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Characterising Disease Suppressive Members Of The Oak Microbiome

Jones, Marcus

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CHARACTERISING DISEASE SUPPRESSIVE MEMBERS OF THE OAK MICROBIOME

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MARCUS H JONES

500481435

Supervisor

PROF. JAMES MCDONALD

School of Natural Sciences

Bangor University

Gwynedd, Wales, United Kingdom

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DECLARATION

I hereby declare that this thesis is the results of my own investigations, except where otherwise stated. All other sources are acknowledged by bibliographic references. This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree unless, as agreed by the University, for approved dual awards.

Yr wyf drwy hyn yn datgan mai canlyniad fy ymchwil fy hun yw'r thesis hwn, ac eithrio lle nodir yn wahanol. Caiff ffynonellau eraill eu cydnabod gan droednodiadau yn rhoi cyfeiriadau eglur. Nid yw sylwedd y gwaith hwn wedi cael ei dderbyn o'r blaen ar gyfer unrhyw radd, ac nid yw'n cael ei gyflwyno ar yr un pryd mewn ymgeisiaeth am unrhyw radd oni bai ei fod, fel y cytunwyd gan y Brifysgol, am gymwysterau deuol cymeradwy.

CONTENTS

ABSTRACT

Tree ecosystems are fundamental to the overall function of the plant, promoting growth and maintaining health, however newly emerging diseases and decline syndromes are increasing in frequency. Current analysis has determined predisposition to disease can be contributed by (a)biotic factors, most commonly affected by drought-stress caused by the increased frequency of drought associated with climate change. Acute oak decline (AOD) is a recently described decline syndrome affecting mature native UK oak trees (*Quercus robur* and *Quercus petraea*) greater than 50 years. The specific cause of AOD remains unknown, but *Brenneria goodwinii, Gibsiella quercinecans*, and *Rahnella victoriana* have been associated with symptoms and causal agents of the syndrome. This study focuses on characterising the microbiome (rhizosphere) of healthy and AOD-symptomatic oak trees to determine members of the oak microbiome with AOD-suppressive phenotypes. Bacteria across 11 distinct genera were recognised with phenotypic properties suppressing the growth of AOD pathogenic strains of *B. goodwinni, G. quercinecans, and R. victoriana*, and identified through bacterial 16S rRNA gene sequencing. Comparison of three major compartments of *Q. robur* using location data of sampled bacteria (foliage, rhizosphere, and stem) associated bacterial 16S rRNA gene sequences with individual compartments, suggesting which major compartment of *Q. robur* has the most AOD-suppressive properties. Bacteria suppressing *G. quercinecans* growth was most abundant in the foliage and rhizosphere, composed of 72.27% and 72.70% respectively, of total successfully sequenced bacteria; bacteria suppressing *R. victoriana* growth was most abundant in the stem, composed of 77.78% of total successfully sequenced bacteria.

1. INTRODUCTION

1.1. State of the World's Trees

An evaluation of the current condition of global tree cover, conducted in early 2000, estimated a minimum decline in tree cover of 50% with declining trends remaining common in the present day (Ahrends *et al.*, 2017). Trees have been utilised by humans for centuries, providing raw materials that supported the foundations of society. Natural resources harvested from forests have become commonplace in the beauty industry, construction, foodstuffs, health and hygiene, logistics, and medicine (Assies., 1997; Jantan *et al.,* 2015) which exacerbates abiotic stressors on forest ecosystems driven by anthropogenic demand. In 2020, the estimated value of wood products imported into the UK was £8.3 billion (UK Wood Production and Trade: provisional figures - Forest Research, 2021) despite the value of the gross domestic product (GDP) declining (Coronavirus and the impact on output in the UK economy – ONS., 2021).

The unprecedented scale of forest fires globally in 2020 emphasises the urgency of immediate environmental action, with rates of deforestation estimated at 10 million hectares per year (Muller Eva, 2018); however, pyrophytic tree species such as lodgepole pine (*Pinus contorta*) utilise forest fires for seed dispersal due to high concentrations of nutrients and elements found in regenerated soils (Petruzzello, 2021). Climate change is a major factor in understanding the potential scale of damage to forested areas. Previous studies concluded that stress-induced mortality is increasing worldwide (Eamus *et al.*, 2013) and drought being the ultimate abiotic factor threatening forests, excluding direct human actions such as deforestation. Despite this, forests remain subjected to mounting stress due to the increased frequency of these extreme weather events alongside the long term changes climate change threatens (e.g., global sea level rise), ultimately increasing worldwide tree mortality rates

(Eamus *et al.*, 2013), with numerous emerging diseases and decline syndromes linked to tree mortality (e.g., Acute Oak Decline, Ash Dieback).

1.2. Trees and their Microbiomes

Early ancestral plants evolved approximately 450 million years ago (Hassani, Durán and Hacquard, 2018) and consequently since have evolved into thousands of tree species known today. Covering a potential global area greater than four billion hectares (Friedlingstein *et al.*, 2019), trees dominate the most biodiverse areas of the planet, most notable of these being forests equating to 31% of global land cover (Muller Eva, 2018). Forest ecosystems support fundamental life processes through regulation of the biosphere; nutrient cycling, soil formation, and carbon storage processes maintain homeostasis. Trees sustain communities of microorganisms living on and within their host tissues, and this collection of microorganisms, their interactions and activities are commonly called the microbiome (Berg *et al.*, 2020). Tree microbiomes are critical for promoting growth and host immunity, stress tolerance, nutrient acquisition, and pathogen defence.

Trees are dominated by microorganisms of which three main areas of the plant are classified as individual microbiomes: the rhizosphere, a region of soil directly influenced by root exudates and rich in microbial diversity (Turner, James and Poole, 2013); the caulosphere (stems), and the phylloplane (foliage or leaves). The definition of a microbiome has been debated since the 17th century, while a recent paradigm shift has left a universal definition unclear (Berg *et al.*, 2020). Modern definitions have included theories of co-evolution and holobiont theory, revising a definition considering microorganisms and macro-organisms (hosts) as one unit (Berg *et al.*, 2020). Ultimately, described as a collection of microorganisms in a specific niche, their interactions, and theatre of activity. Moreover, each microbiome is further described by microbial species, diversity, and polymicrobial interactions within microbiota communities and between hosts. Tree microbiomes do not

significantly differ from a typical plant since these ecosystems are primarily conserved. Microbiomes of plant species can collectively be referred to as a holobiont defining the host and its associated microbial communities (Simon *et al.*, 2019); the collection of microorganisms and their genes, which provide an extended phenotypic and metabolic repertoire for the host Nevertheless, microorganism communities vary in the rhizosphere, stem, and foliage, in density and composition, with species richness gradually declining outside the rhizosphere towards the caulosphere. Host-adapted microbiome communities colonising the plant exterior (phyllosphere) are more specialised with reduced diversity (Hacquard *et al.*, 2015) in contrast to communities colonising the rhizosphere.

Generally, species diversity and richness are key in preserving an ecosystem which remains true for the rhizosphere. Interactions between distinct species and species-host associations contribute to the overall stability of the rhizosphere as well as broader ecosystem functions (Hacquard and Schadt, 2015), indicating significance to microbiota diversity in maintaining plant health. Additionally, Hacquard and Schadt propose concepts suggesting habitat and soil type have larger effects on microbiome structure rather than host genetics, signifying the importance of microbial species associations and interactions. Beneficial impacts of this are shown in earlier studies (Wolf *et al.*, 2002; Berg *et al.*, 2010) describing two species of the *Stenotrophomonas* genus showing a strong association with plant hosts producing osmoprotective substances compatible with cellular functions. Alternatively, studies by Denman., *et al.,* have related polymicrobial interactions as factors of disease (Denman *et al.*, 2018) in acute oak decline (AOD) symptomatic trees due to microbial composition shift of the host-microbiome (Broberg *et al.*, 2018).

1.3. Importance of Oak Trees in the UK

The UK is common to multiple species of oak trees with more ancient oaks than the rest of Europe combined (Hight *et al.*, 2019), however, only two species are native, Pedunculate oak (*Q. robur*) and Sessile oak (*Q. petraea*). *Q. robur*, commonly named English oak, is a well-known and beloved symbol of national heritage, adopted in the iconic logos of UK charities and organisations such as the National Trust and Woodland Trust. Both *Q. robur* and *Q. petraea* remain a frequent sight in rural areas and national parks, protected by the legislation (*Tree Preservation Orders Act 1947*) with tree preservation orders (TPOs) enforced by local authorities to protect trees, groups of trees, and woodlands of particular architectural, cultural, and historical significance. Included in these categories are ancient trees and woodlands which are classified by a period of existence; individual oaks or areas of oak woodland must have existed prior 17th century (>400 years) to attain ancient status. Currently, over 14,000 ancient oaks are recorded in UK woodland (Hight *et al.*, 2019).

Oaks are considered a keystone species in the UK, a concept introduced by Robert T. Paine in 1969, to which species have a disproportionate effect on the environment relative to their abundance. The decline of oaks has formerly resulted in the decline of biodiversity; recent studies listed 2300 species associated with *Q. petraea* and *Q. robur*, 320 of which are obligate to native oak species (Mitchell *et al.*, 2019). Pathogenic species are of concern to the health of ancient oaks, becoming major drivers of loss of biodiversity (Harvell *et al.*, 2002) and consequently causing global deterioration in the oak population. However, the aforementioned stresses (e.g., frequency of temperature variation) of global warming are projected to have less significant impacts on temperate tree species such as *Q. petraea* and *Q. robur* (Vasseur *et al.*, 2014), highlighting the importance of high diversity in oak microbiomes, in particular the rhizosphere.

1.4. Tree Diseases and Decline Syndromes

Three principal factors triggering tree diseases (abiotic, biotic, and decline) linked with a plethora of microorganisms associated with pathogenesis and transmission (Manion, P.D., 1981). Bacteria and fungi are agents of tree disease commonly found in forest soil communities, identified as causing canker diseases, root rots, and wood decay (Manion, P.D., 1981), conversely forming host-microbe symbiotic relationships, with specific associations with host regions (such as the rhizosphere and phyllosphere) and host species. As previously mentioned, stress-induced mortality such as drought is increasing worldwide, enabling opportunistic microorganisms to colonise host microbiomes, potentially becoming pathogenic by shifting microbiome composition.

1.5. Acute Oak Decline

AOD is recognised as a recently described decline syndrome affecting native oak species across the UK (Brady *et al.*, 2017). Contrary to previously described tree diseases and declines, AOD does not conform to Koch's postulates (Falkow, 1988), instead being caused by a polymicrobial complex described as a pathobiome. Three bacteria species (*B. goodwinii, G. quercinecans,* and *R. victoriana*) isolated from AOD symptomatic trees are identified as causal agents of acute symptoms and mortality (Sapp *et al.*, 2016; Brady *et al.*, 2017; Broberg *et al.*, 2018; Moradi-Amirabad *et al.*, 2019) and can be fatal for trees within three to five years of becoming symptomatic. The severity of AOD is linked to predisposition due to abiotic stresses, with the most prevalent abiotic causal agent identified as drought (Eamus *et al.*, 2013). Infected oaks are identifiable by symptoms of outer bark weeping, inner bark necrosis and lesion formation, and the presence of larval galleries associated with the bark-boring beetle species *Agrilus biguttatus* (Denman *et al.*, 2014; Sapp *et al.*, 2016), with *A. biguttatus* considered as a causal agent of AOD.

Primary studies identified *B. goodwinii* dominating the lesion microbiome, today, further recognised as the key biotic causal agent of lesions and inner bark necrosis (Denman *et al.*, 2018) within the caulosphere; *G. quercinecans* was also detected in the lesion microbiome producing necrotising enzymes and classified as an opportunistic pathogen of AOD. Comparably, *B. goodwinii* loses viability outside host tissue (i.e., rainwater, forest soil), yet *G. quercinecans* will survive outside of the oak host suggesting a wider ecosystem distribution (Pettifor *et al.*, 2020) and introduction into host tissue a significant role in the oak microbiome to pathobiome transition. The role of *R. victoriana* in the pathobiome remains unclear (Broberg *et al.*, 2018; Doonan *et al.*, 2019).

Additionally, metagenomic analysis of symptomatic host tissue revealed that 67-95% of predicted genes in AOD lesion microbiomes were bacterial compared to 0.6-6% of genes in non-symptomatic host tissue being derived from bacteria, demonstrating a clear distinction in microbiome composition compared to non-symptomatic hosts (Broberg *et al.*, 2018). Further distinction is shown with a total 627 individual genes identified in all symptomatic tissues, not present in non-symptomatic tissues; 99% of genes bacterial derived including proteins involved in virulence (Broberg *et al.*, 2018). *R. victoriana* and *G. quercinecans* were the second and fourth most abundant genomes in the lesion microbiome with a mean genome percentage of 2.1% and 0.3%, respectively; in addition, metagenome analysis revealed key functions of the lesion microbiome (e.g., carbohydrate metabolism, membrane transport, etc.) are associated with genes encoded in the genomes of *B. goodwinii*, *G. quercinecans* and *R. victoriana* (Denman *et al.*, 2018).

1.6. Suppression of Disease in Medical Application

It is typical to associate diverse microbiota with healthy microbiota in plants as well as humans. Aforementioned concepts of high species diversity and richness of microbiota in the rhizosphere are known to have disease-suppressive properties applicable in medical

application of faecal transplants in the human gut post-infection or post-operation. The complexity of the human gastrointestinal tract enables more than 100 trillion bacterial and archaeal cells to colonise and survive in a mutual relationship with each host (Mondot *et al.*, 2013) achieving symbiosis. Gut microbiota are believed to have co-evolved in humans similar to the above-mentioned co-evolution theory in plants. Previous estimates suggested gut microbiota outnumber human cells by 1:10 (Mondot *et al.*, 2013), however recent revisions have concluded this estimate to be 1:1 (Sender, Fuchs and Milo, 2016). Gut microbiota provide and sustain vital functions humans cannot, for instance, protection against enteropathogens (Lozupone *et al.*, 2012), whilst maintaining physiological homeostasis. Disturbances leading to shifts in microbial composition and diversity, comparable to pathobiome transition, decrease the resilience capacity of the gut microbiome (Mondot *et al.*, 2013) and microbiome composition correlates with several diseases including autism spectrum disorder (ASD), cancer, Crohn's disease, diabetes, inflammatory bowel disease (IBD), and obesity (Lozupone *et al.*, 2012; Mondot *et al.*, 2013; Saraswati and Sitaraman, 2014; Lee, Yacyshyn and Yacyshyn, 2019; Chen *et al.*, 2020). The similarities in effects of disturbances in human gut microbiota compared to oak rhizosphere and phyllosphere, therefore, suggest concepts of microbiome transplantation as equivalent treatment for symptoms of AOD and human gastrointestinal diseases.

1.7. Suppression of Disease in Plants

Plant health is predominantly dependent on the role of plant genes and associations with microbiota (Smith, Handelsman and Goodman, 1999), however, the mechanisms and antagonistic microorganisms involved in disease suppression are less well understood (Noble and Coventry, 2005). The importance of identifying such genes carries potential. Shifting agricultural dependence away from pesticide use and understanding plant health in natural microbiome communities (Smith, Handelsman and Goodman, 1999) has obvious environmental benefits, in turn securing food security through more sustainable farming

practices. Additionally, decline syndromes and plant diseases could decrease in severity with newly developed treatments utilising the disease suppressive properties of the microbiome.

Disease suppressive soils are the most important examples of microbiome-mediated defence and pathogenic protection (Expósito *et al.*, 2017) where pathogens of bacteria and fungi persist in the rhizosphere but do not establish in causing disease. Most pathogenic microorganisms actively penetrate the plant apoplast (Hückelhoven, 2008) in an attempt to gain access to intracellular nutrients, however higher populations of bacteria with disease suppressive phenotypes (e.g. Actinomycetales) limit populations of pathogenic bacteria such as *P. cinnamomi* (Broadbent and Baker, 1974) in disease suppressive soils. These soils are focal points for greater exchange of nutrients and metabolites such as calcium, magnesium, and nitrogen inside the rhizosphere, promoting plant health by tolerance against abiotic stresses. Hückelhoven further demonstrates the role of cell wall defence reactions, partitioning microbial pathogens from plant metabolites and intracellular nutrients (Hückelhoven, 2008) while highlighting the importance of plant-microbe recognition. Plants lack an immune system as understood in animal-based life; therefore, recognition of pathogenic microorganisms is vital in maintaining plant health.

Cao *et al.,* describe pathogenesis as universal across phylogenies with pathogenic mechanisms and host defences used in parallel in plants, invertebrates, and mammals (Cao, Baldini and Rahme, 2001), further implying a common evolutionary origin. This suggests all plant phylogenies, at a gene level, share mechanisms for pathogenic suppression and in addition highlights the importance of the role of plant genes and associations with microflora. Aforementioned, soils also yield disease suppressive effects at small and large scales. Effective treatment for soil-borne diseases such as Fusarium patch, red thread, damping-off, etc., depend on the application of top-dressing with compost (Noble and Coventry, 2005), with inclusion rates of ~20% to sustain disease-suppressive effects. Suppression of plant diseases is attributed to the activity of antagonistic organisms continuously recolonising the

rhizosphere (Hadar and Papadopoulou, 2012), therefore application of disease suppressive soils would require accurate prediction and control for measurement of disease suppression.

1.8. Aims and objectives of this thesis

This research aimed to investigate the prevalence of disease suppressive microorganisms in the microbiota of healthy oak trees across three major compartments, focusing on identifying Using samples collected from a prior study, a collection of approximately 1400 bacterial isolates obtained from the foliage, rhizosphere, and stem of healthy *Q. robur,* were screened in agar-based competition assays to determine their ability to suppress three bacteria associated with AOD lesions – *B. goodwinii* (from aforementioned study), *G. quercinecans* and *R. victoriana.* Suppressive isolates underwent DNA extraction, bacterial 16S rRNA gene PCR, DNA sequencing and BLASTn analysis in order to explore the taxonomic diversity of microbiota that can suppress bacteria associated with AOD.

2. MATERIALS AND METHODS

2.1. Characterising oak isolates with suppressive phenotypes against AOD-associated bacteria

2.1.1. Culturing of associated AOD bacterial strains

Bacterial strains of *G. quercinecans* (FRB97^{T}) and *R. victoriana* (BRK18a) from glycerol stocks stored at -80°C were inoculated onto potato-dextrose agar (PDA) and nutrient agar (NA) following manufacturer's instructions, 39 g/L, and 28 g/L respectively, using quadrant streaking following aseptic technique. Strains were incubated at room temperature (T° = 21°C) for a 48 hour period, and growth was confirmed by visual identification of colonies. This was repeated for a total of 48 hours when formation of single circular beige colonies was recorded for both bacterial strains. It was noted after 24 hours that the formation of bacterial colonies occurred more quickly using PDA as a growth medium for both strains

post reanimation, therefore PDA at 39 g/L was exclusively used for subsequent generations (sub-cultures) of colonies and qualitative q-tray experiments in 2.1.3. Nutrient broth (NB) at 13 g/L concentration was used for sub-cultures using liquid medium and where measurements of optical density (OD_{600}) were required as an indicator of growth rate. All cultures were maintained at 4°C post incubation period and sub-cultured into fresh growth medium 48 hours prior to *in vitro* screening assays to ensure viability.

2.1.2. Culturing of oak microbial isolates

A total of 1824 microbial isolates collected from 15-year-old oak trees (*Q. robur*) were reanimated from glycerol stocks stored at -80°C in 96-well plates onto Q trays (20x20 cm square assay dishes) containing PDA (39 g/L). Scienceware replicator, 96-well, pin-replicators were used to transfer glycerol stocks from 96 well plates onto Q trays and had been treated using aseptic technique prior to use; disinfected with 2% bleach solution for 10 minutes, washed thoroughly with distilled water, and UV treated for a minimum of 20 minutes before inoculation of oak isolates. PDA (39 g/L) was used as growth medium where 1,824 oak isolates were inoculated directly from glycerol stocks using pin-replicators and incubated for 48 hours at T°C. Templates of 96-well plates were used to record the position each isolate coded by plate and well, i.e., AA A1, AU A1. Each individual plate was labelled 'A' and 'U' determined by origin from previous study in addition to chronological order (i.e., A, B, C, etc.); each individual well was labelled by row and column, i.e., A1, B2, C3. A single colony for each isolate was observed after the incubation period and maintained at 4°C preceding use for in-vitro qualitative assays in 2.1.3.

2.1.3. *In vitro* screening assays of suppressive oak isolates

50ml Falcon tubes containing 20mL NB (13 g/L) were treated under ultraviolet (UV) light for a minimum of 20 minutes. Five individual colonies of *G. quercinecans* and *R. victoriana* from

2.1.1 were inoculated into NB per each Falcon tube and incubated in a shaking incubator at optimal growth temperature, 26°C and 28°C respectively, at 150 rpm for 24 hours for optimal quantity of bacterial cells. OD_{600} of bacterial strains was measured after 24 hours using a Jenway 7305 single beam UV/visible spectrophotometer and recorded in Table 1.

Table 1 – OD_{600} of G. quercinecans and R. victoriana in NB after 24 hours of incubation at 26°C and 28°C respectively. Three separate measurements were taken per each incubation number of NB, G. quercinecans, and R. victoriana, and a mean taken as value of OD.

Incubation (repeat) number	**Optical Density (OD_{600})**		
	NB	*G. quercinecans*	*R. victoriana*
1	0.058	0.304	0.803
2	0.064	0.294	0.794
3	0.059	0.297	0.799

3 mL of *G. quercinecans* and *R. victoriana* culture was evenly spread onto the agar surface of q-trays containing PDA and held under aseptic conditions at T°C for 30 minutes until the culture had absorbed. Colonies of oak microbial isolates were then sub-cultured onto the surface of the inoculated plate using pin-replicators in 2.1.2 and incubated at T°C for 48 hours. Following 48 hours of growth, plates were visually inspected for clear circular zones in the growth of *R. victoriana* or *G. quercinecans* were identified around individual isolates following incubation and recorded as inhibition zones (Figure 1, 3). These isolates were recorded as having suppressive growth phenotypes against the respective bacterial strain then sub-cultured into 96-well plates containing 200 µL NB (13 g/L) and incubated for 48 hours, then maintained at -20°C.

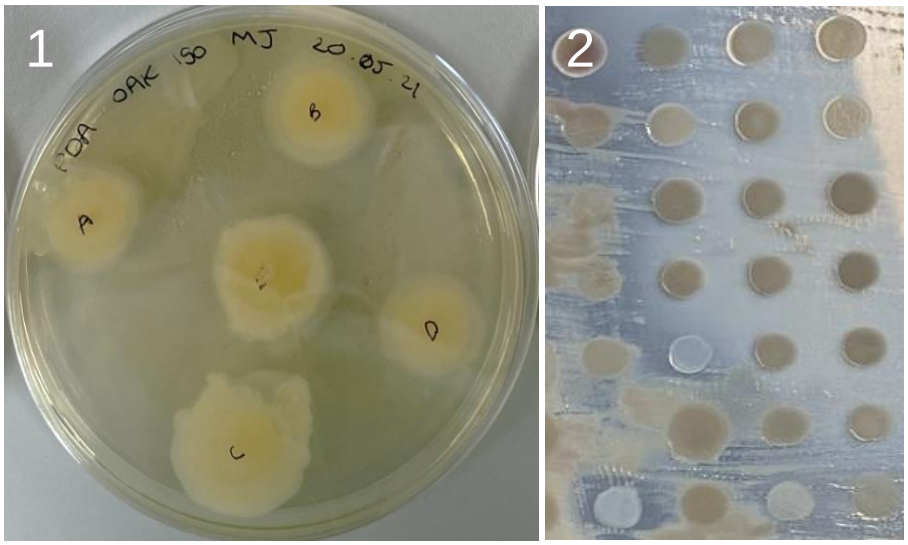

Figure 1 – In vitro assays of healthy oak isolates displaying suppressive growth phenotypes against G. quercinecans and R. victoriana.

1. *Control; growth of five oak isolates (A- E) with known growth suppressive phenotypes on PDA.*

2. *Q tray inoculated with healthy oak isolates from glycerol stocks and AOD associated bacteria. Colonies of isolates seen as small beige circles; presence of clear zones would indicate inhibition of AOD associated bacteria, beige patches show growth of pathogenic bacteria.*

Subsequent experiments in Figure 1 (1 and 2) were used to compare oak isolates previously identified with suppressive phenotypes. The Q tray protocol from 2.1.3. was later adapted to use Petri dishes containing 25mL PDA (39 g/L) and 200 µL of *G. quercinecans* and *R. victoriana* culture respectively, evenly distributed across each plate 30 minutes prior to addition of suppressive oak isolates. A total of 288 isolates, five per plate, were inoculated from NB (13 g/L) in 50 µL quantities and incubated for 48 hours at T°C. Figure 1 (1) was used as a control to confirm the viability of isolates using PDA (39 g/L) without the presence of AOD associated bacteria.

2.2. Taxonomy of oak isolates with suppressive phenotypes

2.2.1. Taxonomic identification of suppressive bacterial isolates using 16S rRNA gene PCR and sequencing

The bacterial 16S rRNA gene was amplified using the 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'- GGTTACCTTGTTACGACTT-3') primer set (Lane, 1991). Master mix contained MyTaq RED Mix (Bioline), primers 27F (10 µM) and 1492R (10 µM), and pure H_2O was prepared following manufacturers protocol (Bioline MyTaq Red Mix); a total 48 µL of master mix and 2 µL cell culture (see 2.1.3.) per oak isolate was added into an individual aliquot on each 96-well plate. PCR reaction conditions are summarised in Table 2 for a total of 35 denaturation cycles.

Table 2 – PCR reaction conditions for bacterial 16S rRNA using 27F and 1492R primers. Denature, Primer annealing, and Extension stages were repeated for 35 cycles, total run time of 2 hours 36 minutes.

PCR Stage	Temperature (°C)	Duration (minutes)
Initial denature	94	5:00
Denature	94	0:40
Primer annealing	52	0:45
Extension	72	1:30
Final extension	72	10:00
Hold	12	∞

2.2.2. Agarose gel electrophoresis of 16S rRNA gene products

5 µL of PCR amplicons from 2.2.1 were loaded into a 1% (w/v) agarose gel containing 1X TAE alongside a 5 µL DNA ladder and 2 µL of SafeView nucleic acid stain for visualisation, and ran at 100 V (150 mA) for 45 minutes. Positive PCR amplicons were identified using ultraviolet wavelength light, highlighted in Figure 2, and corresponding positive PCR reaction products were stored at -20°C until sequenced.

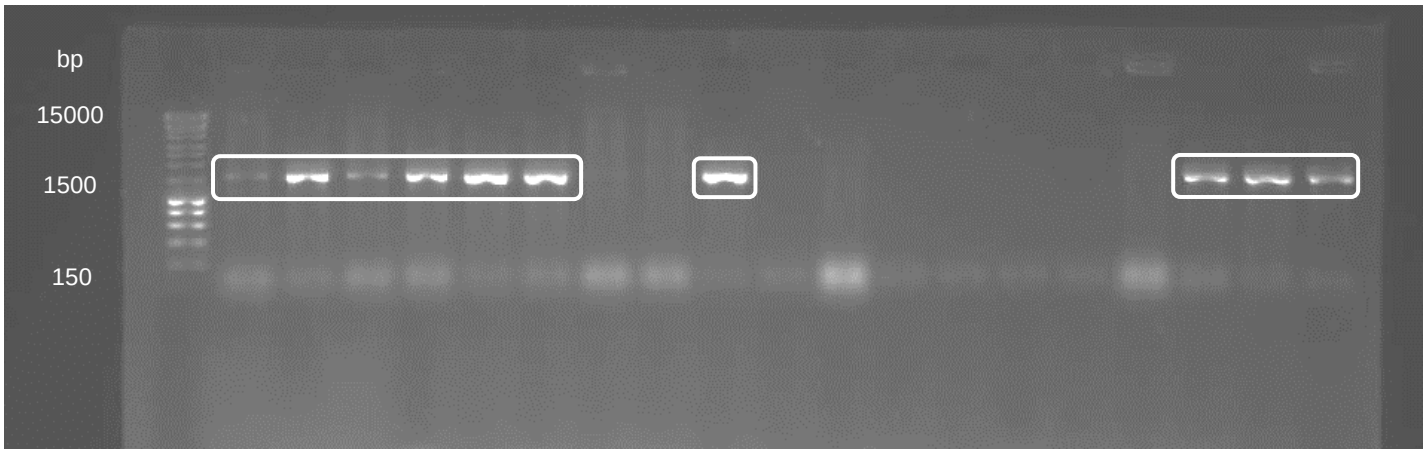

Figure 2 – 1% (w/v) agarose gel loaded with 1 Kb Plus DNA Ladder (left). Positive bacterial 16S rRNA PCR amplicon fragments (highlighted) located at reference band at 1500 bp.

Dilution series of oak isolate cultures were created with NB (13 g/L) in two concentrations subsequent to unsuccessful bacterial 16S rRNA gene amplification. Dilutions of 10^{-1} and 10^{-2} were used following PCR reaction conditions in Table 2. Negative PCR amplicons at 10^{-1} dilution were further diluted to 10^{-2} concentration until positive PCR amplicons were observed (Figure 2) and diluted reaction products stored at -20°C until sequenced.

2.2.3. 16S rRNA gene sequencing and taxonomic identification of oak isolates with suppressive phenotypes

Positive bacterial 16S rRNA gene PCR amplicons were prepared for 16S rRNA gene Sanger sequencing services provided by Genewiz following company protocol. Positive amplicons were sequenced in the forward direction only using 27F primer of which successful PCR

amplicons provided DNA segments of approximately 800 bp to 1500 bp in length. Geneious Prime software was utilised to read, trim, and quality control DNA segments prepared for genetic identification by nucleotide BLAST search via NCBI. FASTA files of successful DNA segments (positive PCR amplicons) were uploaded to rRNA/ITA databases for 16S ribosomal RNA sequences (Bacteria and Archaea) optimised for highly similar sequences (megablast).

3. RESULTS AND DISCUSSION

3.1. Identification of oak isolates with suppressive phenotypes against *B. goodwinii, G. quercinecans, R. victoriana*

3.1.1. In-vitro assays defining healthy oak isolates with suppressive properties

Screening of 1,824 healthy oak isolates identified a total of 576 individuals with growth suppressive phenotypes characterised by antagonistic behaviour on PDA plates. Antagonistic behaviour was defined by opportunistic growth of isolates in the presence of AOD pathogenic bacterial strains, in some assays demonstrating inhibition of pathogenic bacteria growth. Previous research discovered 113 of screen isolates were capable of suppressing growth of *B. goodwinii* (Bg) in-vitro*,* however, this study focuses on 454 isolates identified with suppressive growth phenotypes against *G. quercinecans* (Gq), and 65 isolates identified with suppressive growth phenotypes against *R. victoriana* (Rv). Numerous isolates were also identified with growth suppressive phenotypes against more than one of the mentioned three bacterial strains, identifying 29 isolates capable of suppressing Bg and Gq growth, 23 capable of suppressing Gq and Rv growth, three capable of suppressing Bg and Rv growth, and two capable of suppressing growth of all bacterial strains. The primary focus of this study was to identify potential strains, species, and/or genus of healthy oak isolates with AOD-suppressive properties, therefore, it remains undetermined the underlying mechanisms of which the growth of Bg, Gq, and Rv was inhibited and suppressed.

Additionally, all assays were conducted in-vitro and conditions for opportunistic bacteria were controlled to be idyllic, therefore it cannot be concluded that observed behaviours of disease-suppression would be observed in-vivo due (a)biotic factors such as heat disturbances (Hartmann *et al.*, 2015).

3.1.2. Classification of suppressive oak isolates at Genus level

288 of 576 individual isolates from 3.1.1. were randomly selected for Sanger sequencing of the bacterial 16S rRNA gene domain using 27F and 1492R primer set (Lane, 1991). A total 221 of 288 individual isolates sampled produced positive bacterial 16S rRNA gene PCR product which was genetically sequenced using Sanger 16S rRNA gene sequencing, resulting in a total of 157 individual successful reads. 11 distinct bacterial genera were identified (Table 3) being associated as healthy oak isolates with growth suppressive phenotypes against *B. goodwinii*, *G. quercinecans*, and *R. victoriana* (Figure 3). *Rahnella/Yersinia* genus contained the greatest number of associated oak isolates with n=60; *Acinetobacter, Kluyvera/Klebsiella,* and *Serratia* genera contained the fewest number of associated oak isolates with n=1; moreover, Table 4 highlights the *Yersiniaceae* family containing the greatest genera of associated suppressive oak isolates (n=61), inclusive of the genus *Serratia.*

Table 3 – List of distinct genera identified from Sanger sequencing of bacterial 16S rRNA gene from oak isolates with associated AOD-suppressive properties; n = total number of isolates within each genus.

Genus	Total of Individuals (n)
Acinetobacter	1
Buttiauxella	16
Citrobacter	2
Curtobacterium	4
Erwinia	8
Kluyvera/Klebsiella	1
Pantoea	18
Pseudomonas	39
Rahnella/Yersinia	60
Serratia	1
Stenotrophomonas	7

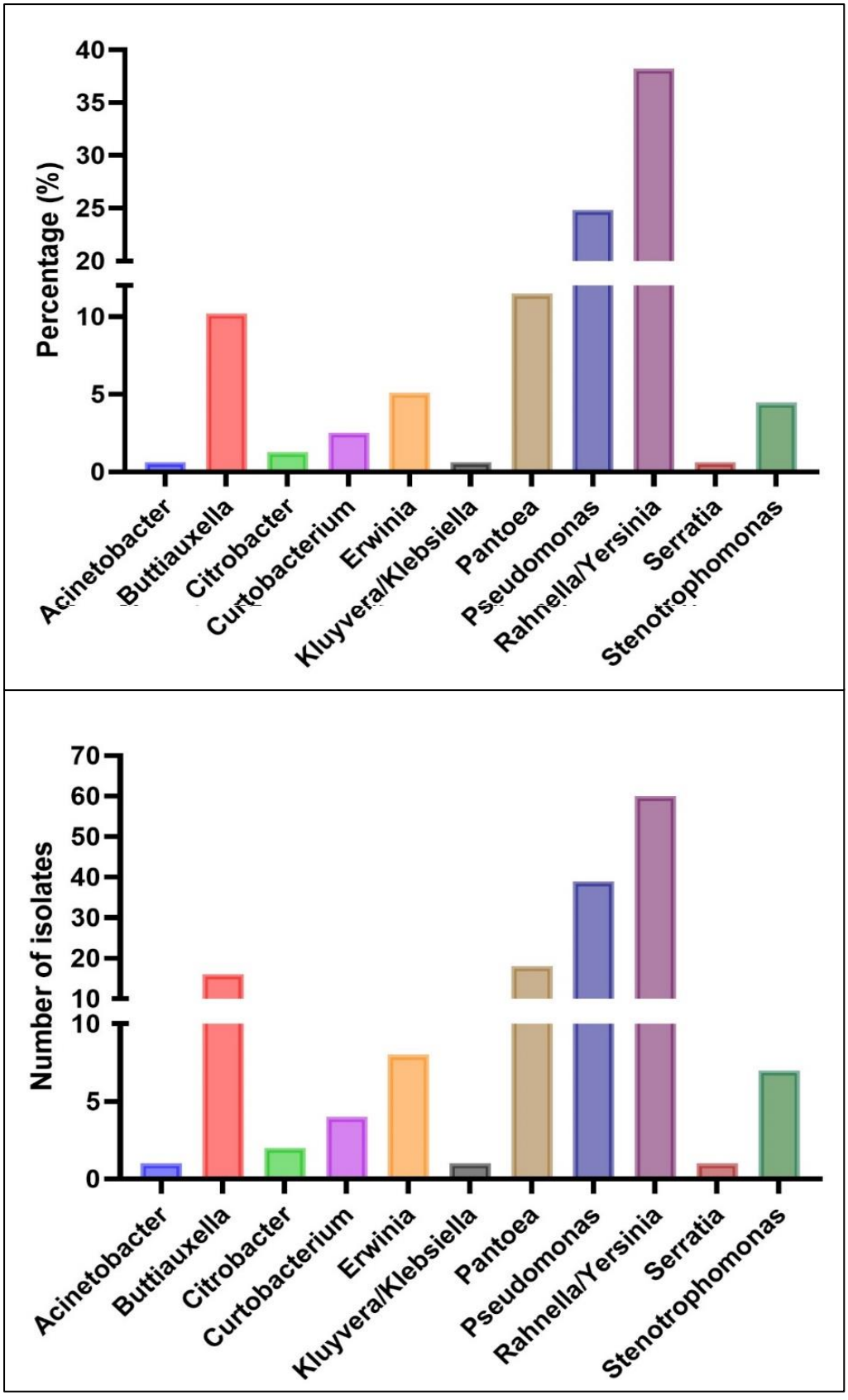

Figure 3 – 1) Distinct genera of associated AOD-suppressive isolates as a percentage of total number of isolates, i.e., Rahnella/Yersinia equates to 38.22% of total isolates; 2) Distinct genera of associated AOD-suppressive isolates as a proportion of total number, i.e., Rahnella/Yersinia, n=60.

Table 4 – Genetic identification of oak isolates identified with suppressive growth phenotypes against associated pathogenic AOD bacterial strains. Strains BRK18a and FRB97^T previously isolated from samples taken alongside 'healthy' oak isolates.

Family	Genus	Identity (%)	Accession	AOD pathogenic bacterial strain(s)
Moraxellaceae	*Acinetobacter*	98.01 - 99.01	NR_117626.1	*R. victoriana (BRK18a)*
Enterobacteriaceae	*Buttiauxella*	100.00	NR_041968.1	*B. goodwinii / G. quercinecans (FRB97^T)*
Enterobacteriaceae	*Buttiauxella*	96.29	NR_041968.1	*B. goodwinii*
Enterobacteriaceae	*Buttiauxella*	96.31 - 96.89	NR_041968.1	*B. goodwinii*
Enterobacteriaceae	*Buttiauxella*	97.77 - 97.88	NR_025330.1	*B. goodwinii*
Enterobacteriaceae	*Buttiauxella*	99.20 - 99.30	NR_025328.1	*B. goodwinii / G. quercinecans (FRB97^T)*
Enterobacteriaceae	*Buttiauxella*	98.29 - 99.24	NR_041968.1	*G. quercinecans (FRB97^T) / R. victoriana (BRK18a)*
Enterobacteriaceae	*Buttiauxella*	98.16 - 99.08	NR_041968.1	*B. goodwinii*
Enterobacteriaceae	*Buttiauxella*	99.04 - 99.52	NR_041968.1	*R. victoriana (BRK18a)*
Enterobacteriaceae	*Buttiauxella*	98.07 - 98.55	NR_041968.1	*R. victoriana (BRK18a)*
Enterobacteriaceae	*Buttiauxella*	99.09 - 99.68	NR_041968.1	*R. victoriana (BRK18a)*
Enterobacteriaceae	*Buttiauxella*	98.35	NR_041968.1	*R. victoriana (BRK18a)*
Enterobacteriaceae	*Buttiauxella*	97.05 - 97.79	NR_041968.1	*R. victoriana (BRK18a)*
Enterobacteriaceae	*Buttiauxella*	97.61 - 98.53	NR_041968.1	*G. quercinecans (FRB97^T) / R. victoriana (BRK18a)*

Enterobacteriaceae	*Buttiauxella*	97.63 - 98.36	NR_041968.1	*R. victoriana (BRK18a)*
Enterobacteriaceae	*Buttiauxella*	98.51 - 99.39	NR_025328.1	*R. victoriana (BRK18a)*
Enterobacteriaceae	*Buttiauxella*	98.49 - 99.38	NR_041968.1	*G. quercinecans (FRB97^T) / R. victoriana (BRK18a)*
Enterobacteriaceae	*Citrobacter*	98.75	NR_028894.1	*B. goodwinii*
Enterobacteriaceae	*Citrobacter*	98.61	NR_028894.1	*G. quercinecans (FRB97^T) / R. victoriana (BRK18a)*
Microbacteriaceae	*Curtobacterium*	99.71 - 100	NR_115034.1	*B. goodwinii*
Microbacteriaceae	*Curtobacterium*	98.97 - 99.25	NR_042315.1	*B. goodwinii*
Microbacteriaceae	*Curtobacterium*	99.52 - 99.71	NR_104839.1	*G. quercinecans (FRB97^T)*
Microbacteriaceae	*Curtobacterium*	99.05 - 99.37	NR_115034.1	*G. quercinecans (FRB97^T)*
Erwiniaceae	*Erwinia*	99.54 - 99.63	NR_104932.1	*G. quercinecans (FRB97^T)*
Erwiniaceae	*Erwinia*	100.00	NR_104932.1	*B. goodwinii / G. quercinecans (FRB97^T)*
Erwiniaceae	*Erwinia*	100.00	NR_104932.1	*B. goodwinii / G. quercinecans (FRB9(^{T})*
Erwiniaceae	*Erwinia*	99.80 - 99.90	NR_104932.1	*B. goodwinii / G. quercinecans (FRB97^T)*
Erwiniaceae	*Erwinia*	99.13 - 99.23	NR_118858.1	*B. goodwinii / R. victoriana (BRK18a)*
Erwiniaceae	*Erwinia*	98.90 - 99.00	NR_118858.1	*B. goodwinii*
Erwiniaceae	*Erwinia*	98.06 - 98.29	NR_114078.1	*B. goodwinii / G. quercinecans (FRB97^T)*

Erwiniaceae	*Erwinia*	98.14 - 99.11	NR_118858.1	*R. victoriana (BRK18a)*
Enterobacteriaceae	*Kluyvera/Klebsiella*	98.00 - 98.47	NR_028803.1	*B. goodwinii / G. quercinecans (FRB97^T)*
Erwiniaceae	*Pantoea*	98.91 - 99.10	NR_041978.1	*B. goodwinii*
Erwiniaceae	*Pantoea*	98.27	NR_041978.1	*B. goodwinii*
Erwiniaceae	*Pantoea*	97.78 - 97.97	NR_041978.1	*B. goodwinii*
Erwiniaceae	*Pantoea*	97.91 - 98.21	NR_041978.1	*B. goodwinii*
Erwiniaceae	*Pantoea*	98.84 - 99.13	NR_041978.1	*B. goodwinii / G. quercinecans (FRB97^T)*
Erwiniaceae	*Pantoea*	98.23 - 98.51	NR_041978.1	*B. goodwinii*
Erwiniaceae	*Pantoea*	98.81 - 99.11	NR_041978.1	*B. goodwinii*
Erwiniaceae	*Pantoea*	96.36 - 96.64	NR_041978.1	*G. quercinecans (FRB97^T)*
Erwiniaceae	*Pantoea*	99.26 - 99.58	NR_041978.1	*G. quercinecans (FRB97^T)*
Erwiniaceae	*Pantoea*	98.55 - 98.84	NR_041978.1	*G. quercinecans (FRB97^T)*
Erwiniaceae	*Pantoea*	97.99 - 98.27	NR_041978.1	*B. goodwinii*
Erwiniaceae	*Pantoea*	98.47 - 98.76	NR_041978.1	*G. quercinecans (FRB97^T)*
Erwiniaceae	*Pantoea*	98.29 - 98.67	NR_041978.1	*B. goodwinii*
Erwiniaceae	*Pantoea*	98.70 - 99.00	NR_041978.1	*B. goodwinii*

Erwiniaceae	*Pantoea*	97.35 - 97.74	NR_116797.1	*B. goodwinii / G. quercinecans (FRB97^T)*
Erwiniaceae	*Pantoea*	97.70 - 98.09	NR_116797.1	*B. goodwinii / G. quercinecans (FRB97^T)*
Erwiniaceae	*Pantoea*	98.66 - 99.17	NR_041978.1	*B. goodwinii*
Erwiniaceae	*Pantoea*	97.56 - 98.58	NR_116797.1	*G. quercinecans (FRB97^T) / R. victoriana (BRK18a)*
Pseudomonadaceae	*Pseudomonas*	99.91 - 100	NR_156986.1	*G. quercinecans (FRB97^T)*
Pseudomonadaceae	*Pseudomonas*	99.72	NR_025164.1	*G. quercinecans (FRB97^T)*
Pseudomonadaceae	*Pseudomonas*	99.81	NR_025164.1	*G. quercinecans (FRB97^T)*
Pseudomonadaceae	*Pseudomonas*	99.81	NR_025164.1	*G. quercinecans (FRB97^T)*
Pseudomonadaceae	*Pseudomonas*	99.81	NR_025164.1	*G. quercinecans (FRB97^T)*
Pseudomonadaceae	*Pseudomonas*	99.81	NR_025164.1	*G. quercinecans (FRB97^T)*
Pseudomonadaceae	*Pseudomonas*	99.63	NR_025164.1	*G. quercinecans (FRB97^T)*
Pseudomonadaceae	*Pseudomonas*	99.81	NR_025164.1	*B. goodwinii*
Pseudomonadaceae	*Pseudomonas*	98.57 - 98.77	NR_025103.1	*B. goodwinii*
Pseudomonadaceae	*Pseudomonas*	99.72 - 99.81	NR_025164.1	*G. quercinecans (FRB97^T)*
Pseudomonadaceae	*Pseudomonas*	99.77	NR_025174.1	*B. goodwinii*
Pseudomonadaceae	*Pseudomonas*	99.71 - 99.81	NR_025164.1	*G. quercinecans (FRB97^T)*

Pseudomonadaceae	*Pseudomonas*	99.53	NR_025164.1	*B. goodwinii*
Pseudomonadaceae	*Pseudomonas*	99.71 - 99.81	NR_025164.1	*B. goodwinii*
Pseudomonadaceae	*Pseudomonas*	99.49 - 99.59	NR_025164.1	*B. goodwinii*
Pseudomonadaceae	*Pseudomonas*	99.71 - 99.81	NR_025164.1	*B. goodwinii*
Pseudomonadaceae	*Pseudomonas*	99.71 - 99.81	NR_025164.1	*B. goodwinii*
Pseudomonadaceae	*Pseudomonas*	99.61 - 99.71	NR_025164.1	*B. goodwinii*
Pseudomonadaceae	*Pseudomonas*	99.71 - 99.81	NR_025164.1	*B. goodwinii*
Pseudomonadaceae	*Pseudomonas*	99.31	NR_025164.1	*G. quercinecans (FRB97^T)*
Pseudomonadaceae	*Pseudomonas*	99.70 - 99.80	NR_025164.1	*B. goodwinii / G. quercinecans (FRB97^T)*
Pseudomonadaceae	*Pseudomonas*	99.44	NR_114223.1	*B. goodwinii / R. victoriana (BRK18a)*
Pseudomonadaceae	*Pseudomonas*	99.91	NR_116899.1	*B. goodwinii*
Pseudomonadaceae	*Pseudomonas*	99.53	NR_116899.1	*B. goodwinii / G. quercinecans (FRB97^T)*
Pseudomonadaceae	*Pseudomonas*	99.81	NR_025164.1	*B. goodwinii*
Pseudomonadaceae	*Pseudomonas*	99.62	NR_025164.1	*G. quercinecans (FRB97^T)*
Pseudomonadaceae	*Pseudomonas*	99.60 - 99.80	NR_025164.1	*R. victoriana (BRK18a)*
Pseudomonadaceae	*Pseudomonas*	99.02 - 99.11	NR_025164.1	*R. victoriana (BRK18a)*

Pseudomonadaceae	*Pseudomonas*	99.80	NR_025164.1	*G. quercinecans (FRB97^T)*
Pseudomonadaceae	*Pseudomonas*	99.72	NR_025164.1	*G. quercinecans (FRB97^T)*
Pseudomonadaceae	*Pseudomonas*	99.81	NR_025164.1	*G. quercinecans (FRB97^T)*
Pseudomonadaceae	*Pseudomonas*	99.13 - 99.33	NR_025164.1	*G. quercinecans (FRB97^T)*
Pseudomonadaceae	*Pseudomonas*	99.62 - 99.81	NR_025164.1	*G. quercinecans (FRB97^T)*
Pseudomonadaceae	*Pseudomonas*	99.72 - 99.81	NR_025164.1	*G. quercinecans (FRB97^T)*
Pseudomonadaceae	*Pseudomonas*	99.31 - 99.48	NR_025164.1	*G. quercinecans (FRB97^T)*
Pseudomonadaceae	*Pseudomonas*	99.31 - 99.49	NR_025164.1	*G. quercinecans (FRB97^T)*
Pseudomonadaceae	*Pseudomonas*	99.22 - 99.40	NR_025164.1	*G. quercinecans (FRB97^T)*
Pseudomonadaceae	*Pseudomonas*	99.57 - 99.74	NR_025164.1	*G. quercinecans (FRB97^T)*
Pseudomonadaceae	*Pseudomonas*	99.39 - 99.57	NR_025164.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella*	98.15 - 98.29	NR_146849.1	*B. goodwinii / G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella*	99.68 - 99.79	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella*	99.44 - 99.52	NR_146849.1	*B. goodwinii*
Yersiniaceae	*Rahnella*	99.80 - 99.90	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella*	99.90 - 100.00	NR_146849.1	*B. goodwinii*

Yersiniaceae	*Rahnella*	98.26 - 99.80	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella*	97.60 - 97.70	NR_146849.1	*B. goodwinii*
Yersiniaceae	*Rahnella*	99.90 - 100	NR_146849.1	*B. goodwinii*
Yersiniaceae	*Rahnella*	99.39 - 99.49	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella/Yersinia*	98.19 - 99.71	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella/Yersinia*	98.66 - 99.80	NR_146849.1	*B. goodwinii*
Yersiniaceae	*Rahnella/Yersinia*	98.91 - 99.88	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella/Yersinia*	98.47 - 100	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella/Yersinia*	98.22 - 99.80	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella/Yersinia*	98.07 - 99.61	NR_146849.1	*B. goodwinii*
Yersiniaceae	*Rahnella*	100.00	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella*	100.00	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella*	100.00	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella*	99.82	NR_146849.1	*B. goodwinii / G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella*	99.91	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella*	100.00	NR_146849.1	*G. quercinecans (FRB97^T)*

Yersiniaceae	*Rahnella*	100.00	NR_146849.1	*B. goodwinii / G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella*	100.00	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella*	100.00	NR_146849.1	*B. goodwinii*
Yersiniaceae	*Rahnella*	100.00	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella*	100.00	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella*	100.00	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella*	99.71	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella*	100.00	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella*	99.19	NR_146849.1	*B. goodwinii / G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella*	100.00	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella*	100.00	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella*	100.00	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella*	100.00	NR_146849.1	*B. goodwinii / G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella*	100.00	NR_146849.1	*B. goodwinii / G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella*	100.00	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella*	100.00	NR_146849.1	*G. quercinecans (FRB97^T) / R. victoriana (BRK18a)*

Yersiniaceae	*Rahnella*	100.00	NR_146849.1	*B. goodwinii*
Yersiniaceae	*Rahnella*	100.00	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella/Yersinia*	98.32 - 99.81	NR_146849.1	*G. quercinecans (FRB97^T) / R. victoriana (BRK18a)*
Yersiniaceae	*Rahnella/Yersinia*	98.09 - 99.54	NR_146849.1	*G. quercinecans (FRB97^T) / R. victoriana (BRK18a)*
Yersiniaceae	*Rahnella/Yersinia*	98.03 - 99.33	NR_146849.1	*G. quercinecans (FRB97^T) / R. victoriana (BRK18a)*
Yersiniaceae	*Rahnella/Yersinia*	98.37 - 99.90	NR_146849.1	*B. goodwinii*
Yersiniaceae	*Rahnella/Yersinia*	98.37 - 99.90	NR_146849.1	*B. goodwinii / G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella/Yersinia*	98.51 - 99.81	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella/Yersinia*	97.30 - 98.87	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella/Yersinia*	98.17 - 99.63	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella/Yersinia*	98.16 - 99.69	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella/Yersinia*	98.14 - 99.62	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella/Yersinia*	98.28 - 99.81	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella/Yersinia*	98.29 - 99.82	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella/Yersinia*	98.85 - 99.76	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella/Yersinia*	97.89 - 99.46	NR_146849.1	*G. quercinecans (FRB97^T)*

Yersiniaceae	*Rahnella/Yersinia*	98.43 - 99.91	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella/Yersinia*	98.31 - 99.81	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella/Yersinia*	98.43 - 99.91	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella/Yersinia*	98.57 - 99.71	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella/Yersinia*	98.11 - 99.70	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella/Yersinia*	98.28 - 99.81	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Rahnella/Yersinia*	97.03 - 98.33	NR_146849.1	*G. quercinecans (FRB97^T)*
Yersiniaceae	*Serratia*	99.14 - 99.23	NR_025339.1	*B. goodwinii*
Xanthomonadaceae	*Stenotrophomonas*	98.37 - 98.88	NR_116366.1	*G. quercinecans (FRB97^T)*
Xanthomonadaceae	*Stenotrophomonas*	98.05 - 98.50	NR_112030.1	*R. victoriana (BRK18a)*
Xanthomonadaceae	*Stenotrophomonas*	98.66 - 98.76	NR_112030.1	*R. victoriana (BRK18a)*
Xanthomonadaceae	*Stenotrophomonas*	98.17 - 98.27	NR_112030.1	*R. victoriana (BRK18a)*
Xanthomonadaceae	*Stenotrophomonas*	97.05 - 97.56	NR_112030.1	*R. victoriana (BRK18a)*
Xanthomonadaceae	*Stenotrophomonas*	97.98 - 98.42	NR_112030.1	*G. quercinecans (FRB97^T) / R. victoriana (BRK18a)*
Xanthomonadaceae	*Stenotrophomonas*	98.05 - 98.59	NR_112030.1	*R. victoriana (BRK18a)*

3.2. Distribution and composition of suppressive oak isolates across major oak tree compartments

Oak isolates in Table 4 (see 3.1.2) were compared across three major compartments of *Q. robur*, foliage, rhizosphere, and stem, to map the microbiome composition of bacteria genera with AOD-suppressive phenotypes. Pathogenic AOD-associated bacteria was compared in each compartment showing the distribution of suppressive oak isolates throughout oak tree microbiomes. Oak isolates suppressive against *G. quercinecans* were generally abundant in the foliage and rhizosphere microbiomes, contributing to 72.27% and 72.70%, respectfully, of successfully sequenced isolates (see 3.1.2). Additionally, oak isolates suppressive against *R. victoriana* were generally abundant in the stem microbiome, contributing to 77.78% of successfully sequenced isolates.

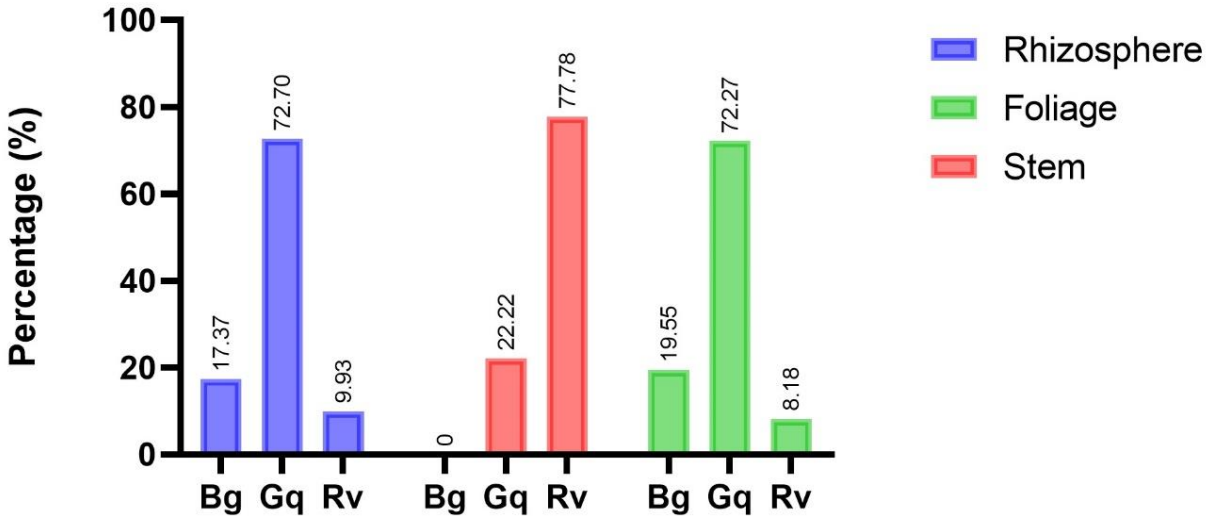

Figure 4 – Distribution of suppressive oak isolates against B. goodwinii, G. quercinecans, and R. victoriana, in major oak tree compartments – foliage, rhizosphere, and stem. Percentage of the sum of oak isolates suppressive against Bg, Gq, and Rv, in each microbiome.

3.2.1. Microbial composition of oak microbiomes

Microflora in the rhizosphere of disease suppressive soils is recorded to be fast-growing, heat-tolerant, and highly adaptive to available substrates (Palaniyandi *et al.*, 2013; van der Voort *et al.*, 2016; Expósito *et al.*, 2017) to be effective in disease suppression. As formerly stated, the bacterial composition of main oak tree compartments varies in AOD-suppressive bacteria, therefore comparisons between communities of pathogenic AOD-associated bacteria and individual growth-suppressive bacteria were investigated.

Figure 5 highlights the composition of a theoretical AOD-suppressive microbiome in relation to equivalent AOD-associated bacteria as a proportion of successful bacterial 16S rRNA gene sequences (Table 4) (see 3.1.2.).

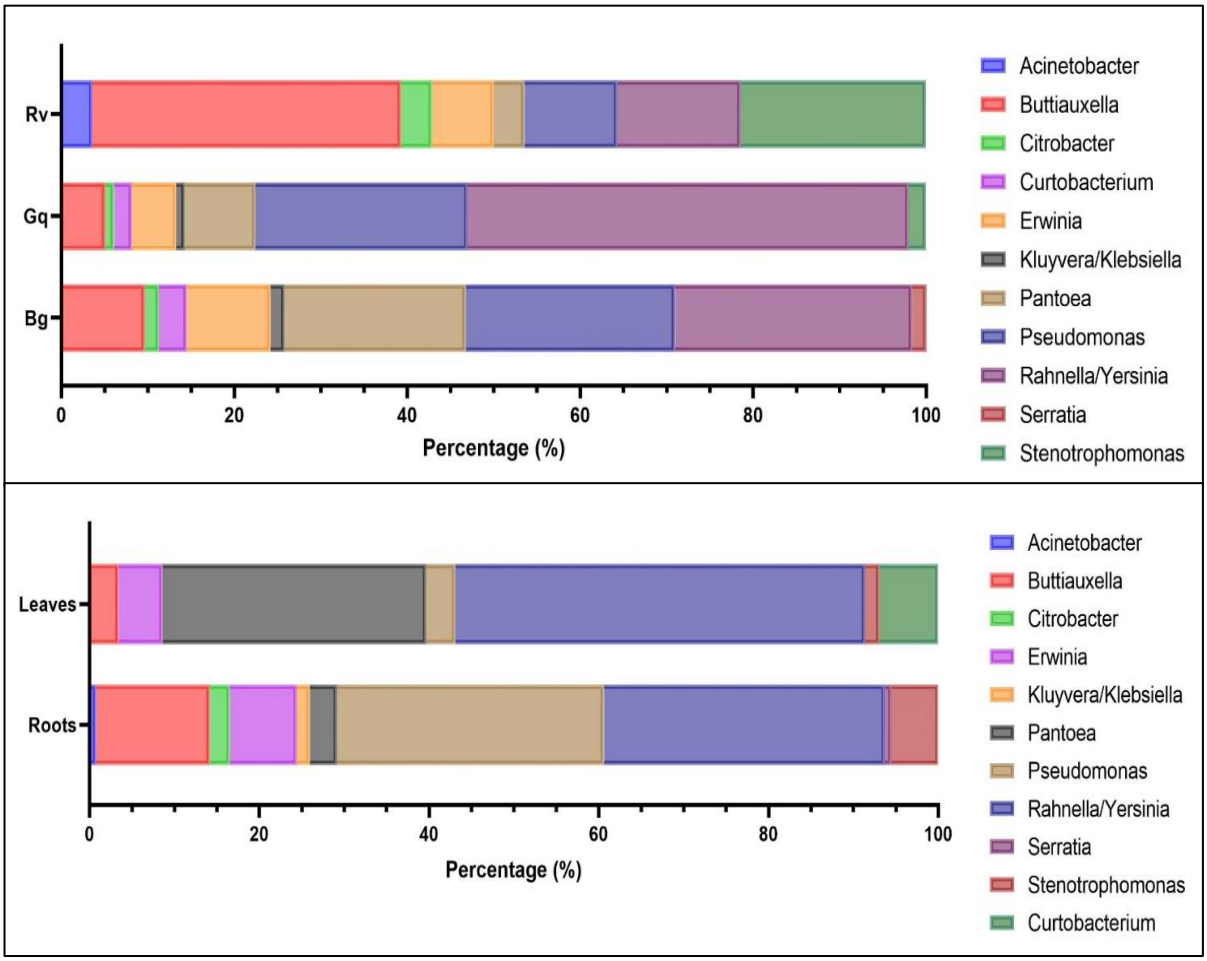

Figure 5 – Genus composition (as percentage) of AOD-suppressive bacteria against B. goodwinii, G. quercinecans, and R. victoriana.

The *Rahnella/Yersinia* genus is dominant in the theoretical *B. goodwinii* and *G. quercinecans* suppressive microbiomes, notably of which are equally diverse in genera (n=11). Initial *in vitro* experiments observing the optimal growth patterns of *R. victoriana* indicated the pathogenic strain BRK18a would outcompete pathogenic strains of *B. goodwinii* and *G. quercinecans* and multiply at greater growth rates (see Table 1); greater growth rate for non-pathogenic *Rahnella* strains could produce AOD-suppressive phenotypes. In comparison, *Buttiauxella* genus is dominant the theoretical *R. victoriana* suppressive microbiome, and less diverse in genera (n=10), however *Rahnella/Yersinia* genus was identified in all theoretical AOD-suppressive microbiomes and further study would be needed to determine the role of *Rahnella* and polymicrobial interactions within microbiota communities. Despite this, it can be proposed that creating AOD suppressive soil microbiomes, suppressing the growth of *B. goodwinii* and *G. quercinecans* can be attributed to bacterial species and strains within the *Rahnella* genus, excluding known pathogenic strains of *R. victoriana*. In this instance, these soils would be defined as having general suppressiveness which can be attributed to the collective microbial community (soil composition) and associated with competition for available resources (Expósito *et al*., 2017).

4. CONCLUSION

The microbiome of the *Q. robur* and *Q. petraea* contains multiple genera of bacteria with favourable phenotypes suppressing the growth of pathogenic AOD-associated bacteria (*B. goodwinii, G. quercinecans*, and *R. victoriana*). *In vitro* experiments identified 576 oak isolates successfully inhibited and suppressed further growth of pathogenic AOD-associated bacteria. 221 of 576 oak isolates provided genetic sequences which were identified using Sanger sequencing of the bacterial 16S rRNA gene. 27F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1492R (5’- GGTTACCTTGTTACGACTT-3’) primers provided almost entire read coverage of the 16S rRNA gene and segment lengths of ~1500 bp, however, actual segment

length ranged from 800 bp to 1500 bp and read exclusively in the 27F direction. These strains were categorised as AOD-suppressive microbiota of the oak tree microbiome.

Composition of three major oak tree compartments, foliage, rhizosphere, and stem microbiomes was mapped using previous bacterial strains from 16S rRNA gene segments. Analysis using NCBI databases confirmed 11 distinct genera which were associated as a theoretical AOD-suppressive microbiome for each compartment; percentage of the sum of suppressive strains against each pathogenic strain was further compared suggesting *G. quercinecans* is suppressed by almost 75% of sequenced strains in the foliage and rhizosphere microbiomes, and *R. victoriana* is suppressed by >75% of sequenced strains in the stem microbiome. Future study could test the effectiveness of AOD-suppression and practical application of artificial AOD-suppressive microbiomes to reduce symptoms of AOD and prevent further distribution across the UK.

5. BIBLIOGRAPHY

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