwi bacterial canker. In fact, these findings may aid to explain why male plants are known to be more susceptible to the disease. As expected, the reported rise in diversity in diseased orchards was accompanied by the dominance of the *Pseudomonadales* order, which includes pathovar Psa, demonstrating how efficient was the colonization of the plants by these microorganisms.

Table XIX Alpha diversity indexes determined for each orchard and for each gender. Dmg: Margalef index; H': Shannon index; J': Pielou's evenness index; 1-D: Simpson diversity index.

	(H')	(J')	(λ)	(Dmg)
Female Health	0.95	0.59	0.49	1.12
Male Health	1.47	0.76	0.68	1.65
Female Disease	0.89	0.32	0.52	1.82
Male Disease	1.94	0.53	0.79	4.79

3.2.7.2. Diversity between orchards

The Jaccard index allowed comparing the diversity between the healthy and diseased orchards to determine the percentage of shared taxa between them (Table XVIII). The higher the obtained percentage, the more similar two populations were. According with this analysis, it was possible to infer that the populations were quite distinct in between orchards, with values of similarity of 12.25%. These results reinforced our previous conclusions on the dramatic effect in microbiota diversity induced most likely by the presence of Psa. According with this analysis, female plants were more similar (17.85%) than male plants (7.94%), meaning that the former shared more taxa than the later. This finding supports our assumptions that the higher susceptibility of male plants to Psa infection may be related with the reported dramatic change in the microbiota diversity.

3.2.8. Analysis of bacterial richness

Rarefaction curves were constructed for each of the 4 samples (FH, MH, FD and MH) to determine if the richness determined for each sample was representative of the bacterial diversity existing in each sample.

Through the analysis of the rarefaction curves of each sample (Figs.31 and 32) we observe that all samples show a tendency to saturation point. Even though for samples female health and male health a low number of sequences were analyzed (compared to the higher number of sequences of samples female disease and male disease), apparently the bacterial population diversity covered was enough to establish a representative structural bacterial diversity in each sample.

.

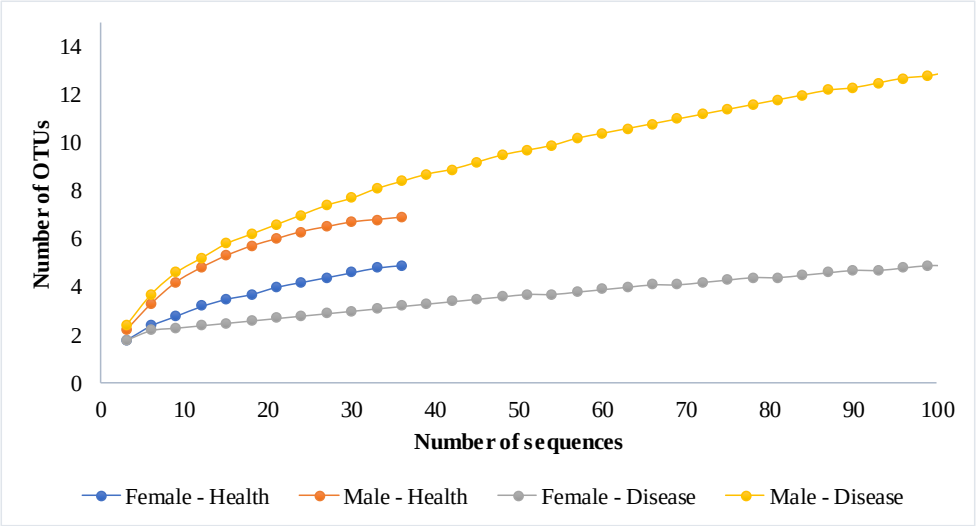

Figure 31 Rarefaction curve of observed OTU's richness for each sample and using an OTU's threshold of ≥97% identity.

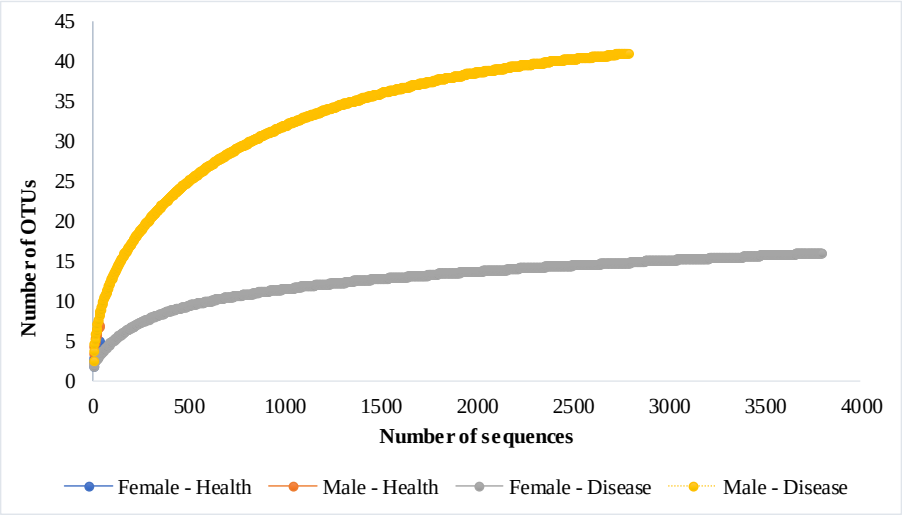

Figure 32 Magnification of scale from previous rarefaction curve above in order to observe the 'plateau' of curves from orchard disease.

3.3. The impact of disease in the microbial diversity

Our study provides new insights about the structural bacterial diversity on leaves of *Actinidia deliciosa* plants, determined by culture -dependent and -independent methods. This was performed on leaves samples from healthy and disease orchards (kiwifruit bacterial canker) to determine the impact of the presence of Psa in the structural bacterial diversity of diseased kiwifruit leaves.

Several fragilities may be identified in this study like the low number of isolates that were classified phylogenetically in the culture-dependent methods, and the low

number of bacterial 16S rRNA gene sequences obtained for all samples in the culture-independent methods. All this could lead to faulty results on the characterization of the structural bacterial diversity of the samples. Nevertheless, statistical analyses of the results provided evidence that a comfortable coverage of the bacterial diversity was achieved, and that the results and the conclusion presented are reliable.

A greater number of OTU's was identified by the culture-dependent methods (49 taxa) than by the culture-independent methods (43 taxa). The richness level determined for all samples, shown an even distribution of the populations obtained by culture-dependent methods (female health -19 species; male health – 23 species; female disease - 22 species and male disease 23 species) while by culture-independent methods the distribution was uneven (female health -5 species; male health – 7 species; female disease - 16 species and male disease - 40 species). This difference between methods results can be explained by the fact that culture-dependent methods provide results about the culturable microbial populations of the environment, that most of the time are minorities that normally are not detected by culture independent methods. While culture-independent methods tend to provide information about the "true" structural bacterial diversity of an environment, since is based on total DNA of the environment. Nevertheless, the existence of major populations was observed in both methodologies, and shifts on the structural bacterial diversity and relative abundance was observed between healthy orchards samples and the diseased orchards samples.

In both approaches (culture -dependent and -independent methods) the existence of putative phytopathogenic bacterial populations was observed in higher concentration in diseased orchards samples, i.e. populations belonging to genera *Pseudomonas*, *Serratia*, *Pantoea* and *Erwinia,* among others. Also in both approaches (more pronounced on culture-independent methods results), it was observed that male plants tend to have higher diversity than female plants, being this difference more obvious on diseased plants.

4 Conclusions

Culture-dependent methods provided evidence that the presence of *Pseudomonas* populations was more pronounced on diseased plants than on healthy plants. Only *Pseudomonas* populations from diseased plants were confirmed as being Psa. Apparently the presence of Psa provided conditions for the development of other potential pathogenic bacterial populations in diseased plants, this being more pronounced on male plants.

Culture-independent methods provided evidence that the presence of *Pseudomonas* populations was limited to diseased plants. Apparently, the presence of such populations (most probably Psa) in diseased plants provided conditions for the development of other potential pathogenic bacterial populations in diseased plants.

The overall results obtained provide evidence that, although Psa is most probably the agent for the kiwifruit bacterial canker, the presence of other pathogenic populations may also contribute for the disease and aid on the deteriorating condition of the diseased plant.

There are some evidences of gender differentiation on the kiwifruit bacterial canker. The diseased male plants present higher bacterial diversity than diseased female plants. Furthermore, although the structural diversity of the putative pathogenic bacterial populations is identical between disease plants of both genders, the relative abundance is not. This could explain the conception that male plants are more susceptible to the disease, since the relative abundance of the putative pathogenic populations in male plants was more even than in female plants, enhancing the destructive role of those populations on male plants.

5 References

Abelleira, A., Lopez, M., Penalver, J., Aguín, O., Mansilla, P., Picoaga, A., García, M.J. (2011) First report of bacterial canker of kiwifruit caused by *Pseudomonas syringae* pv. *actinidiae* in Spain. *Plant Disease* 95(12): 1593.

Abelleira, A., Ares, A., Aguín, O., Picoaga, A., López, M., Mansilla, P. (2014). Current situation and characterization of *Pseudomonas syringae* pv. *actinidiae* on kiwifruit in Galicia (northwest Spain). *Plant Pathology* 63(3): 691–699.

Al-Awadhi, H., Dashti, N., Khanafer, M., Al-Mailem, D., Ali, N., Radwan, S. (2013). Bias problems in culture-independent analysis of environmental bacterial communities: a representative study on hydrocarbonoclastic bacteria. *SpringerPlus* 2(1): 369.

Bae, C., Wook, S., Yu, H., Song, R., Young, B. (2015). Infection processes of xylem - colonizing pathogenic bacteria : possible explanations for the scarcity of qualitative disease resistance genes against them in crops. *Theoretical and Applied Genetics*.

Balestra, M., Mazzaglia, A., Quattrucci, A., Renzi, M., Rossetti, A. (2009). Current status of bacterial canker spread on kiwifruit in Italy. *Australasian Plant Disease Notes* 4(1): 34–36.

Balestra, M., Renzi, M., Mazzaglia, A. (2010). First report of bacterial canker of *Actinidia deliciosa* caused by *Pseudomonas syringae* pv. *actinidiae* in Portugal. *New Disease Reports 22*: 10.

Balestra, G. M., Renzi, M., & Mazzaglia, A. (2011). First report of *Pseudomonas syringae* pv. *actinidiae* on kiwifruit plants in Spain.*New Disease Reports* 24(10).

Bastas, K. K. (2012). First Report of Bacterial Canker of Kiwifruit Caused by *Pseudomonas syringae* pv. *actinidiae* in Turkey. *Plant Disease 96*(3).

Berg, G. (2009). Plant-microbe interactions promoting plant growth and health: Perspectives for controlled use of microorganisms in agriculture. *Applied Microbiology and Biotechnology* 84(1): 11–18.

Berg, G., Grube, M., Schloter, M., Smalla, K. (2014). Unraveling the plant microbiome: Looking back and future perspectives. *Frontiers in Microbiology* pp:1–7.

Berlec, A. (2012). Novel techniques and findings in the study of plant microbiota: Search for plant probiotics. *Plant Science 193–194*. http://doi.org/10.1016/j.plantsci.2012.05.010

Bikel, S., Valdez-Lara, A., Cornejo-Granados, F., Rico, K., Canizales, S., Soberón, X., Ochoa, A. (2015). Combining metagenomics, metatranscriptomics and viromics to explore novel microbial interactions: Towards a systems-level understanding of human microbiome. *Computational and Structural Biotechnology Journal* 13: 390–401.

Birtel, J., Walser, C., Pichon, S., Bürgmann, H., Matthews, B. (2015). Estimating bacterial diversity for ecological studies: methods, metrics, and assumptions. *PloS One*, 10(4): 1-23.

Blaser, J., Cardon, G., Cho, K., Dangl, L., Donohue, J., Green, L., Brodie, L. (2016). Toward a Predictive Understanding of Earth's Microbiomes to Address 21st Century Challenges. *American Society for Microbiology* 7(3): 1–16.

Bodenhausen, N., Bortfeld, M., Ackermann, M.,Vorholt, J. (2014). A Synthetic Community Approach Reveals Plant Genotypes Affecting the Phyllosphere Microbiota. *PLoS Genetics* 10(4): 1-12.

Bringel, F., & Couée, I. (2015). Pivotal roles of phyllosphere microorganisms at the interface between plant functioning and atmospheric trace gas dynamics. *Frontiers in Microbiology* 6(486):1–14.

Bulgarelli, D., Schlaeppi, K., Spaepen, S., Themaat, E., Schulze, P. (2013). Structure and functions of the bacterial microbiota of plants. *Annual Review of Plant Biology* 64: 807–38.

Busby, E., Soman, C., Wagner, R., Friesen, L., Kremer, J., Bennett, A., Dangl, L. (2017). Research priorities for harnessing plant microbiomes in sustainable agriculture. *PLoS Biology*, *15*(3): 1–14.

Cameron, A., & Sarojini, V. (2014). *Pseudomonas syringae* pv. *actinidiae*: Chemical control, resistance mechanisms and possible alternatives. *Plant Pathology* 63(1): 1–11.

Clarridge, E., & Alerts, C. (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clin. Microbiol. Rev.* 17(4): 840–862.

Copeland, J., Yuan, L., Layeghifard, M., Wang, W., Guttman, D. (2015). Seasonal community succession of the phyllosphere microbiome. *Mol Plant-Microbe Interact* 28(3): 274–285.

Costa, J., & Tiago, I. (n.d.). Assessing the structural and functional diversity of soil microorganism's. Centre for Functional Ecology, Department of Life Sciences, University of Coimbra.

Cox, J., Cookson, M., Moffatt, F. (2013). Sequencing the human microbiome in health and disease. *Human Molecular Genetics*, 22(1): 88–94.

DGAV. (2013). Plano de Ação Nacional para o Controlo da *Pseudomonas syringae* pv . *actinidiae* do *Kiwi*.

EPPO. (2012). Revision of EPPO Standard PM 1/2 EPPO A1 and A2 Lists of pests recommended for regulation as quarantine pests. Standards approved by EPPO Council in 2012-09.

EPPO. (2014). PM 7/120 (1) *Pseudomonas syringae* pv. *actinidiae*. *EPPO Bulletin, 44*(3), 360–375. http://doi.org/10.1111/epp.12171

EPPO. (2017a). Global Database. *Pseudomonas syringae* pv. *actinidiae* (PSDMAK).Available in: < https://gd.eppo.int/taxon/PSDMAK/distribution>Access to 20 March 2017.

EPPO. (2017b). *Pseudomonas syringae* pv. *actinidiae*. Bacterial canker of Kiwifruit. Available in: <

https://www.eppo.int/QUARANTINE/Alert_List/bacteria/P_syringae_pv_actinidiae.htm> Access to 29 May 2017.

European Comission. (2014). In order to evaluate the situation and official controls for *Pseudomonas syringae* pv. *actinidiae*. 1-23. http://doi.org/10.1163/1571809042388581

Everett, R., Taylor, K., Romberg, K., Rees, J., Fullerton, R., Vanneste, L., Manning, A. (2011). First report of *Pseudomonas syringae* pv. *actinidiae* causing kiwifruit bacterial canker in New Zealand. *Australian Plant Disease Notes* 6(1): 67–71.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap, 39(4): 783–791.

Ferguson, A. R, & Huang, H. (2007). Genetic Resources of Kiwifruit: Domestication and Breeding. *Horticultural Reviews* 33: 1-121.

Ferguson, A. R. (2013). Kiwifruit. The Wild and the Cultivated Plants. *Advances in Food and Nutrition Research* 68, 15–32.

Ferrante, P., & Scortichini, M. (2015). Redefining the global populations of *Pseudomonas syringae* pv. *actinidiae* based on pathogenic, molecular and phenotypic characteristics. *Plant Pathology 64*(1): 51–62.

Fujikawa, T., & Sawada, H. (2016). Genome analysis of the kiwifruit canker pathogen *Pseudomonas syringae* pv. *actinidiae* biovar 5. *Nature Publishing Group* pp.1–11.

FAOSTAT. (2017). Statistics division. Available in: < http://faostat3.fao.org/browse/Q/QC/E> Access to 25 May 2017

Garcia, E. (2015). Variabilidade genética e fenotípica de *Pseudomonas syringae* pv . *actinidiae*, agente causal do Cancro da Actinídea , na Região de Entre Douro e Minho.

Goodwin, S., Mcpherson, D., Mccombie, R. (2016). Coming of age : ten years of next-generation sequencing technologies. *Nature Publishing Group* 17(6): 333–351.

Gopal, M., & Gupta, A. (2016). Microbiome selection could spur next-generation plant breeding strategies. *Frontiers in Microbiology* 7: 1–10.

Gopal, M., Gupta, A., Thomas, V. (2013). Bespoke microbiome therapy to manage plant diseases. *Frontiers in Microbiology* 4: 10–13.

Hall, T. A. (1999). Bioedit a user-friendly biological sequence alignment editor and analysis program for windows. *Nucleic Acid Symposium Series* pp 1–4.

Hill, M. O. (1973). Diversity and evenness: a unifying notation and its consequences. *Ecology* 54(2): 427–432.

Huang, Y., Niu, B., Gao, Y., Fu, L., Li, W. (2010). CD-HIT Suite : a web server for clustering and comparing biological sequences. *Bioinformatics Applications Note* 26(5): 680–682.

Ibarbalz, M., Pérez, V., Figuerola, M., Erijman, L. (2014). The bias associated with amplicon sequencing does not affect the quantitative assessment of bacterial

community dynamics. *PLoS ONE*, *9*(6): 1-6

Imam, J., Singh, K., Shukla, P. (2016). Plant Microbe Interactions in Post Genomic Era : Perspectives and Applications. *Frontiers in Microbiology* 7: 1–15.

INE. (2015). *Estatísticas agrícolas 2005 a 2014*. Lisboa, Portugal

Jaccard, P. (1912). The distribution of the flora in the alpine zone. *New Phytologist* 11(2): 37–50.

Jukes, T. H., & Cantor, C. R. (1969). This Week ' s Citation CIassic.

Khandan, N., Worner, P., Jones, E., Villjanen, H., Gallipoli, L., Mazzaglia, A., Balestra, M. (2013). Predicting the potential global distribution of *Pseudomonas syringae* pv. *actinidiae* (Psa). *New Zealand Plant Protection* 66: 184–193.

Koh, J., Cha, J., Chung, J., Lee H. (1994) Outbreak and spread of bacterial canker in kiwifruit. Korean. *Plant Pathololology* 10:68–72.

Kroll, S., Agler, M., Kemen, E. (2017). Genomic dissection of host-microbe and microbe-microbe interactions for advanced plant breeding. *Current Opinion in Plant Biology* 36:71–78.

Leps, J., & Smilauer, P. (2003). *Multivariate Analysis of Ecology Data using CANOCO*. Cambridge University Press.

Li, X., Li, J., Soejarto, D. (2009). Advances in the study of the systematics of *Actinidia* Lindley. *Frontiers of Biology in China* 4(1): 55–61.

Lindow, S., & Brandl, M. (2003). Microbiology of the Phyllosphere. *Applied and Environmental Microbiology* 69(4): 1875–1883.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Buchner, A., Bode, A. (2004). ARB : a software environment for sequence data. *Nucleic Acid Research* 32(4): 1363–1371.

Madugan, T., Martinko, M., Stahl, D., Clark, .P. (2013). Brock Biology of Microorganisms. *Pearson Education* 16(12): 1-1155.

Marcelletti, S., Ferrante, P., Petriccione, M., Firrao, G., Scortichini, M. (2011). *Pseudomonas syringae* pv. *actinidiae* draft genomes comparison reveal strain-specific features involved in adaptation and virulence to *Actinidia* species. *PLoS ONE* 6(11)

McCann, C., Rikkerink, A., Bertels, F., Fiers, M., Lu, A., Rees J..(2013). Genomic Analysis of the Kiwifruit Pathogen *Pseudomonas syringae* pv *actinidiae* Provides Insight into the Origins of an Emergent Plant Disease. *PLoS Pathog 9*(7):1-19

Mizrahi, O., Davenport, R., Gilad, Y. (2013). Taxonomic Classification of Bacterial 16S rRNA Genes Using Short Sequencing Reads: Evaluation of Effective Study Designs. *PLoS ONE* 8(1): 1–14.

Morris, E., Bardin, M., Kinkel, L., Moury, B., Nicot, P. (2008). Expanding the

Paradigms of Plant Phatogen Life History and Evolution of Parasitic Fitness beyond Agricultural Boundaries. *PLoS ONE Pathogens* 5(12): 1-7

Moura, L., Garcia, E., Aguín, O., Ares, A., Abelleira, A., Mansilla, P. (2015). Identificação e caracterização de *Pseudomonas syringae* pv . *actinidiae* (Psa) na Região do Entre Douro e Minho (Portugal) in Entre Douro and Minho region (Portugal). *Revista de Ciências Agrárias 38*(2): 196–205.

Müller, A., Obermeier, M., Berg, G. (2016). Bioprospecting plant-associated microbiomes. *Journal of Biotechnology 235*: 171–180.

Müller, T., Ruppel, S. (2014). Progress in cultivation-independent phyllosphere microbiology. *FEMS Microbiology Ecology* 87(1): 2–17.

Myer, R., Kim, S., Freetly, C., Smith, L. (2016). Evaluation of 16S rRNA amplicon sequencing using two next-generation sequencing technologies for phylogenetic analysis of the rumen bacterial community in steers. *Journal of Microbiological Methods* 127: 132–140.

Pat Schloss. (2017) morthur. Department of Microbiology and Immunology. The University of Michigan Medical School. Available in: < http://www.mothur.org >Access to 22 June 2017

Parnell, J., Berka, R., Young, H., Sturino, M., Kang, Y., Dileo, V. (2016). From the Lab to the Farm: An Industrial Perspective of Plant Beneficial Microorganisms. *Frontiers in Plant Science* 7: 1–12.

Pielou, C. (1966). Species-diversity and pattern-diversity in the study of ecological succession. *Journal of Theoretical Biology* 10(2): 370–383.

Pieterse, J., Van der Does, D., Zamioudis, C., Leon-Reyes, A., Wees, S. (2012). Hormonal Modulation of Plant Immunity. *Annual Review of Cell and Developmental Biology* 28: 489–521.

Pinto, A. H., Neves, M., Ana, C., Manteigas, M., Abrunhosa, B., Saltão, B., … Batista, V. (2014). Relatório da execução do plano de ação nacional para o controlo da *Pseudomonas syringae* pv. *actinidiae* do kiwi (psa) na drapcentro.

Phytobiomes 2015: Designing a new Paradigm for Crop Improvement. Available in: < http://www.phytobiomes.org/activities/pages/phytobiomes-2015.aspx> Access to 25 April 2017

Rainey, F., Ward-rainey, N., Kroppenstedt, R. (1996). The Genus *Nocardiopsis* Represents a Phylogenetically Coherent Taxon and a Distinct *Actinomycete* Lineage: ProDosal of Nocardiomaceae fam . nov . *Systematic Bacteriology* pp.1088–1092.

Rastogi, G., & Rajesh, K. (2011). Molecular Techniques to Assess Microbial Community Structure, Function, and Dynamics in the Environment.pp 1-516

Rastogi, G., Coaker, G., Leveau, J. (2013). New insights into the structure and function of phyllosphere microbiota through high-throughput molecular approaches. *FEMS Microbiology Letters* 348(1): 1–10.

Rinke, C., Schwientek, P., Sczyrba, A., Ivanova, N., Anderson, I., Cheng, J., Woyke, T. (2013). Insights into the phylogeny and coding potential of microbial dark matter. *Nature* 499(7459): 431–437.

Rosselli, R., Romoli, O., Vitulo, N., Vezzi, A., Campanaro, S., Pascale, F., Squartini, A. (2016). Direct 16S rRNA-seq from bacterial communities: a PCR-independent approach to simultaneously assess microbial diversity and functional activity potential of each taxon. *Scientific Reports* 6(1): 32165.

Rout, M. (2014). The Plant Microbiome. *Advances in Botanical Research* 1(69):.279-309.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology Evolution 4: 406-425.

Schlaeppi, K., & Bulgarelli, D. (2015). The Plant Microbiome at Work. *Molecular Plant-Microbe Interactions* 212(3): 212–217.

Schloss, P., Westcott, S., Ryabin, T., Hall, J., Hartmann, M., Hollister, E., Weber, C.. (2009). Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology* 75(23): 7537–7541.

Scortichini, M. (1994). Occurrence of *Pseudomonas syringae* pv. *actinidiae* on kiwifruit in Italy. *Plant Pathology* 43(6): 1035–1038.

Serizawa, S., Ichikawa, T., Takikawa, Y., Tsuyumu, S., Goto, M. (1989). Occurrence of Bacterial Canker of Kiwifruit in Japan: Descriotion of Symptoms, Isolation of the Pathogen and Screening of Bactericides. *Annals of the Phytopathological Society of Japan* 55: 427–436.

Sessitsch, A., & Mitter, B. (2015). 21st Century agriculture: Integration of plant microbiomes for improved crop production and food security. *Microbial Biotechnology* 8(1): 32–33.

Silva. High quality ribosomal RNA databases. (2017). Available in < https://www.arb-silva.de/projects/living-tree> Access to 9 June.

Simpson, G. (1960). Notes on the measurement of faunal resemblance. *American Journal of Science* 258(2): 300 –311.

Solden, L., Lloyd, K., Wrighton, K. (2016). The bright side of microbial dark matter: Lessons learned from the uncultivated majority. *Current Opinion in Microbiology* 31: 217–226.

Stonehouse, W., Gammon, C., Beck, K., Conlon, C., Hurst, P., Kruger, R. (2013). Kiwifruit: our daily prescription for health. *Canadian Journal of Physiology and Pharmacology* 91(6): 442–7.

Strange, R., & Scott, P.. (2005). Plant disease: A Threat to Global Food Security. *Annual Review of Phytopathology* 43: 83–116.

Turner, S., Pryerb, M., Vivian,. Palmera, J.. (1999). Investigating Deep Phylogenetic Relationships among Cyanobacteria and Plastids by Small Subunit rRNA Sequence Analysis. *Society of Protozoologists* 46(4): 327–338.

Turner, T., James, E., Poole, P.. (2014). The Plant Microbiome. *Advances in Botanical Research* 69: 279–309.

van Overbeek. (2017). Modifying the plant microbiome to make plants more resistant to pests and diseases. University & Research Wageningen. Available in: < https://www.wur.nl/en/project/Modifying-the-plant-microbiome-to-make-plants-more-resistant-to-pests-and-diseases-.htm> Access to 12 March.

Vanneste, J. L. (2013). Recent progress on detecting, understanding and controlling *Pseudomonas syringae* pv. *actinidiae*: A short review. *New Zealand Plant Protection 66*: 170–177.

Vanneste, J. L., Poliakoff, F., Audusseau, C., Cornish, D., Paillard, S., Rivoal, C., Yu, J. (2011). First Report of *Pseudomonas syringae* pv. *actinidiae* , the Causal Agent of Bacterial Canker of Kiwifruit in France. *Plant Disease* 95(10): 1311–1311.

Vorholt, J. (2012). Microbial life in the phyllosphere. *Nature Reviews. Microbiology*, 10(12): 828–40.

Wang Z, Tang X, Liu S,. (1992). Identification of the pathogenic bacterium for bacterial canker on *Actinidia* in Sichuan. Journal of Southwest Agricultural University.

Whipps, J., Hand, P., Pink, D., Bending, G. (2008). Phyllosphere microbiology with special reference to diversity and plant genotype. *Journal of Applied Microbiology* 105(6): 1744–1755.

Wiedmann-al-ahmad, M., Tichy, H., Schon, G. (1994). Characterization of *Acinetobacter* Type Strains and Isolates Obtained from Wastewater Treatment Plants by PCR Fingerprinting. *Society of Microbiology* 60(11): 4066–4071.

Willey, J., Sherwood, L., Woolverton, C. (2014). Prescott's Microbiology. *Mc Graw Hill* 9*:1-1139*.

Williams, J. G., Kubelik, A. R., Livak, K. J., Rafalski, J. A., & Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are usefil as genetic markers, *18*(22), 6531–6535.

Woese, C. R. (1988). Bacterial evolution. *Canadian Journal of Microbiology* 34(4): 547–551.

Wu, D., Hugenholtz, P., Mavromatis, K., Pukall, R., Dalin, E., Ivanova, N., Eisen, J.. (2009). A phylogeny-driven genomic encyclopaedia of *Bacteria* and *Archaea. Nature* 462: 1056–1060.

Yashiro, E., Spear, R., Mcmanus, P.. (2011). Culture-dependent and culture-independent assessment of bacteria in the apple phyllosphere. *Applied Microbiology* 110: 1284–1296.

6 Supplementary data

Table I.I Organic buffers used on ABM2 and R2A medium.

Composition	Medium pH	Weight (g/L)
TAPS (N-Tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid)	8,5	9,732
ACES (N-[2-Acetamido]-2-aminoethane-sulfonic acid)	7	7,288
MES (2-[N-Morpholino]ethanesulfonicacidhydrate,4-Morpholineethanesulfonic acid	5,5	7,808

Table I.II Macronutrients composition 10x concentrated.

Composition	
Nitriloacetic acid	1g/l
Calcium sulfate	0,6 g/l
Magnesium Sulfate Heptahydrate	1g/l
Sodium Chloride	0,8 g/l
Potassium Nitrate	1,03 g/L
N_2NO_3	6,89 g/l
N_2HPO	1,11 g/l

Table I.III Micronutriments composition 100x concentrated.

Composition	
Manganese (II) sulfate monohydrate	0,22 g/l
Zinc sulfate Heptahydrate	0,05 g/l
Boric acid	0,05 g/l
Cobalt(II) Chloride Hexahydrate	0,0046 g/l
Copper(II) sulfate pentahydrate	0,0025 g/l
Sodium Molybdate Dihydrate	0,0025 g/L

Table IX.I TAE (50x) stock solution.

Reagents	**Volumes**
Tris/base	121 g
Glacial acetic acid	28,55 ml
Aqueous solution EDTA (0,5M)	50 ml
H_2O Mili-Q sterile (sterilized by autoclaving (121ºC, 20 min)	500 ml

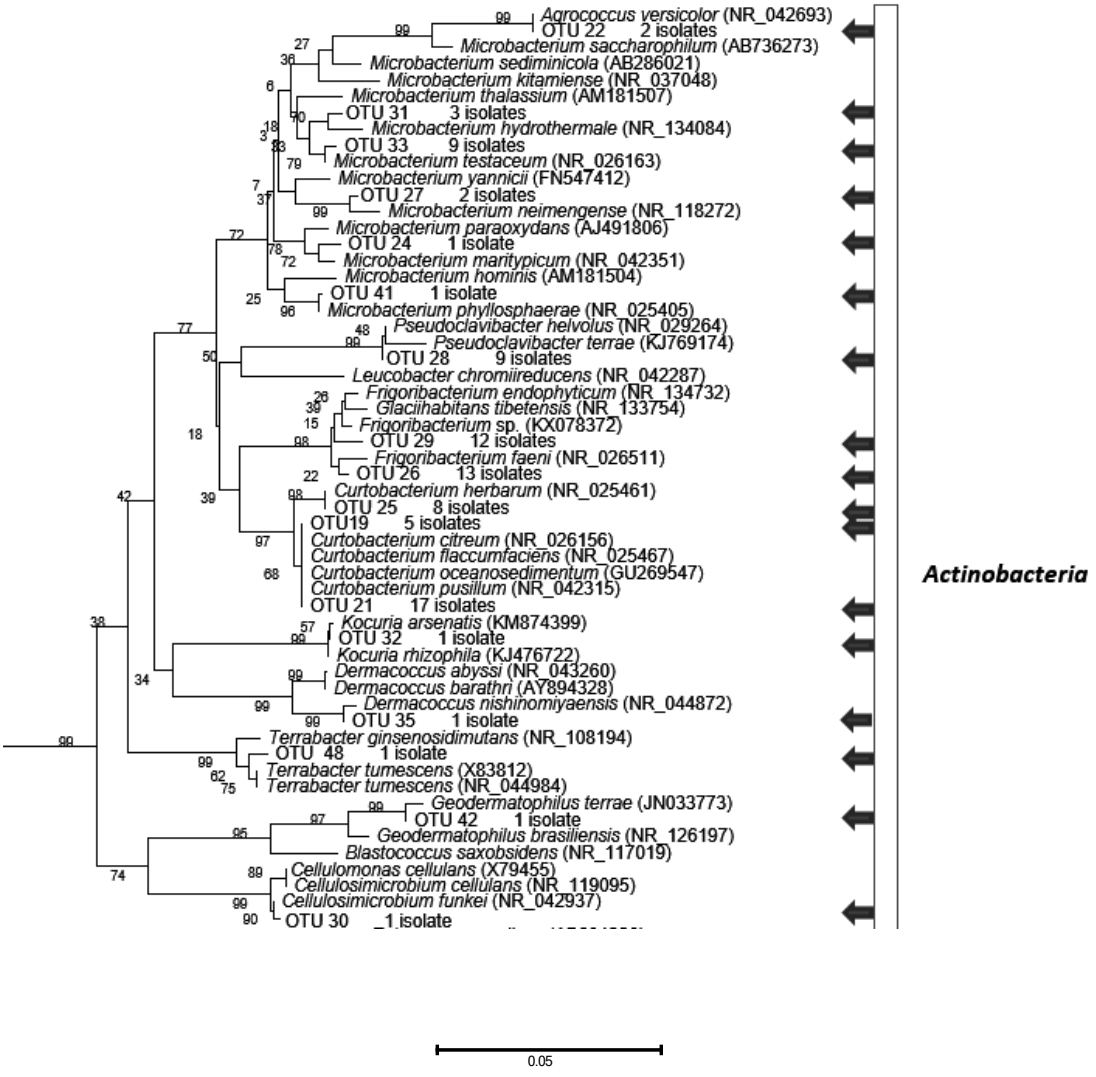

Figure 33 Phylogenetic dendrogram based on a comparison of the 16S rRNA gene sequences of representative strains of each OTU and the closest phylogenetic relatives. The trees were created using the neighbor-joining algorithm. The numbers on the tree branches indicate the percentages of bootstrap sampling, derived from 1000 replications. OTUs placement is indicated with an arrow. Scale bar, 5 inferred nucleotide substitutions per 100 nucleotides.

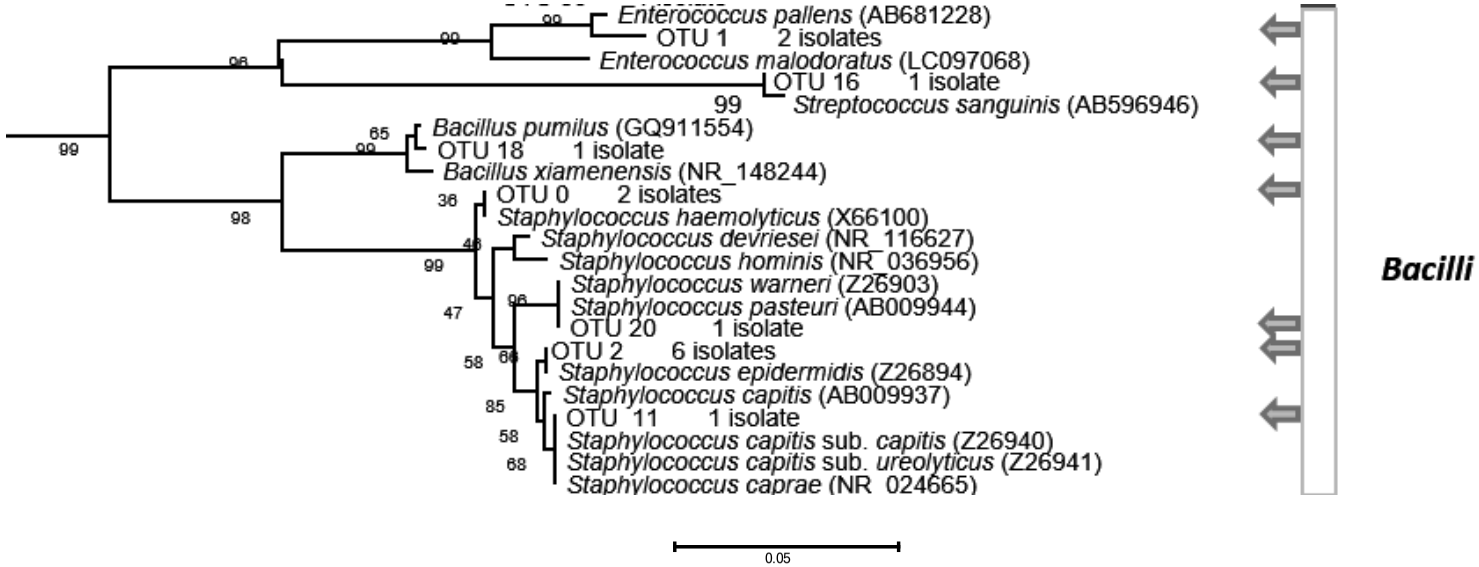

Fig.33 Continued.

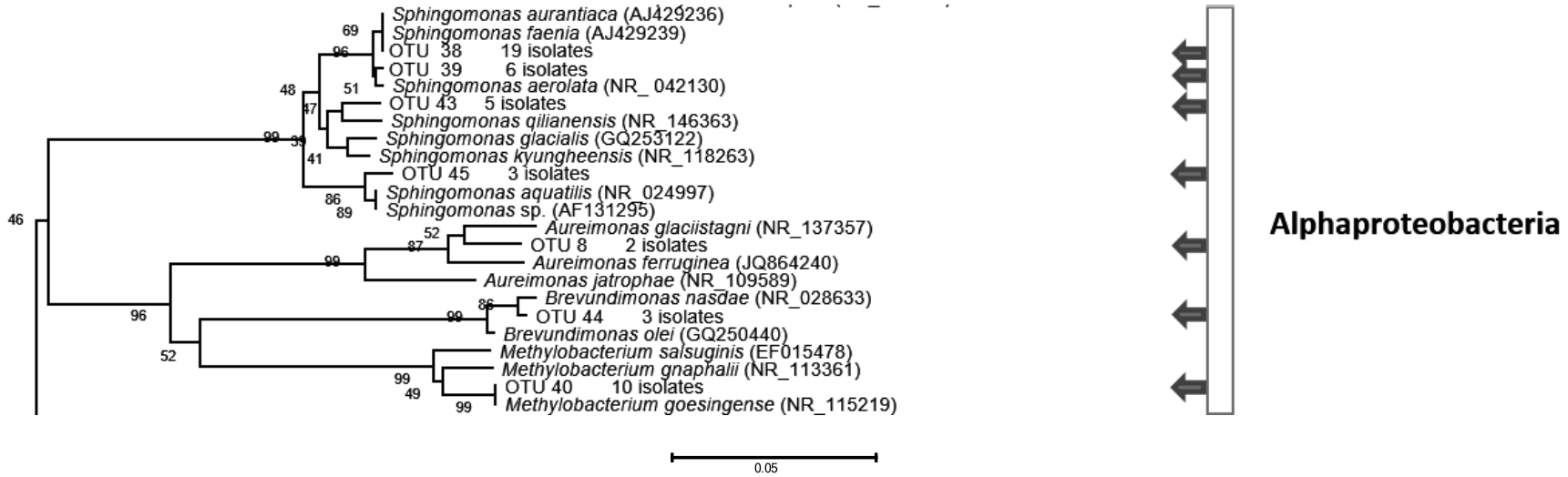

Fig.33 Continued.

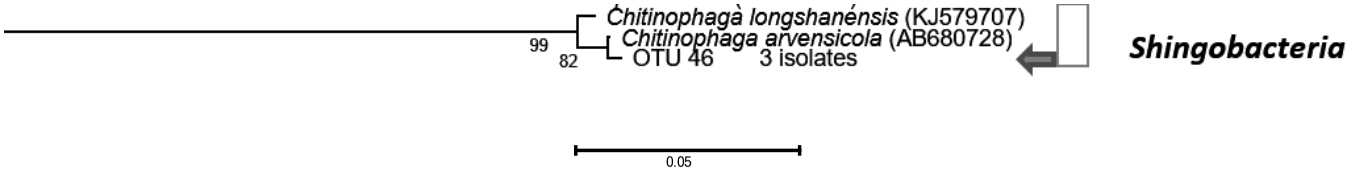

Fig.33 Continued.

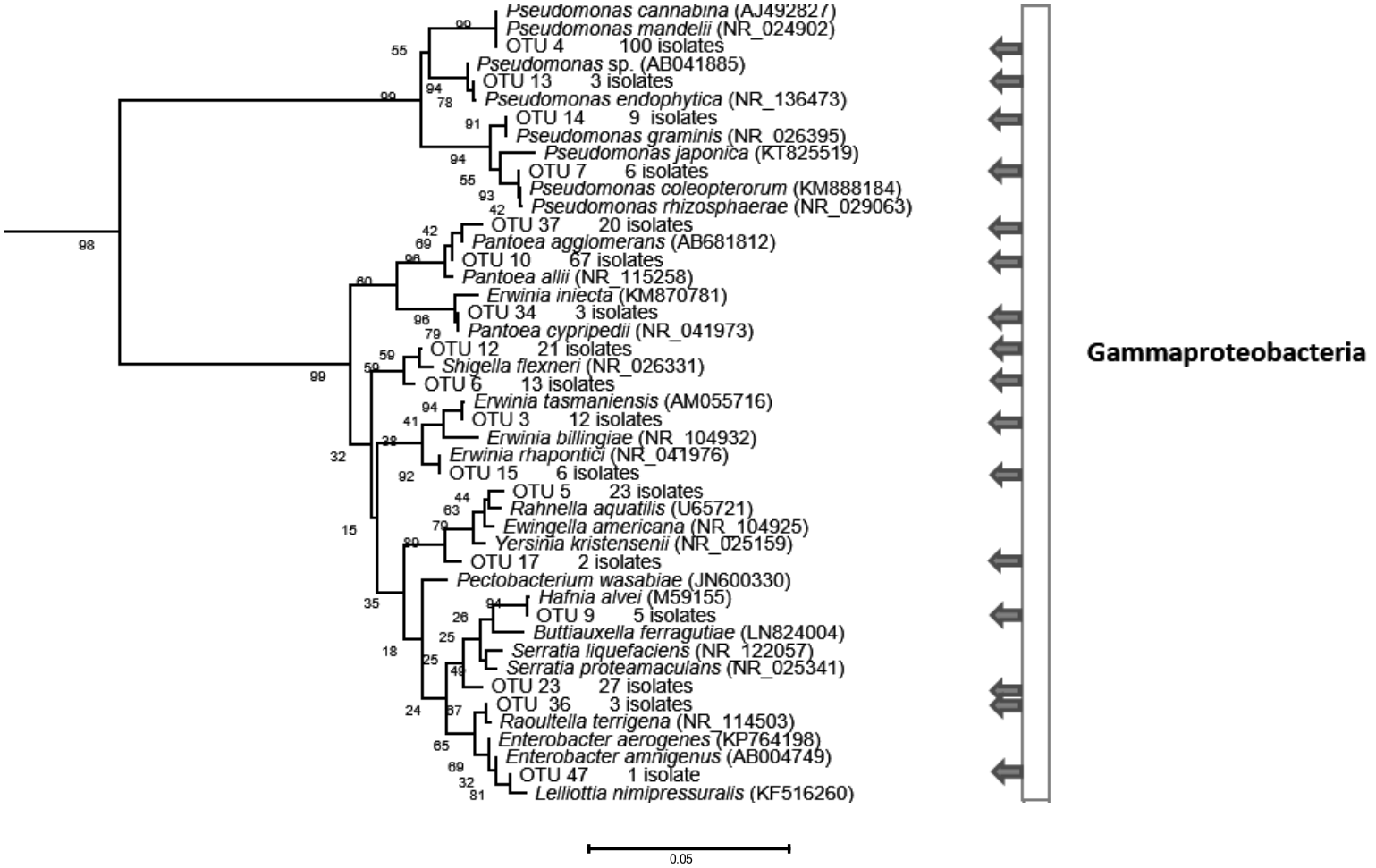

Fig.33 Continued.