Uncovering pathogens in the soil microbiome using the *Galleria mellonella* virulence model

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I certify that all material in this dissertation which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: ……

Abstract

Environmental reservoirs of disease can harbour opportunistic pathogens that are harmful to human health. Anthropogenic effects such as climate change and antimicrobial resistance are likely to cause increase exposure to, and harm from, these pathogens. It is thus vital to study these environmental reservoirs in order to be prepared appropriately and prevent these diseases from emerging or re-emerging. Studies into the prevalence and diversity of opportunistic pathogens in the environment are largely focused on water sources. Microbial communities in soil are known to harbour a diverse range of species, yet are relatively poorly understood. Studies typically screen for specific 'indicator pathogens', or test the virulence of individual pathogens. These methods overlook a great deal of pathogenic species that can be present. Recent advances in the *Galleria mellonella* model system have shown that it can be used to screen for pathogens in microbial communities taken from environmental samples. We used the *G. mellonella* model to screen for potentially harmful pathogens in soil microbial communities. We frequently detected high levels of virulence, suggesting that soil often harbours pathogenic species. We were subsequently able to isolate pathogens from these soils and characterise their virulence, finding most of them to be highly virulent. 16s sequencing determined that the majority of pathogens found were either *Serratia liquefaciens* or *Providencia alcalifaciens,* both of which are known human pathogens. A highly virulent *Aeromonas hydrophila* was also identified. AMR profiling of these isolates found lower levels of antibiotic resistance than has typically been reported from clinical isolates. This study emphasises the importance of soil as a reservoir for pathogens, and provides a glimpse at the range of potentially harmful species that it contains.

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Author's declaration

This dissertation is based on a collaborative research project undertaken by myself and Jacopo Ferraresso, under the supervision of Dr Michiel Vos. Experimental design was a collaborative effort involving all parties. Jacopo and myself collaboratively collected the environmental samples, extracted soil communities, isolated clones from hemocoel, performed all virulence assays, extracted and analysed 16s rRNA, assayed AMR profiles and ran qPCR. Jacopo and I performed many of the data analyses together, with some subsequent additional independent analysis by myself. Jacopo and I also collaborated in creating most of the figures including figures 3, 6-11, 16, S1, S3 and S4, although I have subsequently redesigned some of these and created additional figures. The content of the introduction, chapter 2, and discussion sections was written solely by myself, with comments from all parties. Koleta Michalek also assisted with some preliminary sample collection and virulence assays. We aim to publish our findings in a collaborative paper including further analyses performed by colleagues at the University of Bath. The contents of this dissertation were not used by any of my collaborators for independent dissertations or theses, and to date have not been submitted in any other form to my knowledge. I would like to thank all my collaborators for their work and comments on this dissertation.

Acknowledgements

Firstly I would like to thank my supervisor Michiel Vos for his continued support and expert advice throughout my project. I could also not have done the project without the help of my lab partner Jacopo Ferraresso, who was vital in performing a great deal of the lab work and subsequent analysis. Koleta Michalek was also instrumental in getting the project up-and-running. Many thanks to all of the members of the Coastal Pathogens lab group for their advice and assistance, in particular Lihong Zhang who provided guidance in all things PCR-related, Anne Leonard for her help with AMR assays, Elze Hesse for her assistance with R script, and Luke Lear for regular general advice and support. A personal thanks to David Walker-Sünderhauf and Hugh White for their constructive comments on this dissertation, and all the staff at the Environment and Sustainability Institute for their persistent efforts to keep everything working smoothly on a daily basis. I would also like to thank my pastoral tutor Matthew White for his advice on effective time management.

List of abbreviations

ABR	Antibiotic resistance
AMR	Anti-microbial resistance
Bt	*Bacillus thuringiensis*
CFU	Colony-forming units
EID	Emerging infectious disease
ESBLs	Extended-spectrum beta lactamases
FIB	Faecal indicator bacteria
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
PRR	Pattern recognition receptors
UTI	Urinary-tract infection
WHO	World Health Organisation

Introduction

Infectious diseases have been a constant threat to human health throughout history (Balloux and van Dorp, 2017). These diseases are caused by the growth and replication of microorganisms within the body of a host organism, and are usually either bacterial, fungal or viral in nature (Jones *et al.*, 2008). These organisms proliferating inside a host can lead to a huge variety of problems as they compete with host cells, produce toxic compounds, physically and chemically interfere with bodily functions, or directly destroy host cells (Wilson, 2002; van Baarlen *et al.*, 2007). In this introduction, I provide a brief introduction into what pathogens are, the differences between obligate and opportunistic pathogens, and give details on some notable infectious diseases, both historical and currently emerging**.** I discuss the effects that anthropogenic changes are having on infectious diseases, and highlight why it is increasingly important to study environmental reservoirs of disease. Finally I talk about the traditional methods for studying these environments, and how new model systems and techniques can improve upon them.

What is a pathogen?

Pathogens, informally known as germs, are defined as any microorganism that has the potential to cause disease (Smilack, 1976). Usually, these microorganisms are either simple cellular life such as bacteria or fungi, or mobile genetic elements such as viruses (Jones *et al.*, 2008). If these pathogenic microorganisms are able to enter the internal systems of another organism, they have the potential to cause great harm to it. Organisms that are colonised by such pathogens are known as hosts, and the potential harm that the pathogen can cause is known as virulence (Bull, 1994). Virulence is affected by the range of 'virulence factors' that the pathogen can produce, which include many toxic chemicals, destructive enzymes, and compounds which help the pathogen evade or damage their host's immune system (Chen, 2004). To defend against pathogens, host organisms have immune systems, which include a wide range of physical, chemical, and molecular defences (Medzhitov and Janeway, 2000).

The problem of opportunistic pathogens

Some pathogenic organisms are 'obligate pathogens', which means that at some point in their life-cycle they need to infect a host organism to survive and proliferate (Balloux and van Dorp, 2017). Examples of this include viruses, all of which are obligate pathogens due to their need to use a host cell for replication, along with the pathogens responsible for diseases such as tuberculosis and leprosy (Jagielski *et al.*, 2016). However, many pathogenic species are in fact 'free-living' organisms, perfectly capable of surviving without the need for a host organism. These facultative or 'opportunistic' pathogens typically only cause virulence in hosts with weakened immune systems (Balloux and van Dorp, 2017). An example of this is the bacterium *Staphylococcus aureus,* which is commonly present on human skin. Normally this bacterium is a commensal, meaning it can survive on our skin with no ill effect to us. However, if the skin is breached *S. aureus* can enter the body, where it has the potential to cause serious skin and wound infections, bacteremia and toxic shock syndrome (see *Table 1*) (Tong *et al.*, 2015). As well exploiting as physical breaches to a host's immune system, opportunistic pathogens can be particularly dangerous to hosts with reduced internal immune function, a state known as immunodeficiency or immunosuppression (Smith, 1973). This state can be caused by a variety of factors including long-term conditions such as HIV infection (Fauci, 1988), genetic predisposition (Buckley *et al.*, 1997), or the onset of old age (Saltzman and Peterson, 1987). It can also be affected by factors such as malnutrition (Bourke, Berkley and Prendergast, 2016), or be deliberately induced for certain medical procedures such as organ transplantation (Borel, 1998). Because of the free-living nature of opportunistic pathogens, there also exist a great number of environmental populations of them, which can act as reservoirs of disease (Gerba, 2015). This complicates efforts to manage the disease, as infections can arise without host-to-host transmission. Therefore it is necessary to maintain a good understanding of what pathogens are present in the environment, and I will discuss this issue in more detail later in this introduction.

Notable examples of bacterial diseases

Pathogens by nature encompass an extremely diverse range of organisms, however the vast majority of infectious human diseases are caused by species of bacteria (Jones *et al.*, 2008), and it is these which we will focus upon. Here I

briefly describe some examples of notable bacterial diseases that pose a significant threat to human health.

One of the most infamous pandemics in human history is the bubonic plague, also known as 'The Black Death' or simply 'The Plague'. This disease ravaged Europe in the 1300's, causing an enormous loss of life, estimated to be 20-60% depending on the country (Cook, 2004). The bacterium responsible, *Yersinia pestis*, is believed to have been carried by rats, and causes large pustules and buboes to form on infected humans, which can spread the disease further (Perry and Fetherston, 1997). It is confirmed to be responsible for other devastating outbreaks across history, including the Plague of Justinian which contributed to the fall of the Roman Empire (Wagner *et al.*, 2014). Fortunately, it is rare for a pathogen to exhibit the levels of virulence and infectivity that The Plague reached, and thanks to the development of modern medicine, bacterial infections can now usually be treated quickly with antibiotics (Perry and Fetherston, 1997). Plague infections in humans are now rare, although not unheard of, as the bacterium still persists in some wild mammal populations (Perry and Fetherston, 1997)

An example of a less-virulent, but still dangerous bacterial disease is Salmonellosis, often known as food poisoning or gastroenteritis. Salmonellosis is caused by bacteria of the *Salmonella* genus, which can persist for weeks in the intestines of animals including humans (Crum-Cianflone, 2008). Disease most commonly arises when fecally-contaminated food is consumed without being correctly prepared; undercooked meat and eggs in particular are common examples (Crum-Cianflone, 2008). *Salmonella* infection can be asymptomatic, or it can cause severe abdominal cramps, diarrhoea and dehydration, and if complications arise, even mortality (Acheson and Hohmann, 2001). However, *Salmonella* infections can be easily avoided with proper hygiene and sterilisation of foodstuffs.

Cholera, caused by the bacterium *Vibrio cholerae*, provides a strong example of the threat that can be posed by environmentally-sourced bacteria. Cholera is most commonly contracted by drinking water contaminated with human faeces, and can cause severe vomiting and diarrhoea, leading to extreme dehydration and even death (WHO, 2019). The bacterium can be removed from water sources by adequately sterilising them before consumption, and as such the disease is very rare in developed countries but is still a serious threat in developing regions

(Griffith, Kelly-Hope and Miller, 2006; Ali *et al.*, 2012). Infections are suspected to be underreported due to the negative connotations an outbreak will have, but studies attempting to correct for this estimate 2.9 million cases and 95,000 deaths per year occur in endemic countries (Ali *et al.*, 2015). Historically cholera is very important to the study of spatial epidemiology, as John Snow's work during the 1854 outbreak in London was instrumental in stopping the spread of the disease (Shiode *et al.*, 2015).

Emerging infectious diseases

While the examples of bacterial diseases mentioned above pose significant health risks to humans, they are reasonably well-understood and can generally be easily avoided or treated. However, there are many diseases that are currently more difficult to deal with, and reported incidences of some of these are on the rise. These 'emerging infectious diseases' (EIDs) are defined as "*those whose incidence in humans has increased within the past two decades or threatens to increase in the near future*" (van Doorn, 2014). This may be due to either novel or previously undetected infections. There are also cases of known infections currently increasing in frequency, defined as re-emerging diseases (National Institutes of Health, 2007). In recent years a worrying number of diseases are re-emerging due to the phenomenon known as antimicrobial resistance, which will be discussed later in this chapter. Urbanised areas are particularly likely to be hotspots for EIDs due to the density and frequency of contact of populations within them (Neiderud, 2015). This year to date (at the time of writing, 2019) the World Health Organisation has reported many significant disease outbreaks including Cholera, Listeriosis, Gonorrhoea, Carbapenem-resistant *Pseudomonas aeruginosa*, and numerous viral infections (WHO, 2019).

The influence of anthropogenic effects on EIDs

Anthropogenic changes often have a large part to play in disease emergence and re-emergence. Many outbreaks of disease have been caused by the human introduction of infectious agents to susceptible populations, a prominent historical example being the movement of boats bearing Plague carriers (Tognotti, 2013). It is thus important to remain aware of potential ways that humans could affect disease dynamics in the future, and I will describe some of these here.

One of the most pressing and wide-scale examples of this is climate change (Khasnis and Nettleman, 2005; Cavicchioli *et al.*, 2019). Global temperature shifts are predicted to have a huge effect on both hosts and pathogens for a variety of reasons (Wu *et al.*, 2016). For example, many microorganisms (particularly bacteria) will be capable of utilising warmer temperatures to directly speed up their generation time, although this will not necessarily favour pathogenic species, and temperatures could even become hot enough to inhibit growth (Wu *et al.*, 2016). Host species will also be affected significantly, as changing temperatures significantly increase to the strain on their immune systems, making it easier for infectious agents to gain a foothold in the body (Raffel *et al.*, 2006). Vector populations will also be affected; there is evidence of increasing populations of mosquitoes in recent years (Watts *et al.*, 2018). There will be many environmental effects of climate change, for example the destruction of many ecosystems threatens to shift the species ranges of both hosts and pathogens. This will increase the likelihood that susceptible host species become exposed to novel diseases, while also granting novel pathogens new opportunities to spread (Sorci, Cornet and Faivre, 2013). Such incidents are already beginning to occur, such as a recent outbreak of Anthrax in Russia, thought to have been started by an infected carcass becoming exposed from permafrost due to a heatwave (www.bbc.co.uk/news/world-europe-36951542).

Another prominent factor is the ever increasing human population. By itself this creates more potential hosts living in close proximity to one-another, especially in growing cities, where the density and proximity of hosts increase the chance of epidemic or pandemic EIDs developing (Neiderud, 2015). Population growth also leads to increasing globalization and urbanization, which creates further problems. For example modern transportation is expediting the spread of disease, as our increasingly interconnected world allows pathogens to move from one area to another very quickly. Air travel appears to be one of the main vectors of this type of spread (Colizza *et al.*, 2006). Not only does this increase the rate at which they can spread, but it also allows for pathogens to reach hosts that have little-to-no immunity to them, potentially causing widespread epidemics. Malaria has already been noted to have been ‘imported’ to various countries on a number of occasion due to the travel of infected individuals, as has Yellow Fever, Dengue and West Nile Virus (Tatem, Rogers and Hay, 2006).

The ability of modern medicine to keep diseases in check has also been undermined in recent years. This is in part due to 'vaccine hesitancy', the distrust of established medical procedures partly thanks to misinformation spread by discredited scientists (Wakefield *et al.*, 1998). This has unfortunately led to many easily avoidable infections occurring, and has recently been implicated in the current return of measles in the UK, causing the loss of its W.H.O. status as an eliminated disease in the country (www.gov.uk/government/publications/measles-confirmed-cases).

The rise of antimicrobial resistance

Another major anthropogenic factor allowing for the re-emergence of diseases is the spread of antimicrobial resistance (AMR); the evolution of so-called 'superbugs'. This phenomenon is the result of microorganisms evolving to become less susceptible to compounds that are used to treat infections (WHO, 2020). This is a major problem in modern-day healthcare, and it is predicted that by 2050 AMR infections will be the most common cause of death world-wide (*Figure 1,* O'Neill, 2016). The most well-known example of AMR is bacterial pathogens becoming antibiotic-resistant, becoming much harder to treat through clinical means. Most antimicrobial compounds are produced naturally by environmental microorganisms, and those that produce them or are regularly exposed to them usually have some innate resistance to them (Martinez, 2008). Others are naturally susceptible, but can acquire genes coding for resistance through a number of means. The most common method for this is horizontal gene transfer, which is the process of acquiring genetic information from the surrounding environment or unrelated bacterial species (Thomas and Nielsen, 2005). Bacteria regularly do this through a process known as conjugation, whereby the bacteria link to one another using a 'pilus' and exchange genetic information in the form of plasmids, a type of mobile genetic element (Llosa *et al.*, 2002). Alternatively, genetic information may be transferred by transduction; where mobile genetic elements such as bacteriophages incorporate genes from their host and transfer them between cells (Ochman, Lawrence and Groisman, 2000). Some bacteria are also capable of acquiring genes from the environment around them through a process known as bacterial transformation, which can allow them to acquire and use genes from dead cells, even of another species (Riffith, 1928). Alternatively, it is also possible for random genetic mutations to

cause new mechanisms of antimicrobial resistance, although the exact rates at which this can happen are subject to a complex array of factors (Martinez and Baquero, 2000).

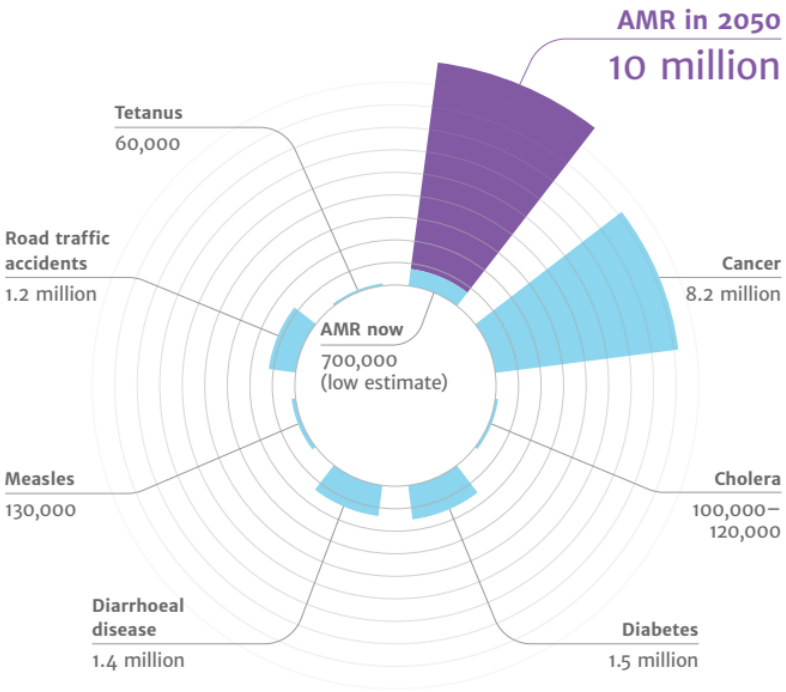


Figure 1: Predicted deaths due to AMR. The Review on Antimicrobial Resistance predicts that deaths due to AMR will exceed all other causes of mortality by 2050 (O'Neill, 2016).

Once a resistance gene is present in a pathogen it is subject to the rules of natural selection. The chance for a resistant phenotype to emerge is limited by any fitness costs that the change incurs, and as such resistant strains will often be outcompeted by the wild-type (unmodified) strains (Martinez and Baquero, 2000). The costs incurred partly depend on the location of the mutation in the genome, and can often be significantly lower if the gene is located on a plasmid (Vogwill and MacLean, 2015) - a type of mobile-genetic-element - or if secondary mutations increase fitness without affecting the resistance gene (Melnyk, Wong and Kassen, 2015). However, the selection pressures change when in the presence of antimicrobials, where resistant strains will have a fitness advantage and encourage the proliferation of the resistance gene (Skalet *et al.*, 2010).

In recent years we have become increasingly reliant on antibiotics in both healthcare situations and in farming practices, particularly in the developed world.

While use of these amazing compounds has led to a ‘golden age’ where most diseases pose little threat, this could easily change. Because of the widespread prevalence of antimicrobials in so many environments, these is now huge selection pressure on pathogens to become resistant (WHO, 2020). This was most publicised during the rise of methicillin-resistant *Staphylococcus aureus* (MRSA) infections in hospitals from its initial discovery in 1968 to the rise of ‘epidemic MRSA’ in the 1980’s and early 1990’s (Duerden *et al.*, 2015), causing a huge public health risk. This kind of resistance is becoming increasingly common in pathogenic species, and is a trend that is likely to only get worse. Indeed, many dangerous diseases such as tuberculosis are now displaying simultaneous resistance to more than one antibiotic, leaving few options for treatment available (Millard, Ugarte-Gil and Moore, 2015). Strains of Vancomycin-resistant *Enterococci*, which are resistant to even antibiotics of last resort, are becoming increasingly common too (McGuinness, Malachowa and DeLeo, 2017).

Which environments are typically disease reservoirs?

It is important to maintain awareness of the spread of disease-causing organisms, such that we are able to adequately prepare countermeasures for them if infections arise. This is especially difficult for opportunistic pathogens because unlike obligate pathogens they can exist in natural environments in the absence of host species (Balloux and van Dorp, 2017). Therefore there exist persistent environmental sources aka ‘reservoirs’ of opportunistic pathogens with the potential to become infectious (Berg, Eberl and Hartmann, 2005; Gerba, 2015). This makes it important to perform surveillance of environments which could be potential disease reservoirs. Pathogens in general are capable of surviving in a wide range of reservoirs, which can include host populations of humans and animals (Haydon *et al.*, 2002), and in man-made environments such as hospitals (Dancer, 2011), and even on the surfaces of mobile phones (Brady *et al.*, 2009). These are all important areas for research into disease transmission. However, for the purposes of this study I will be focusing on external, environmental reservoirs.

Water sources are one of the most vital areas in which to maintain environmental surveillance, as many pathogenic species are able to survive and even thrive in water (Ramírez-Castillo *et al.*, 2015). Some level of exposure to waterborne

pathogens is unavoidable, particularly in water sources which are used for bathing or drinking. If correctly treated most pathogenic species can be removed from water before humans are exposed to it (LeChevallier, 2013). However this is not always done, and unhygienic or poorly sanitised water is thought to contribute to 3.2% of deaths globally (WHO, 2010). Water is known to transmit a wide variety of dangerous diseases, some of which include cholera, typhoid and dysentery (Cabral, 2010). Pathogenic species such as *Salmonella*, *Escherichia coli*, *Legionella pneumophila,* and *Pseudomonas aeruginosa* have also been found in drinking water (Falkinham, Pruden and Edwards, 2015). Studies also show that microplastics in water are providing novel substrates for pathogens to grow on (Keswani *et al.*, 2016), potentially compounding the issue.

It is also possible for the air to transmit pathogenic species. Airborne pathogens can spread quickly and cause widespread outbreaks, as seen with severe acute respiratory syndrome (SARS) in Asia (Gumel *et al.*, 2004), or seasonal influenza strains, which are estimated to cause 3-5 million severe illnesses and 290-650 thousand deaths annually (WHO, 2018). Studies have shown that the microbial species present in the air are affected by seasonal variation; for example in the U.S. *Pseudomonas, Burkholderia, Rhizobiales*, and *Sphingomonadales* are found in the summer, while *Bacteroidales, Clostridiales*, and *Fusobacteria* are more common in winter (Bowers *et al.*, 2011). Air pollution itself can be hazardous to health, and so there are many studies into its effects, however in general the majority of microorganisms found in polluted air are non-pathogenic (Cao *et al.*, 2014).

However, one natural environment in particular has received relatively little attention with regards to the pathogens it contains: soil. Soil environments can be a persistent source of diseases and need to be studied and understood (Berg, Eberl and Hartmann, 2005). Soil pathogens have the potential to infect the food that we eat as well as spreading directly into humans (Baumgardner, 2012). Natural microbial communities can contain billions of cells and thousands of species in every gram of soil matrix (Pursey *et al.*, 2018), and due to the nutrient-rich nature of soil it is probable that it is home to many pathogenic microbes that have as yet gone undiscovered. While some studies have been done into the pathogens that can be present in the roots of food crops, relatively little is known about the species and virulence of opportunistic pathogens in soils in general. As

the primary focus of our study, soil microorganisms will be discussed in more detail in chapter 2.

Traditional methods for detecting environmental pathogens

To detect pathogens in environmental reservoirs a number of screening methods have been developed. Here I will briefly describe a few of them, their merits and drawbacks.

The detection and quantification of specific bacteria which typically reside in human and animal gastrointestinal tracts is often used as a proxy for water quality (Anderson, Whitlock and Harwood, 2005; Cabral, 2010; de Brauwere, Ouattara and Servais, 2014). These 'faecal indicator bacteria' (FIB) such as *Escherichia coli* are usually filtered from the water sample by passing it through a filter membrane, then cultured on nutrient broth (Quilliam *et al.*, 2011). This method can also be used for sediment samples, but they must first be thoroughly mixed with a sterile buffer solution, then the supernatant can be processed in a similar way to water samples (Anderson, Whitlock and Harwood, 2005). Following a period of incubation, growing microbial colonies can be visibly detected. Colonies can then be investigated further to determine their identity, using variables such as colony size and colour, or microscopy (Váradi *et al.*, 2017). If looking for a particular species such as *E. coli* then a specific nutrient agar can be used to select for it, which may also contain chemical 'inhibitors', typically antibiotics, which exclude or at least slow the growth of any other species (Lagier *et al.*, 2015). FIB by themselves are unlikely to cause particularly serious diseases, but detecting and quantifying their numbers can infer the presence of other, more dangerous faecal pathogens (Schriewer *et al.*, 2015), along with providing an estimate of how sanitary the sample is. This detection method is quick and simple, requiring relatively little complex equipment.

Another prominent environmental screening technique is the use of molecular biology to determine which species are present in a sample by detecting specific genetic markers of interest (Quilliam *et al.*, 2011). This method is often used in combination with an initial stage of pathogen culture prior to DNA extraction, although some kits allow for direct extraction of nucleic acid from environmental samples (Tsai and Olson, 1991; Quilliam *et al.*, 2011). Polymerase chain reaction (PCR) is then used to amplify regions of nucleic acid using primers for a specific

gene, usually one that is highly conserved (Fincher, Parker and Chauret, 2009). Once amplified, nucleic acid can be detected through a number of means. Qualitative PCR simply determines the presence or absence of specific genes via gel electrophoresis, in which nucleic acids present can be visually differentiated (Lee *et al.*, 2012). There is also quantitative PCR (qPCR) aka real-time PCR, which can determine the relative frequency of a specific gene within a sample by detecting a fluorescent marker and comparing the fluorescence to a control (Ponchel *et al.*, 2003). PCR-based methods can detect a wider range of pathogens with a higher degree of accuracy than FIB methods, including those that would be unculturable in the lab (Cai, Caswell and Prescott, 2014), but is significantly more time-consuming and resource-intensive.

Metagenomics approaches can also be used to detect a relatively wide range of environmental pathogens. This involves the extraction of all DNA from an environmental sample (the metagenome), and transplanting fragments of this DNA into clonal vectors such as plasmids, which can then be used as a 'DNA library' (Daniel, 2005). These DNA libraries can then be screened for specific nucleotide sequences or metabolic activity. One way of doing this is via detecting specific 'genetic fingerprints' (Maron *et al.*, 2005). During sequence analysis, microsatellites – specific conserved sequences of DNA that repeat themselves – can be used as a marker to detect the presence of a particular pathogen which is known to contain that genetic sequence (Ellegren, 2004). Microsatellite arrays can be used to screen for the presence of many pathogens at once (Wang *et al.*, 2002; McLoughlin, 2011), and can be used to detect specific resistance or virulence genes. They can even detect DNA markers without the need for PCR (Call, Borucki and Loge, 2003).

Limitations of traditional detection techniques

While the practises of detecting 'indicator pathogens' and specific gene sequences are certainly useful in providing some indication of how pristine a sample is, there are also inherent drawbacks to these approaches. For example, the process of DNA extraction itself can damage and degrade sequences, showing false negatives (Van Burik *et al.*, 1998). It can also release chemicals from the samples that interfere with the PCR process (Lu *et al.*, 2015). In the case of fungal pathogens, PCR based methods cannot distinguish between active and dormant species, though there are alternative methods such as fluorescence in

situ hybridisation (FISH) that can (Tsui *et al.*, 2011). For metagenomics-based approaches, considerable time and money needs to be invested in creating and maintaining DNA libraries, and researching and developing suitable detection probes, although these costs are steadily decreasing (Kunin *et al.*, 2008; Teeling and Glockner, 2012). As well as the possibility of false negatives, genetic methods can also provide false positives. Some of the species and genes detected in this way can in fact be apathogenic, since it is not possible to directly assess the virulence of any species detected, only look for virulence genes (Thoerner *et al.*, 2003). However, the biggest problem with all of these traditional techniques is that they are reliant on detecting very specific elements within a sample, be it indicator pathogens or genetic sequences. This limits all of these methods to detection of a subset of known elements and renders them ineffective at detecting novel pathogens. Therefore these studies only provide a small snapshot of the potential pathogenic load of each sample; the total pathogen load could be much greater.

The *Galleria mellonella* model system

Another important approach in the study of pathogens is the inoculation of model organisms with specific pathogen isolates to directly assess their virulence. This method uses organisms that share a similar immune response to humans, and are easily bred and contained. Mouse or zebrafish models are typically used (e.g.: Barman *et al.*, 2008; Bergeron *et al.*, 2017), however there are significant financial, ethical and logistical limitations to such studies (Tsai, Loh and Proft, 2016). Due to the number of experimental replicates required to accurately assess virulence, large populations of model organisms are required. Sufficient numbers of the model organism must also be obtained on a regular basis, either through reproduction or purchase from an external supplier. Another model organism which studies have begun to use as a more easily manageable, ethically sound, and cheaper substitute for mammalian infection studies is the larvae of the wax moth *Galleria mellonella* (Champion, Wagley and Titball, 2016).

G. mellonella larvae have been shown to have a surprisingly similar innate immune system to mammals (Ramarao, Nielsen-Leroux and Lereclus, 2012). Many components of the innate immune system are highly conserved between mammals and insects, with some of the mechanisms involved being ancient in origin (Vilmos, 1998; Hoffmann, 1999). In immune responses, cells' external

membranes provide a first line of defence against invading elements, with peptides and antimicrobial agents present on these surfaces (Alberts *et al.*, 2017). In both insects' cuticles and mammals' epidermis the main structural component is protein, and pathogens either take advantage of physical breaches or create their own using proteinases, the latter method being more common in insects than mammals (Scully and Bidochka, 2006). Intracellular pathogens then use a range of virulence factors to breach cell membranes, disable host defence molecules and acquire resources. There are strong similarities in the mechanisms used between insect and mammalian pathogens, with it being common in both cases for proteases to be utilised (Leger, Charnley and Cooper, 1987; Cowell *et al.*, 2003). Bacterial toxins seek to exploit similar targets across both mammalian and insect hosts. For example, both the insect midgut and mammalian intestine microvillar surfaces have glycoconjugate receptors which are bound by toxins from both insect and mammal pathogens, typically Cry toxins and Ctx toxins respectively (Cuatrecasas, 1973; Jurat-Fuentes, Gould and Adang, 2002).

Both insects and mammals have innate immune systems, which work to disrupt and destroy any pathogens which make it inside the host organism. While there are differences in the mechanism of action of some of these components, the basic principles remain the same across both host types. Both produce anti-microbial substances to inhibit and damage invading cells (Vilmos, 1998). Both also produce phagocyte cells which consume and destroy invaders, in the case of insects these are known as haemocytes, which are analogous to mammalian neutrophils (Bergin *et al.*, 2005). Both types of phagocyte have similar mechanisms of action: they bind to the invading cell, envelop it and destroy it with chemicals and protein complexes (Scully and Bidochka, 2006). In both insects and mammals, the immune system detects highly conserved microbial components to recognise pathogens. These 'pathogen-associated molecular patterns' (PAMPs) are detected by pattern recognition receptors (PRRs) which trigger an immune response. Insect and mammal PRRs have been shown to have a number of similarities (Khush and Lemaitre, 2000; Kimbrell and Beutler, 2001). Pathogens of both host types have also evolved similar methods of immune evasion by changing the PAMPs present on their surface (Scully and Bidochka, 2006).

The most important factor when determining whether a model organism is suitable as a proxy for mammalian infection studies is whether there is a comparable mortality rate for both host organisms. Studies have shown that this indeed appears to be the case for *Galleria*; there are strong correlations in mortality rates between *G. mellonella* and mice when injected with infectious agents such as *Pseudomonas aeruginosa*, *Listeria* and *Candida albicans* (Jander, Rahme and Ausubel, 2000; Brennan *et al.*, 2002; Mukherjee *et al.*, 2010). Indeed, there is evidence to suggest that bioassays using *Galleria* are also able to identify virulence factors that are involved in mammalian pathogenesis (Jander, Rahme and Ausubel, 2000).

While the aforementioned similarities between the *G. mellonella* model and traditional mammalian models show that it is a viable alternative, *Galleria* have also been shown to have a number of significant advantages over traditional models. *Galleria* reproduce relatively quickly, allowing for large sample sizes to be studied at once, improving the accuracy and consistency of studies (Tsai, Loh and Proft, 2016). *Galleria* are also much easier to contain than mammalian model organisms, requiring no large-scale facility to house them, and can be kept at temperatures as low as 15°C without ill effect (Ramarao, Nielsen-Leroux and Lereclus, 2012). Another major advantage is that as they are an invertebrate model, studies involving *Galleria* require no ethical approval (Tsai, Loh and Proft, 2016). This makes studies more expedient, and allows for high-throughput studies that would be ethically questionable in mammalian models. Additionally, the usage of *Galleria* as a replacement for mammalian models contributes to a reduction in mammalian suffering (Scully and Bidochka, 2006), and as such is being promoted by the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3RS.org.uk), among others.

Typical use of the *Galleria* model is to infect larvae with a predetermined volume of a specific strain of pathogen through either intrahemocoelic injection or force-feeding (Ramarao, Nielsen-Leroux and Lereclus, 2012). Larvae can then be incubated at 37°C (human internal body temperature) with no adverse effects, allowing for studies on human pathogens. Following incubation, the survival rates of *Galleria* are measured, and mortality can be easily judged due to the strong degree of melanisation exhibited upon death (Ramarao, Nielsen-Leroux and Lereclus, 2012). While it is true that *Galleria* lack the adaptive immune system

present in vertebrates, this immune system has evolved to protect against repeat infections by the same pathogen. The innate immune system is the primary defence against novel pathogens, and thus studies using insect models are still relevant when carrying out experiments such as mortality assays for novel pathogens.

Using the *Galleria* model to detect environmental pathogens

Studies are now beginning to develop the *Galleria* model system even further. Hernandez *et al.* (2019) recently showed that it is possible to use *G. mellonella* larvae as a bioindicator for microbial water and sediment quality by inoculating *G. mellonella* with both water sample extracts and soil washes containing entire microbial communities. The presence or absence of pathogens within these microbiomes was easily detectable via a simple assay of *Galleria* mortality. A number of pathogenic microbial communities were detected in this way. Subsequently, virulent pathogens from those communities were selectively enriched, isolated and studied further. This type of virulence assay has the significant advantage over traditional screening techniques in that it can directly screen for virulence from environmental pathogens without any prior knowledge of what pathogen species are present, and without the need to detect any specific genetic elements within it; allowing for the detection of novel pathogens that would otherwise go undetected. Indeed Hernandez *et al.* found a strain of *Proteus mirabilis* which carried genes from *Salmonella,* and a species of *Vibrio injenensis* that had never been detected outside of Korea before.

In this study, my collaborators and I used the *G. mellonella* model system in a manner similar to Hernandez *et al.*; using a whole-community inoculation approach to detect and study pathogens within the understudied environmental reservoir of soil.

Chapter 2 Bacterial pathogens in soil

Pathogens in soil

Soil is an environmental reservoir of disease in which studies are severely lacking. It is well-known to be a nutrient-rich environment teeming with bacteria and other microorganisms. For example a single gram of forest soil has been estimated to contain $4x10^7$ prokaryotic cells, and a single gram of cultivated soil and grasslands to contain $2x10^9$ prokaryotic cells (Daniel, 2005). These microorganisms are also extremely diverse, due to the extremely heterogeneous nature of soil (Fierer and Jackson, 2006). The solid components of soil can vary widely in their composition, from the materials they are comprised of (such as sand, silt, clay, and organic matter) to the particle size and plants growing in them, all of which have a large effect on the microbial diversity (Garbeva, van Veen and van Elsas, 2004). Additionally, many soils experience regular fluctuations in temperature, water availability and pH, creating greater habitat differentiation within those soils and allowing for a huge range of potential niche habitats for microbes (Fierer and Jackson, 2006). A large proportion of the species present are endemic to the soil, although some are ubiquitous - being found in many other environments too. There is evidence to suggest that pathogens can often be introduced into soil via the deposition of contaminated fertiliser or animal faeces (Park *et al.,* 2012).

Studies into pathogens in soil microbiomes typically focus on control of plant pathogens, particularly those used as crops (Weller *et al.*, 2002; Dordas, 2008; Sankaran *et al.*, 2010). While this is certainly an important field of study, and essential for food security, there is relatively little knowledge as to which soil pathogens could potentially cause a direct threat to human health. For these pathogens to have the potential to infect humans they must be capable of growing at 37°C (human internal body temperature) and studies suggest that this is quite common in soil bacteria (Berg *et al*, unpublished). Bacteria in general are one of the most common types of human pathogen, and in the interest of time it was decided that our study focus on these. Some examples of clinically relevant bacterial pathogens that are known to be contracted directly from soil include, but are not limited to, those in *Table 1*.

Table 1: Examples of clinically relevant soil-borne bacterial pathogens

Species	Disease	Symptoms	Source
Clostridium tetani	Tetanus	Muscle spasms, leading to seizures. Potentially fatal	Spores known to persist in soils for long periods of time
Clostridium botulinum[1]	Botulism	Muscle paralysis, can lead to asphyxiation	Often found in soils, infection commonly occurs via ingestion of contaminated food
Bacillus cereus[2]	Gastroenteritis. Can lead to bacteremia, meningitis, and pneumonia	Vomiting and diarrhoea	Common in the rhizosphere, often food-borne
Bacillus anthracis[3]	Anthrax	Initially flu-like, but often causes fatal internal damage if inhaled or ingested	Low concentrations found in soil, but meat from grazing animals can contain dangerous levels. Notoriously used as a bioweapon
Listeria monocytogenes[4]	Gastroenteritis. Can lead to meningitis, pneumonia and cervical infections	Flu-like symptoms, vomiting and diarrhoea	Ubiquitous in soil. Common in food-borne infections
Pseudomonas aeruginosa[5]	Pneumonia, sepsis, UTI's	Haemorrhaging and necrosis involving lingering toxins	Ubiquitous in soil and many other environments
Serratia marcescens[6]	Many including: pneumonia, UTI's, sepsis, and meningitis	Various due to the range of diseases caused	Soil, water, plants, animals and nosocomial infections
Staphylococcus aureus[7]	Skin and wound infections, bacteremia, food poisoning, toxic shock syndrome	Fever, joint pain or effusion, erythema and purulent drainage	Commonly nosocomial but also present in soil
Escherichia coli[8]	Very commonly associated with food poisoning	Diarrhoea, stomach cramps, vomiting and fever	Infections usually food or water borne. Soil deposition often from water or faecal matter
Salmonella spp.[9]	Food poisoning, gastrointestinal disease, potentially typhoid	Diarrhoea, fever, abdominal cramps. Typhoid can lead to complications	Digestive tracts; faeces contaminates water and soil
Enterobacter spp.[10]	UTI's, respiratory tract infection, bacteremia, soft tissue infections, and many others	Widely varied depending on site of infection	Intestinal tracts and skin of humans and animals, water, soil and plants

Examples of known bacterial pathogens that are found in soil. References as follows. 1: (Baker *et al.*, 2009), 2: (Granum and Lund, 2006), 3: (Spencer, 2003), 4: (Rees, Doyle and Taylor, 2017), 5: (Gellatly and Hancock, 2013), 6: (Li *et al.*, 2011; Cristina, Sartini and Spagnolo, 2019), 7: (Brandt *et al.*, 1999; Tong *et al.*, 2015), 8: (Yang *et al.*, 2017), 9: (Eng *et al.*, 2015), 10: (Sanders and Sanders, 1997; Loiwal *et al.*, 1999). Additional references: (Brandt *et al.*, 1999; Baumgardner, 2012; Tong *et al.*, 2015)

The significance of rhizosphere bacteria

Within environmental reservoirs there are hot-spots of microbial activity. In soil it appears that the most active area is the layer of soil influenced by root metabolism, dubbed the rhizosphere by Hiltner in 1904 (Hartmann, Rothballer and Schmid, 2008). Plants create an increase in the turnover of organic nutrients around them thanks to both their own physical and chemical processes, and also the fact that they draw in other organisms such as herbivores and detrivores which also accelerate the process. Thanks to this, the rhizosphere has a high concentration of nutrients and microbes competing for them (Garbeva, van Veen and van Elsas, 2004). Plants have a complex relationship with soil bacteria, some having symbiotic relationships which aid the uptake of soil nutrients and help to fixate nitrogen (Frey-Klett, Garbaye and Tarkka, 2007), while other microbes serve as plant pathogens or have a neutral effect. This high concentration of microbes in the environment causes strong inter- and intra-species selection pressures. Rhizosphere bacteria must adapt to these pressures, and this often includes the creation and dispersal of antimicrobial compounds to combat one another (Hibbing *et al.*, 2010). There is also a greater chance for horizontal gene transfer of resistance genes to occur in such environments due to the high concentration of bacteria, aiding the spread of resistance genes. We hypothesise that these factors in a diverse and competitive environment such as the rhizosphere have the potential to select for virulent, resistant and quick-growing pathogens. Due to the sheer diversity of the rhizosphere and lack of studies in this area, it is possible that these pathogens have hitherto gone undetected; thus soil could potentially harbour dangerous opportunistic human pathogens.

Aims of this study

To briefly reiterate, the majority of studies into soil pathogens are interested in those that are plant pathogens, particularly those of crops. Those studies that do look at human pathogens are generally focused on those that can be transmitted to humans from poorly prepared food, particularly those found in the roots of crops. There has been relatively little investigation into what pathogenic species occur in other types of soil, and this is the knowledge gap that this study investigates.

Our aims were to:

- Determine whether virulent human pathogens are present in soil microbial communities using *G. mellonella* assays.
- Analyse whether soil type and location affect the prevalence of virulent microbial communities.
- Determine which soil pathogens are the most virulent using further *G. mellonella* assays.
- Identify the microbial species present in pathogenic soils using 16s sequencing, paying particular regard to the most virulent isolates.
- Compare how similar our isolated soil pathogens are to reported human infections by those species in terms of antimicrobial resistance and 16s sequence.

Materials and Methods

Soil sample collection

We collected a total of 40 soil samples from locations in Cornwall, UK in 2019 as described in *Table 2*. Sites were chosen at random from easily reachable grassy and wooded areas within walking distance of the University of Exeter Campus, with the exception of samples from Wheal Jane ('A' samples), the general area of which was deliberately chosen to obtain metal-contaminated soil samples. Exact coordinates, descriptions of each location and maps of each of the sampling sites are detailed in *Table S1 and Figure S1*. At each site, the topsoil was removed to a minimum 5cm depth, and a universal tube (30ml, Thermo Scientific) was filled completely with the exposed soil using a sterile metal spoon. Disposable gloves were worn to avoid contamination of the samples, and both gloves and spoon were sterilised with ethanol before and after collecting each sample. Collection of rhizosphere soil samples ('G' samples) was achieved via extraction of a section of turf with soil still attached, which was transported in a sterile plastic bag. A second, paired sample was then taken from the exposed area in the same manner as previously described. Rhizosphere soil was removed from the roots in a Category II extraction hood via shaking and further loosened with sterile tools if needed. Two samples taken from river bank soil were collected in a similar manner to the initial samples, however no topsoil removal was necessary as they were collected directly from the exposed mud of the river banks and bed.

Table 2: Sampling areas

Sampling date (2018)	Location	Approximate coordinates	Site designations	Number of samples
August	Falmouth, Cornwall, UK	50.154, -5.072	GO, BO, SO and PO	4
September	University of Exeter, Penryn, Cornwall, UK	50.172, -5.121	P & C	15
October	"	"	G	14 (paired samples)
November	Wheal Jane, Cornwall, UK	50.231, -5.128	A	7

Sampling dates and approximate locations from which all 40 environmental soil samples were taken. Exact coordinates and maps of each individual sampling site can be found in *Table S1 and Figure S1*.

Extraction of bacterial communities from environmental samples

All soil samples were brought to the lab for processing on the same day as collection, with all further methods being undertaken in a Category II extraction hood to avoid contamination. Bacterial extraction methods were based on Hernandez *et al.* (2019). Each soil sample was removed from the universal tube and gently mixed with the sterile spoon to homogenise it. 10g of each sample was then placed into a fresh universal tube. 10ml of sterile M9 buffer solution (12.8 g L-1 Na_2HPO_4-$7H_2O$; 3 g L-1 KH_2PO_4; 0.5 g L-1 NaCl; 0.1 g L-1 NH_4Cl) was added to this, and the mixture vortexed on a Stuart® Vortex mixer for two minutes until the sample was fully homogenised. This solution was then centrifuged at 500rpm (56g) for 15 minutes in a Heraeus Megafuge 40R centrifuge (Thermo Scientific) to separate out the solid matter. The aqueous layer was poured into a new sterile universal tube and centrifuged at 3500rpm (2739g) for a further 30 minutes to pellet bacteria. The supernatant was discarded, and the pellet containing bacteria re-suspended in 1ml of M9 buffer. All centrifugation steps were performed at 4°C to limit the growth of bacteria during the process.

G. mellonella injection with whole soil communities

Boxes of *Galleria mellonella* larvae in the last instar before pupation were obtained from UK Waxworms Ltd (www.uk-waxworms.co.uk) and stored in a cold room at 4°C prior to injection. All larvae used for this study were used within two weeks of purchase. For each sample to be injected, ten larvae were placed in a petri dish and kept on ice for a minimum of ten minutes prior to injection to render the larvae immobile and unconscious. A Hamilton 50µl syringe (model 80950) was sterilised via flushing with 70% ethanol, then flushed with M9 to remove residual ethanol. 10µl of each microbial community extracted from our soil samples was injected into the hind right proleg of ten larvae. Each person performing injections also injected a further ten larvae with 10µl of M9 as a control for mortality caused by the injections themselves. Ten additional larvae were left uninjected to control for any natural mortality. All larvae were then incubated at 37°C for 24hrs and mortality recorded. The criteria for determining mortality were to first identify larvae that had become visually immobile, these were easily detected as they were usually also heavily melanised. Larvae were then touched on the head with forceps, and if no movement was detected they were deemed deceased. Following the mortality assay all larvae involved were immediately frozen at -80°C for a minimum of one hour prior to disposal. Microbial communities that caused >50% mortality were deemed to be 'virulent'. These samples were then passed through a Corning® 0.2µm sterile syringe filter before being injected into fresh larvae using the same procedures, to exclude any mortality due to anything the size of a bacterium or larger. Filtered samples that still caused mortality would have been assumed to be caused by chemicals present and excluded from further analysis, however this did not apply to any of our samples.

Pathogen clone isolation

Virulent soil communities causing >50% larvae mortality in were injected into ten fresh larvae per sample as per the above procedure, and monitored regularly during incubation at 37°C. Individuals showing clear signs of being heavily infected were selected for hemocoel extraction. Level of infection was judged by the degree to which the larvae melanised (a strong indicator of infection), with very dark but still motile larvae being chosen, due to the fact that hemocoel becomes difficult to extract post-mortem. A minimum of two infected larvae were

selected per soil sample and placed in an Eppendorf tube at 4°C prior to hemocoel extraction. Larvae were then placed on ice for at least ten minutes prior to extraction. Forceps, blade forceps and cotton buds were sterilised with ethanol. Each larva was sterilised around the hind right proleg (the injection site) using a cotton bud, and an incision was made at the injection site – enough to allow hemocoel to exude without puncturing the gut. Hemocoel was allowed to exude, collected with a pipette, and mixed in 400µl M9. Larvae were then immediately euthanised by freezing them at -80˚C.

Hemocoel extractions were diluted $x10^{-1}$, $^{-2}$ and $^{-3}$ in LB broth, plated on LB agar, and incubated overnight at 37˚C. Following colony growth, distinct colony morphologies on each plate were identified, and a minimum of one of each morphological type observed (*Figure S2* and *Table S2*) was streaked on fresh LB agar and incubated overnight to try and isolate it. Following successful isolation of each morphology an overnight culture of each was prepared by inoculating a single colony in 10ml LB broth and incubating at 37°C. Freezer stocks of these clones were made using 0.7ml of 50% glycerol and 0.7ml of overnight culture. Each overnight culture was plated on LB agar at a range of dilutions and incubated at 37°C overnight. The plate with the highest number of CFU's that could still be visually distinguished by eye was counted with the aid of a Stuart® SC6PLUS colony counter. Counts were scaled up proportionally to the amount they were diluted by to estimate the CFU concentration in 1ml of overnight culture.

Naming conventions used for isolated soil clones

All clonal soil isolates obtained from *G. mellonella* larvae hemocoel were named using the following methods, in the order in which they are stated. Firstly, the site from which its soil sample was taken e.g. P1. Secondly, the arbitrary number of the larva from which hemocoel was extracted from for that soil community, i.e. 1st, 2nd etc. Thirdly, an arbitrary number based on the order in which the clone was obtained from each plated hemocoel extraction. For example P11.1 refers to a clone isolated from site P1, the first hemocoel extraction for that community, and the first clone isolated from that hemocoel. This naming nomenclature was extended for 'G' samples, to indicate whether the sample was from the grass-root soil (R) or from the paired bulk soil sample (S). For example G6R3.3 refers to a clone isolated from site G6, grass-root soil, third hemocoel extraction, and the

third clone isolated from that hemocoel. A final name extension was added in one particular case where a clone displayed a different morphology to that originally recorded, to indicate its morphotype. This was clone SO1.1/6, the '/6' indicating that the morphology now resembled type 6. Morphological types for each clone are otherwise not indicated in their name, and can instead be found in *Table S2.*

G. mellonella injection with clonal soil isolates

Overnight cultures of each clone isolated from virulent (>50% larvae mortality) soil communities were diluted in M9 buffer to 10^5 CFU and injected into fresh larvae as per the method described above, with the same M9 and no-injection controls. After incubation for a period of twelve hours mortality was recorded. If mortality in any control group was >2 the assay was deemed invalid and was repeated with fresh larvae. Clones causing >50% larvae mortality were deemed 'virulent clones' and selected for further study. Overnight cultures of selected virulent clones were grown overnight from freezer stocks in LB media and diluted in M9 buffer to bring the CFU (per injection) to approximately 10^3, 10^5 and 10^7, this being calculated from the previously grown plates. 20 larvae were injected with 10µl of each diluted overnight culture in the same manner as previously described, along with M9 and no-injection control groups. After an initial ten hour period of incubation larvae mortality was recorded hourly until either all larvae expired, or a period of 37 total hours had passed since injection (Hernandez *et al.*, 2019). If mortality in either control group was >2 the experiment was repeated with fresh larvae. LD_{50} was determined as the lowest CFU treatment that caused >50% larvae mortality. The same protocol was also used in a similar assay to compare virulence between distinct clones, but using just the 10^3 CFU treatment.

Species identification of isolated clones using 16s rRNA

DNA was extracted from overnight cultures of each clone using a "DNeasy® UltraClean® Microbial Kit" (QIAGEN). Each extracted DNA sample was amplified using the following PCR protocol and reagents. 25µl of Green master mix (DreamTaq, Thermo Scientific™), 5µl of primer mix (consisting of 16s27f: 5'-AGAGTTTGATYMTGGCTCAG-3' and 16s1492r: 5'-TACCTTGTTAYGACTT-3') at 4.5µM concentration, 0.5µl of BSA, 18.5µl of PCR-grade water and 1µl of the extracted DNA. Thermocycler used was a Veriti 96-well thermal cycler (Applied Biosystems). Thermocycler protocol was one cycle of 95°C for five minutes, 30

cycles of 95°C for 30 seconds, 48°C for 30 seconds and 72°C for 90 seconds, then one cycle of 72°C for ten minutes. Samples were then held at 4°C until collection. PCR product was run on a 1% agarose gel with 5µl ETBR per 100ml for 35 mins at 120V. Gels were imaged in a Syngene G: Box alongside positive and negative controls and a DNA ladder. PCR product was purified using a NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). The concentration of purified PCR product was quantified using Fluorometric Quantification with a Qubit™ dsDNA HS assay kit in a Qubit® 2.0 flurometer (Invitrogen) and diluted appropriately using PCR-grade H^2O. DNA was sent for sequencing at Eurofins Genomics (NGS-Laboratory, Anzinger Str. 7a, 85560, Ebersberg, Germany) using the 'TubeSeq' service with the aforementioned 16s27f primer. Sequence data was then trimmed in MEGA (www.megasoftware.net) to remove poor-quality reads based on provided sequence quality documentation. Trimmed sequences were compared to the NCBI database "16S ribosomal RNA sequences (Bacteria and Archaea)" using the nucleotide BLAST program (blast.ncbi.nlm.nih.gov /blast.cgi) running the Megablast algorithm to determine the closest species match. BLAST results were sorted by total score, coverage, E-value and percentage identity, and the top results were recorded. Trimmed sequences were subsequently submitted to the NCBI GenBank database for accessioning.

Phylogenetic analysis of 16s genes

We conducted a phylogenetic analysis for four major clades found in our environmental samples; *Bacillus, Serratia, Providencia and Enterococcus.* 16s rRNA sequences were obtained from the NCBI GenBank database for several species within each genus to further clarify the evolutionary relationships of our environmental clones. One additional 16s sequence was added as an outgroup for each analysis. All analyses were conducted in MEGA-X (Kumar *et al.*, 2018). Sequences were aligned using ClustalW with default settings. Evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, Nei and Kumar, 2004) with 1000 bootstrap replicates. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option).

Antimicrobial resistance profiling

Clones isolated from soil were further examined and differentiated using antimicrobial-resistance testing. We tested 32 clones that had previously been identified as either *Serratia liquefaciens, Providencia alcalifaciens* or *Aeromonas hydrophila.* We used the EUCAST (www.eucast.org, 2019) disk diffusion method for antimicrobial susceptibility testing (Version 6.0, January 2017) along with the following reference strains: *Escherichia coli* (ATCC® 25922™), *Escherichia coli* (ATCC® 35218™), and *Klebsiella pneumoniae* (ATCC® 700603™). Overnight cultures of each of our clones and reference strains were diluted using 0.85% NaCl solution to 0.5 McFarland standard based on optical density at 600nm. Both our clones and the reference strains were then plated on Muller-Hinton (MH) agar using swabbing in multiple directions to ensure an even spread. 13 different antibiotic discs were added on top of the cultures, and plates were incubated at 37°C for 16-20 hrs. The time between starting dilutions and placing the prepared plates in the incubator was kept to a maximum of 15 minutes to minimise cell growth. Antibiotics used were Oxoid™ antimicrobial susceptibility disks (Thermo Scientific™): Cefoxitin (30µg), Cefpodoxime (10µg), Trimethoprim (5µg), Piperacillin / tazobactam combination (36µg), Tigecycline (15µg), Ceftazadime (10µg), Chloramphenicol (30µg), Amoxycillin / clavulanic acid combination (30µg), Aztreonam (30µg), Ciprofloxacin (5µg), Gentamicin (10µg), Ertapenem (10µg) and Azithromycin (15µg). Following incubation the diameter of the inhibition zone (‘halo’) around each antibiotic disc was measured by eye to the closest millimetre using a ruler. Care was taken to consistently measure only the zone where zero growth could be detected. Inhibition zones were compared to the relevant EUCAST Clinical Breakpoint Tables (v.9.0, 2019) for each species that we tested; *A. hydrophila* to the *Aeromonas* spp. tables, *S. liquefaciens* and *P. alcalifaciens* to the *Enterobacteriales* tables, and interpreted as being either sensitive, resistant, or intermediately resistant to each antibiotic.

Results

Environmental soil samples were collected from locations in Cornwall, UK, to assess the prevalence and identity of opportunistic pathogens in the soil. We used the *Galleria mellonella* model system as a screen for pathogenic species in soil communities, as this species has been shown to have a similar innate immune system to mammals. Soil communities that caused >50% *G. mellonella* larvae mortality were investigated further; clones were isolated from these soil communities for study and their virulence was assayed. Detailed larvae mortality was recorded across a range of time-points for particular clones of interest. Isolated clones were further characterised by morphology, 16s sequencing and AMR profiling. *Figure 1* shows each stage of the study, the results from each of these stages are detailed below.

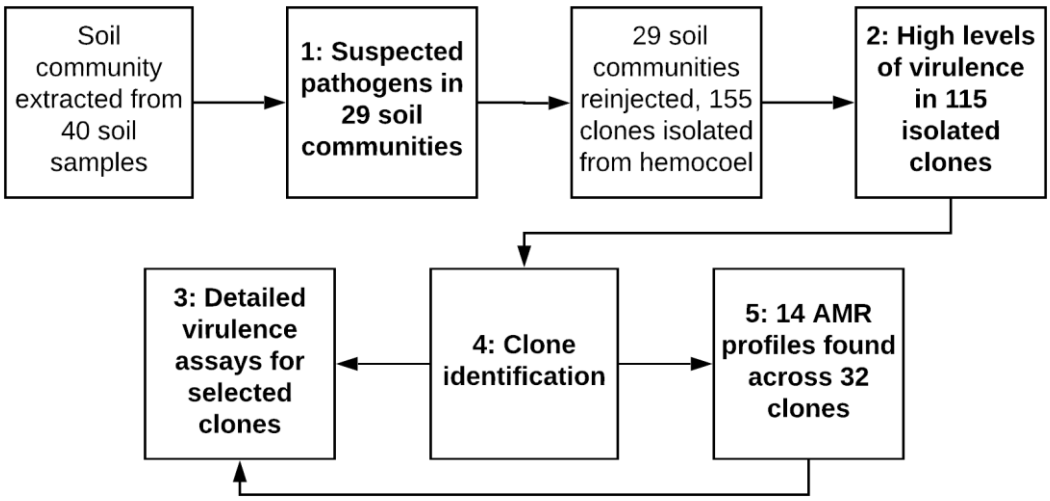


Figure 1: Workflow diagram of the whole project. An overview of the stages of this project. Numbers used at each stage are indicated. Boxes in bold text refer to a specific heading in this results section, indicated by the number preceding it. The exact number of clones isolated from each hemocoel extraction can be found in *Table S4*.

1: Suspected pathogens in 29 soil communities

After collecting our soil samples and extracting the microbial communities from them, we used the *G. mellonella* model to test for virulence. Our soil communities frequently caused high levels of larvae mortality (*Figure 3: A*). Both the M9 and no-injection control groups typically showed no larvae mortality, but 1-2 deaths were recorded in a control group in two of the assays. Of all 40 soil communities, 29 caused larvae mortality of >50% (*Figure 3: B*) – these will henceforth be referred to as 'virulent' soil communities. To examine whether soil community virulence was affected by soil type, sample sites were categorised into one of three types depending on the location they were taken - grassy areas, around tree roots, or from heavy-metal contaminated land at Wheal Jane. We excluded one sample, C4, which did not fall into any of these categories. Data was not normally distributed, and so we used a non-parametric test, finding no significant difference in larvae mortality between these types of areas (Kruskal-Wallis rank sum test, $\chi^2 = 0.55061$ and $p > 0.75$ on 2 d.f, *Figure 4*). We also compared the larvae mortality between paired samples taken from both grass roots and the bulk soil directly below them (sampling event 'G'). We found that there was a significant difference in larvae mortality between these two treatments; the samples taken from the grass roots caused higher levels of larvae mortality than those taken from bulk soil (paired t-test., $t = 2.5205$ and $p < 0.05$ on 6 d.f., *Figure 5)*.

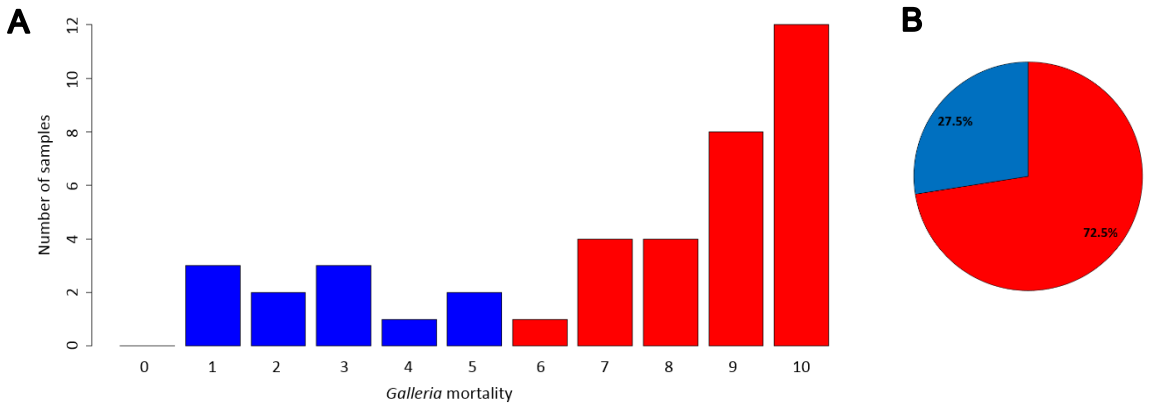


Figure 3: Soil community virulence assays. Mortality of ten larvae following inoculation with 10µl of each soil microbial community and incubation at 37°C for 24hrs. A: Samples frequently caused high levels of mortality; those in red caused >50% larvae mortality and were suspected to harbour pathogens. B: 72.5% of samples tested fell into this category. Clones from these communities were isolated for further study. Full results for each sampling site are in *Table S3*.

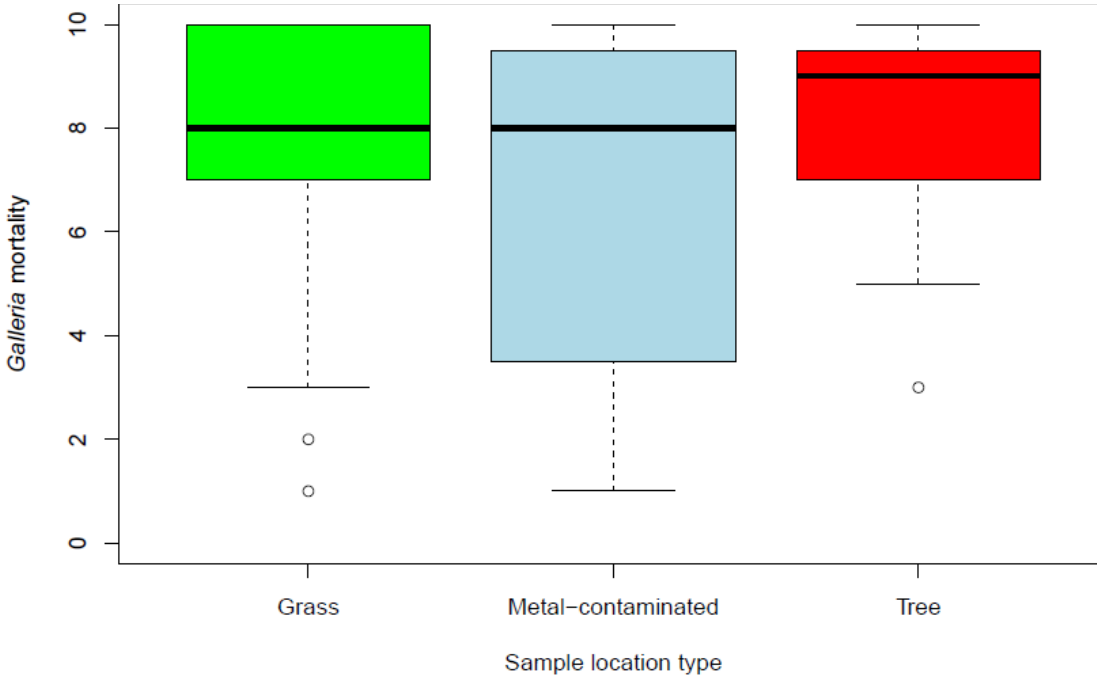


Figure 4: Soil community virulence sorted by sample soil type. Soil community virulence assays (*Figure 3)* separated by the location each soil sample was obtained; 25 from grassy areas, seven from metal-contaminated soil and seven from around tree roots. We found no significant effect of location type on larvae mortality.

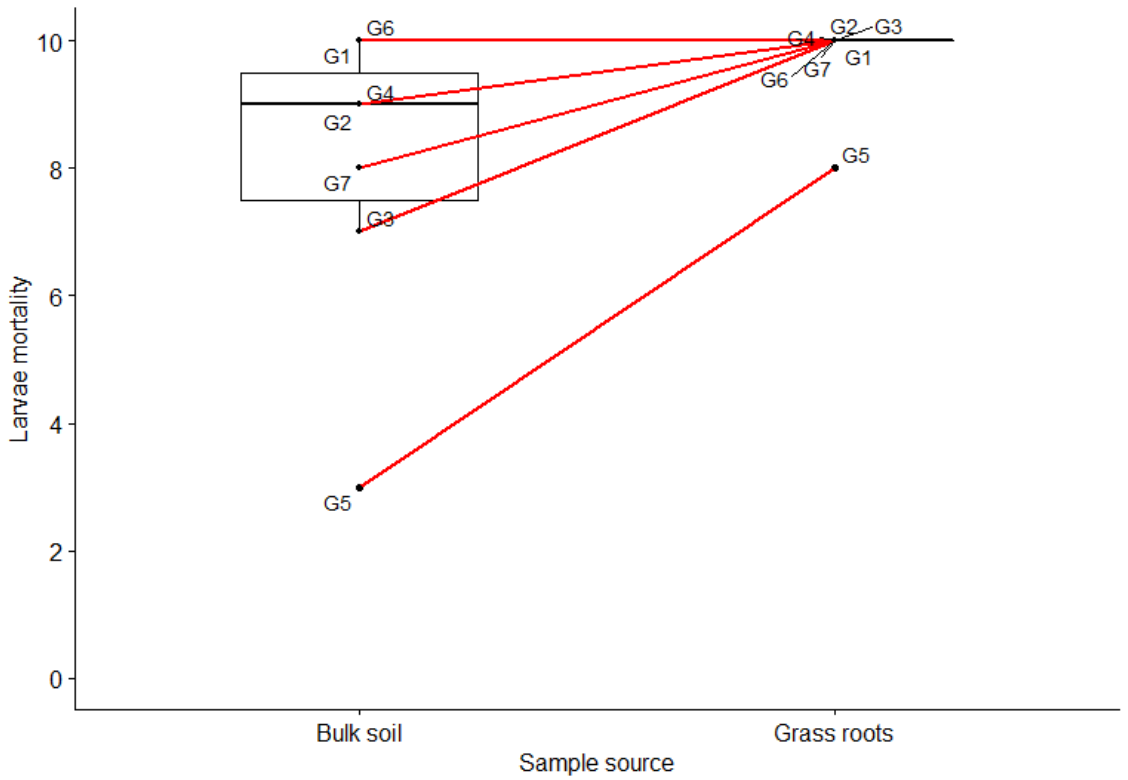


Figure 5: Soil community virulence of paired root-soil samples. Mortality of ten larvae following injection with microbial communities from seven paired soil community samples. Red lines connect paired samples. There was a significant difference in larvae mortality between communities from grass roots and those from bulk soil. All but one of the grass-root communities caused 100% mortality. Samples from one site (G5) caused the lowest mortality for both treatments (shown as outliers).

2: High levels of virulence in 115 isolated clones

29 of our extracted soil communities were suspected to likely harbour pathogens; those causing >50% mortality in *G. mellonella*. We isolated clones from these soil communities to investigate the pathogens responsible. A minimum of two hemocoel extractions were performed per soil community to increase the likelihood of isolating at least one instance of any pathogens present. Clones were distinguished visually based on morphology, size and colour into one of eleven morphological types (*Figure S2* and *Table S2*). At least one of each morphological type present in each hemocoel extraction was isolated. A total of 66 hemocoel extractions were performed, with at least two clones being isolated from each extraction in all but three instances (*Table 3*). In total we successfully isolated 155 clones from the hemocoel of reinjected *G. mellonella*; the exact

number of clones isolated from each hemocoel extraction can be found in Table S4.

Table 3: Numbers of clones isolated from *G. mellonella* hemocoel

Number of clones isolated	1	2	3	4	5
Frequency	3	45	11	6	1

Summary of the frequency at which single or multiple clones were isolated from larvae hemocoel. The exact number of clones isolated from each hemocoel extraction and the proportion found to cause >50% larvae mortality can be found in *Table S4.*

Larvae mortality was assayed for individual isolated clones, and high levels of mortality were frequently recorded (*Figure 6*). M9 and no-injection controls typically showed no larvae mortality, but 1-2 deaths were recorded in a control group in two of the assays. 115 (72.4%) of the clones tested caused >50% larvae mortality, and the most frequent result was 100% mortality. Clones causing >50% larvae mortality were selected for further identification and analysis. There were 13 instances in which only a single clone from a hemocoel extraction caused >50% larvae mortality, and 48 instances where two or more clones caused >50% larvae mortality (*Table S4*). There were also five hemocoel extractions from which all isolated clones caused ≤50% mortality. *Figure 7* shows the results of these clone virulence assays separated by sampling site.

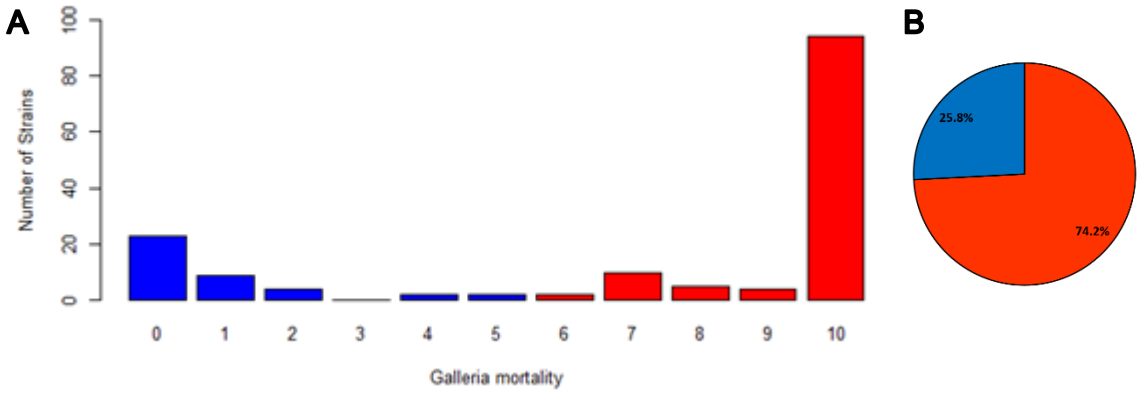


Figure 6: Virulence of isolated clones. Mortality of ten larvae following injection with 10^5 CFU of each clone isolated from soil communities that caused >50% larvae mortality (*Figure 3*). Following incubation at 37°C for 12hrs modal larvae mortality was 100% (A). Samples highlighted in red caused >50% larvae mortality and were selected for further study. 74.2% of the clones tested fell into this category (B).

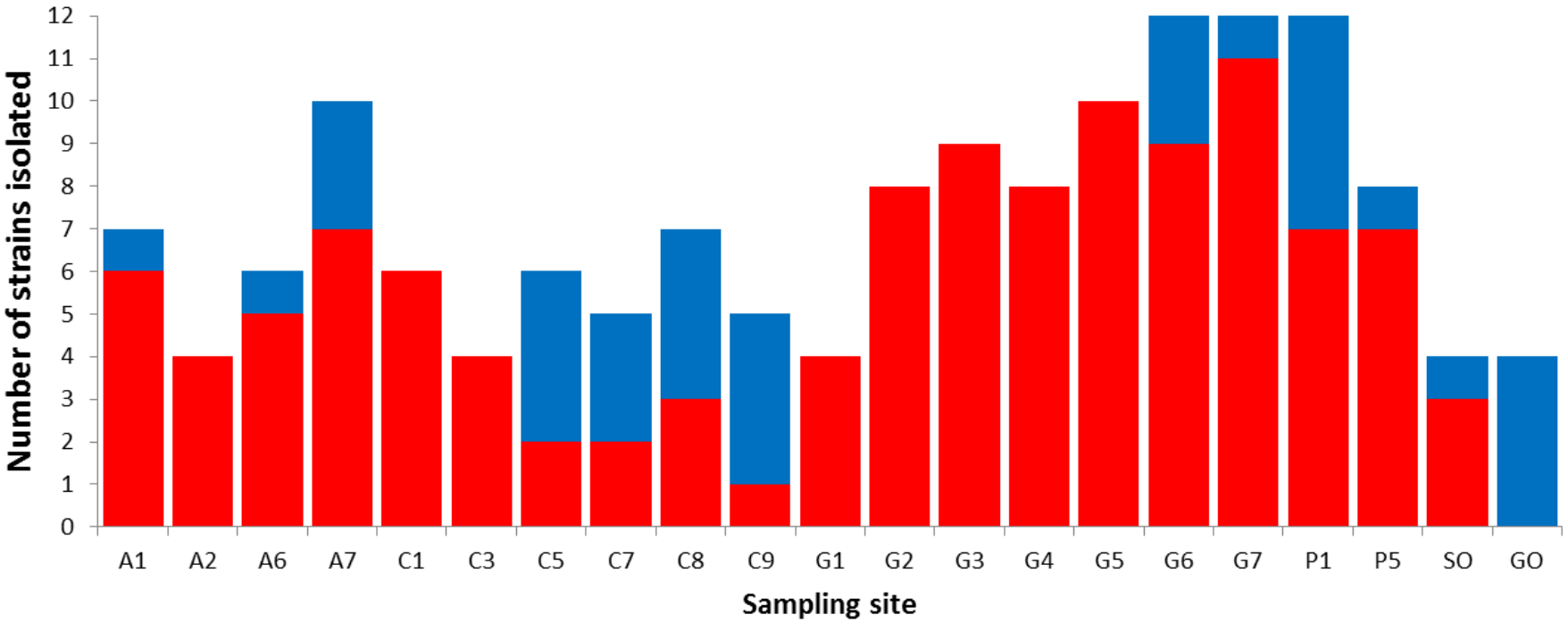


Figure 7: Number of clones isolated from each sampling site. The number of clones isolated from larvae hemocoel from each soil community that caused >50% larvae mortality (*Figure 3*). Indicated in red are clones that caused >50% larvae mortality following injection of 10^5 CFU of each clone, and incubation at 37°C for 12hrs. Those in blue caused ≤50% mortality. This data is further represented in *Table S4* and *Figure S3*.

3: Detailed virulence assays for selected clones

To examine the virulence of our clones in more detail we compared larvae mortality over time for clones found to cause >50% larvae mortality from the first two sampling events (*Table S1)*. We selected eight clones, choosing where possible those that showed differences in morphology, virulence and growth rate. Larvae mortality was recorded hourly between 10-37 hours post-inoculation for three CFU concentrations of each clone. No larvae mortality was recorded in the control groups. We plotted the data as survival curves (*Figure 8*) with the intent to examine them using Kaplan-meier survival analysis, however we later decided not to do this analysis due to time constraints. We did however compare the time taken for each clone dilution to cause 100% larvae mortality. By this metric, five of the clones (*Figure 8: A-E*) were similar to one another, with the lowest CFU treatment causing 100% larvae mortality within approximately 15 hours and higher CFU treatments causing 100% mortality sooner. 16s sequencing later determined that all five of these clones were *P. alcalifaciens* (*Table 4*). Two further clones (*Figure 8: F-G*) took longer to reach 100% mortality, with the 10^3 CFU of clone F not reaching 100%. These two clones also caused more gradual larvae mortality than the *P. alcalifaciens*, and both were later identified as belonging to the *Bacillus* genus, though they could not be identified to species level (*Table 4*). The final clone tested (*Figure 8: H*) showed lower virulence still, with neither the 10^3 or 10^5 CFU treatments causing 100% mortality by the end of the experiment. Unfortunately this clone could not be successfully identified. We also calculated that the LD_{50} for clones A-G was 10^3 CFU, and 10^7 for clone H.

Clones that were found to have distinct differences in their AMR resistance profiles - and therefore likely to be genetically distinct - were tested to examine their relative virulence over 37hrs, in the same manner as those tested in *Figure 8*. One representative clone from each AMR profile type described in *Table 5* was tested. For this assay we decided to only use the lowest CFU inoculation (10^3 CFU) as this treatment had previously shown the greatest variation between clones (*Figure 8*). One larvae died in the M9 control group after nine hours, no other mortality was recorded in the controls. We compared the time taken for each clone to cause 100% larvae mortality, and found that the single *Aeromonas hydrophila* clone was the most virulent, causing 100% mortality within twelve hours (*Figure 9: A*). Clones of both *P. alcalifaciens* (*Figure 9: B*) and *S.*

liquefaciens (*Figure 9: C*) showed consistency in the time taken for their respective species to cause 100% larvae mortality (15-18hrs and 17-20hrs, respectively). All clones tested caused 100% larvae mortality within 20 hours, with the exception of one clone of *S. liquefaciens* (G7R1.1) which did not reach 100% larvae mortality within the experimental timeframe. We subsequently analysed the survival data looking for similarities and differences both within and between each genus (*Figure 9: D*). *Providencia* isolates displayed indistinguishable virulence to one-another, as did the *S. liquefaciens* isolates (pairwise comparisons using log-rank test on survival fitted by Kaplan-meier method, $p>0.25$ for all comparisons). One *Serratia* clone (G7R1.1) was markedly less virulent than the other *Serratia* clones (pairwise comparisons using log-rank test on survival fitted by Kaplan-meier method, $p<0.05$). We also found significant differences between the *Aeromonas*, *Providencia* and *Serratia* (*Figure 9: D*); using a Cox proportional hazards model the hazard ratio for *Aeromonas* was higher than other genera, while for *Serratia* it was the lowest (Cox proportional hazards model, n=208, 2d.f, $p<0.001$ between all genera).

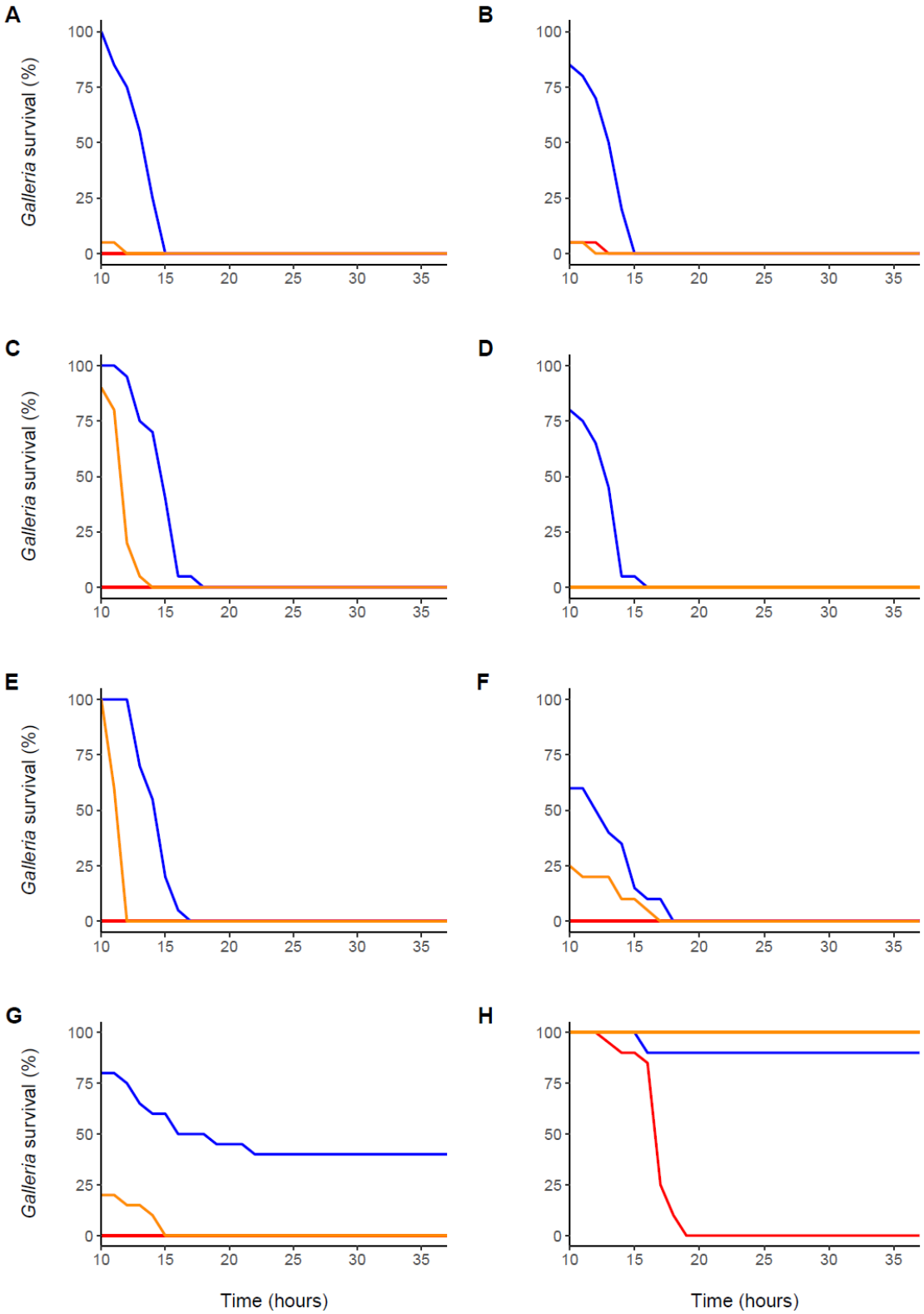


Figure 8: *Galleria* survival curves for eight pathogenic clones. Survival of 20 *G. mellonella* following inoculation with 10^3 (blue), 10^5 (orange) and 10^7 (red) CFU of each clone. Clones A-E were *P. alcalifaciens* (P11.1, P11.2, P54.2, P14.1, and P51.1 respectively). F and G were both *Bacillus* spp. (P54.1 and P12.1, respectively), and H (SO1.1) was not successfully sequenced.

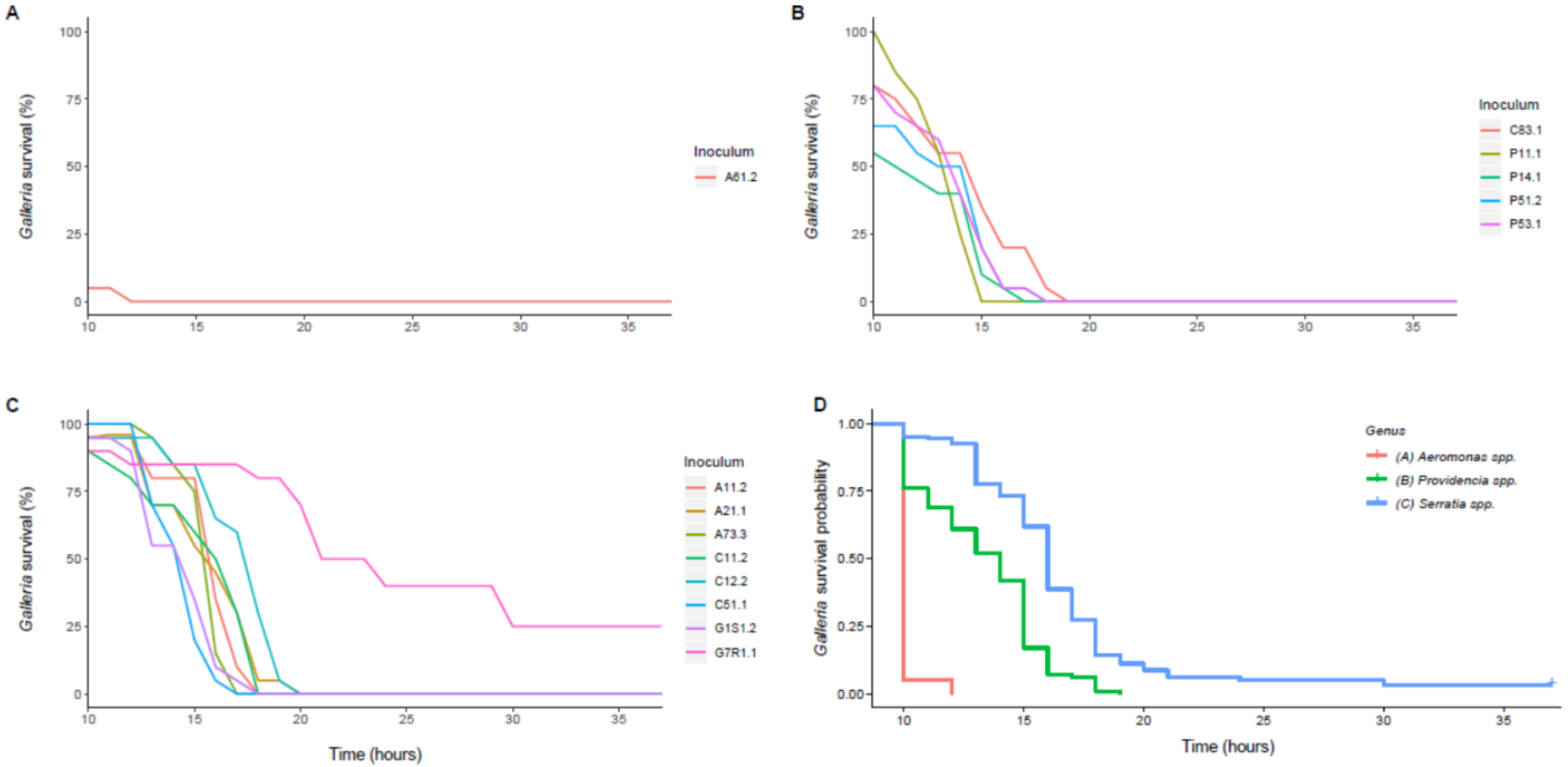


Figure 9: *Galleria* survival curves for AMR-profiled clones. Survival of 20 larvae following inoculation with 10^3 CFU of (A) *Aeromonas hydrophila*, (B) *Providencia alcalifaciens*, and (C) *Serratia liquefaciens* clones isolated from soil. One of each AMR resistance profile found is represented. D shows the probability of larvae survival at each time point for all three bacterial species, fitted using the Kaplan-meier method. LD50 for all clones was 10^3 CFU

4: Clone identification

All isolated clones were visually separated into one of eleven morphological types (*Figure 10*). Each morphotype's defining characteristics are described in *Figure S2*, along with a photographic reference. The most abundant morphological type was 4-5, accounting for 68% of all clones. This morphology was initially thought to be two distinct types (recorded as either 4 or 5) but subsequent platings could not be distinguished from one another. The only distinction initially found between these two morphologies was colony size, but this was found to vary slightly for both morphologies. As such morphology 4 and 5 were deemed the same and the groups merged.

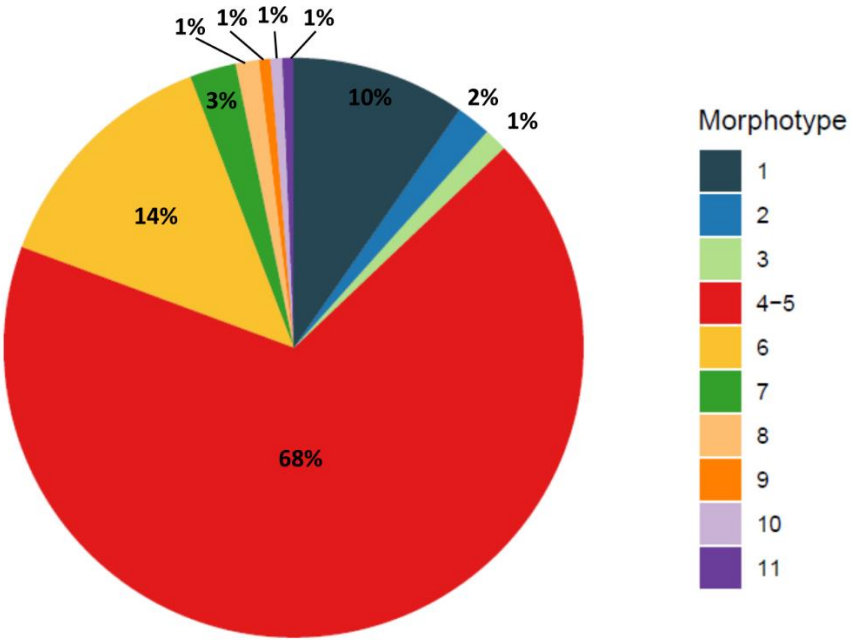


Figure 10: Clone morphologies. Clones isolated from larvae hemocoel were plated on LB agar and differentiated by their visual appearance, and separated into one of eleven morphological types. Two of these types (4 & 5) were later merged as they were indistinguishable on further platings. Descriptions and images of each morphology can be found in *Figure S2* and the morphology of each clone found is in *Table S2*.

Clones that displayed significant differences between one another in terms of virulence, morphology and location found were selected for 16s rRNA sequencing. We preferentially selected to sequence clones that caused >50% larvae mortality, but a few that did not were also selected. All clones that were

successfully sequenced are shown in *Table 4*, along with their closest species match. All trimmed 16s sequences used for identification were sent to the NCBI GenBank database, accession numbers are included in *Table 4* for all but three sequences; two of these were rejected due to them being of insufficient length, and one was rejected for having chimeric sequences. These sequences can instead be found in *Table S5*. Following 16s sequencing we determined that many clones were *Serratia* spp., predominantly *Serratia liquefaciens*, but also *Serratia plymuthica* and *Serratia nematodiphila*, along with several which could not be identified to the species level. We were able to identify several clones as *Providencia alcalifaciens*, and *Providencia rustigianii*. Several members of the *Bacillus* genus were also found, but we were unable to accurately define these to the species level using BLAST results, except in the case of *Bacillus megaterium*. *Enterococcus* spp. including *Enterococcus casseliflavus* were also present, but were also difficult to define to species level. Other species found include *Paenibacillus lautus*, *Citrobacter gillenii*, *Raoultella terrigena* and *Aeromonas hydrophila*. The relative frequency of each of these can be found in *Figure 11*.

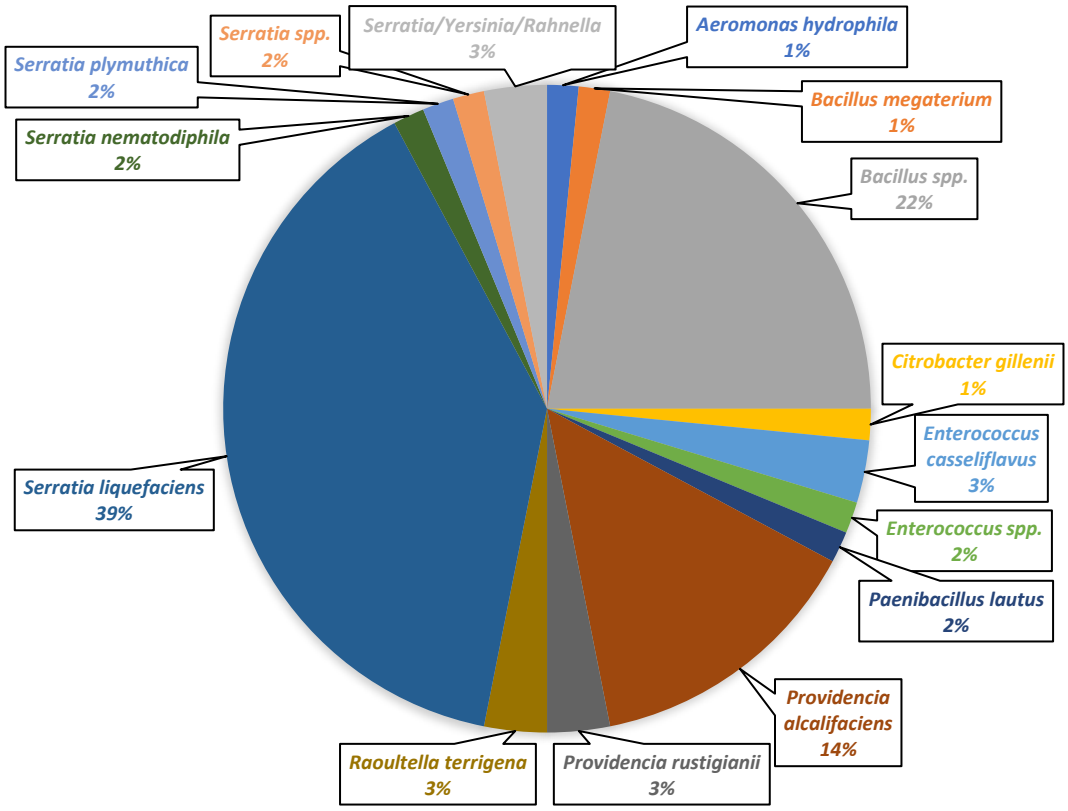


Figure 11: Species identified. 16s rRNA sequencing was used to determine the species of a minimum of one of each morphology found, along with any showing variance in virulence or growth rate. Details on specific results can be found in *Table 4*.

112 Table 4: Species identification using 16s rRNA sequencing

Site	Clone name	Closest match in GenBank 16s database	% Match (base pairs used)	% Coverage	E-value	GenBank accession number
A1	A11.2	*Serratia liquefaciens**	98.6 (783)	100	0	MN619709
	A12.2	*Bacillus* spp.	100 (104)	100	2E-49	Too short**
	A12.3	*Serratia liquefaciens**	99.61 (776)	100	0	MN619710
A2	A21.2	*Serratia liquefaciens**	99.42 (518)	100	0	MN619711
A6	A61.2	*Aeromonas hydrophila**	100 (840)	100	0	MN619712
	A62.2	*Enterococcus* spp.	96.04 (99)	100	1E-41	Too short**
	A63.1	*Serratia / Yersinia / Rahnella**	99.78 (451)	100	0	MN619713
	A63.2	*Serratia liquefaciens**	99.51 (822)	100	0	MN619714
A7	A71.2	*Bacillus megaterium*	99.28 (417)	100	0	MN619715
	A71.3	*Paenibacillus lautus*	100 (299)	100	2E-157	MN619716
	A72.2	*Bacillus* spp.	100 (439)	100	0	MN619717
	A72.3	*Serratia / Yersinia / Rahnella**	99.51 (408)	100	0	MN619718
	A73.3	*Serratia liquefaciens**	99.76 (822)	100	0	MN619719
C1	C11.2	*Serratia liquefaciens**	98.94 (659)	100	0	MN619720
	C12.2	*Serratia liquefaciens**	98.96 (672)	100	0	MN619721
	C13.1	*Serratia liquefaciens**	98.78 (655)	100	0	MN619722
	C13.2	*Serratia liquefaciens**	99.24 (785)	100	0	MN619723
C3	C31.1	*Bacillus* spp.*	100 (664)	100	0	MN619724
	C31.2	*Bacillus* spp.*	100 (767)	100	0	MN619725
	C32.1	*Serratia liquefaciens**	99.7 (670)	100	0	MN619726
C5	C51.1	*Serratia liquefaciens*	99.85 (654)	100	0	MN619727
	C52.1	*Serratia* spp.*	99.61 (507)	100	0	MN619728
C7	C71.1	*Bacillus* spp.	99.82 (548)	100	0	MN619729
	C71.2	*Bacillus* spp.	100 (498)	100	0	MN619730
	C72.1	*Serratia* spp.	99.52 (832)	100	0	MN619731
	C72.3	*Bacillus* spp.*	99.85 (666)	100	0	MN619732
C8	C81.1	*Serratia liquefaciens**	98.47 (653)	100	0	MN619733
	C81.2	*Serratia liquefaciens*	99.23 (653)	100	0	MN619734
	C82.1	*Serratia liquefaciens*	99.39 (654)	100	0	MN619735
	C83.1	*Providencia rustigianii**	100 (831)	100	0	MN619736
	C83.2	*Providencia rustigianii*	100 (654)	100	0	MN619737
C9	C91.1	*Enterococcus casseliflavus*	100 (499)	100	0	MN619738
	C91.3	*Enterococcus casseliflavus*	100 (651)	100	0	MN619739
	C92.1	*Serratia plymuthica*	99.75 (533)	76	0	MN619740
G1	G1R1.1	*Serratia liquefaciens**	99.85 (654)	100	0	MN619741
	G1S1.2	*Serratia liquefaciens**	99.85 (653)	100	0	MN619742
G2	G2S1.1	*Serratia nematodiphila**	100 (655)	100	0	MN619743
G3	G3R1.1	*Serratia liquefaciens**	99.7 (670)	100	0	MN619744
	G3S1.1	*Serratia liquefaciens**	99.85 (653)	100	0	MN619745
	G3S1.3	*Bacillus* spp.*	100 (664)	100	0	MN619746
G4	G4R1.1	*Providencia alcalifaciens**	99.69 (654)	99	0	MN619747
G5	G5R1.1	*Serratia liquefaciens**	99.55 (660)	100	0	MN619748

	G5R1.3	*Bacillus* spp.*	99.74 (775)	100	0	MN619749
	G5R2.1	*Bacillus* spp.*	99.85 (672)	100	0	MN619750
	G5R4.2	*Serratia liquefaciens**	99.85 (653)	100	0	MN619751
G6	G6R1.1	*Raoultella terrigena*	100 (318)	100	7E-168	MN619752
	G6R1.3	*Serratia liquefaciens*	100 (365)	100	0	MN619753
	G6R2.1	*Serratia liquefaciens*	100 (339)	100	2E-179	MN619754
	G6R3.2	*Citrobacter gillenii*	100 (449)	100	0	MN619755
	G6R3.3	*Raoultella terrigena*	100 (309)	100	7E-163	MN619756
G7	G7R1.1	*Serratia* spp.*	99.65 (570)	100	0	MN619757
	G7R2.2	*Serratia liquefaciens**	99.84 (613)	100	0	MN619758
	G7S1.2	*Serratia liquefaciens**	99.54 (655)	100	0	MN619759
P1	P11.1	*Providencia alcalifaciens**	99.88 (849)	100	0	MN619760
	P11.2	*Providencia alcalifaciens**	100 (832)	100	0	MN619761
	P12.1	*Bacillus* spp.*	99.42 (863)	100	0	Chimeric**
	P12.2	*Bacillus* spp.*	100 (667)	100	0	MN619762
	P13.1	*Bacillus* spp.*	99.55 (880)	100	0	MN619763
	P13.2	*Serratia liquefaciens**	98.93 (654)	100	0	MN619764
	P14.1	*Providencia alcalifaciens**	100 (652)	99	0	MN619765
P5	P51.1	*Providencia alcalifaciens**	100 (653)	99	0	MN619766
	P51.2	*Providencia alcalifaciens**	99.85 (656)	100	0	MN619767
	P52.1	*Providencia alcalifaciens**	99.88 (828)	100	0	MN619768
	P53.1	*Providencia alcalifaciens**	100 (680)	99	0	MN619769
	P54.1	*Bacillus* spp.*	99.89 (884)	100	0	MN619770
	P54.2	*Providencia alcalifaciens**	100 (666)	99	0	MN619771
SO	SO1.1/6	*Serratia* spp.*	99.64 (828)	100	0	MN619772

The closest species match for the 16s sequence of each clone when compared to the GenBank database. In cases where several species were equally likely the closest genus is stated. A63.1 and A72.3 had equal matches for multiple species of *Serratia, Yersinia, and Rahnella*. Coordinates, descriptions and maps of each sampling site can be found in *Table S1* and *Figure S1*. *Clones marked with an asterisk caused >50% larvae mortality (*Figure 6)*. **Sequences for A12.2 and A62.2 could not be accessioned due to being of insufficient length, and the sequences for P12.1 contained chimeric sequences. These sequences can instead be found in *Table S5.*

To further investigate the identity of our isolated clones, we conducted a phylogenetic analysis using 16s rRNA sequences for each major clade found in our environmental samples: *Bacillus, Serratia, Providencia and Enterococcus* (*Figure 12, 13, 14,* and *15*, respectively). Our sequences were compared to their closest BLAST matches along with several additional species from within their clade; clinically relevant and commonly occurring species were favoured for this. All sequences were aligned using ClustalW in MEGA-X (Kumar *et al.*, 2018). Due to short lengths for some of our 16s sequences, some sequences had to be omitted as they could not be aligned with one another; these are indicated in the figures where appropriate. Trees were constructed using the Neighbour-Joining method (Saitou and Nei, 1987), and evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, Nei and Kumar, 2004) and are in the units of the number of base substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). Trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Sequences from GenBank contain their accession numbers and strain names, sequences from our isolated clones state the clone name and closest sequence match from BLAST in brackets.

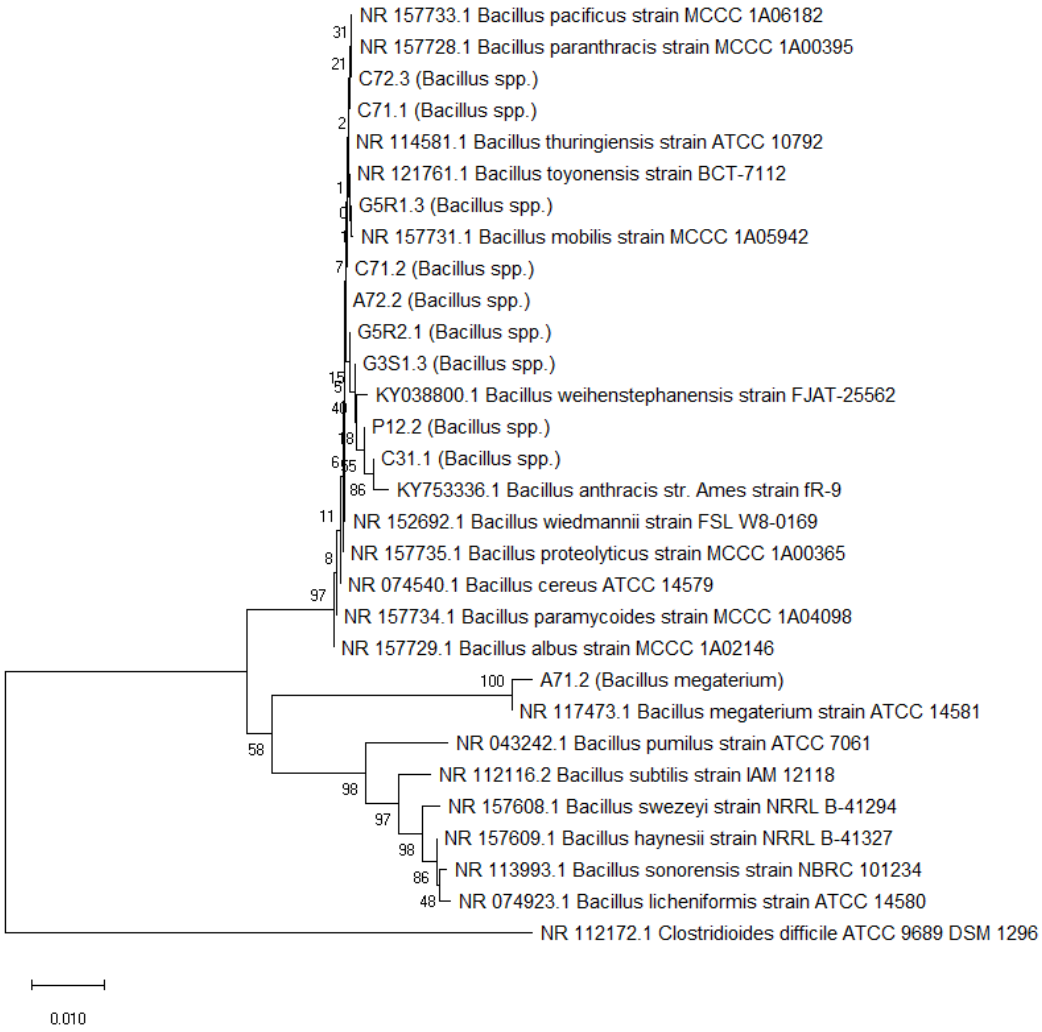


Figure 12: **Evolutionary relationships of *Bacillus* clones.** Neighbour-joining tree of environmental *Bacillus* based on 16s rRNA sequences and 1000 bootstrap replicates *Clostridioides difficile* (strain U 5/41) was used as on outgroup. Sequences for A12.2, C31.2, A71.3, P12.1, P54.1 and P13.1 were omitted as they did not sufficiently overlap with the rest of the sequences. The closest GenBank matches to our environmentally isolated clones are indicated in brackets.

Figure 13: **Evolutionary relationships of *Serratia* clones.** Neighbour-joining tree of environmental *Serratia* based on 16s rRNA sequences and 1000 bootstrap replicates *Pseudomonas aeruginosa* (strain DSM 50071) was used as on outgroup. Sequences from A72.3 and A63.1 were omitted as they did not sufficiently overlap with the rest of the sequences. The closest GenBank matches to our environmentally isolated clones are indicated in brackets.

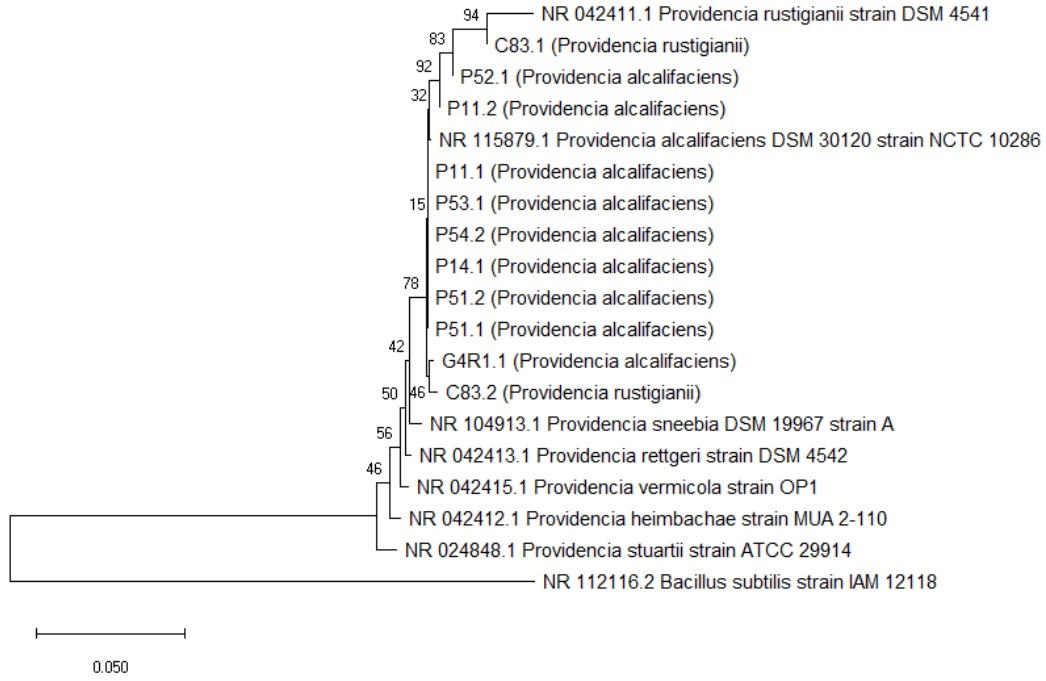


Figure 14: **Evolutionary relationships of *Providencia* clones.** Neighbour-joining tree of environmental *Providencia* based on 16s rRNA sequences and 1000 bootstrap replicates. *Bacillus subtilis* (strain IAM 12118) was used as on outgroup. The closest GenBank matches to our environmentally isolated clones are indicated in brackets.

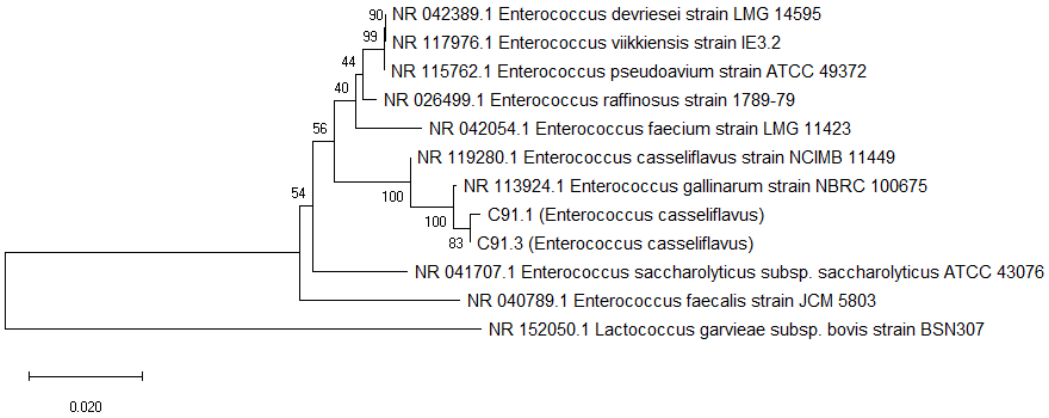


Figure 15: Evolutionary relationships of *Enterococcus* clones. Neighbour-joining tree of environmental *Enterococcus* based on 16s rRNA sequences and 1000 bootstrap replicates. *Lactococcus garviae* subspecies bovis (strain BSN307) was used as on outgroup. Sequence for A62.2 was omitted due to being <100 base pairs. The closest GenBank matches to our environmentally isolated clones are indicated in brackets.

5: 14 AMR profiles found across 32 clones

Antimicrobial resistance is an important factor when it comes to the treatment of pathogenic bacteria, and so we examined how resistant our environmentally isolated clones were to 13 different antibiotics and antibiotic combinations (*Table 5*) using EUCAST standard protocols. These antibiotics were chosen to cover a wide range of clinically-relevant classes. The clones tested were susceptible to the majority of these antibiotics. 10/21 clones of *S. liquefaciens* were susceptible to all antibiotics, while the remaining clones showed some resistance to 1-2 antibiotics. All *P. alcalifaciens* tested were resistant to Azithromycin, with some intermediate resistances to other antibiotics also found. Across all the clones there were notably multiple instances of resistance to penicillin-based antibiotics at both intermediate and fully resistant levels, the Amoxycillin / clavulanic acid treatment in particular being the least effective antibiotic. Comparison of these resistance profiles to clinical strains investigated by other studies reveals some significant differences. *S. liquefaciens* typically show susceptibility to most of the antibiotics tested, with the exception of Cefoxitin (I/R), Amoxycillin / clavulanic acid (R), and Azithromycin (R); our clones being less than fully resistant to these antibiotics suggests that they are not the same as the clinical strains. *P. alcalifaciens* strains also show some major differences, with some sources noting either I or R levels of resistance for Trimethoprim, Chloramphenicol and Amoxycillin / clavulanic acid, while Azithromycin resistance appears to be typical for the species. Insufficient information could be found on *A. hydrophila* resistances to highlight any significant differences, or lack thereof, in our single isolated clone.

Table 5: AMR profiling.

Resistance profiles	Cefoxitin	Cefpodoxime	Trimethoprim	Piperacillin / tazobactam	Tigecycline	Ceftazidime	Chloramphenicol	Amoxycillin / clavulanic acid	Aztreonam	Ciprofloxacin	Gentamicin	Ertapenem	Azithromycin	No. clones found with this profile
A. hydrophila	S	S	S	S	S	S	S	I	S	S	S	S	S	1
	R^{1}	-	-	-	-	S^{1}	S^{1}	-	-	S^{1}	-	-	-	N/A
P. alcalifaciens	S	S	S	S	S	S	S	S	S	S	S	S	R	5
	S	S	S	S	I	S	S	I	S	S	S	S	R	2
	S	S	S	S	I	S	S	I	S	S	S	I	R	1
	S	S	S	S	I	S	S	S	S	S	S	S	R	1
	S	S	S	S	S	S	S	I	S	S	S	S	R	1
	$S^{2,3}$	S^{2}	$S/I/R^{2}$	S^{2}	-	S^{2}	$S/I/R^{2}$ I^{3} S^{4}	$S/I/R^{2}$	$S^{2,3}$	$S^{2,3}$	$S^{2,3,4}$	-	R^{2}	N/A
S. liquefaciens	S	S	S	S	S	S	S	S	S	S	S	S	S	10
	S	S	S	S	S	S	S	I	S	S	S	S	S	5
	I	S	S	S	S	S	S	S	S	S	S	S	S	1
	S	S	S	R	S	S	S	R	S	S	S	S	S	1
	S	S	S	S	S	S	S	R	S	S	S	S	S	1
	S	S	S	S	S	I	S	I	S	S	S	S	S	1
	S	R	S	S	S	S	S	R	S	S	S	S	S	1
	I	I	S	S	S	S	S	S	S	S	S	S	S	1
	I/R^{5}	S^{5}	S6	$S^{5,7}$ S/I^{8}	S^{6}	$S^{5,6}$	$S^{5,6}$	I/R^{5} $R^{6,8}$	$S^{5,6}$	$S^{5,6,8}$	$S^{5,6,7,8}$	$S^{5,6}$	$R^{5,6}$	N/A

Antibiotic susceptibility profiles of clonal soil pathogens tested against 13 antibiotics using EUCAST protocol. Each row represents a distinct resistance profile. The number of clones of each profile found is indicated on the right. S: susceptible, R: resistant, I: intermediate resistance, - : no data available. Beneath the AMR profiles we found are previously reported resistance profiles for the species based on the following references. 1: (Palú *et al.*, 2006), 2: (Stock and Wiedemann, 1998), 3: (Wang *et al.*, 2014), 4: (Chander, Goyal and Gupta, 2006), 5: (Stock, Grueger and Wiedemann, 2003), 6: (Mahlen, 2011), 7: (Engelhart *et al.*, 2003), 8: (Traub, 2000).

Discussion

The aims of this study were to screen environmental soil samples for the presence of virulent human pathogens, to determine whether soil type and location affected the occurrence of these pathogens, identify the species present, and determine which were the most virulent, subsequently comparing them to reported clinical strains. We used the *Galleria mellonella* model system as a virulence screen, and were able to screen for the presence of pathogens in 40 soil communities taken from locations in Cornwall, UK, in a manner similar to Hernandez *et al.* (2019). *G. mellonella* injected with extracted soil communities frequently showed high levels of mortality. The majority of soil communities obtained caused mortality of >50% in *Galleria*, and the modal result was total mortality (*Figure 3).* This finding suggests that species of human pathogens are common in soil communities, and highlights the importance of studying and understanding them. We were subsequently able to selectively isolate and enrich environmental clones from these samples and characterise them in further assays, the results of which will be discussed here.

The effect of soil type on the frequency of pathogens

Soil bacteria need to obtain essential resources such as vitamins and minerals from the soil. Iron in particular is a vital resource, and there is competition between species and individual bacteria to obtain it. To do so, bacteria use extra-cellular complexes known as siderophores, which bind to the iron and allow it to be taken into the cell. In pathogenic species, these same siderophores are an important virulence factor as they are similarly used to gather iron from within the host species (Hider and Kong, 2010). In environments where iron resources are scarce, the production of siderophores is upregulated (Boiteau *et al.*, 2016), and it follows that in these environments selection would favour bacteria which produce high numbers of siderophores. It is for this reason that we suspected certain soil types to contain higher frequencies of virulent pathogens than others. In particular, we suspected soil communities from rhizosphere soil to cause more *Galleria* mortality than soil communities from elsewhere; in the rhizosphere the increased inter and intra-species competition for iron could select for higher levels of siderophore production. Inversely, we suspected that microbes in iron-rich soils would likely produce fewer siderophores due to the abundance of resources, and thus fewer virulent pathogens would be found there.

In comparing the microbial communities we took from grassy areas, tree roots, and metal-contaminated soil samples we could find no significant differences in *Galleria* mortality (*Figure 4)*. This result does not support our hypothesis, however this could be due to a low statistical power. Our samples were not systematically taken with this analysis in mind, and as such we had seven samples from near tree-roots, seven from metal-rich soils (including two from river-bed soil), but 25 from grass. Future studies seeking to investigate this potential relationship may find a significant difference if they were to collect equal samples of each soil type, and ideally greater numbers of samples.

A similar analysis looked at paired the samples taken from grass roots and bulk soil in the same location (*Figure 5)*, again asking whether there were more pathogens present in soil communities from one of these soil types. In this case, we did find a significant difference in *Galleria* mortality, which was significantly higher in the communities from grass-roots. This supports the idea that rhizosphere communities contain more virulent species than other soil communities. However we were not able to identify whether the higher frequencies of pathogens noted was due to increased selection for siderophore production as hypothesised. Future studies could quantify siderophore production levels to examine whether this is indeed the case.

Community virulence compared to clone virulence

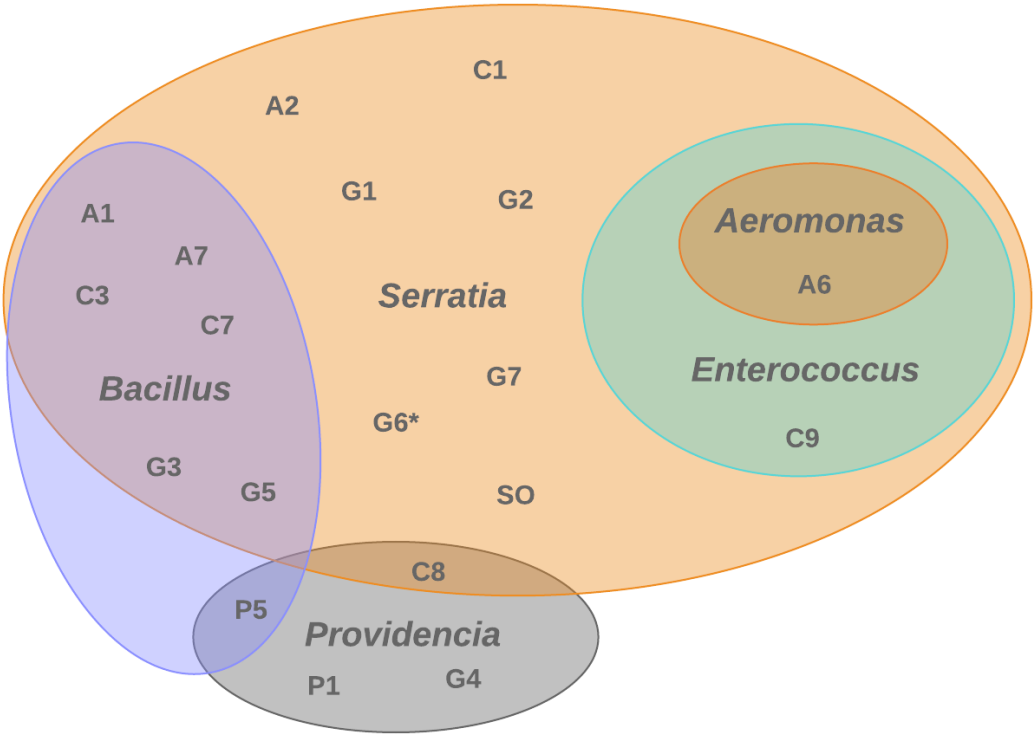
Clones isolated from the soil communities that caused >50% *Galleria* mortality generally also caused high levels of mortality (*Figure 6*). Of 155 clones isolated, 115 (74.2%) caused mortality of >50%, with the 94 of these (60.6%) causing 100% mortality. This shows that in most cases the virulence is not caused by a single species from the community, but rather that the majority of the community causes high levels of virulence. This finding is explored further in *Figure 7*, where we see that in most cases all clones isolated from a site caused >50% mortality, and similarly in *Table S4* - most or all of the clones isolated from each hemocoel extraction were found to cause >50% mortality. There are exceptions however, and this suggests that non-pathogenic species are coexisting with pathogens in the soil. Intriguingly, none of the clones isolated from site GO caused more than 10% mortality, despite the community sample causing 90% mortality. This could be due to a number of factors, such as a synergistic effect between multiple species, or potentially it could be that the causal pathogen was not isolated from

the community. Future experiments could co-inoculate clones in *Galleria* to investigate possible synergistic, or antagonistic relationships between them.

On the prevalence and diversity of pathogenic soil species

The clones we isolated from the 29 soil communities that caused >50% *Galleria* mortality were identified via 16s rRNA sequencing. The genera detected were predominantly *Serratia, Providencia* and *Bacillus*, with the most common species being *Serratia liquefaciens* and *Providencia alcalifaciens* (*Figure 11*). 21 of the soil communities were found to contain *Serratia* species, *Bacillus* species were present in eight, and *Providencia* species were in four. We also found *Enterococcus* in two samples, and *Aeromonas, Citrobacter* and *Raoultella* each in one sample. Looking at the sites from which each genus was found (*Figure 16*) gives us some idea of the patterns of genus co-occurrence within the soil. *Serratia* spp. were present in all but three sites, and were frequently found in isolation. *Providencia* were also found in isolation, but also co-occurred with *Serratia* and *Bacillus*. *Bacillus* by contrast was never found in isolation, and was predominantly found with *Serratia.* Interestingly, *Enterococcus* was only found in combination with *Serratia*, and *Aeromonas* was only found in a single site (A6) which contained both *Serratia* and *Enterococcus*. This could suggest some form of mutualistic relationship between the three genera.

It is interesting to note the common occurrence of *Serratia* across the majority of sampling sites, as these have not been noted as being particularly prevalent in soils previously. Berg, Eberl and Hartmann (2005) did note the occurrence of *Serratia* spp. in the rhizosphere of oilseed rape, but found no instances where it occurred outside that environment; our study clearly shows that this is not the case. Janssen (2006) describes the dominant soil bacterial taxa found in 16S rRNA libraries, and notes that Gammaproteobacteria, the clade to which *Serratia* belongs, has a mean prevalence of 8.1% across 194 samples, but does not indicate the prevalence of specific genera. Other studies have looked at overall species richness (Lozupone and Knight, 2007), soil microbial community structure (Fierer *et al.*, 2005) or the prevalence of specific genes in specific soil types (Byrne-Bailey *et al.*, 2011), but little information could be found on typical species prevalence based on 16s in uncultivated soils.



298

299 **Figure 16: Sites where each genus was identified.** Sampling sites from which
300 each genus of bacteria was detected. Overlapping clades indicate that clades co-
301 occurred in the soil sample sites indicated. Only sites from which the community
302 samples caused >50% *Galleria* mortality and clones were successfully
303 sequenced were included. *We also detected a *Citrobacter* and *Raoultella* in
304 infections following inoculation from the site G6 sample.

305 Detailed virulence assays of detected pathogens

306 *Galleria* mortality was recorded over 37hrs following inoculation with 10^3, 10^5 and
307 10^7 CFU of eight pathogenic clones (*Figure 8*). These clones were chosen from
308 clones from the first two sampling events that caused >50% larvae mortality
309 (*Table S1*), preferentially choosing clones that displayed differences in
310 morphology, virulence and growth rate, to attempt to examine a range of
311 pathogens. Based on the time taken for these clones to cause 100% larvae
312 mortality, the five clones later identified as *P. alcalifaciens* show the least
313 virulence of the species tested, with the 10^7 CFU treatment causing 100%
314 mortality within 13 hours, and the 10^3 CFU treatment doing so within 18 hours
315 (*Figure 8*). The two *Bacillus* clones (F & G) analysed had noticeably different
316 survival curves to both the *P. alcalifaciens* and to one-another (*Figure 8*). Clone
317 F showed a similar time to the *P. alcalifaciens* clones for 100% mortality to be

reached, however survival at earlier time points was generally lower than the *P. alcalifaciens* (60% after 10 hours for the 10^3 CFU treatment), suggesting higher virulence in the first few hours following inoculation. The 10^3 CFU treatment for Clone G did not reach 100% mortality within our experimental timeframe, which could possibly indicate that it was a different species of *Bacillus* to clone F. Clone H was the least virulent of the eight clones tested, with only the 10^7 CFU treatment reaching 100% mortality, while the 10^3 treatment caused none at all. This suggests it was a different species, or at least a different genotype, to all the other seven clones tested, but unfortunately it was not successfully sequenced. These results go some way to showing that there is consistency in virulence within the species found.

Galleria mortality curves were recorded for clones identified as *Aeromonas hydrophila, Providencia alcalifaciens,* and *Serratia liquefaciens*, following AMR profiling (*Figure 9*). One representative clone of each AMR resistance profile was chosen for inoculation, and only the 10^3 CFU treatment was used as this had shown the greatest difference between clones in the previous experiment. We compared the time taken to cause 100% mortality for each clone, finding that *A. hydrophila* was the most virulent. All *P. alcalifaciens* clones caused 100% mortality within 15-18hrs, while all *Serratia* did so within 17-20hrs, with the exception of one (G7R1.1). Analysing the survival data using the Kaplan-meier method provided further evidence that of the three species represented, *A. hydrophila* was the most virulent, followed by *P. alcalifaciens,* and *S. liquefaciens*. Intra-species variation was generally not significant (pairwise comparisons using log-rank test on survival fitted by Kaplan-meier method, $p>0.25$ for all comparisons), with the exception of one clone of *S. liquefaciens*, G7R1.1, which caused lower mortality (pairwise comparisons using log-rank test on survival fitted by Kaplan-meier method, $p<0.05$). G7R1.1 was subsequently sent for whole-genome-sequencing (results not included in this document) and identified as a singular isolate of *Serratia plymuthica*. Incidentally, our 16s sequencing also identified a single *S. plymuthica* (C92.1) from the other clones found (*Table 4*) which showed no virulence in its' mortality assay. These findings suggest that *S. plymuthica* may have lower virulence than *S. liquefaciens*, though we could find no existing literature to support this hypothesis, possibly due to the rarity of *S. plymuthica* infections. Finally a Cox proportional hazards model was used to further examine the differences between the survival curves of the three genera

tested, finding that *Aeromonas* was significantly more virulent than the others, while *Serratia* was the least virulent of the three (Cox proportional hazards model, n=208, 2d.f, p<0.001 between all genera).

The relevance of *Serratia* to human infection

Serratia species and *Serratia marcescens* in particular are known to be significant human pathogens (Mahlen, 2011). *S. marcescens* is a well-known opportunistic pathogen of the immunocompromised in hospitals, and although it generally displays low virulence it can cause UTI's, conjunctivitis, respiratory tract infections and bloodstream infections (Cristina, Sartini and Spagnolo, 2019) and was infamously used by the U.S. military as to experimentally test susceptibility to, and viability of, biological warfare attacks (Mahlen, 2011). Infections by *S. liquefaciens*, the bacterium most commonly found in our environmental samples, are relatively rare compared to *S. marcescens* infections, but are still the *"second most common Serratia species involved in human infections"* (Mahlen, 2011). Various reports have found *S. liquefaciens* in bacteremia, UTI's, endocarditis, meningoencephalitis and abscesses. Typically infections appear to be from an environmental source, although hospital acquired infections have been noted when equipment has become contaminated (Ikumapayi *et al.*, 2016). *S. liquefaciens* has even been noted to infect immunocompetent people (Gutiérrez-González, Peteiro and Toribio, 2014). Worryingly, *S. liquefaciens* has increasingly been noted as a cause of transfusion-related sepsis via contaminated blood, and fatality rates in such cases are high (Roth *et al.*, 2000). Because of its rarity an accurate diagnosis of *S. liquefaciens* can take significantly longer than other species, delaying effective treatment (Helvaci *et al.*, 2019). However if correctly diagnosed *S. liquefaciens* is usually susceptible to a wide variety of antibiotics (Traub, 2000; Stock, Grueger and Wiedemann, 2003; Samonis *et al.*, 2017), and the spread of infection is relatively easy to combat so long as adequate hygiene standards are maintained (Engelhart *et al.*, 2003). The clones we isolated from soil communities seem to be more susceptible to Azithromycin, Cefoxitin and Penicillin combination treatments than is generally reported, with the most common antibiotic profile found being susceptible to all antibiotics tested (*Table 5*). This could be due to a lack of resistance genes acquisition, or the loss of resistance genes due to selective pressure from the environment, and suggests that environmentally acquired *S. liquefaciens*

infections would be easily treatable. Our 16s results also suggest that *S. plymuthica* and *S. nematodiphila* were present, in our environmental samples. *S. plymuthica* has been noted as a human pathogen (Carrero *et al.*, 1995), albeit far more rarely than other *Serratia,* whereas *S. nematodiphila* is rare amongst the *Serratia* in that it has not yet been reported as a human pathogen (Erem *et al.*, 2019), instead being known to form symbioses with an entomopathogenic nematode (Zhang *et al.*, 2009). The presence of both of these species is therefore likely to pose a minimal threat to human health.

The relevance of *Providencia* to human infection

Providencia infections in humans seem to be relatively rare in general and are often nosocomially acquired. *Providencia* are opportunistic human pathogens, and infections are typically due to either *Providencia stuartii*, which can cause UTI's, or *Providencia rettgeri*, which is often implicated in cases of diarrhoea (Yoh *et al.*, 2005). *P. alcalifaciens*, which we found, seems to be less common, but is also a causative agent of diarrhoea (Haynes and Hawkey, 1989) and seems to often be acquired through contaminated food (Murata *et al.*, 2001; Shah *et al.*, 2015). *Providencia* spp. in general seem to be prone to acquiring resistance to antibiotics, particularly ESBL's (Overturf, Wilkins and Ressler, 1974; Tumbarello, 2004). The fact that *Providencia* can be a commensal gut bacterium also means that it's potential as a pathogen can be overlooked.

The relevance of *Aeromonas* to human infection

Aeromonas species have been emerging as human pathogens over the last few decades (Batra, Mathur and Misra, 2016). Typically they are strongly associated with water, and human infection occurs via ingestion of contaminated food sources such as fish and prawns (Wang and Silva, 1999; Vivekanandhan, Hatha and Lakshmanaperumalsamy, 2005; Praveen *et al.*, 2016). *Aeromonas* is most commonly recovered from the gastrointestinal tract and known to cause acute gastroenteritis, it is also often associated with diarrhoea, although doubts remain as to whether it is the causative agent in these cases (Janda and Abbott, 2010). Rarely, it has also been known to cause sepsis, meningitis and necrotizing soft tissue infections, which can potentially be fatal (Parras *et al.*, 1993; Lin and Cheng, 1998; Richards *et al.*, 2015). The fact that we only isolated a single clone of *A. hydrophila* from our environmental samples suggests that it is relatively rare

in the soil microbiome, however this sample was taken from close to a river, and could possibly be an indication that the water source itself was contaminated, potentially warranting further investigation. This site was also one of the heavy-metal contaminated sites, though it is unclear whether this has any bearing on the presence of *A. hydrophila.*

The relevance of *Enterococcus* to human infection

It is also interesting to note the occurrence of *Enterococcus* spp. and specifically *Enterococcus casseliflavus* in our environmental samples. *Galleria* injected with all identified clones of *Enterococcus* showed no mortality whatsoever, and while *Enterococcus* are relatively avirulent, they are known to be opportunistic pathogens in humans (Reid, Cockerill and Patel, 2001). Commonly found in UTI's, *Enterococcus* spp. also have the potential to cause potentially life-threatening diseases. In cases where *Enterococcus* infections lead to bacteremia the mortality rate can be between 26% and 46%, and from endocarditis can be between 9-15% (Agudelo Higuita and Huycke, 2014). Additionally, *Enterococcus* spp. typically display high levels of antibiotic resistance, with many strains being resistant to multiple drugs including Vancomycin (CDC, 2013). Thus the strains we detected could still potentially pose a threat to human health, and it is important to note that soil could be a potential source. It is unclear as to why there was no *Galleria* mortality, it is possible that the effects of *Enterococcus* spp. infection were not detectable in the time allowed by the experiment, or the conditions for optimal growth were not met. Indeed, it seems probable that detection of *Enterococcus* spp. from our environmental samples would not have happened if they had not co-occurred with other, detectably virulent pathogens. Alternatively, these species could suggest contamination from the natural microbiome of *G. mellonella*; *Enterococcus gallinarum / saccharolyticus* is known to be a common gut commensal of insects and has been found to be prevalent in *Galleria* (Allonsius *et al.*, 2019). Indeed, our phylogenetic analysis of *Enterococcus* (*Figure 15*) suggests that our clones are quite closely related to *E. gallinarum*, providing evidence to suggest contamination of our samples with internal gut flora.

The relevance of *Bacillus* to human infection

A number of virulent *Bacillus* were detected in our soil samples. We compared the 16s sequences we obtained to a number of *Bacillus* spp. (*Figure 12*). These phylogenetic analyses suggest that all of the *Bacillus* isolated that could not be identified to species level were members of the *Bacillus cereus* group. This could be a potential cause for concern, as some species in that group are highly pathogenic. *B. cereus sensu stricto* is a known human pathogen, often responsible for food poisoning and known to cause vomiting and diarrhoea (Granum and Lund, 2006). *B. anthracis* is closely related to *B. cereus*, and is an extremely potent human pathogen that has been used as a bioweapon (Spencer, 2003). *B. thuringiensis* (or Bt) is a potent entomopathogen, and is often used as a biological pesticide due to the Cry toxins it produces. The effects of Bt in mammals are still the subject of some debate (Rubio-Infante and Moreno-Fierros, 2016). The main differences between these species are the presence of plasmid-borne toxins in both *B. anthracis* and *B. thuringiensis* (Ivanova *et al.*, 2003). It would be wise for future studies to clarify whether the clones we isolated are one of these species, or one of the less harmful members of the *B. cereus* group.

Potential risk factors for infection

While we detected the presence of the aforementioned pathogen species, they do not directly pose a threat to human health while contained within soil. However there are many potential ways in which these pathogens could infect a human host and cause disease. Here we briefly mention some potential routes to infection.

One potential avenue for infection has already received a fair amount of study, that of infected food crops. Several studies have found examples of bacterial species mentioned in *Table 1* in a variety of plant rhizospheres including oilseed rape, potatoes, maize, wheat and rice (Berg, Eberl and Hartmann, 2005). Pathogens such as these can be taken in through plants roots and internalised, particularly in unhealthy or damaged plants (Hirneisen, Sharma and Kniel, 2012). Fruit and vegetables can then serve as a vector for important pathogens such as *E. coli* and *Salmonella* (Berger *et al.*, 2010).

Another source of infection includes gardening and farming accidents which often happens as a result of skin penetration by splinters, thorns or tools. Such

accidents can cause deep tissue wounds, contaminated by soil or faecal matter, and are known to be a source of tetanus, caused by the bacterium *Clostridium tetani* (Baker *et al.*, 2009). While *C. tetani* is a dangerous pathogen, infections can generally be avoided through adequate precautions such as protective gloves and vaccination.

Geophagia (the direct ingestion of soil) is also a significant route of infection. It is often associated with unhygienic children (Shivoga and Moturi, 2009; Gotkowska-Płachta and Korzeniewska, 2015) who unwittingly intake soil matter, but it can also result from less obvious situations such as outdoor sports. Indeed there are anecdotal reports of large groups of people being infected by accidental soil ingestion at muddy events such as motorcycle races and obstacle courses (*The Telegraph*, 2015). Geophagia can also be due to mental illness or in certain cultures it can actually be a deliberate traditional practice (Woywodt and Kiss, 2002).

A more indirect route of infections can be found in the leaching of soil pathogens into other environments such as water systems and even the air. Depending on the use and management of the land, soil can slowly be damaged and eroded by a number of factors including irrigation, evaporation, wind and agricultural practices (Wall, Nielsen and Six, 2015). In this way soil pathogens could spread to and contaminate other environmental reservoirs. Appropriately managing land use to avoid this erosion is important to prevent such contamination (Wall, Nielsen and Six, 2015)

Comments on the *Galleria mellonella* virulence model

This study is consistent with Hernandez *et al.* (2019) in showing that the *Galleria* model can be used to selectively isolate and enrich pathogens from the soil, and identify novel pathogens that would be missed by more traditional techniques. The *Galleria* model is still relatively new compared to traditional mammalian model systems, and is still being developed in some major areas. For example the immune system of *Galleria* is still being studied in order to further understand it (Pereira *et al.*, 2018). On a practical level, while *Galleria* can be bought in bulk from external suppliers for relatively little expense, this does come with a trade-off: there could potentially be issues with the consistency of studies when using *Galleria* acquired from outside sources. Suppliers do not breed the larvae under

standardised conditions, and often use antibiotics or preservative agents to keep the *Galleria* fresh and sterile, which may bias the results of experiments using them (Allonsius *et al.*, 2019). To remedy this, efforts are being made to create lab-reared strains with consistent breeding conditions and without using any antibiotics or preservatives in the process such as TruLarv™ (*Bio Systems Technology*), although this is currently an expensive option for high - throughput experiments such as virulence assays. There is also a lack of standardised experimental conditions and procedures between individual research labs that needs to be addressed to improve consistency of studies (Tsai, Loh and Proft, 2016). More research is also needed into the microbiome and genetics of *Galleria* to understand them and the microbes that they carry more thoroughly.

Limitations of the project

It is important to note the limitations of such a study to clarify any misconceptions and lay the groundwork for future experiments. One important element to mention is that the numbers of each species detected do not accurately correspond to the proportions in which they would naturally exist within the soil. This was never an intended outcome of our study, instead being simply to detect and characterise some of the species present. Due to the way in which we performed hemocoel extractions, isolating each morphology at least once, many of the clones isolated were likely replicates of one another. This means that data such as that presented in *Figure 11* is simply a representation of the frequency of species successfully recovered, and not the original frequencies. Future studies seeking to determine these relative frequencies would benefit from a more systematic approach to pathogen isolation, or simply performing metagenomics analysis on whole-community samples.

Additionally, some pathogenic species may simply not have been successfully extracted from the soil samples. Our method for extracting the soil communities may have left some microbes still attached to soil particles, potentially omitting some species from our analysis. It is also interesting to note the complete absence of any species of fungi from our samples. Soil, and the rhizosphere in particular, certainly contains many fungal species (Frac *et al.*, 2018), so this seems somewhat anomalous. One explanation for this could be the type of growth media used; while LB is able to culture a wide range of microbial species, there are also many which do not grow on it. The range of bacterial species found

would also almost certainly vary depending on the growth media, and future experiments would do well to use several media types for this reason.

It is important to clarify that, our method of selecting microbial communities and clones causing >50% *Galleria* mortality for further study was an arbitrary cut-off point, used simply for the purpose of isolating and identifying the most virulent pathogens. This is not to say that communities and clones displaying less virulence were not dangerous. Those causing less virulence in our assays could still have severe detrimental effects, and future studies may wish to investigate this further. For example, *Enterococcus* species are known human pathogens (Reid, Cockerill and Patel, 2001), and yet in our assays no *Galleria* mortality was recorded from any of the *Enterococcus* found. One possible explanation for this is that slow-acting pathogens would likely not have been found within the timescales we used. It is likely that assays run over a greater amount of time would be able to detect a greater range of pathogens for this reason.

It is also unfortunate that some of our 16s sequences were of poor quality, several clones could not be identified due to the quality of their reads, either due to being too short or having too many uncertain bases in the sequence. Of those that were successfully sequenced, three could not be accepted for accessioning by GenBank – two were too short and one was determined to contain chimeric sequences. These issues could be due to a number of factors including our handling of the DNA extractions and amplifications, errors in the sequencing itself, or something that happened in transit. It is possible that under different circumstances we could have improved sequence quality, and thus have been able to identify our clones with a greater degree of accuracy.

Concluding remarks

In this study we detected pathogenic bacteria very frequently across all the soil types and locations sampled. The vast majority of these bacteria were also found to be highly virulent when isolated from their soil communities. The species detected include several known human pathogens of clinical relevance, including *S. liquefaciens*, *P. alcalifaciens*, *A. hydrophila* and species in the *B. cereus* group. The extreme biodiversity of soil environments suggests that samples taken from other soil types and locations would vary greatly in the diversity of pathogen species, and it would be interesting to determine whether the species we found

584 are a localised phenomenon or typical of soil environments in general. We hope
585 that future experiments will be able to clarify this. While this study cannot hope to
586 provide a comprehensive overview of all the pathogenic species present in soil,
587 it provides a small glimpse at the pathogenic potential of species in the soil
588 microbiome, which thus far have been relatively unknown. We hope that this
589 highlights the needs for further studies in this area and helps in building a more
590 complete understanding of the soil as a reservoir for pathogenic species.

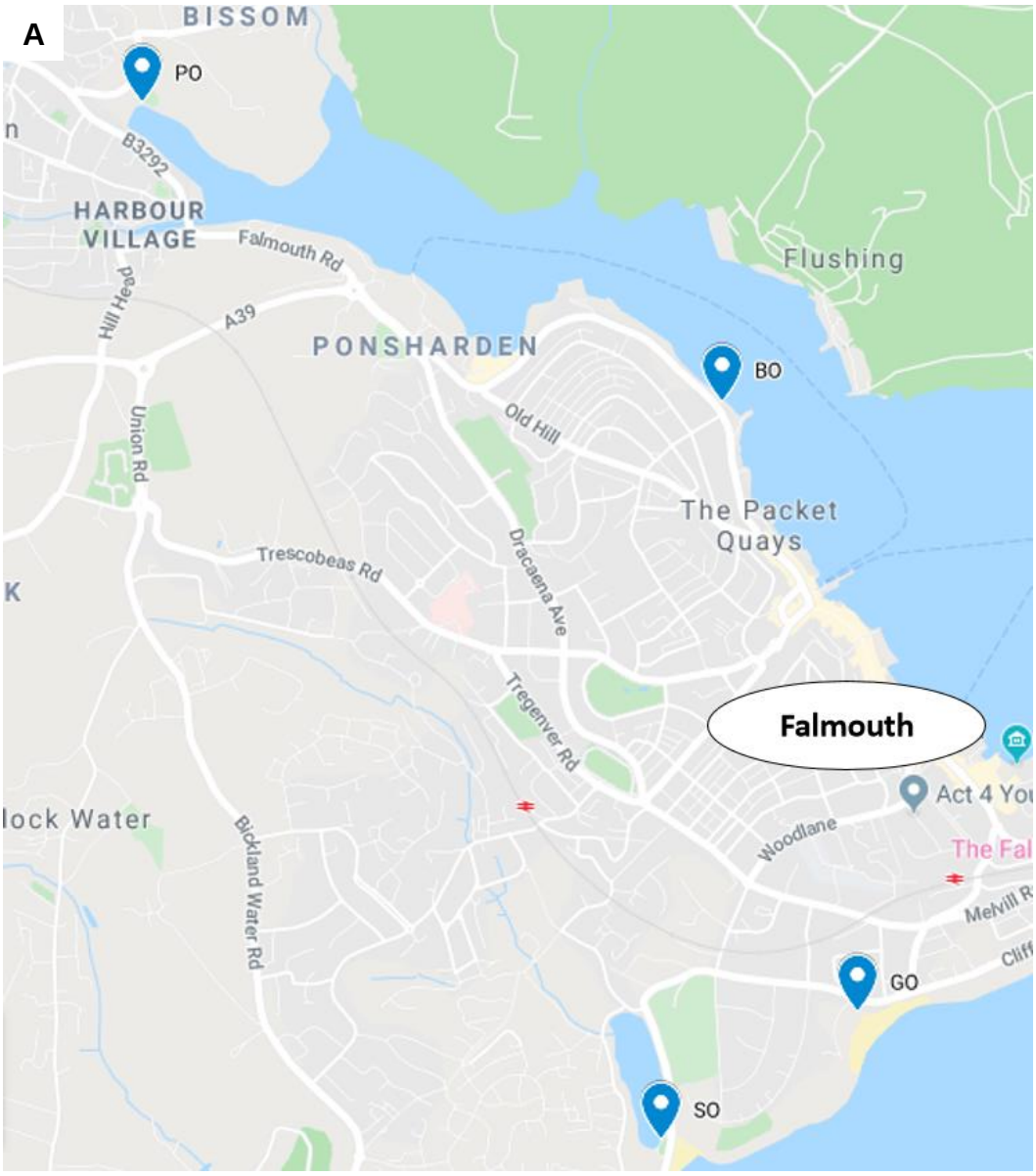
Supplementary material

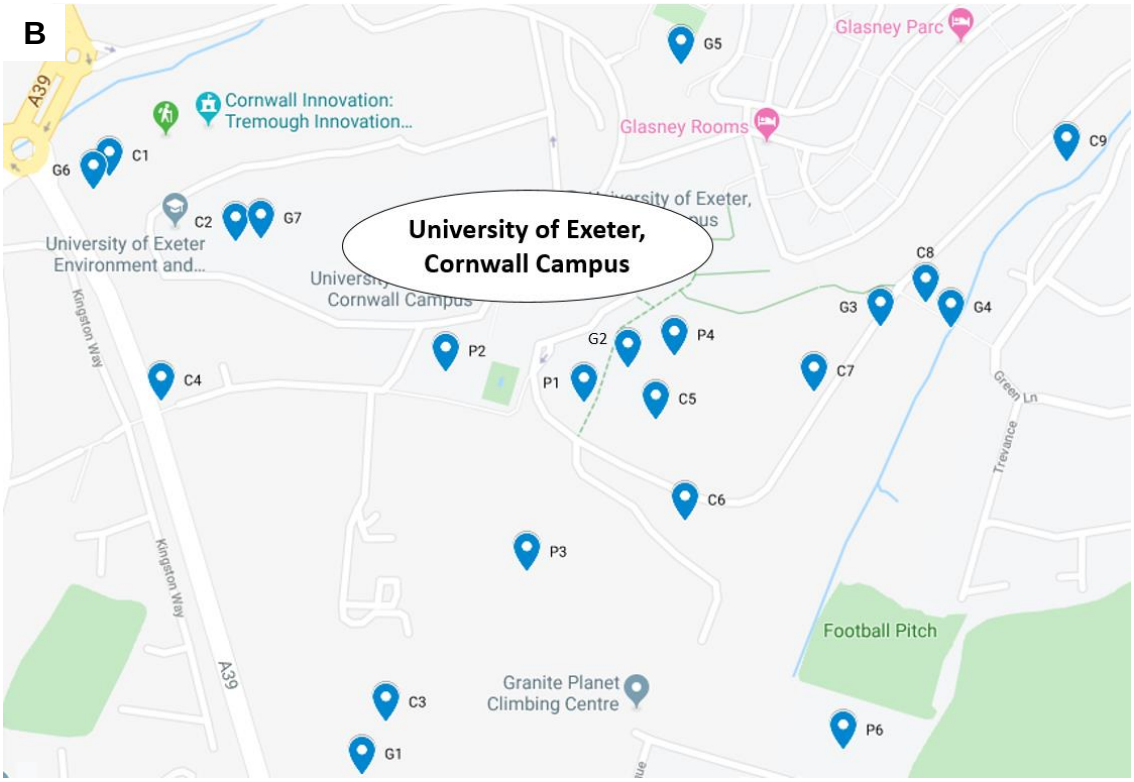
Table S1: Sampling Sites

Sampling date	Sample name	Coordinates	Location description	Location type
29/08/2018	GO	50.14386, -5.06939	Patch of grass near Gyllingvase beach	Grass
	BO	50.16017, -5.07415	Grass verge near Greenbank hotel	Grass
	SO	50.13619, -5.07767	Coastal path near Swanpool beach	Grass
	PO	50.16961, -5.09872	Close to Penryn estuary	Grass
17/09/2018	P1	50.16987, -5.1237	Woods in front of Tremough House	Tree
	P2	50.17003, -5.12522	Tremough House gardens	Grass
	P3	50.16922, -5.12468	Vegetable garden near the Performance Centre	Grass
	P4	50.17028, -5.12323	Open field in front of Tremough House gardens	Grass
	P5	50.17238, -5.11719	Sunken garden by pedestrian entrance to campus	Grass
	P6	50.16804, -5.12098	Sports field behind industrial estate	Grass
10/09/2018	C1	50.17131, -5.12859	Unused grass plot next to Tremough Innovation Centre	Grass
	C2	50.17076, -5.12716	Beneath the Eucalyptus in front of the Environment and Sustainability Institute	Tree
	C3	50.16736, -5.12592	Open field close to the campus Sports Centre parking lot	Grass
	C4	50.16985, -5.12781	Old Cornish stone wall by the A39 entrance to campus	Unclassified*
	C5	50.16985, -5.12329	Wood in front of Tremough House	Tree
	C6	50.16922, -5.12307	Forested area next to the Performance Centre	Tree
	C7	50.16999, -5.12126	Grass verge near main path, close to trees	Tree
	C8	50.17055, -5.12027	Grass verge at path intersection	Grass
	C9	50.1715, -5.11858	Grass verge close to main path through campus	Grass
22/10/2018	G1R&S	50.16766, -5.12574	Open field close to the campus Sports Centre parking lot	Grass
	G2R&S	50.16977, -5.12286	Forested area in front of Tremough House	Tree
	G3R&S	50.17033, -5.12053	Grass verge by main path	Grass
	G4R&S	50.17035, -5.11996	Footpath over small stream	Grass
	G5R&S	50.17209, -5.12262	Campus playing field	Grass
23/10/2018	G6R&S	50.17131, -5.12859	Unused grass plot next to Tremough Innovation Centre	Grass
	G7R&S	50.17093, -5.12698	In front of the Environment and Sustainability Institute	Grass
05/11/2018	A1	50.23242, -5.13296	Wheal Jane river bank	Metal-contaminated

A2	50.23242, -5.13297	Wheal Jane river bank	Metal-contaminated
A3	50.22996, -5.12738	Wheal Jane	Metal-contaminated
A4	50.22996, -5.12713	Wheal Jane	Metal-contaminated
A5	50.23013, -5.12725	Wheal Jane	Metal-contaminated
A6	50.23009, -5.12706	Wheal Jane	Metal-contaminated
A7	50.23022, -5.12713	Wheal Jane	Metal-contaminated

Exact locations of each sampling site as determined via GPS coordinates, and a brief description of the site. Each site was categorised into one of three types based on the area: areas of grass, tree roots, or heavy-metal contamination. *Site C4 could not be categorised as any of these and was omitted from analyses using these categories.

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A39
Cornwall Innovation:
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G6
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C2
G7
University of Exeter
Environment and...
University of Exeter,
Cornwall Campus
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C4
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G2
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P1
C5
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Glasney Parc
Glasney Rooms
C9
C8
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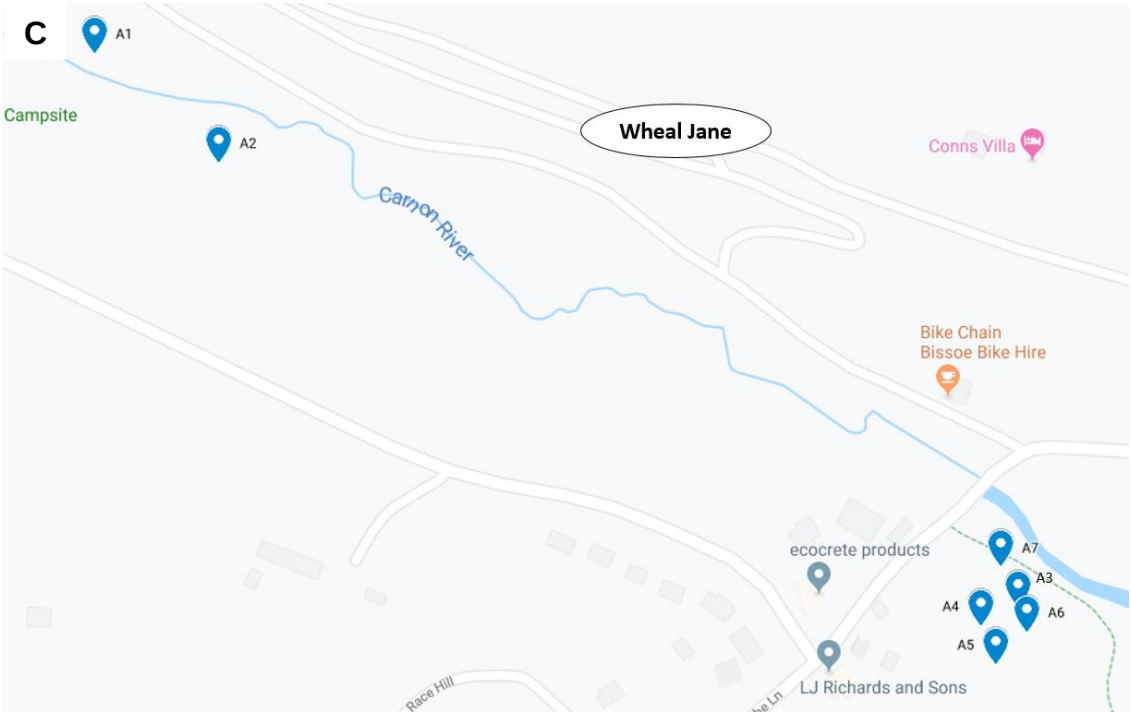
G4
C7
Green Ln
Trevance
C6
P3
Football Pitch
Granite Planet
Climbing Centre
C3
G1
P6

600


C
A1
Campsite
A2
Wheal Jane
Conns Villa
Carnon River
Bike Chain
Bissoe Bike Hire
ecocrete products
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A3
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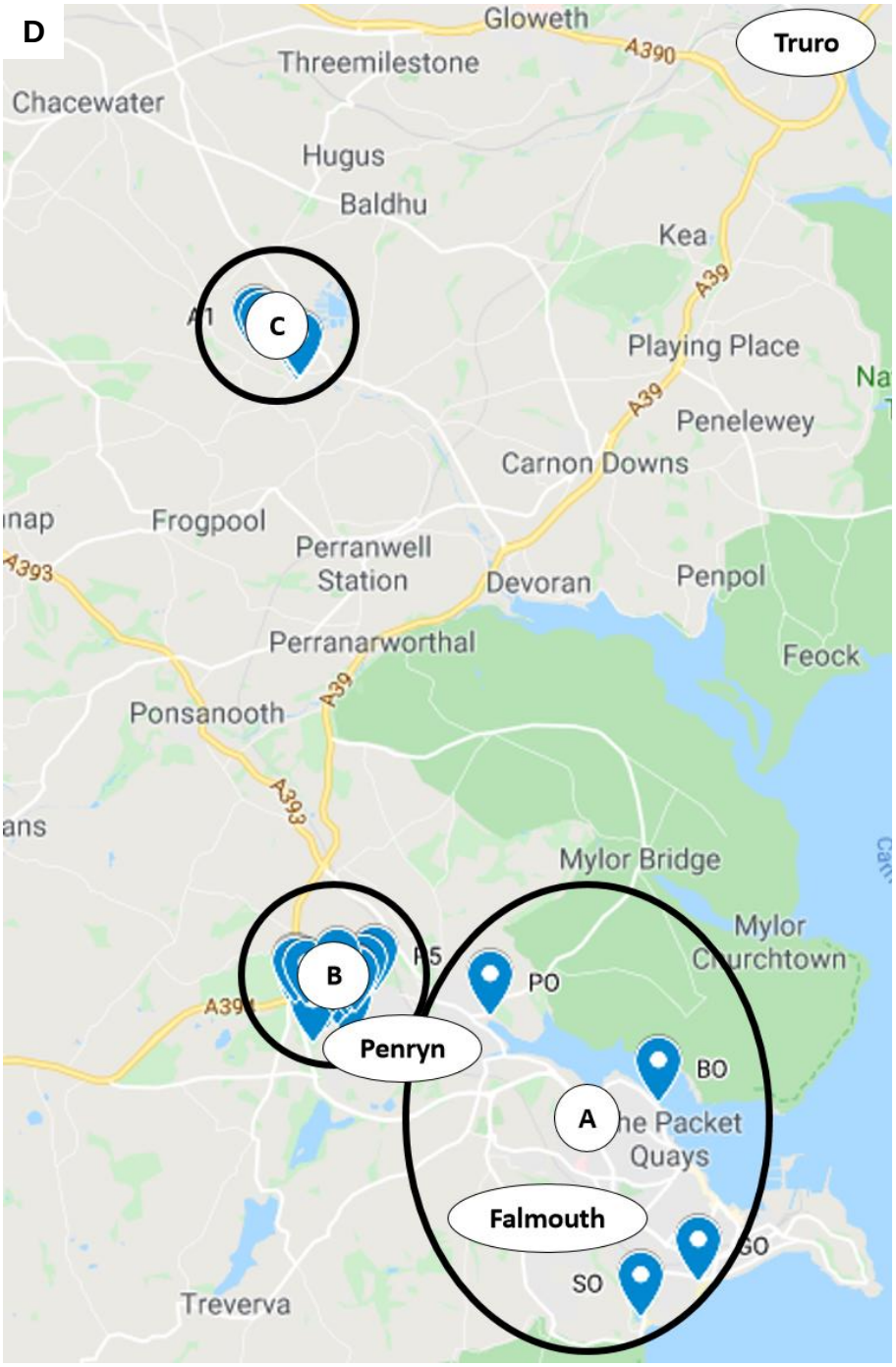
Race Hill
LJ Richards and Sons

601



602

603 **Figure S1: Maps of sampling sites.** Maps indicating the locations of our
604 sampling sites. A) Falmouth, Cornwall, UK. B) University of Exeter, Cornwall
605 Campus, Penryn, UK. C) Wheal Jane, Cornwall, UK. Map D shows where maps
606 A, B, and C are located in a wider context. Major towns and cities are highlighted.
607 Original images c/o Google Maps 2019 (maps.google.com).

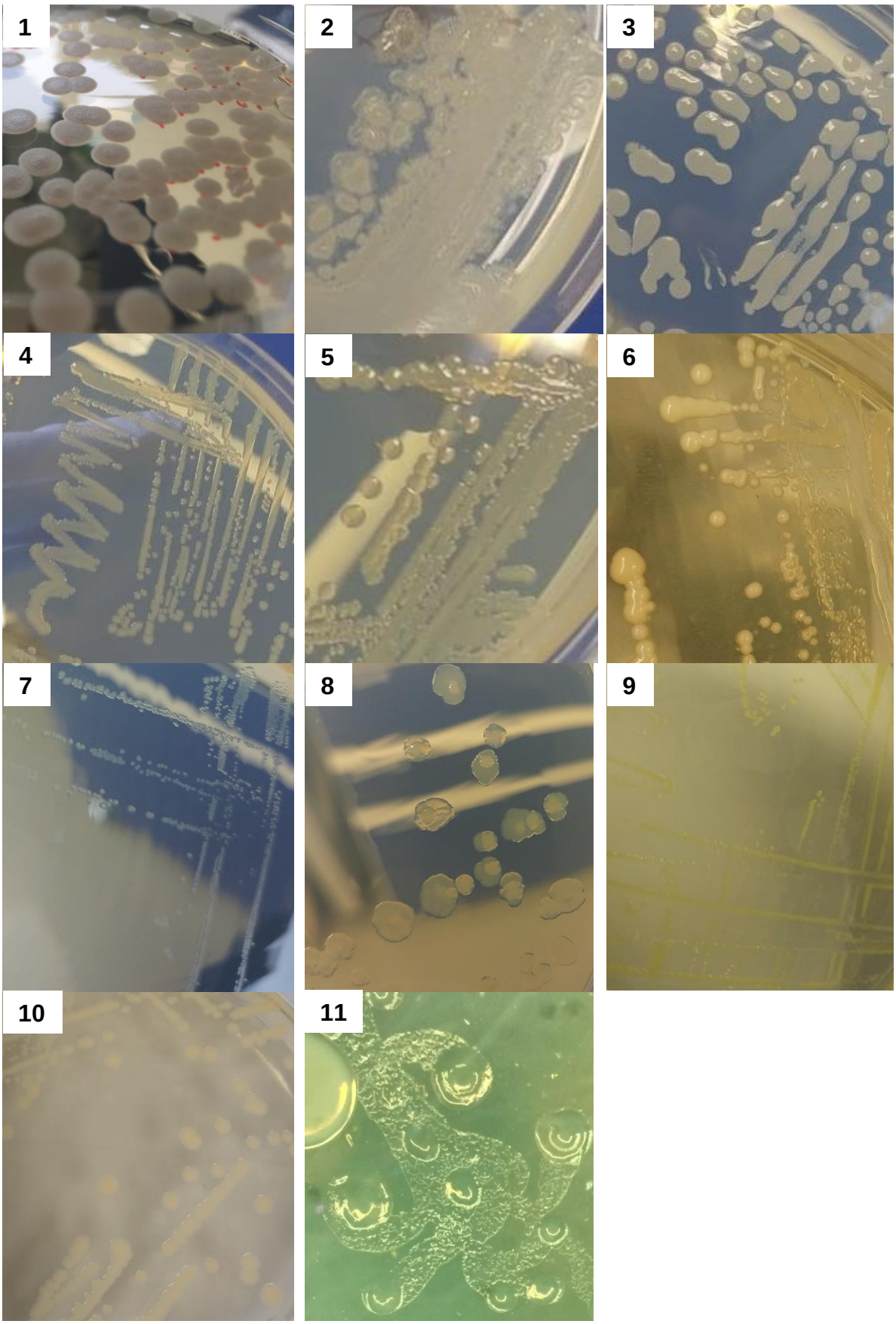


Figure S2: Images and descriptions of clone morphologies. Microbial colonies isolated from soil samples using *Galleria* hemocoel extraction. Eleven

visually distinct colony morphologies were found. A brief description of each follows.

1 – Wide, flat, slightly ‘fuzzy’ colonies. Sequencing determined that these were typically species of *Bacillus.*

2 – Hard, closely-spaced, ‘wrinkly’ colonies. Found to be either *Bacillus lichenformis*, *Providencia alcalifaciens*, or another undetermined Bacillus species.

3 – ‘Blobby’ colonies with a wet appearance, slightly whiter than other morphs. Only 2 examples of this were found and they were not chosen for sequencing.

4 and 5 – White, round colonies. While initially we seperated 4 and 5 based on slight variations in size, later platings showed that the two were in fact indistinguishable from one another and thus were merged. These were the most common colony morphology and were typically *Providencia alcalifaciens*.

6 – Round colonies with a distinct whitish colour. We found that they were *Serratia* species.

7 – Very small round colonies, somewhat transparent. Isolates caused no mortality. Determined to be *Enterrococcus casseliflavus*.

8 – ‘Egg-shaped’ colonies with a distinct two-tone colour. These were *Serratia nematodiphila*.

9 – Very small round colonies, quite similar to #7 except for colouration. Unfortunately 16s sequencing results were poor and this morph could not be identified.

10 – Large, round, white colonies. Somewhat similar to #4/5, except noticably larger and more opaque. Found only once and identified as *Aeromonas hydrophila*.

11 – a singular isolate that ‘swarmed’ across the plate. Exclusively found alongside the large white colony shown, and inseperable from it. Sequencing revealed it to be *Paenibacillus latus*.

Table S2: Morphology of each clone

Morphology number	Clones displaying this morphology
1	P11.3, P11.4, P11.5, P12.1, P12.3, P12.4, P13.1, P54.1, C31.1, C31.2, C72.1, C72.3, G3S1.3, G5R1.2, A72.2
2	P11.2, P12.2, A12.2
3	P54.3, SO1.2
4-5	P11.1, P13.2, P14.1, P51.1, P51.2, P51.3, P52.1, P53.1, P54.2, GO1.1, GO1.2, GO1.3, GO1.4, C11.1, C11.2, C12.1, C12.2, C13.1, C13.2, C32.1, C32.2, C51.1, C51.2, C52.1, C52.2, C53.1, C53.2, C71.1, C71.2, C72.2, C81.1, C81.2, C82.1, C82.2, C83.1, C83.2, C91.2, C92.1, C92.2, G1S1.1, G1S1.2, G1R1.1, G1R1.2, G2S2.1, G2S2.2, G2R1.1, G2R1.2, G2R2.1, G2R2.2, G3S1.1, G3S1.2, G3S2.1, G3S2.2, G3R1.1, G3R1.2, G3R2.1, G3R2.2, G4S1.1, G4S1.2, G4S2.1, G4S2.2, G4R1.1, G4R1.2, G4R2.1, G4R2.2, G5R1.1, G5R1.4, G5R2.1, G5R2.2, G5R3.1, G5R3.2, G5R4.1, G5R4.2, G6R1.1, G6R1.3, G6R2.1, G6R2.2, G6R3.1, G6R3.2, G6R3.3, G6S1.1, G6S2.2, G6S3.1, G6S3.2, G7R1.2, G7R1.3, G7S1.1, G7S1.2, G7S2.1, G7S2.2, G7S3.1, G7S3.2, SO1.1, A11.1, A11.2, A12.1, A13.1, A13.2, A61.1, A62.1, A63.1, A71.1, A71.2, A71.4, A72.1
6	G7R1.1, G7R2.1, G7R2.2, SO1.1/6, A12.3, A21.1, A21.2, A22.1, A22.2, A63.2, A72.3, A73.1, A73.2, A73.3
7	C83.3, C91.1, C91.3, A62.2
8	G2S1.1, G2S1.2
9	G6S2.4
10	A61.2
11	A71.3

Each clone isolated from our soil communities was categorised based on the morphologies found in *Figure S2.* Here we indicate which morphology each of our clones displayed.

Table S3: Soil community mortality rates

Sample name	GO	BO	SO	PO	P1	P2	P3	P4	P5	P6
Galleria mortality / 10	9	1	7	9	9	3	7	2	9	4

Sample name	C1	C2	C3	C4	C5	C6	C7	C8	C9	G1R
Galleria mortality / 10	10	5	6	1	10	3	9	7	8	10

Sample name	G2R	G3R	G4R	G5R	G6R	G7R	G1S	G2S	G3S	G4S
Galleria mortality / 10	10	10	10	8	10	10	10	9	7	9

Sample name	G5S	G6S	G7S	A1	A2	A3	A4	A5	A6	A7
Galleria mortality / 10	3	10	8	10	8	2	5	1	10	9

Mortality of ten *Galleria* following injection with 10µl of the microbial community extracted from each soil sample and incubation at 37°C for 24hrs.

Table S4: Numbers of clones isolated from each hemocoel extraction

Sampling site	Hemocoel extraction	Total clones isolated	Clones causing >50% mortality
P1	1	5	3
	2	4	2
	3	2	2
	4	1	1
P5	1	3	3
	2	1	1
	3	1	1
	4	3	2
GO	1	4	0
SO	1	2	2
C1	1	2	2
	2	2	2
	3	2	2
C3	1	2	2
	2	2	2
C5	1	2	2
	2	2	0
	3	2	0
C7	1	2	0
	2	3	2
C8	1	2	1
	2	2	1
	3	3	1
C9	1	3	1
	2	2	0
G1S	1	2	2
G1R	1	2	2
G2S	1	2	2
	2	2	2
G2R	1	2	2
	2	2	2
G3S	1	3	3
	2	2	2
G3R	1	2	2
	2	2	2
G4S	1	2	2
	2	2	2
G4R	1	2	2
	2	2	2
G5R	1	4	4
	2	2	2
	3	2	2
	4	2	2
G6R	1	3	1
	2	2	1
	3	4	2
G6S	1	2	1
	2	4	3
	3	2	1
G7R	1	3	2
	2	2	2
G7S	1	2	2
	2	2	2
	3	2	2
A1	1	2	2
	2	3	2
	3	2	2
A2	1	2	2
	2	2	2
A6	1	2	2
	2	2	1
	3	2	2
A7	1	4	2
	2	3	2
	3	3	3

The exact number of clones isolated from each site and hemocoel extraction. Where possible we performed multiple hemocoel extractions for each sampling site to detect as many pathogens as possible. Similarly, we aimed to isolate at least one of every morphology found in each hemocoel extraction. Also indicated is the number of clones from each site and extraction which were later found to cause >50% *Galleria* mortality following injection of 10^5 CFU of each clone, and incubation at 37°C for 12hrs.

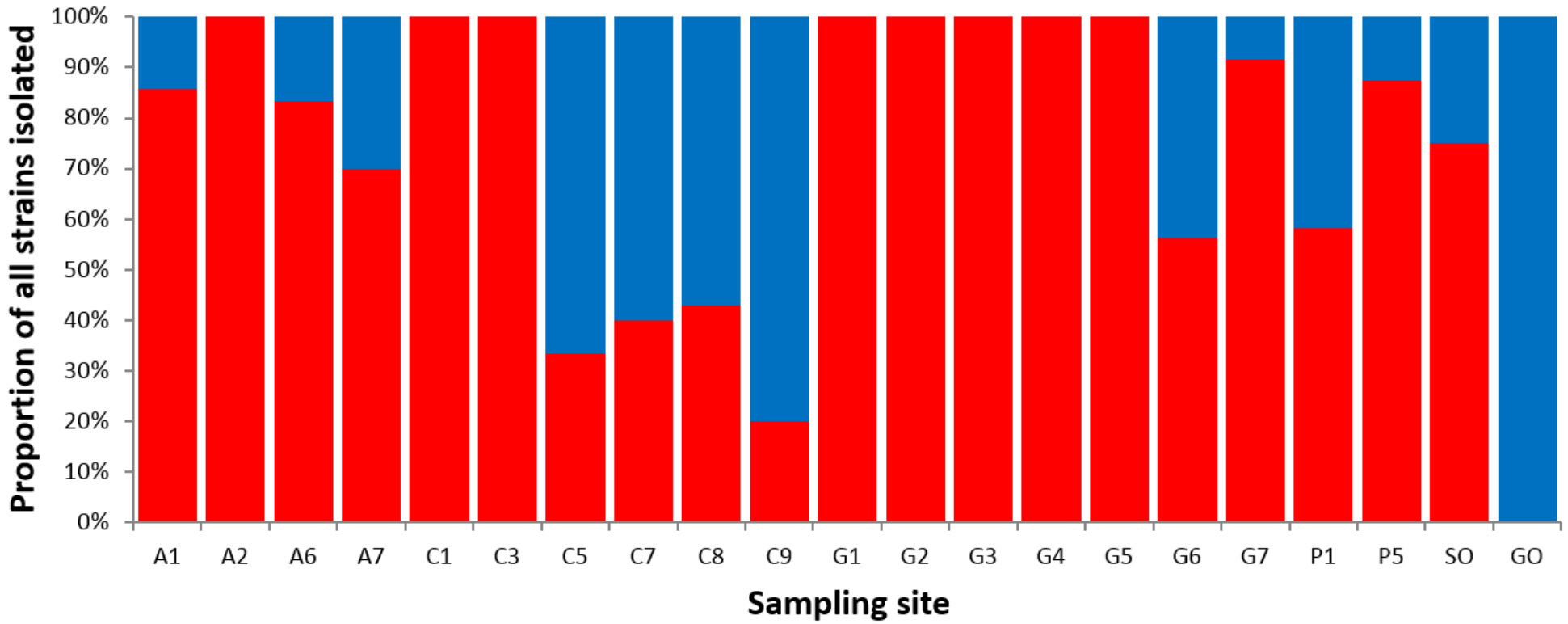


Figure S3: Proportional number of clones isolated from each sampling site. Clones were isolated from *Galleria* hemocoel from each soil community that caused >50% *Galleria* mortality (Figure 3). Indicated in red are the proportion of clones found to cause >50% *Galleria* mortality following injection of 10^5 CFU of each clone, and incubation at 37°C for 12hrs. Those in blue caused ≤50% mortality. This data is further represented in *Figure 7* and *Table S4*.

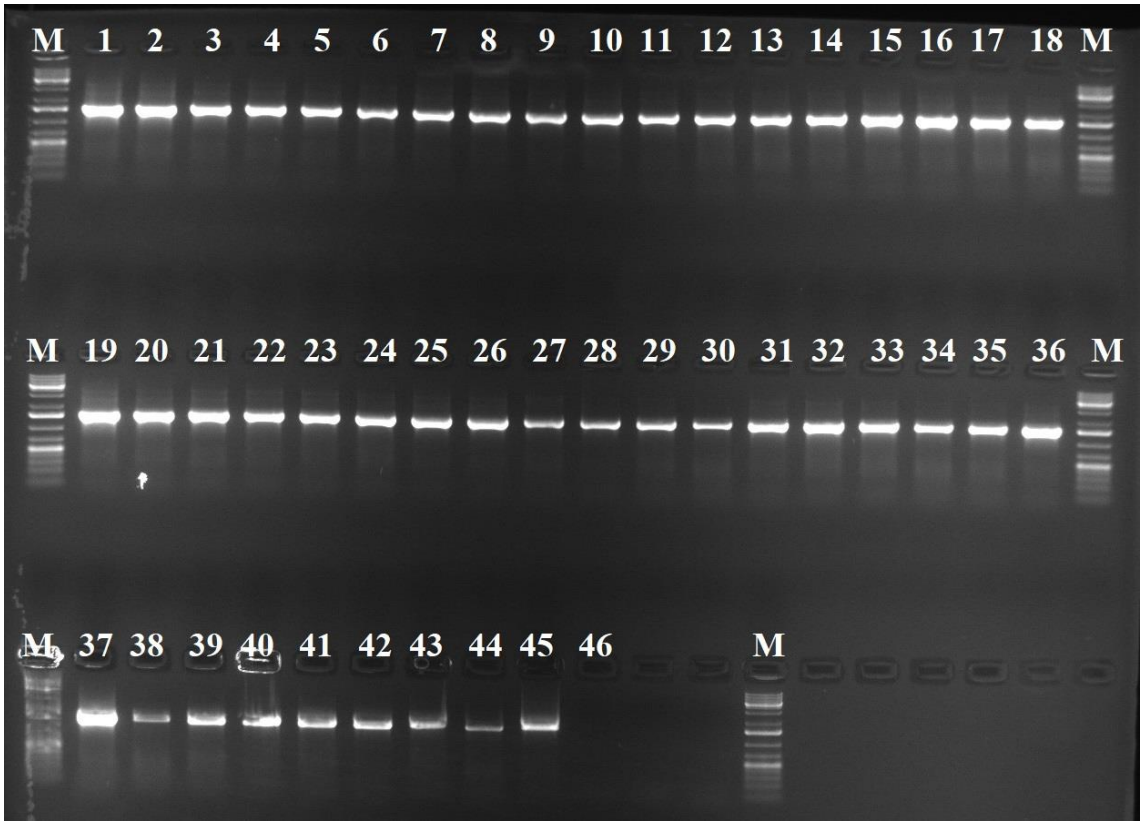
661 Table S5: Non-accessioned 16s sequences

Clone name	Reason not accepted	Trimmed 16s sequence used
A12.2	Too short	AGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAG ACTGGGATAACTCCGGGAAACCGGGGCTAATAC- CGGATGCTTGATTGAACCGCATGGTTCA
A62.2	Too short	AGTCGAACGCTTTTTCTTTCCCGGAGCTTGCTCNCCGAAAGANA AAGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCAT CAGAAGGGGATA
P12.1	Contains chimeric sequences	GGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACC GCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCATGTA GGCGAGTTGCAGCCTACAATCCGAACTGAGAACGGTTTTATGAG ATTAGCTCCACCTCGCGGTCTTGCAGCTCTTTGTACCGTCCATT GTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGA CGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTA GAGTGCCCAACTTAATGATGGCAACTAAGATCAAGGGTTGCGCT CGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGA CAACCATGCACCACCTGTCACTCTGCTCCCGAAGGAGAAGCCC TATCTCTAGGGTTTTCAGAGGATNNNAAGACCTGGTAAGGTTCT TCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGG GCCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCGGCCGTACTCC CCAGGCGGAGTGCTTAATGCGTTAACTTCAGCACTAAAGGGCG GAAACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGGACTA CCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCA GTGTCAGTTACANNCCAGAAAGTCGCCTTCGCCACTGGTGTTCC TCCATATCTCTACGCATTTCACCGCTACACATGGAATTCCACTTT CCTCTTCTGCACTCAAGTCTCCCAGTTTCCAATGACCCTCCACG GTTGAGCCGTGGGCTTTCACATCAGACTTAAGAAACCACCTGCG CGCGCTTTACGCCCAATAATTCCGGATAAC

662 These sequences were not accepted to the NCBI GenBank for the reasons
663 described above. All other 16s sequences used were accessioned, their
664 accession numbers can be found in *Table 5.*

Figure S4: Agarose gel electrophoresis images

Agarose gels used to check the successful amplification of 16s and pfs gene targets following PCR. Reagents, amplification cycles and gel electrophoresis protocol are as stated in the methods section unless otherwise indicated.



Checking for amplification of 16s DNA following PCR (20/11/2018). Sequences are from the following clones:

Reference no.	1	2	3	4	5	6	7	8
Source clone	P11.1	P11.2	P12.1	P12.2	P13.1	P13.2	P14.1	P51.1

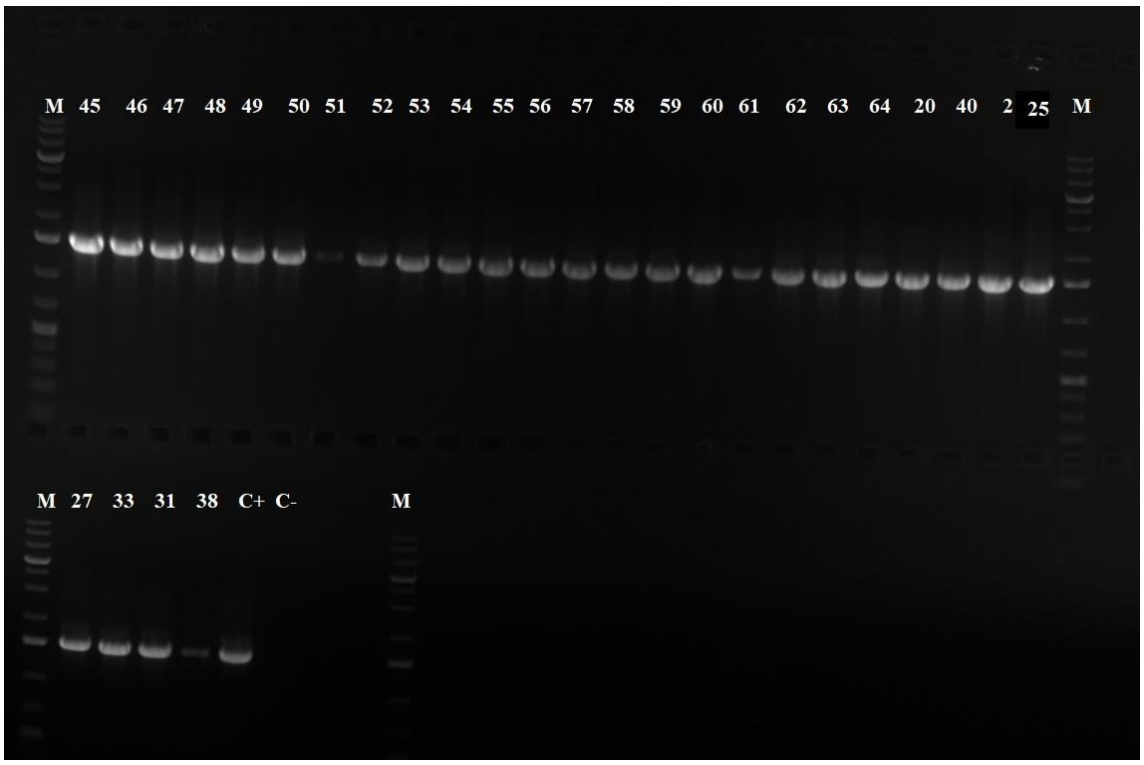
Reference no.	9	10	11	12	13	14	15	16
Source clone	P51.2	P53.1	P54.1	C11.2	C12.2	C13.1	C31.1	C31.2

Reference no.	17	18	19	20	21	22	23	24
Source clone	C32.1	C51.1	P54.2	C72.1	C72.3	C81.1	C81.2	C82.1

Reference no.	25	26	27	28	29	30	31	32
Source clone	C83.1	C83.2	C91.2	C91.3	G1R1.1	G1S1.2	G2R1.2	G2S1.1

Reference no.	33	34	35	36	37	38	39	40
Source clone	G2S2.2	G3R1.1	G3S1.1	G3S1.3	G4R1.1	G4S1.1	G5R1.1	G5R3.2

Reference no.	41	42	43	44	45	46
Source clone	G5R4.2	G7R1.1	G7R2.2	G7S1.2	E. coli	C-



673

674 Checking for amplification of 16s DNA following PCR (23/12/2018). Sequences
675 are from the following clones:

Reference no.	45	46	47	48	49	50	51
Source clone	P52.1	C13.2	C82.2	G5R1.2	G5R1.3	G5R2.1	G6R1.2

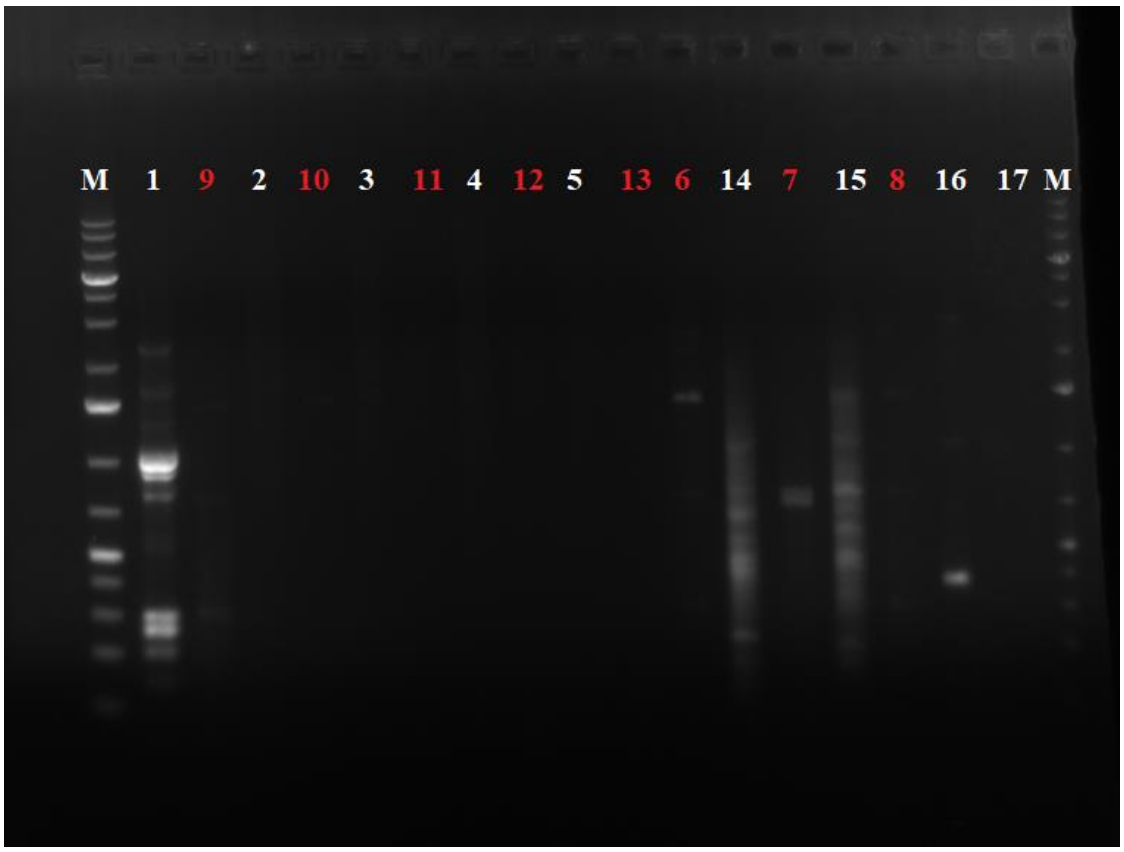
Reference no.	52	53	54	55	56	57	58
Source clone	G6R3.4	G6S2.1	A83	G7R1.3	SO1.1/6	A11.2	A12.3

Reference no.	59	60	61	62	63	64	20
Source clone	A21.2	A61.2	A63.2	A71.4	A72.3	A73.3	C72.1

Reference no.	40	2	25	27	33	31	38
Source clone	G5R3.2	P11.2	C83.1	C91.2	G2S2.2	G2R1.2	G4S1.1

Reference no.	65	66
Source clone	C+	C-

676



677

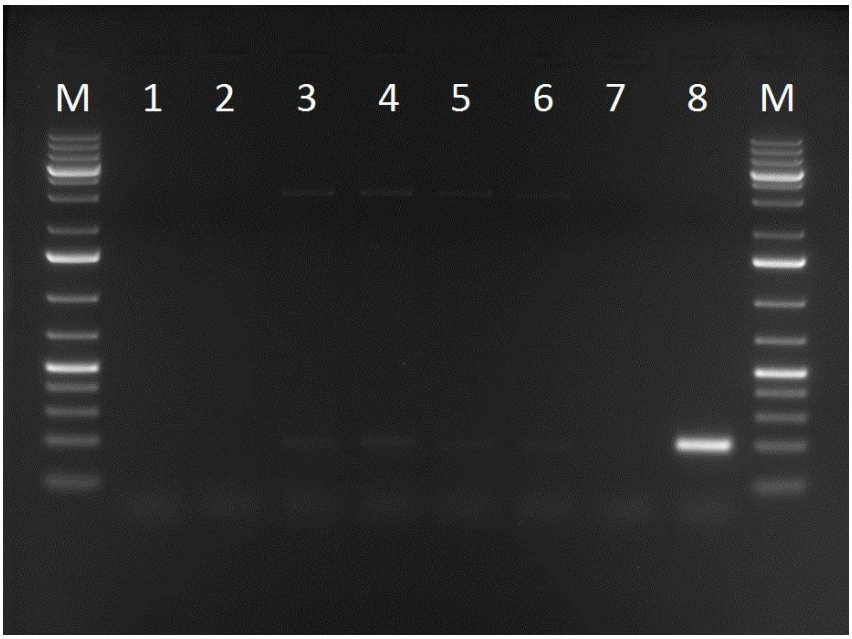
678 Checking for amplification of *Serratia* DNA following PCR using *Serratia*-specific
679 pfs primer (12/3/2019). Sequences are from the following clones:

Reference no.	1	2	3	4	5	6	7
Source clone	A61.2	C83.1	P11.1	P51.2	G4R1.2	A11.2	C12.2

Reference no.	8	9	10	11	12	13	14
Source clone	C51.1	G1S1.2	G7R1.1	P12.1	C31.2	C91.3	Coli

Reference no.	15	16	17
Source clone	P3	G3	-

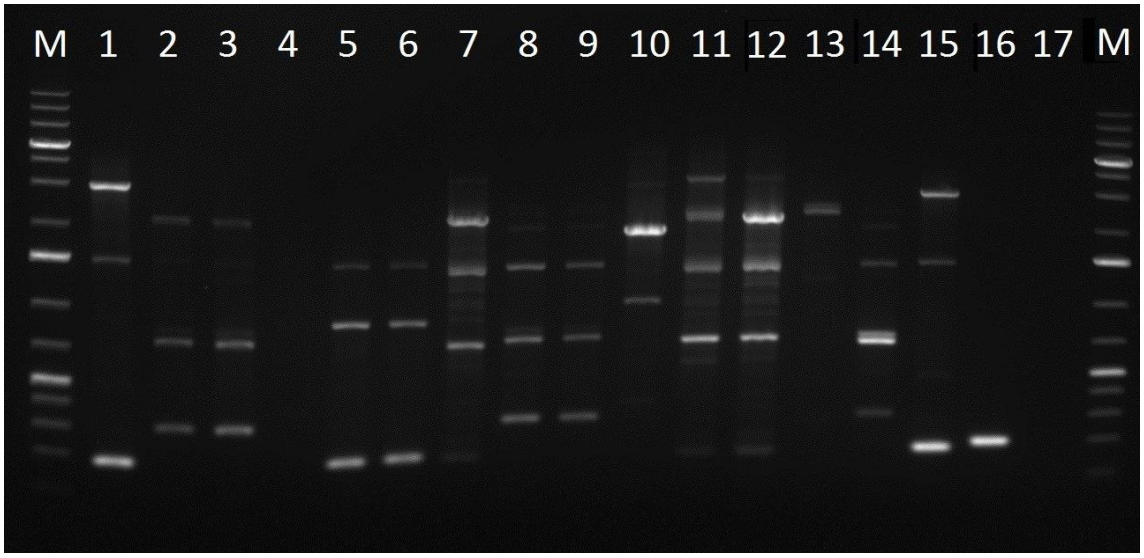
680



681

Checking for amplification of *Serratia* DNA following PCR using *Serratia*-specific pfs primer (26/3/2019). Sequences are from the following clones:

Reference no.	1	2	3	4	5	6	7	8
Source clone	A12.2	A71.2	C52.1	C52.2	C53.1	C53.2	C-	C+

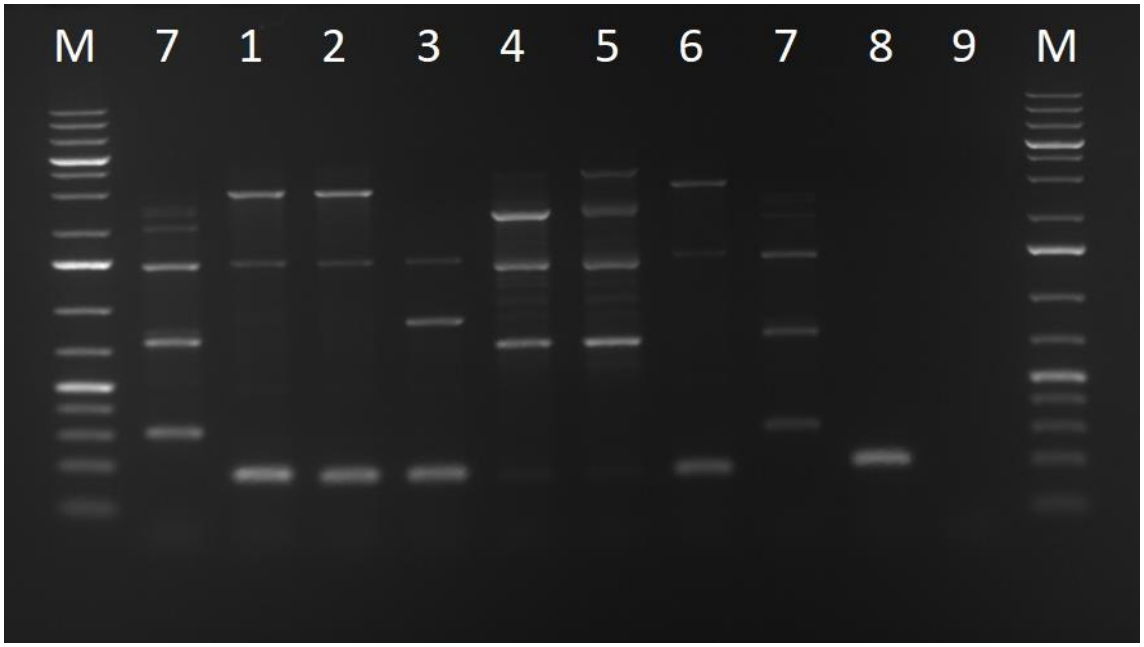


Checking for amplification of *Serratia* DNA following PCR using *Serratia*-specific pfs primer (27/3/2019). Sequences are from the following clones:

Reference no.	1	2	3	4	5	6	7
Source clone	C72.1	C81.2	C82.1	C83.2	C92.1	C92.2	G6R1.1

Reference no.	8	9	10	11	12	13	14
Source clone	G6R1.3	G6R2.1	G6R3.2	G6R3.3	G6S1.1	G6S2.4	G6S3.2

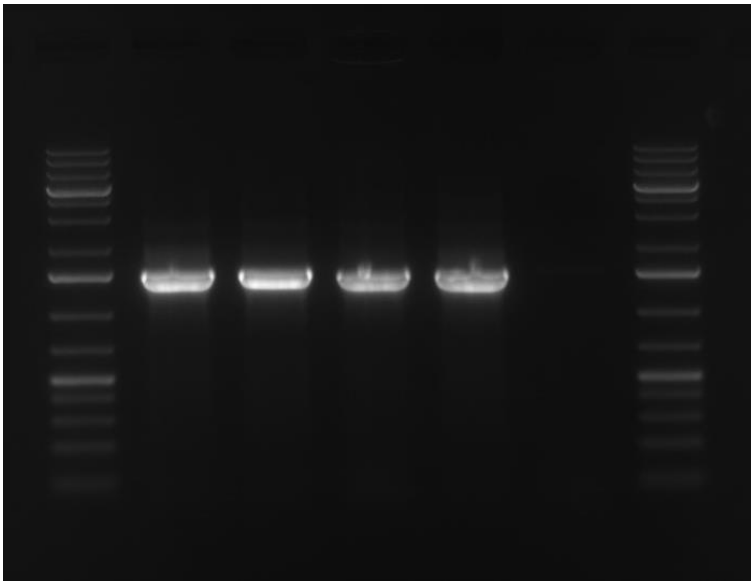
Reference no.	15	16	17
Source clone	G7R1.2	C+	C-



Checking for amplification of *Serratia* DNA following PCR using *Serratia*-specific pfs primer (4/4/2019). Sequences are from the following clones:

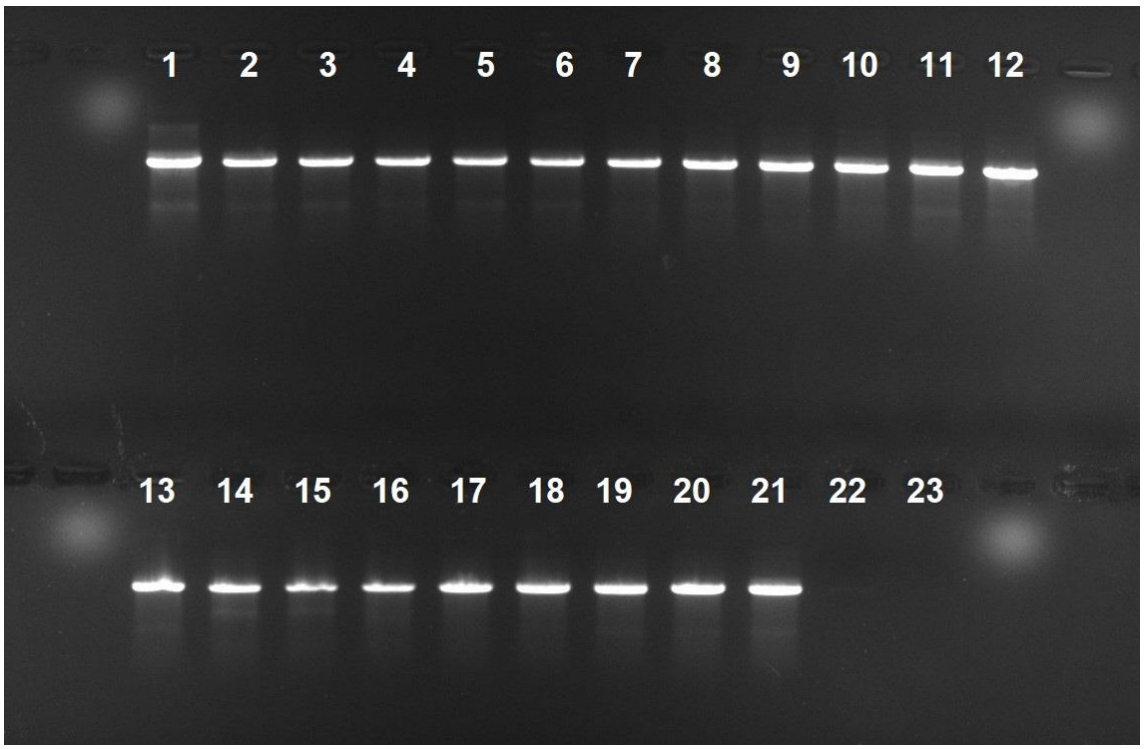
Reference no.	1	2	3	4	5	6	7
Source clone	C52.1	C72.1	C92.1	G6R1.1	G6R3.3	G7R1.2	A11.2

Reference no.	8	9
Source clone	C+	C-



Checking 16sF+R primers (freshly diluted from stock) (17/4/2019). Sequences are from the following clones:

Reference no.	1	2	3	4	5
Source clone	A12.2	A62.2	Colony	C+ (C81.2)	C- (H20)



Checking remaining 16s extractions from 11-4 (18/4/2019). Sequences are from the following clones:

Reference no.	1	2	3	4	5	6	7
Source clone	A71.2	A71.3	A72.2	C52.1	C71.1	C71.2	C91.1

Reference no.	8	9	10	11	12	13	14
Source clone	C92.1	G6R1.1	G6R1.3	G6R2.1	G6R3.2	G6R3.3	G6S2.4

Reference no.	15	16	17	18	19	20	21
Source clone	G6S3.2	P11.1	P11.2	P54.2	CS.1b*	FL*	C+ (C81.2)

Reference no.	22	23					
Source clone	C- (H20)	C- (H20)					

*These clones being sequenced in aid of other experiments

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