Testing the effects of *Bdellovibrio* on Wheat *(Triticum aestivum)* and as a Food Security Agent in Mushrooms (*Agaricus bisporus*)

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

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Abstract

Bdellovibrio bacteriovorus is a naturally soil-dwelling, Gram-negative predatory bacterium that attaches to, invades, and replicates within a wide range of other Gram-negative bacterial species, killing such prey in the process. A small number of previous studies testing the effect of *B. bacteriovorus* against known Gram-negative plant pathogens have suggested that *B. bacteriovorus* has potential as a 'food security agent' against Gram-negative bacterial infections in crop plants.

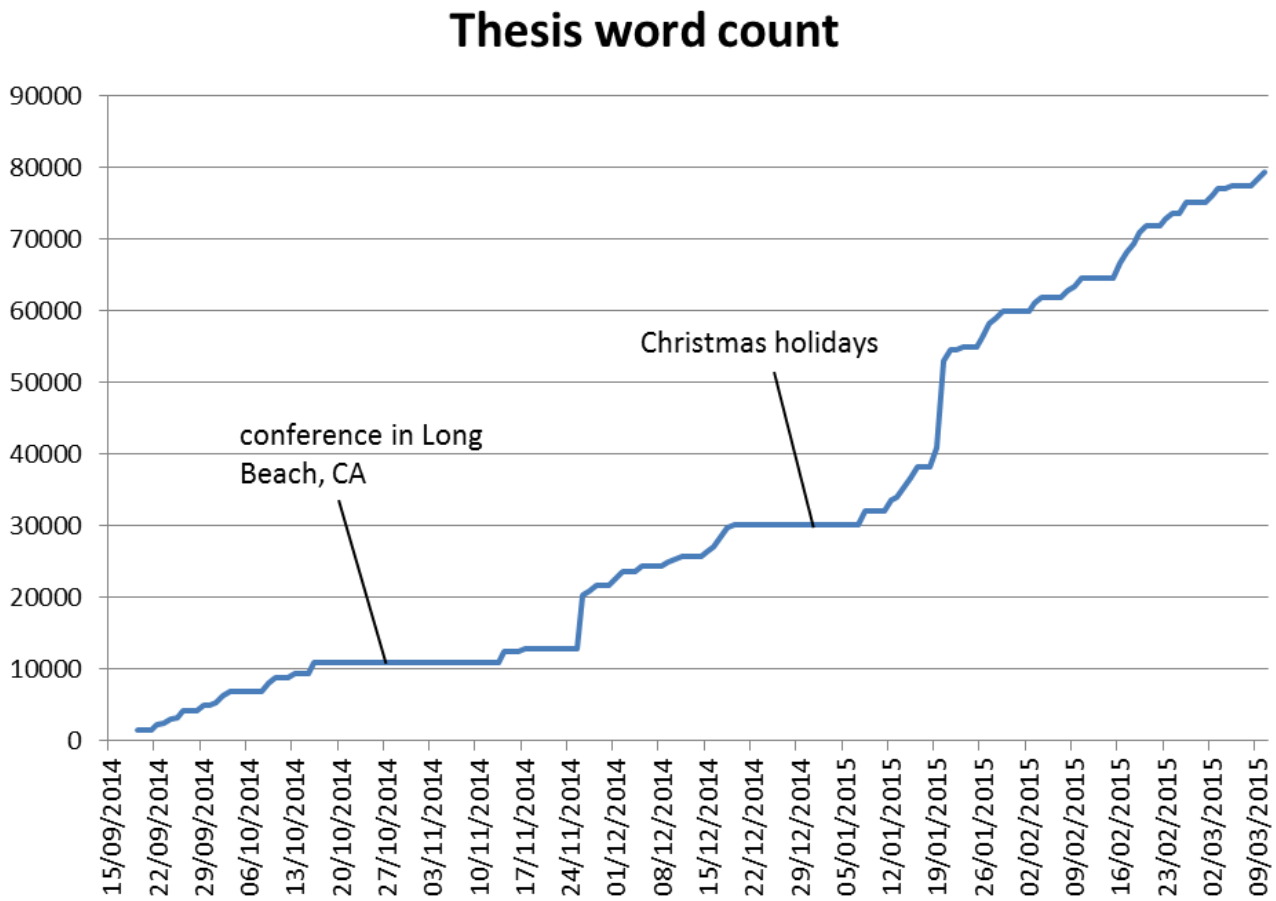
My project built on this knowledge by screening a range of known Gram-negative bacterial plant pathogens and Plant Growth-Promoting Rhizobacteria (PGPRs) for susceptibility to *Bdellovibrio* predation *in vitro*; testing predation-susceptible strains in a simple, semi-sterile *in vivo* system on the surface of *Agaricus bisporus* mushrooms; and finally testing the effect of *Bdellovibrio* addition in a more complex, natural *Triticum aestivum* (wheat) soil rhizosphere mesocosm.

An *in vitro* prey strain growth assay showed that susceptibility to *B. bacteriovorus* predation varied between a range of 20 Gram-negative (mostly *Pseudomonas*) bacterial pathogen/PGPR species, isolated from a range of different host crops or soil environments. Four of these species (*Pseudomonas avellanae* 48, *P. syringae pv. phaseolicola, P. tolaasii* 2192^{T} and *P. agarici* 2289) were highly susceptible to predation, and three species (*B. vietnamiensis* G4, *P. marginalis* 667, and *Pectobacterium atrosepticum* SCRI1143) showed apparent resistance to predation. *P. tolaasii* 2192^{T}, causes dark, pathogenic lesions on post-harvest mushroom host crops; *In vivo* co-inoculation tests on the surface of *A. bisporus* mushrooms showed that lesions were significantly reduced with *B. bacteriovorus* treatment, which was due to *B. bacteriovorus* predatory killing and reduction of prey cell numbers, preventing symptoms. *B. bacteriovorus* also preyed upon and killed a putative pathogenic *Pseudomonas* species isolated from a grey lesion on an organic, garden mushroom, but some likely commensal species isolated

from mushroom tissue showed resistance to predation. These data together suggest that *B. bacteriovorus* could be used commercially to prolong the shelf life of mushrooms, reducing crop losses through spoilage, with minimal negative effects on mushroom PGPR species.

Finally, inoculating *B. bacteriovorus* into the soil around young winter wheat plants in a natural pot soil mesocosm was found to increase plant growth and grain yield at harvest; this was contrary to my initial hypothesis that *B. bacteriovorus* would reduce wheat plant growth, by preying upon and killing PGPR species such as *P. fluorescens* that reduce wheat plant infection with *Gaeumannomyces graminis var. tritici,* the yield-reducing take-all fungal pathogen of wheat. The soil was found to be low in nitrogen; thus *B. bacteriovorus* inoculation could have increased wheat growth due to *B. bacteriovorus* death in the soil and subsequent release of nutrients including nitrogen. However, some *B. bacteriovorus* cells survived in the soil where they could prey upon some Gram-negative bacterial species, reducing their numbers. Some of the wheat growth and yield-producing effects of *B. bacteriovorus* may be due to the predation of species that are associated with late flowering, and therefore grain development, in wheat, allowing time for more grain to develop. Alternatively, it could be due to processes performed by *B. bacteriovorus* in the soil that are not related to predation, such as production of the plant hormone IAA, or *B. bacteriovorus* colonisation of the roots and predation of root-associated pathogenic bacterial species.

Further studies are required to identify the mechanisms behind these unexpected crop yield-promoting effects, and the extent of any nutrient 'boost' effect due to death of *B. bacteriovorus* in the wheat soil, to determine whether *B. bacteriovorus* could be used as a pre-harvest growth and yield-promoting agent. Although most studies of *B. bacteriovorus* so far have focussed on its predatory activity, it likely performs other functions in its natural soil habitat, which may underlie some of the growth and yield-promoting effects shown here. However, these data show that *B. bacteriovorus* could be used commercially as a 'food security agent' when used as a post-harvest treatment to prevent crop spoilage and loss (as for *A. bisporus* mushrooms).



The final word count was 84,630, which is closest in length to *Harry Potter and the Chamber of Secrets*, by J.K. Rowling.

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Abbreviations

AHL	*N*-Acyl Homoserine Lactone
ANOVA	Analysis of Variance
Bdellovibrio	*Bdellovibrio bacteriovorus*
BH	Benjamini-Hochberg
BLAST	Basic Logical Alignment Search Tool
CFU	Colony-Forming Units
CSV	Critical Significance Value
Cu	Copper
DAPG	2,4 Diacetylphloroglucinol
DoF	Degrees of Freedom
EPS	Exopolysaccharide
EM	Electron Microscope/Microscopy
GH-1	Great Harpenden-1
GMI	Garden Mushroom Isolate
HD	Host-Dependent
HGCA	Home-Grown Cereals Authority
HI	Host-Independent
IAA	Indole-3-Acetic Acid
K	Potassium
LPS	Lipopolysaccharide
ML	Maximum Likelihood
MOP	Muriate of Potash
N	Nitrogen
NGS	Next-Generation Sequencing
OD	Optical Density
P	Phosphorus
PAMP	Pathogen-Associated Molecular Pattern
PC	Principal Co-ordinate
PCoA	Principal Co-ordinates Analysis
PCR	Polymerase chain reaction
PCWDE	Plant Cell Wall Degrading Enzyme
PFU	Plaque-Forming Units
PRR	PAMP Recognition Receptor
qPCR	Quantitative Polymerase Chain Reaction
QIIME	Quantitative Insights Into Microbial Ecology
QS	Quorum Sensing
QTL	Quantitative Trait Loci
rDNA	Ribosomal Deoxyribonucleic Acid
REML	Restricted Maximum Likelihood
RRes	Rothamsted Research
RT-PCR	Reverse transcription polymerase chain reaction
SED	Standard Error of the Difference
SEM	Scanning Electron Microscope/Microscopy
S-layer	Surface layer
SMI	Supermarket Mushroom Isolate
SNP	Single Nucleotide Polymorphism
TAB	Take-All Building

TAD	Take-All Decline
TAI	Take-All Index
TEM	Transmission Electron Microscope/Microscopy
T-RFLP	Terminal Restriction Fragment Length Polymorphism
TSP	Triple Super Phosphate
T1SS	Type I Secretion System
T2SS	Type II Secretion System
T3SS	Type III Secretion System
T6SS	Type VI Secretion System
Zn	Zinc

1 Introduction

My PhD project was funded under a global food security initiative in a tripartite collaboration between Rothamsted Research, The University of Nottingham and the University of Reading to test the ability of the naturally predatory soil bacterium *Bdellovibrio bacteriovorus* to combat soil-borne bacterial agents that reduce crop growth or end-point yield, for example by post-harvest food spoilage. *Bdellovibrio bacteriovorus* bacteria are well known for their *in vitro* and applied capacity to prey upon animal and human pathogens, but this project sought to investigate their role and potential applications in their original soil habitat as 'food security agents'. As *B. bacteriovorus* was the main focus of my project, Prof Liz Sockett (University of Nottingham) provided expertise and supervision throughout. Initially, Dr Rob Jackson (University of Reading) provided a range of Gram-negative, crop-associated (pathogen and commensal) species to screen *in vitro* for susceptibility to *B. bacteriovorus* predation, carried out at the University of Nottingham (and supervised by Prof Liz Sockett). Dr Jackson also supervised some initial *in vivo* tests in Chapter 3, but more comprehensive *in vivo* tests on a simple biotic mushroom surface in Chapter 4 were primarily supervised by Prof Sockett, with advice from Dr Jackson. Finally, an investigation of *B. bacteriovorus* predatory activity and other effects on the infection of wheat with the fungal pathogen *G. graminis* var. *tritici* in a complex pot soil mesocosm was devised, with fungal pathogen expertise from Prof Kim Hammond-Kosack and Dr Vanessa McMillan (Rothamsted Research, RRes) and soil metagenomic analysis expertise from Dr Penny Hirsch and Dr Ian Clark.

Firstly, I will contextualise my PhD project by explaining the Global Food Security rationale behind this research.

1.1.1 **Challenges in Global Food Security**

The global human population is increasing, and is predicted to rise to 9.1 billion in 2050 [1]. Most population growth takes place in developing countries, and these populations are becoming increasingly urban: By 2050, 70% of people worldwide are expected to live in urban communities [2]. Recent estimates show that this expanding, city-dwelling population will require global food production to double , to keep up with expansion whilst making up the deficit in chronic malnutrition, estimated to affect 805 million people in 2014 [3]. However, population expansion will simultaneously reduce the amount of land available for farming crops and animals, due to increased living space requirements [1]; 38% of global land is used for agriculture, 12% of which is used for growing crops [4], and expansions in agricultural land would compete with the housing land space requirements of the increasing human population[5].

1.1.1.1 ***Growing more crops in a smaller space***

Most remaining land is unsuitable for cultivation in terms of soil quality and climactic conditions [4], and global temperature increase due to climate change will further reduce potentially suitable land for cultivating crops through localized drought; urbanisation itself is associated with intensive agricultural practices, long-distance food transport, and refrigerated food storage, which are all contributing factors to climate change and arable land reduction [6]. To ensure an adequate food supply in future, a greater number of crops, with higher yields and reduced post-harvest spoilage, will therefore need to be grown in the space that is already used or available for farming, and the practical implications of this, such as growing crops closer together, increases the risk of plant pathogen transmission and poor crop growth.

1.1.1.2 ***Reducing pre-harvest crop infection***

Bacterial plant diseases are an acute threat to global food security. *Pseudomonas* pathovar infections in particular are responsible for significant crop losses, causing diverse infections in a range of plant species (Figure 1): for example, brown blotch disease in mushrooms, caused by *Pseudomonas*

tolaasii (Figure 1a), frequently results in crop losses of around 8%, and in some cases up to 50% (though some of these losses are post-harvest, explained in Section 1.1.1.3) [7]. Similarly, *Pseudomonas syringae pv. phaseolicola*, which causes halo blight in bean (Figure 1c), is responsible for total yield losses of up to 42% [8]. Few chemicals, antibiotics and disinfectants exist to prevent infections from taking hold in their crop hosts, without being toxic to humans. Furthermore, those that exist are not effective: chlorinated compounds, for example, are the most common means of *P. tolaasii* control, but still have undesirable side effects [7, 9]. A novel and effective agent for disease prevention is needed to combat *Pseudomonas* infections, and thus maximise crop yields and increase food security.

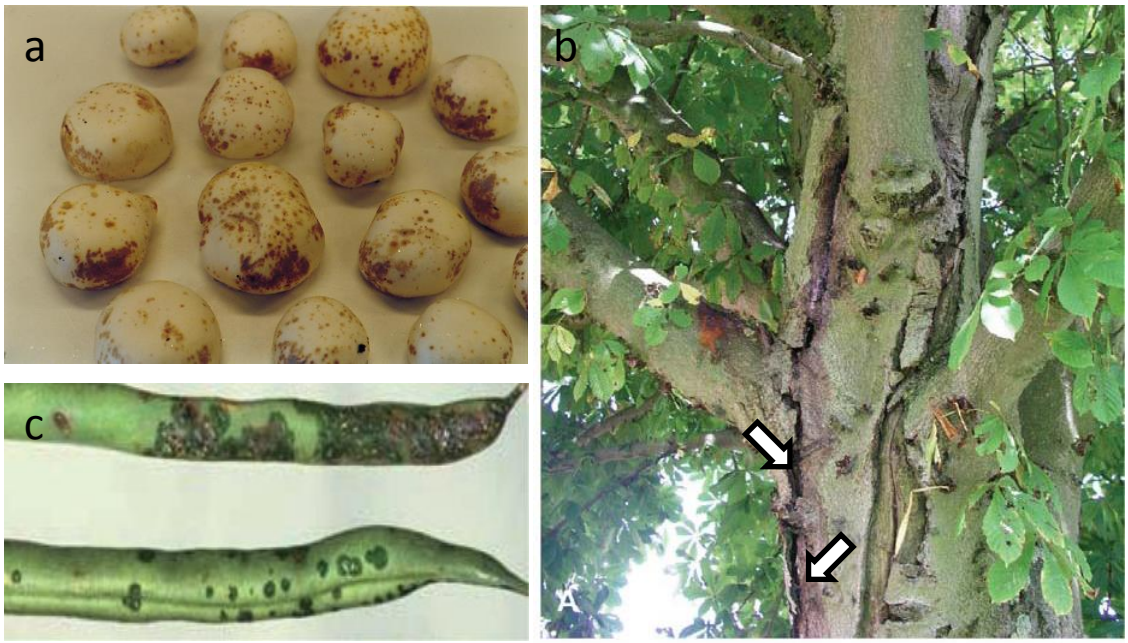


Figure 1 . *Pseudomonas* pathovars cause diverse infections in a wide range of plants, including (a) brown blotch disease in cultivated mushrooms caused by *P. tolaasii* [9] (b) canker (lesions indicated with arrows) in horse chestnut trees caused by *P. aesculi* [10], a disease related to canker in hazelnut caused by *P. avellanae* 48 and (c) halo blight in bean caused by *P. syringae pv. phaseolicola* [8].

In addition to reducing the amount of arable land available, climate change is predicted to have an effect on the ability of pathogens such as *Pseudomonas* to thrive in crop and soil environments; for example, a 3-year metagenomic study in perennial ryegrass (*Lolium perenne*) found that the long-term

elevated CO_2 levels associated with climate change stimulate the growth of *Pseudomonas* populations in the rhizosphere soil; this may include crop pathogenic species, which could increase crop infection levels [11]. Additionally, a relatively recent increase in global shipping of plants and crops introduces exotic new strains into new parts of the world [10, 12]. A recently documented case was the introduction of *Ralstonia solanacearum,* a close phylogenetic relative of *Pseudomonas* and a highly infective plant pathogen with over 200 host species, into greenhouses in the US that had received imports of geraniums grown in Guatemala [12]. These imports have now been stopped; unless an effective agent for disease control is available, restrictive containment measures like these are the only method of preventing destructive pathogen spread.

The destructive effects of *Pseudomonas* transfer to new ecosystems are similarly documented. *P. syringae pv. aesculi,* the causal agent of horse chestnut bleeding canker (Figure 1b), was introduced to Europe from India in 2002/2003. The effects in Europe have been widespread: in 2007, a national survey found that 70% of horse chestnut trees in England show symptoms of the disease, which include foliar discolouration and large, bleeding bacterial cankers on the trunk, often resulting in death. In Indian horse chestnut trees, by contrast, infection results only in minor leaf lesions [10]. This is due to *P. s. pv. aesculi* and Indian horse chestnut co-evolution, resulting in host development of specific resistance mechanisms. These are absent in European horse chestnut trees that have had no previous exposure to the pathogen. This example is particularly relevant to global food security, as many crop plants are now grown outside of their ecosystem of origin, away from pathogens that naturally infect them [12].

More recently in 2014 and 2015, bacterial canker of kiwifruit, caused by *Pseudomonas syringae pv. actinidae,* was reported for the first time in Greece and New Zealand [13, 14]. It has also previously spread to several other countries in Europe, South America and Australasia after its primary identification in Asia in the 1980s [15]. *P. syringae* pathogens in general can survive for an extended period of time in fruit seeds, and thus in the fruit and

seeds exported to other countries, which may have facilitated this spread [15]. Copper compounds, which release Cu^{2+} ions that bind to and disrupt the functions of proteins, and Streptomycin antibiotic that inhibits bacterial protein synthesis, can be used to treat the disease, reducing symptoms by 70-80% [16]. However, development of resistance to the antibiotics may occur, and as the Copper compounds are applied as a spray, runoff into the environment is problematic, so there is a need for the development of new treatments [16].

Thus, the spread of pathogens to new ecosystems is facilitated by high global plant and crop trade (and pathogen populations may increase with climate change) which results in crop damage that could have devastating effects on food security.

1.1.1.3 *Reducing bacterial spoilage of post-harvest crops*

Bacterial pathogens are also an issue in post-harvest spoilage of crops; as some infected crops are asymptomatic until after they are harvested, and the spread of bacteria in a crop has therefore not been detected and inhibited, this can cause major crop loss and wasting of resources required for growth, both of which translate as an economic loss for the grower. For example, brown blotch disease of mushrooms caused by *P. tolaasii*, shown in Figure 1a, is often detected in mushroom crops after harvest, and is spread during the harvesting process on the hands of mushroom pickers. Similarly, *Erwinia carotovora* causes soft rot of onions (*Allium cepa*); this disease is only detectable in crops at harvest or post-harvest, also resulting in lost crops, wasted resources, and large economic losses, as onions are grown in large amounts worldwide. These examples demonstrate that the threat bacterial pathogens pose to food security are compounded by their ability to evade detection, and new methods of preventing the establishment of disease during live crop growth and post-harvest are necessary to maximise crop yield.

1.1.1.4 *Promoting commensal, plant-growth promoting (PGPR) bacterial species*

In contrast to bacterial pathogens, some species of bacteria perform beneficial functions to their plant host, such as nitrogen fixation around roots and preventing the establishment of other bacterial or fungal diseases on the host. An example of this is the inhibition of take-all fungus (*Gaeumannomyces graminis* var. *tritici*) infections on wheat crops by bacteria in the rhizosphere. Previous studies have shown that the bacterial colonisation of wheat roots is altered with take-all infection: it is hypothesized that commensal bacterial species influence the ability of wheat to tolerate take-all infection, thus influencing their overall survival [17]. Micronutrient deficiency, for example deficiency of Zinc (Zn) in soil, increases the size of lesions caused by take-all on wheat. In one study, under conditions of Zn deficiency in soils, the number of fluorescent *Pseudomonas* reached higher cell numbers in the rhizospheres of take-all infected wheat crops than uninfected wheat crops, suggesting that these species may influence tolerance to take-all infection through modification of micronutrient availability to their host [17].

Other studies have demonstrated the successful experimental use of fluorescent *Pseudomonas* species such as *Pseudomonas fluorescens* to control take-all through their colonisation of wheat roots. The positive impact of some *Pseudomonas* and other commensal species on crop growth is an important consideration in this study, as the use of *Bdellovibrio* as a biocontrol agent for bacterial diseases, such as those described above caused by *Pseudomonas,* may have adverse effects on bacterial species that aid crop plant growth and survival [18, 19]. Natural variations in *Bdellovibrio* levels in soils may also affect PGPR levels. Chapter 5 was initially an investigation into the potential effects of *Bdellovibrio* addition on wheat PGPRs and take-all disease in the wheat rhizosphere**,** and so a more in-depth review of PGPRs in the wheat rhizosphere context is included in the introduction there.

1.1.2 *Bdellovibrio bacteriovorus* are predatory bacteria derived from soil

Bdellovibrio bacteriovorus is a small, Gram-negative deltaproteobacterium with an unusual predatory lifestyle: it attacks, invades and kills other Gram-negative bacteria in order to grow and reproduce, invading and killing its prey in the process. Elsewhere in this text, *Bdellovibrio* should be taken to refer to *B. bacteriovorus,* unless otherwise indicated. *Bdellovibrio* is naturally present at a low concentration in freshwater and terrestrial environments [20, 21], and was originally isolated from soil in by Stolp and Starr (1963) who first noted the predatory activity of *Bdellovibrio* in its ability to lyse lawns of *Pseudomonas* [22]. Since then, *Bdellovibrio* has been shown to prey upon a wide variety of Gram-negative bacteria, including important pathogens of plants, humans and animals [23-25]: this unique ability gives *Bdellovibrio* valuable potential as a biocontrol agent with applications in medical, agricultural and industrial arenas. This report focuses on the potential application of *Bdellovibrio* as 'food security guards' to prevent or treat bacterial infections in crops and other ecologically important plants.

1.1.3 The *Bdellovibrio* predatory and other life cycles

The *Bdellovibrio* predatory life cycle consists of two discrete phases: a free-swimming 'attack' phase (Figure 2i) and a 'growth' phase in the periplasm of the Gram-negative host cell. This cycle could be used to grow at the expense of crop pathogens. There is an alternate cycle called the prey, or host-independent (HI) cycle. In this cycle, there is very slow replication of *Bdellovibrio* on conventional organic nutrients [26]. Although this cycle may be important for *Bdellovibrio* in soil and other environments, it is the predatory cycle that could be optimized to kill crop-pathogenic bacteria as prey.

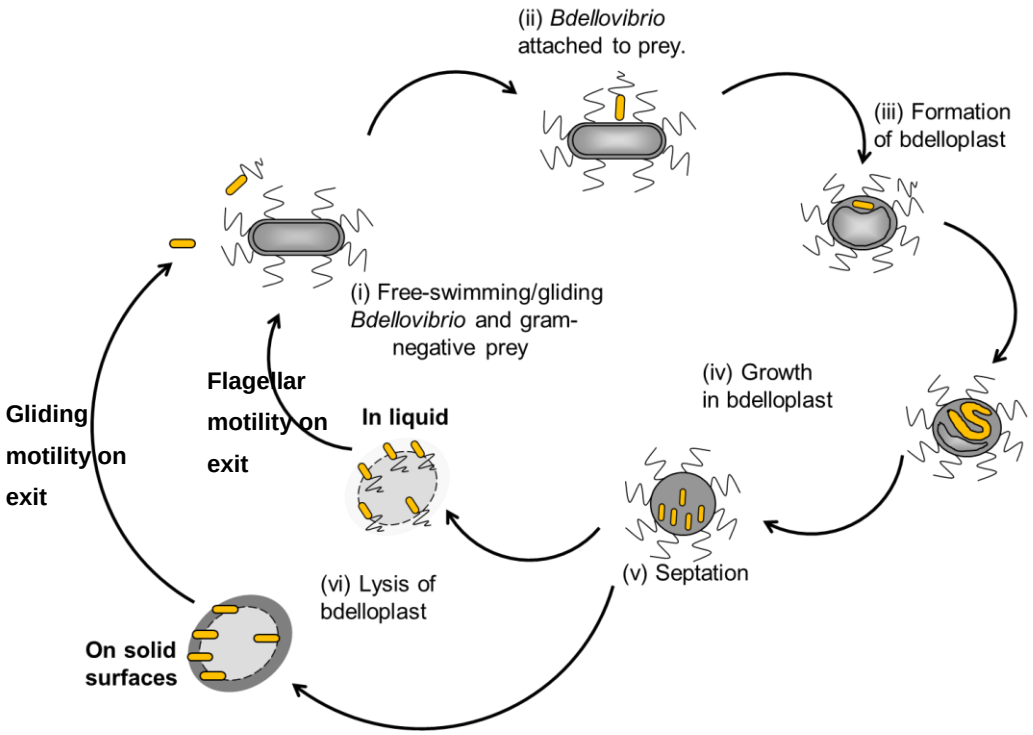


Figure 2 . The predatory life cycle of *Bdellovibrio bacteriovorus* on Gram-negative prey bacteria, adapted from [27]. Attack-phase *Bdellovibrio* attach to the prey cell, create a pore in its outer membrane and enter into the periplasm, forming a Bdelloplast. Here, they grow filamentously, using up host cell resources until they are depleted. *Bdellovibrio* then septates into a variable number of daughter cells, which then lyse the host. The new attack-phase *Bdellovibrio* develop flagella and swim if in liquid, or glide if in dry conditions on a surface, to find more prey and repeat the cycle.

1.1.3.1 *Attack-phase swimming and gliding motility in the host-dependent Bdellovibrio life cycle*

In attack phase, *Bdellovibrio* cells are highly motile: in liquid conditions, swimming speeds of up to 160 µm s^{-1} can be achieved through rotation of a sheathed, unipolar flagellum [28, 29]. Recently, Lambert and co-workers demonstrated that *Bdellovibrio* are also motile without flagella in drier conditions, moving along solid and semi-solid agarose surfaces by gliding [30]. Both types of motility are important for encountering and exiting from potential prey cells, but gliding motility is predicted to be particularly important for prey encounter on natural biotic surfaces, such as plant leaves, mushroom surfaces and soil, where *Pseudomonas* plant pathogens and commensals are found naturally. Additionally, attack-phase *Bdellovibrio* are known to actively move towards bacteria-rich areas through chemotaxis

when swimming with flagella, increasing the chance of prey encounter [31, 32]. Chemotaxis may also be in play during gliding but this has not yet been shown.

1.1.3.2 *Transitioning from attack phase to growth phase inside a Gram-negative prey bacterium*

Collision with and attachment to a potential prey cell marks the transition between attack and growth phase (Figure 2 ii). *Bdellovibrio* reversibly attach at their non-flagellar (leading) pole, initiating a recognition period in which *Bdellovibrio* senses, through an as yet uncharacterized mechanism, whether the attached cell is suitable prey, i.e. a Gram-negative bacterium [33]. If so, *Bdellovibrio* digests a small, temporary pore in the outer membrane, which it squeezes through and re-seals, losing its flagellum in the process. With *Bdellovibrio* established in the prey periplasm, the prey cell is killed: predator and prey together form a rounded structure called a bdelloplast (Figure 2 iii).

1.1.3.3 *Bdellovibrio reproduction inside the prey cell and completion of the predatory cycle*

Inside the bdelloplast, *Bdellovibrio* digests and takes up the contents of the dead prey cell, growing filamentously (Figure 2 iv). Once resources are fully depleted, *Bdellovibrio* septates synchronously into a variable odd or even number of identical daughter cells, dependent on the amount of resources available, shown in Figure 2 v [34]. The number of cells produced is referred to as the 'burst-size'. These attack-phase *Bdellovibrio* lyse the prey cell and develop flagella and swim away if in liquid conditions, or glide away if in dry conditions, to locate more prey and repeat the cycle again (Figure 2 vi).

1.1.4 **Previous studies of *Bdellovibrio* in soil and crop settings**

There are only a small number of studies that focus on in the potential of *Bdellovibrio* to combat Gram-negative pathogens of plants, and an even smaller number that assess the predatory activity of *Bdellovibrio* against important bacterial pathogens of plants in their natural plant host or crop soil context, which are discussed below. However, these few studies

demonstrate that *Bdellovibrio* have predatory activity against a range of plant pathogens, and that there are fewer plant growth promoting bacterial species that may be sensitive to predation. My study is therefore a logical progression of this research area: assessing the effects of adding *Bdellovibrio* to crops and crop soils to measure their effects on crop-pathogen interactions and crop growth and yield.

1.1.4.1 *B. bacteriovorus predation of Gram-negative bacterial rice pathogens*

In 1980, Uematsu extensively studied the predatory capacity of *B. bacteriovorus* isolated from rice paddy water against the rice bacterial leaf blight pathogen *Xanthomonas oryzae*, the range of bacterial species other than *X. oryzae* that these *Bdellovibrio* isolates could prey upon, and the survival of *Bdellovibrio* in the soil and on the rice leaf plant surface [35]. The studies showed primarily that *B.bacteriovorus* could prey upon and kill *X. oryzae* in a prey lawn on a petri dish, and also in rice paddy field water, in which *X. oryzae* cells disappeared within 5 days, measured by live enumeration of the pathogen. On the other hand, when *B.bacteriovorus* was inoculated into sterilised rice paddy soil along with *Erwinia amylovora* (a plant pathogen that causes black necrotic lesions and plant cell wall disintegration), the *E. amylovora* population did not decrease, though *Bdellovibrio* could successfully prey upon and kill *E. amylovora* on a prey lawn in a petri dish, as for *X. oryzae. Bdellovibrio* was found to be present naturally in rice paddy water and soil at 10^{0}-10^{3} cells ml^{-1} or g^{-1} throughout the year, though the population was generally lower in the summer and winter; however, when inoculated onto the rice plant leaf surface and in the rice plant tissue, *B. bacteriovorus* was unable to survive, even in the presence of *X. oryzae* prey, as indicated by a reduction in live *B. bacteriovorus* cells enumerated on an agar prey lawn. Together, these data demonstrate that *Bdellovibrio* could potentially be used as a food security agent against an important bacterial pathogen of rice.

More recently, Song (2003) isolated 6 *B. bacteriovorus* strains from rice paddy water and soils. These isolates were able to prey upon and kill another rice crop pathogen, *Burkholderia glumae,* which causes rice grain rot. The

prey ranges of these *B. bacteriovorus* strains were characterised by testing plaque formation on agar lawns containing single, soil-dwelling potential prey species, including *B. glumae*. The prey range varied between strains, but crucially none of the *B. bacteriovorus* strains formed plaques on lawns of some bacterial species that perform beneficial functions in the rice rhizosphere, including *Azospirillum brasiliense* (nitrogen fixation, discussed in detail in Section 5.1.7.3) and *Pseudomonas fluorescens* (antibiosis of fungal pathogens, discussed in Section). This indicates that *Bdellovibrio* may reduce populations of plant pathogenic bacterial species by predation, with a minimal effect on populations of Plant Growth-Promoting Rhizobacteria species (PGPRs), suggesting that *Bdellovibrio* may be effective when applied as a food security agent to promote crop growth and yield.

1.1.4.2 *The Gram-negative plant pathogenic prey range of Bdellovibrio spp. isolated from soil*

Similarly, Jurkevitch and coworkers (2000) [21] isolated several co-existing *Bdellovibrio* spp. (including *B. bacteriovorus, B. stolpii* and some previously unidentified spp.) from soil and the bean plant rhizosphere on four different Gram-negative soil-dwelling prey species, and characterised their prey ranges by measuring the OD of prey cells in the presence and absence of each *Bdellovibrio* sp. after overnight incubation at 30ºC. As in the rice paddy study conducted by Song (2003), the host ranges of the strains differed, but this study found that some preyed upon different plant pathogenic species to others (for example, the isolate BEP2 could prey upon *E. amylovora* while isolate SRP1 could not, though both could prey upon the tomato pathogen *Pseudomonas syringae* pv. *tomato*), and some also preyed upon (a smaller number of) known PGPR species such as *Pseudomonas putida*. This study showed that there is diversity in *Bdellovibrio* species isolated from the same soil/rhizosphere environment. This diversity enables them to prey upon different Gram-negative species, a fact which could potentially be exploited to use different strains to combat specific plant pathogens in the rhizospheres of different crops.

1.1.4.3 *Bdellovibrio predation of a Gram-negative Pseudomonas pathogen in a bean plant host*

Only one of these studies involved testing *B. bacteriovorus* against a plant pathogen directly on the host plant itself. Scherff (1973) [23] isolated 3 *B. bacteriovorus* strains from the rhizosphere of soybean plants, and inoculated them in combination with the soybean bacterial blight pathogen *Pseudomonas glycinea* onto the leaf surface of the same host plant, using an abrasive inoculation technique. Carborundum-dusted leaf surfaces were rubbed with cheesecloth to create wounds in the leaf tissue, to which 1:1, 9:1 and 99:1 ratios of *B. bacteriovorus*:*P. glycinea* were applied. Scherff found that one of these isolates, Bd-17, inhibited the *P. glycinea*-induced black lesions and systemic toxic symptoms (e.g. chlorosis of the leaves and plant stem). This inhibition was observed at a ratio of 9:1 and 99:1 *Bdellovibrio*:*P. glycinea* in the inoculum, when compared with a *P. gycinea*-only inoculated control.

1.1.5 **Aims of This Study**

Chapter 3- *in vitro* screening to identify predation-susceptible pathogens

- To screen a variety of Gram-negative pathogens of agriculturally important plants, fungi and trees, donated by Dr Rob Jackson (University of Reading), *in vitro* for susceptibility to predation and killing by *B. bacteriovorus* HD100
- To test the suitability of *B. bacteriovorus* predation of susceptible pathogen species, identified from the *in vitro* screening tests, as a means of disease control *in vivo,* by assessing any predator-caused pathogenic symptoms in leaf tissue of the model plant *Nicotiana benthamiana*

Chapter 4- *Pseudomonas tolaasii* predation: from *in vitro* to *in vivo*

Pseudomonas tolaasii is an important crop spoilage pathogen, and can affect the mushroom host at both pre- and post-harvest stages. This was an ideal

first pathogen-host system in which to test *B. bacteriovorus* HD100 as a spoilage control agent: mushrooms are a widely consumed food, the tests are highly replicable and low-cost with a simple initial read-out of brown blotch disease lesion intensity, and the mushroom is easily manipulated and containable in falcon tubes, as *P. tolaasii* is a category 2 organism. My main aims were:

- To assess the effectiveness of *B. bacteriovorus* HD100 as a post-harvest crop spoilage prevention treatment

- To set up an '*in funga*' assay testing *B. bacteriovorus* HD100 predation of *P. tolaasii* on the surface of the button mushroom, *Agaricus bisporus*

- To measure the impact of predation on *P. tolaasii* mushroom pathogen numbers and brown blotch disease symptoms

To then investigate the impact of *B. bacteriovorus* HD100 predation on other pathogenic or commensal, mushroom-endogenous bacterial species, one further aim was:

- To isolate and characterise bacterial species that naturally inhabit both commercially produced and organic, garden-grown mushrooms, and determine their susceptibility to predation by *Bdellovibrio*

Chapter 5- Testing the effects of *B. bacteriovorus* in a complex, crop soil bacterial community

The wheat rhizosphere and its microbial population are important factors affecting wheat crop growth; infection with the take-all fungus *Gaeumannomyces graminis* routinely reduces commercial wheat yields, but is itself suppressed by Gram-negative *Pseudomonas* Plant Growth-Promoting Rhizobacteria (PGPR) species. This part of my project **initially** aimed to quantify the impact of *Bdellovibrio* treatment of agricultural soil in a wheat rhizosphere mesocosm, before crop growth and maturation, on:

- Infection level of the wheat plant with the growth-stunting and yield-reducing take-all fungus

- Phenotypic measures of wheat plant growth, primarily as an indicator of take-all fungal infection levels

- The wheat soil bacterial microbiome, as measured by Next-Generation 16s ribosomal DNA sequence typing, potentially affected by *Bdellovibrio*, and with a potential effect on take-all infection levels

Due to unexpected **positive effects** of *Bdellovibrio* treatment of the wheat rhizosphere **on wheat plant growth**, seemingly not mediated by take-all infection of wheat, further aims were to assess:

- The effect of a heat-killed *B. bacteriovorus* HD100 treatment in soil on wheat plant growth, as above, compared to the live *B. bacteriovorus* HD100 treatment, to assess any effect of additional nutrients added to the soil in dead bacterial cells

- The effect of *B. bacteriovorus* HD100 treatment in two different soils, to determine whether *B. bacteriovorus* HD100 growth-promotion of wheat plants is affected by soil structure

- nitrogen, phosphorus and potassium levels in pre- and post-*B. bacteriovorus* HD100 treated soils

In order to determine a mechanism for *Bdellovibrio* improvement of wheat crop growth and yield.

2 Materials and Methods

2.1 Strains used in this study (in all chapters)

Strain	Description	Reference
Bdellovibrio bacteriovorus predator		
HD100	Wild type isolated from soil in Germany	[22, 36]
Escherichia coli prey for lab analyses/culturing		
S17-1	*thi, pro, hsdR*$^{-}$*, hsdM*$^{+}$*, recA,* integrated plasmid RP4-Tc::Mu-Kn::Tn7	[37]

2.2 Media and Buffers used in this study

The amounts below are given for 1 L media broth/agar or buffer. All media were made in de-ionised water and autoclaved.

YT Broth for culturing *E. coli* S17-1 and *P. putida* Prey

5 g NaCl
5 g DIFCO Yeast Extract
8 g DIFCO Bacto-Tryptone

Adjusted to pH 7.5 with 2 M NaOH
(+10 g Sigma Select A5054 agar for agar plates)

YPSC Broth for culturing host dependent *Bdellovibrio* on overlay plates

1 g DIFCO Yeast Extract
1 g DIFCO Peptone
0.5 g Anhydrous sodium acetate
0.25 g $MgSO_4.7H_20$

Adjusted to pH 7.6 with 2 M NaOH
Sterile $CaCl_2$ from 25 g L^{-1} stock to give 0.25 g L^{-1} after autoclaving

(+10 g Sigma Select A5054 agar for bottom layer of agar plate)
(+6 g Sigma Select A5054 agar for top layer of agar plate)

King's Medium B for culturing *Pseudomonas* isolates

20 g Bacto-Proteose Peptone No. 3
1.5 g $MgSO_4.7H_2O$
1.5 g K_2HPO_4
12.5 ml 80% glycerol

Adjusted to pH 7.2 with 2 M NaOH
(+15 g Sigma Select A5054 agar for agar plates)

Ca^{2+} HEPES buffer

5.94 g HEPES
0.294 g $CaCl_2.2H_2O$

Adjusted to pH 7.8 with 2 M NaOH

Coliform chromogenic agar

55.8 g Brilliance *E. coli*/coliform agar (Thermo Scientific, CM0956)

pH 7.0

2.3 General culturing procedures

2.3.1 *Bdellovibrio bacteriovorus* HD100

On an agar plate: A lawn of *E. coli* prey was prepared by mixing 100 µl *E. coli* S17-1 with 5 ml YPSC top agar (+1% $CaCl_2$), which was poured over a YPSC bottom agar plate and dried for 5 minutes. 100 µl *Bdellovibrio* HD100 from a frozen stock was pipetted on to the lawn of *E. coli* S17-1, tipped gently

to spread it out and dried for 5 minutes. The frozen stock was made from an individual plaque formed from a single cell in a 1 in 10 serially diluted lysate, on a lawn of *E. coli* S17-1. The plate was incubated agar side down at 29°C for 24 hours, then turned agar side up and incubated for 36 hours, until a clear plaque area formed.

In a 2 ml liquid 'lysate': Agar was picked from the plaque area using a P1000 pipette and transferred into a bijou tube containing 2 ml Ca^{2+} HEPES buffer with 150 µl *E.coli* S17-1 liquid culture. This lysate was incubated at 29°C for 15 hours with shaking at 200 rpm.

In a 10 or 50 ml lysate: For a 10 ml lysate, 200 µl of the original 2 ml *Bdellovibrio* HD100 liquid lysate was added to 10 ml Ca^{2+} HEPES buffer with 1 ml *E. coli* S17-1 and incubated at 29°C for 15 hours with shaking at 200 rpm. For a 50 ml lysate, 1 ml of the original 2 ml *Bdellovibrio* HD100 liquid lysate was added to 50 ml Ca^{2+} HEPES buffer with 3 ml *E. coli* S17-1 and incubated at 29°C for 15 hours with shaking at 200 rpm..

2.3.2 ***E. coli* S17-1 prey**

E. coli S17-1 prey, for routine culturing of predatory stocks of *Bdellovibrio,* was streaked out from a frozen stock on a YT media agar plate and incubated for 15 hours at 37°C. 50 ml YT media broth in a 250 ml conical flask was inoculated with one colony of *E.coli* S17-1 from the plate and incubated for 15 hours at 37°C with shaking at 200 rpm.

2.4 Methods used in Chapter 3

2.4.1 **Strains used in this study**

Strain	Description	Reference
Bdellovibrio bacteriovorus predator		
HD100	Wild type isolated from soil in Germany	[22, 36]
Escherichia coli prey for lab analyses/culturing		
S17-1	*thi, pro, hsdR*$^{-}$*, hsdM*$^{+}$*, recA,* integrated plasmid RP4-Tc::Mu-Kn::Tn7	[37]
Gram-negative plant pathogen and PGPR species		
Pseudomonas avellanae 3487	Causes hazelnut canker in leaves, bark and roots	[38]
Burkholderia vietnamiensis G4	Saphrophytic soil organism; PGPR	[39]
Pseudomonas syringae pv. *phaseolicola*	Causes halo blight on bean leaves	[40]
Pseudomonas putida UWC1	Saphrophytic soil organism; PGPR	[41]
Pseudomonas fluorescens SBW25	Saphrophytic soil organism; PGPR, isolated from sugar beet leaf	[42]
Pseudomonas fluorescens LSWS	Saphrophytic soil organism; PGPR, biofilm-forming derivative of *P. fluorescens* SBW25	[43]
Pseudomonas syringae DC3000 NEW	Causes bacterial speck in tomato leaves and fruit	[44]
Xanthomonas campestris pv. *campestris* 8004	Causes black rot in Brassica and Arabidopsis leaves and stem	[45]
Erwinia amylovora CFBP1430	Causes fireblight in apple and pear leaves, trunk and fruits	[46]
Pseudomonas syringae pv. tabaci 11528	Causes wildfire in tobacco leaves	[47]
Pseudomonas entomophila L48	Toxic to insects, isolated from soil	[48]
Pseudomonas corrugata	Causes pith necrosis in tomato leaves and stem	[49]
Pseudomonas marginalis 667	Causes soft rot in diverse hosts and plant tissues	[50]
Pseudomonas cichorii 943	Causes soft rot in lettuce leaves	[51]
Pseudomonas agarici 2289	Causes drippy gill in	[52]

	mushrooms	
Pectobacterium atrosepticum SCRI1043	Causes blackleg in potato leaves, stem and tubers	[53]
Pseudomonas syringae pv. *aesculi* 6617	Causes bleeding canker in horse chestnut trunk and leaves	[10]
Pseudomonas marginalis pv. *pastinaciae*	Causes soft rot in strawberry leaves, flowers, stem and fruit	[54]
Pseudomonas marginalis pv. *marginalis* 247	Causes soft rot in diverse hosts and plant tissues	[55]
Pseudomonas tolaasii 2192^T	Causes brown blotch on mushroom caps and stem	[56]
Pseudomonas viridiflava 2848	Causes leaf lesions in dwarf runner bean	[54]
Tobacco model plant species		
Nicotiana benthamiana	Plant pathogen disease model	[57]

2.4.2 **Bacterial culturing procedures**

The method for culturing predatory *B. bacteriovorus* HD100 on *E. coli* S17-1 prey is described in **Section 2.3,** and was used in this chapter.

***Pseudomonas* pathogenic/PGPR strains**

The *Pseudomonas* strains were streaked out from frozen stocks on King B medium agar plates, which supports the growth and pyocyanin/fluorescein production of *Pseudomonas* strains [58], and incubated at 29°C for 24-48 hours, depending on how quickly the individual strains grew. 50 ml King B medium broth in a 250 ml conical flask was inoculated with a single colony from a *Pseudomonas* stock plate, and incubated for 15 hours at 29°C with shaking at 200 rpm.

2.4.3 **Measuring the effect of *Bdellovibrio* on *Pseudomonas* growth: Optical Density ($OD600_{nm}$) measuring assay in a BMG plate reader**

B. bacteriovorus predation of each pathogen/commensal strain was firstly tested in King's medium B suspension, containing buffer and the test prey strain in a plate reader. 180 µl/well of a 50% v/v King's Medium B, 50% v/v 2 mM $CaCl_2$ 25 mM HEPES pH 7.6 buffer mixture was added to the wells of a clear-bottomed, 96-well Krystal microplate (Porvair Sciences Ltd, Product No.

215006). 1.5ml aliquots of predatory cultures of *B. bacteriovorus* HD100, containing 2.5 x 10^8 PFU ml^{-1}, were prepared and heat killed at 105˚C for 5 minutes and allowed to cool to ambient temperature (21˚C). This heat-killed, cooled culture was then added, in a 3:1 ratio, to a live liquid culture of *B. bacteriovorus* HD100 to give 6.3 x 10^7 PFU ml^{-1} of live *B. bacteriovorus* HD100. This mixture was used as a diluted application of *Bdellovibrio* to achieve a controlled lowered concentration of predator in these experiments. Microplate wells were then set up using either 64 µl of the heat-killed culture alone as a negative control; 64 µl of the heat-killed/live mixture described above; or 64 µl of the original live culture of *Bdellovibrio*. These preparations gave final live *B. bacteriovorus* HD100 cell numbers of 0, 4 x 10^6 or 1.6 x 10^7 PFU, respectively. One set of wells containing these three predatory mixtures was made for each pathogen/commensal test prey strain.

For test prey cells, a liquid culture of each pathogen/commensal strain from Section 2.4.1 was diluted 2 in 5 to give 3.0 x 10^8 CFU/ml^{-1} in 50% v/v King's Medium B, 50% v/v 2 mM $CaCl_2$ 25 mM HEPES pH 7.6 buffer mixture. The final number of each test prey strain in the mixtures is listed in Table 1. 20 µl of these diluted cultures were each transferred to a corresponding set of microplate wells containing the three predator mixtures. The plates were then sealed with a Breatheasy® seal (Diversified Biotech Cat. No. BEM-1) and transferred to a BMG plate reader programmed to incubate and measure the $OD600_{nm}$ of each well, as an indicator of test prey strain growth, immediately, and then every 30 minutes for 24 hours. *B. bacteriovorus* HD100 alone does not produce an $OD600_{nm}$ value due to its small cell size [59].

Table 1 . Cell number of potential prey species added to each microtitre well containing the *B. bacteriovorus* HD100 predatory mixtures for the Optical Density ($OD600_{nm}$) growth assays (n = 3 for each strain).

Strain	cells/well of microtitre plate
Pseudomonas avellanae 48	5.68×10^6
Burkholderia vietnamiensis G4	7.00×10^6
Pseudomonas syringae pv. *phaseolicola*	2.04×10^8
Pseudomonas putida UWC1	5.32×10^6
Pseudomonas fluorescens SBW25	1.08×10^7
Pseudomonas fluorescens LSWS	2.54×10^7
Pseudomonas syringae DC3000 NEW	6.76×10^6
Xanthomonas campestris pv. *campestris* 8004	6.31×10^6
Erwinia amylovora CFBP430	1.50×10^7
Pseudomonas entomophila L48	2.64×10^6
Pseudomonas corrugata	1.29×10^7
Pseudomonas marginalis 667	7.40×10^6
Pseudomonas cichorii 9437	4.52×10^6
Pseudomonas agarici 2289	1.08×10^7
Pectobacterium atrosepticum SCRI1043	3.08×10^6
Pseudomonas syringae pv. *aesculi* 6617	7.48×10^7
Pseudomonas marginalis pv. *pastinaciae*	3.48×10^6
Pseudomonas marginalis pv. *marginalis* 247	2.14×10^6
Pseudomonas tolaasii 2192^T	5.88×10^6
Pseudomonas viridiflava 2848	9.52×10^5

2.4.4 Test inoculation procedure for Tobacco plant leaves

An *in planta* assay was used in pilot work to test the ability of four *Pseudomonas* pathovars and of *B. bacteriovorus* HD100 itself to cause disease symptoms in leaves of the model plant, *Nicotiana benthamiana* (tobacco), when inoculated separately, with a view to using *N. benthamiana* to test the predatory activity of *B. bacteriovorus* HD100 against these pathovars *in vivo* having tested them first *in vitro,* as described in Section 2.4.3. The *N. benthamiana* plants were grown by Dr Rob Jackson (University of Reading) in a plant growth room for 12 weeks, kept at 22°C under a growing light and a fan to circulate air. *P. avellanae* 48, *P. syringae pv. phaseolicola*, *P. agarici* 2289 and *P. tolaasii* 2192^T were grown in liquid

cultures (see Section 2.4.2). Each was diluted with King's medium B to $OD600_{nm}$ = 0.1, to give 7.10 x 10^7, 2.55 x 10^9, 1.35 x 10^8, and 7.35 x 10^7 cells ml^{-1} respectively. A 50 ml *B. bacteriovorus* HD100 lysate was grown (see culturing protocols) and filtered x2 through a 0.45 µm Millipore filter to give 2.5 x 10^8 cells ml^{-1}.

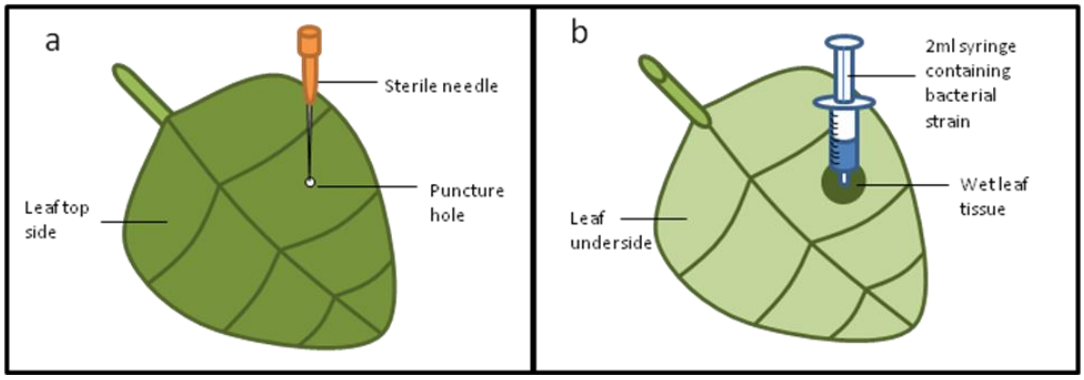


Figure 3 . The tobacco leaf inoculation procedure (RW Jackson Lab, Reading University). (a) For each replicate, one hole per strain was made in a tobacco leaf on a whole, 12-week old plant using a sterile needle. **(b)** A 2 ml syringe containing a suspension of the bacterial strain to be inoculated (at 7.10 x 10^7 to 2.55 x 10^9 cells ml^{-1}) was placed over the hole on the underside of the leaf, forming a seal against a gloved finger on the other side. The syringe plunger was depressed gently until the strain spread through the leaf, creating a wet patch around the hole. The leaf was then wiped with laboratory tissue to remove excess liquid and grown at 22°C under a growing light and fan.

For the inoculations, five holes each were made through the leaf using a 25 g x 5/8" 0.5 x 16 mm sterile needle on two tobacco leaves from one whole, 12-week old plant (Figure 3a). To inoculate leaves, a 2 ml sterile syringe was filled with the strain to be inoculated. With no needle attached, the end of the syringe was placed on the underside of the leaf over one of the holes, forming a tight seal against a gloved finger on the other side. The syringe plunger was gradually depressed until a wet patch spread through the leaf tissue around the hole (Figure 3b). The amount of liquid that was inoculated into the leaf each time was 0.25 ml, and the amount lost by dripping off from the leaf was minimized by depressing the syringe plunger slowly and carefully (~30 seconds for each strain). The leaf was then dabbed dry with laboratory tissue to prevent cross-contamination of plants or inoculation sites on each plant leaf. Each strain was inoculated once on each of two leaves.

Plants were kept in the plant growth room under the same conditions as before and the inoculated areas were monitored daily for the appearance of symptoms such as chlorosis (yellowing) and necrosis (browning) of the leaf tissue. Photographs of the leaves were taken after 4 days, when disease symptoms had appeared, with a digital camera set to an aperture of f=5.6; the shutter speed was adjusted according to the light level at the time the photographs were taken (and which remained constant for all photographs). The zoom function was set to 3 stops and the distance between the camera and leaf was controlled using a tripod. This gave us some preliminary results, shown in Section 3.4.3**,** but the use of *N. benthamiana* as a model plant host was not continued after this.

2.4.5 Testing for the presence of plant pathogen strains and *B. bacteriovorus* HD100 from inoculated *N. benthamiana* plant tissue

Enumerations on King's medium B agar were carried out to determine the number of pathogen cells in tobacco leaf tissue both directly after inoculation (T = 0) and also after disease symptoms had appeared on the leaves (T = 4 days). To do this, directly after inoculation, the inoculated areas in one of the two replicates were all cut out separately using an 8 mm diameter cork borer, sterilized by dipping in ethanol and flaming with a bunsen burner between samples, and each sample of leaf tissue was homogenised thoroughly in 1 ml calcium HEPES (2 mM CaCl2 25 mM HEPES pH 7.6) using a glass pestle and mortar. The resuspensions containing leaf tissue that had been inoculated with each plant pathogen as described in Section 2.4.4, were diluted 1 in 10 to 10^{-3} for the T = 0 samples that had been resuspended directly after inoculation, and to 10^{-6} for the T = 4 day samples. Dilution series were made in triplicate for each resuspended sample, and 100 µl of each dilution was spread on a King's medium B agar plate. All plates were incubated statically at 29ºC for 24 hours, until colonies appeared.

In leaf samples where *B. bacteriovorus* was inoculated alone, described in Section 2.4.4**,** the diluted samples (as above) were also plated, at 100 µl of each dilution per plate, using a YPSC medium overlay technique in a prey

lawn containing 100 µl of a liquid culture of *E. coli* S17-1 cells (which had been grown in liquid YT medium at 37ºC with 200 rpm shaking for 15 h). These overlay plates were incubated statically at 29ºC for 24 hours at the University of Reading, and then were brought back to the University of Nottingham by car and incubated statically at 29ºC for a further 10 days (it is important to note that the plates were at a temperature lower than 29ºC for the duration of their transportation from the University of Reading to the University of Nottingham, which was 3 h). The plates were checked daily for the appearance of cleared prey 'plaques' indicating the presence of live *B. bacteriovorus,* in order to enumerate any *B. bacteriovorus* cells that were present in each sample. However, use of *N. benthamiana* as a model plant host was discontinued in favour of using more readily obtainable, easily-inoculated post-harvest *Agaricus bisporus* mushrooms, as described in methods Section 2.5.5 and results Section 4.4.1**.**

2.4.6 Transmission Electron Microscopy of mixed *Pseudomonas* pathogen-*B. bacteriovorus* HD100 liquid suspensions.

Electron microscopy (EM) was used to visualise *P. avellanae* 48, *P. syringae pv. phaseolicola, P. agaricii* 2289, and *P. tolaasii* 2192^{T}, each in the presence of *B. bacteriovorus* HD100 at the beginning of the predatory cycle. A 10 ml lysate of HD100 (see culturing protocol) was filtered x2 through a 0.45 µm Millipore filter and 2 x 2 ml was centrifuged at 13000 rpm for 2 minutes. The majority of the supernatant was removed and the cells were resuspended in the residual supernatant (20 µl). 2 ml of a liquid culture of each *Pseudomonas* pathovar (Section 2.4.2) was spun down for 2 min at 13000 rpm, then resuspended in 2 ml Ca^{2+} HEPES buffer. This process was repeated twice to wash King's medium B off the cells, as it has particles in it that can obscure an adequate view of the cells. 20 µl of the *Pseudomonas* pathovars were added separately to 20 µl *Bdellovibrio* HD100 preparations and incubated at room temperature for 15 minutes to allow *Bdellovibrio* attachment to *Pseudomonas* cells. 15 µl of each mixture was added to separate formovar/carbon S162 (Agar Scientific) EM grids and incubated for 5 minutes, then gently wiped off with whatman paper. 15 µl of 0.5% w/v

uranyl acetate stain in dH_2O was then added to each EM grid and incubated for 1 min before gently wiping off with Whatman paper. Images were taken at a magnification of 20000 x on a JEOL 1200Ex electron microscope.

2.5 Methods used in Chapter 4

2.5.1 **Strains used in this study**

Strain	**Description**	**Reference**
Bdellovibrio bacteriovorus HD100 predator		
HD100	Wild type isolated from soil in Germany	[22, 36]
Escherichia coli prey for lab analyses/culturing		
S17-1	*thi, pro, hsdR*$^-$*, hsdM*$^+$*, recA,* integrated plasmid RP4-Tc::Mu-Kn::Tn7	[37]
Pseudomonas tolaasii mushroom spoilage pathogen		
2192^T	Type strain, NCPPB No. 2192^T, brown blotch pathogen of mushrooms	[56]
Model button mushroom species		
Agaricus bisporus	Sourced from Tesco, pre-packaged in a plastic tray with film cover	[60]

2.5.2 **Primers used in this study**

Name	**Sequence**	**Description**
16s_8F	AGAGTTTGATCMTGGC	'Universal' forward primer designed to amplify/sequence diverse bacterial 16s rDNA sequences [61]
16s_1492rev	TACCTTGTTAYGACTT	'Universal' reverse

		primer designed to amplify/sequence diverse bacterial 16s rDNA sequences [61]

The Forward primer hybridises to the 8-27 bp region and the reverse primer to the 1510-1492 bp region of *E. coli* rDNA (and were thus named for these positions). The forward primer contains an 'M' base which pairs to either Adenine (A) or Cytosine (C), and the reverse primer contains a 'Y' base which hybridises with either Thymine (T) or C, allowing for hybridisation and priming of 16s rDNA sequences from different bacterial species.

2.5.3 **Bacterial culturing procedures**

Following growth on *E. coli* S17-1 prey as described in Section 2.3, the *B. bacteriovorus* HD100 were filtered by passage twice through Millipore 0.45μm syringe filters to remove any remaining prey. *P. tolaasii* 2192^T was grown in King's Medium B (described in Section 2.2) at 29˚C for 16 hours. When isolating indigenous bacteria from mushrooms Coliform chromogenic agar (Oxoid, product code CM0956, described in Section 2.2) was used, again with incubation at 29˚C.

2.5.4 ***B. bacteriovorus*** **predation of** ***P. tolaasii*** **populations grown in vitro**

B. bacteriovorus predation of *P. tolaasii* was firstly tested in a buffer-*Pseudomonas* King's medium B suspension in a plate reader. 180 µl/well of a 50% v/v King's Medium B, 50% v/v 2 mM $CaCl_2$ 25 mM HEPES pH 7.6 buffer mixture was added to the wells of a clear-bottomed, 96-well Krystal microplate (Porvair Sciences Ltd, Product No. 215006). 1.5ml aliquots of predatory cultures of *B. bacteriovorus* HD100, containing 2.5 x 10^8 PFU ml^{-1}, were prepared and heat killed at 105˚C for 5 minutes and allowed to cool to ambient temperature (21˚C). This heat-killed, cooled culture was then added,

in a 3:1 ratio, to a live liquid culture of *B. bacteriovorus* HD100 to give 6.3 x 10^7 PFU ml^{-1} of live *B. bacteriovorus* HD100. This was used as a diluted application of *Bdellovibrio* to achieve a lowered concentration of predator in these experiments. Microplate wells were then set up using either 64 µl of the heat-killed culture alone as a negative control; 64 µl of the heat-killed/live mixture described above; or 64 µl of the original live culture of *Bdellovibrio*. These preparations gave final live *B. bacteriovorus* HD100 cell numbers of 0, 4 x 10^6 or 1.6 x 10^7 PFU, respectively. For test prey cells, a liquid culture of *P. tolaasii* 2192^T, containing 7.4 x 10^8 CFU/ml^{-1}, was diluted 2 in 5 to give 3.0 x 10^8 CFU/ml^{-1} in 50% v/v King's Medium B, 50% v/v 2 mM $CaCl_2$ 25 mM HEPES pH 7.6 buffer mixture. 20 µl of this diluted *P. tolaasii* 2192^T containing 5.9 x 10^6 CFU was transferred to the microplates containing the predator mixtures. The plates were then sealed with a Breatheasy® seal (Diversified Biotech Cat. No. BEM-1) and transferred to a BMG plate reader programmed to incubate and measure the $OD600_{nm}$ of each well, as an indicator of *P. tolaasii* 2192^T growth, immediately, and then every 30 minutes for 24 hours. *B. bacteriovorus* HD100 alone does not produce an $OD600_{nm}$ value due to its small cell size [59].

2.5.5 Testing the effect of *B. bacteriovorus* predation of *P. tolaasii* on brown blotch lesion intensity on infected mushrooms

Button mushrooms (*Agaricus bisporus*) used in this experiment were sourced from a supermarket and thus were from a non-sterile setting. Wearing gloves to avoid hand contamination, mushrooms were gently wiped clean with laboratory tissue to remove any attached compost and excess surface moisture, but allow the mushroom epidermis to remain intact. Stipes were trimmed flat with a sterile scalpel blade, and each mushroom was placed, pileus side up, in a sterile 50 ml skirted Falcon tube. Bacterial preparations were grown in liquid culture as before, but concentrated before use, by centrifugation in Falcon tubes at 5200rpm, 20min at 25°C in a Sigma 4K15 centrifuge and resuspension in King's Medium B to the appropriate concentration (which was checked by viable counting after the experiments). Concentrations used in the 15µl applications to the mushrooms were as

follows: *P. tolaasii* 2192^T (1.7 x 10^6 Colony Forming Units, CFU, 15 µl^{-1}), *B. bacteriovorus* HD100 (2.9 x 10^6 Plaque-Forming Units, PFU, 15 µl^{-1}) and King's Medium B were applied directly to the mushroom pileus in one of 5 pairwise combinations for the experiment (see Table 2). In later experiments other concentrations of bacteria were used as described.

Table 2 . Treatment conditions applied to mushroom pilei. Details of the 5 pairwise combinations of *B. bacteriovorus* HD100, *P. tolaasii* 2192^T and King's Medium B added to *Agaricus bisporus* mushrooms to test the effect of *B. bacteriovorus* predation of *P. tolaasii* on affected mushroom brown blotch lesion intensity.

Condition	Addition 1 (in 15 µl)	30 min, 21˚C	Addition 2 (in 15 µl)	48 h, 29^{0}C
King's Medium B control	King's Medium B broth	→	King's Medium B broth	→
B. bacteriovorus alone	*B. bacteriovorus* HD100	→	King's Medium B broth	→
P. tolaasii alone	*P. tolaasii* 2192^T	→	King's Medium B broth	→
B. bacteriovorus before *P. tolaasii*	*B. bacteriovorus* HD100	→	*P. tolaasii* 2192^T	→
B. bacteriovorus after *P. tolaasii*	*P. tolaasii* 2192^T	→	*B. bacteriovorus* HD100	→

Mushrooms were incubated statically at 29˚C, in capped Falcon tubes for 48 hours, after which brown blotch lesions appeared on *P. tolaasii* 2192^T

infected samples. Lesions were photographed using a Canon PowerShot A620 digital camera and tripod in a containment hood, with the same standard lighting for each photograph. The aperture was set to F = 5.6 and shutter speed was set to 1/60 sec, to give a good light exposure (± 0 Exposure Value units). The lesion intensity on each mushroom was analysed using ImageJ analysis software (http://rsbweb.nih.gov/ij/): Image J converted each image to 8-bit grayscale, assigning a value of 0-255 to each pixel; the area of mushroom inoculated was selected and the average grayscale value for each pixel (the Pixel Value, PV), was calculated. On this scale, 0 = black and 255 = white, and so the data were transformed using the formula 1/PV to invert the scale, so that darker lesions give higher intensity values.

2.5.6 Visualising *B. bacteriovorus* and *P. tolaasii* interactions on the mushroom surface

Mushrooms under each of the five treatment conditions detailed in Table 2 were visualised using Scanning Electron Microscopy (SEM). Preparation of mushroom samples for imaging was as follows: Samples of mushroom pileus surface tissue W 5 mm x L 5 mm x D 2 mm were cut and stored in 70% ethanol. They were then dehydrated through a graded series of ethanol concentrations (fresh 70% ethanol, followed by 90% ethanol, and finally 2 changes of 100% ethanol) and dried using a Polaron E3000 Critical Point Dryer. The dried samples were mounted onto aluminium stubs using silver paint, and the stubs were gold coated (~10 µm thickness) using a Polaron E5100 SEM Coating Unit. The samples were viewed and photographed under a JEOL JSM 840 Scanning Electron Microscope at 20 kV. Images were false-coloured in Adobe Photoshop by selecting *P. tolaasii* 2192^{T} and *B. bacteriovorus* HD100 cells and using the 'Colorize' function in the 'Hue/Saturation' tool. A pale yellow colour was selected for *P. tolaasii* to provide optimum contrast to the mushroom surface, and blue gave a sharp contrast for the *B. bacteriovorus.*

2.5.7 **Enumerating *P. tolaasii* recovered from infected mushroom tissue**

Mushrooms were pre-treated using methods as above; *B. bacteriovorus* HD100 was applied at either 2.9 x 10^6 or 1.4 x 10^7 PFU 15 μl^{-1} before 1.7 x 10^6 *P. tolaasii* 2192^T in 15μl. Mushroom lesions were photographed in a class II containment hood after 48 hours, as above, and lesion intensities were analysed using ImageJ analysis software. Lesion tissue from each mushroom was then cut out using a sterile scalpel blade. Tissue samples were weighed and homogenised in sterile 2 mM $CaCl_2$ 25 mM HEPES pH 7.6 buffer (1 ml calcium HEPES/0.04 g lesion tissue) using separate glass pestle and mortar sets, (pre-cleaned with ethanol and dried), for samples under each of the different treatment combinations. *P. tolaasii* 2192^T CFU recovered from each sample were enumerated by serial dilution and plating on King's Medium B agar, incubated at 29˚C for 15 hours. Characteristic smooth, beige colonies growing on King's Medium B were counted and recorded as *P. tolaasii*.

2.5.8 **Identifying bacterial species co-isolated from some experimentally infected supermarket mushroom tissue**

When King's Medium B plates were examined and counted to enumerate *P. tolaasii* 2192^T from one batch of six mushrooms (two in each treatment group), a relatively high number of bacterial colonies, some of which were small and clumped together on the King's B medium enumeration plates, were recovered from *P. tolaasii* 2192^T inoculated mushroom tissue pre-treated with *B. bacteriovorus* HD100 compared with tissue inoculated with *P. tolaasii* 2192^T alone. This suggested that other, possibly indigenous, bacteria were present, in addition to the added *P. tolaasii* 2192^T and *B. bacteriovorus* HD100. To test this, 20 single colonies were selected from the small clumped colonies recovered from mushroom tissue pre-treated with *B. bacteriovorus* HD100 at both 2.9 x 10^6 and 1.4 x 10^7 PFU ml^{-1} (taken from two mushrooms from each group). These were plated directly onto Coliform chromogenic agar (Oxoid) and incubated at 29˚C for 15 hours, along with a *P. tolaasii*

2192^{T} control, to distinguish between Pseudomonads and Coliforms. All of these small, clumped colonies were purple on the chromogenic agar, indicating a different identity to *P. tolaasii* 2192^{T}, which gave straw-coloured colonies on chromogenic agar.

Total genomic DNA from each of 3 purple coliform isolates (hereafter referred to as Supermarket Mushroom Isolates 1, 2 and 3) was extracted using a Sigma DNA extraction kit, and 'universal' 16s ribosomal DNA primers (Section 2.5.2) were used in PCR reactions to amplify 16s rDNA sequences which were sequenced by Source Bioscience Life Sciences, using the same primers. The PCR programme used was as follows:

Temperature (ºC)	Time (min:sec)	No. of Cycles
98	05:00	1
98	00:30	30
50	00:30	
72	01:30	
72	10:00	1

The resulting sequences were used to identify the closest match to the 16s rDNA sequences of the isolates using the BLAST online tool, http://blast.ncbi.nlm.nih.gov/Blast.cgi. As the isolates were all most closely related to the same organism in BLAST given the 16s rDNA sequences, a phylogeny was created using only one of the isolates which compared its 16s rDNA sequence those of other, similar species of bacteria, found using the NCBI Gene search tool (http://www.ncbi.nlm.nih.gov/gene/).

The phylogenetic tree construction software used was Phylogeny.fr ([62] http://phylogeny.lirmm.fr/phylo_cgi/index.cgi), using the "one-click" option. In this option, the programme uses optimised and recommended default settings, which are shown in Appendix 1, to construct a Maximum Likelihood (ML) method phylogenetic tree. Briefly, this method compares all possible

phylogenetic arrangements of sequences, for which the likelihood of the data given each arrangement is given a score, and the highest scoring arrangement is selected. This programme uses MUSCLE to align the sequences to one another, Gblocks to eliminate poorly conserved and highly divergent regions in the DNA alignment, PhyML to construct the phylogenetic tree and calculate bootstrap values, and TreeDyn to render the graphical representation of the phylogenetic tree.

2.5.9 **Isolating other bacterial species from garden mushroom lesion tissue and assessing their susceptibility to *Bdellovibrio* predation.**

To determine whether *B. bacteriovorus* HD100 could prey upon other pathogens/commensals of organic mushrooms as well as commercially grown varieties, Prof. Liz Sockett collected fresh garden-grown mushrooms exposed to natural conditions (outdoors, in organic soil with no treatments to reduce the incidence of pathogens). These mushrooms had large, grey lesions on their surface, suggesting the presence of one or more bacterial pathogens; the lesion tissue was cut out and resuspended in sterile calcium HEPES buffer (2 mM CaCl2 25 mM HEPES pH 7.6) using a sterile glass pestle and mortar set using the same method described in Section 2.5.7. The resulting suspension was serially diluted 1 in 10 to 10^{-7}, and 10^{-5} to 10^{-7} dilutions were spread on King's Medium B and LB agar plates at 100 µl/plate, before incubating statically at 29ºC for 15 hours. Two types of media were used in order to increase the range of species that may be isolated from the mushroom tissue.

To test the range of *B. bacteriovorus* HD100 predation on these mushroom-dwelling species, 2 colonies were selected from the King's Medium B plates (Garden Isolates 1 and 2) and 3 from the LB medium plates (Garden Isolates 3, 4 and 5), for contrasting morphologies (colony colour, morphology and size) and were purified by sequentially streaking of single colonies on the appropriate agar (King's Medium B or LB, depending on which each isolate had been initially grown on), inclubating at 29ºC for 15 hours, three times. Garden Isolates (GIs) 2, 3 and 5 were chosen from the initial 5 isolates for

further testing because GI4 and GI2 were similar in colour and morphology and may be identical so only GI2 was included, and GI1 was fluorescent on King's medium B agar and thus was likely a pseudomonad, and I had already tested *Bdellovibrio* predation of a range of *Pseudomonas* pathogens/commensals in Chapter 3, and was interested in testing a broader range of species if possible.

B.bacteriovorus HD100 predation of GI2, 3 and 5 was tested using the optical density measuring assay described in Section 2.4.3. These isolates were added to the wells of the microtitre plates, containing the predatory mixtures of *B. bacteriovorus* HD100, at 1.07 x 10^8 (GI2), 2.23 x 10^7 (GI3), and 4.08 x 10^7 cells/well (GI5), and $OD600_{nm}$ was measured as before, every 30 min over 24 hours, using a BMG microtitre plate reader.

To determine whether any of these three isolates was a potential causal agent of the grey lesions that they were isolated from or alternatively if they were naturally dwelling, possibly commensal species, a preliminary mushroom inoculation assay was carried out in the same way as for *P. tolaasii* 2192^T in Section 2.5.5. In this assay, each of the isolates 2, 3 and 5 as well as a King's medium B only and an LB medium only control, were added to the supermarket mushroom caps. Each isolate was grown from a single colony picked from agar plates of each isolate, prepared as above, in King's medium B (GI2) or LB (GI3 and 5) liquid medium, and incubated for 15 h at 29ºC with 200 rpm shaking. The isolates were then adjusted to $OD600_{nm}$ = 1 before use. As previously, each addition was made in 15 µl/mushroom cap, and 2 additions were made 30 min apart, according to the same protocol as the *P. tolaasii* 2192^T and King's medium B control detailed in Table 2. 8 mushrooms were inoculated with each isolate, and 4 with each medium control- this was conducted as a quick, preliminary test, and the numbers of mushrooms used in each group reflect the amount of mushrooms that could be accommodated in suitable, available containers. As before, mushrooms were incubated at 29ºC for 72 hours, and then caps were photographed and analysed using ImageJ, according to the same standards as in Section 2.5.5.

To determine the species identity of these isolates, the same method of DNA extraction and 16s rDNA sequencing was used as in Section 2.5.8**,** using the generic 16s_8F and 16s_1492REV primers [61] listed in Section 2.5.2, followed by the same method of creating a phylogeny as in Section 2.5.8, using bacterial 16s rDNA sequences from related species.

2.6 Methods used in Chapter 5

2.6.1 **Strains used in this study**

Strain	**Description**	**Reference**
Bdellovibrio bacteriovorus HD100 predator		
HD100	Wild type isolated from soil in Germany	[22]
Pseudomonas putida prey		
	Previously used by Hobley et al. as prey in a *B. bacterivorous* Tiberius genome sequencing study	[63]
Gaeumannomyces graminis var. *tritici* (take-all disease)		
isolate no. 66	Isolated in 2012 from Rothamsted field New Zealand by Gail Canning (Rothamsted Research)	This Study
Triticum aestivum L. (winter wheat)		
var. Cadenza	Low TAB variety	[64, 65]
var. Hereward	High TAB variety	[64, 65]

2.6.2 **Primers used in this study**

Name	**Sequence**	**Description**
Whole-gene 16s ribosomal DNA amplification and sequencing		
16s_8F	5'-AGAGTTTGATCMTGGC-3'	Universal Forward primer for sequencing 16s ribosomal DNA [61]
16s_1492Rev	5'-TACCTTGTTAYGACTT-3'	Universal Reverse primer for sequencing 16s ribosomal DNA [61]
16s Variable (V4) region amplification for soil metagenomic DNA analysis		
Name	Sequence	Description
515f PCR Primer Sequence-Forward Primer	5'-AATGATACGGCGACCACCGAGATCTACAC-TATGGTAATT-GT-GTGCCAGCMGCCGCGGTAA-3'	Forward primer containing a 5' Illumina adapter sequence, a forward primer pad, and linker, followed by the forward primer (different parts delimited by hyphens) [66]
806r PCR Primer Sequence-Reverse Primer, barcoded	5'-CAAGCAGAAGACGGCATACGAGAT-XXXXXXXXXXXX-AGTCAGTCAG-CC-GGACTACHVGGGTWTCTAAT-3'	Reverse primer containing a reverse complement of 3' illumina adapter, a Golay barcode (where the 12 X sequence represents a sequence of bases unique to each sample), reverse primer pad, and linker, followed by the reverse primer (different parts delimited by hyphens) [66]
Reverse Transcription Polymerase Chain Reaction (RT-PCR) amplification of RNA encoding *B. bacteriovorus* hypothetical phytohormone proteins		
Bd0059RT-F	GATTGGTGAACGGGTTTCTG	Forward primer to amplify hypothetical Indole-3-Acetic Acid (IAA) modifying protein-encoding RNA
Bd0059RT-R	GACTGACCAAACGGGACACT	Reverse primer for the above
Bd1990RT-F	AAACCGGCAAGACTCACATC	Forward primer to amplify hypothetical IAA regulated protein-encoding RNA
Bd1990RT-R	AGACGGTCAATACGCTCCTG	Reverse primer for the above
Bd2647RT-F	ATACCTGCGCGAATATCAGG	Forward primer to amplify hypothetical IAA carboxylase protein-encoding RNA
Bd2647RT-R	GATGACCAAAGGCCTCTTCA	Reverse primer for the above

Bd2890RT-F	TTCTATCCGGTGGATTTTGC	Forward primer to amplify hypothetical cytokinin oxidase protein-encoding RNA
Bd2890RT-R	GACAACCCAGTCACGAGTCA	Reverse primer for the above
Bd0452RT-F	TGAGCCAGACACTTTGATCG	Forward primer to amplify hypothetical ethylene forming protein-encoding RNA
Bd0452RT-R	TGATCACACGGTGGGTAGTG	Reverse primer for the above
Bd2540RT-F	ACGACTTATCGCATGGAAGG	Forward primer to amplify hypothetical monoamine oxidase (IAA-producing) protein-encoding RNA
Bd2540RT-R	AGGCCATGTTCATCTTCACC	Reverse primer for the above
Bd2266RT-F	TCACTACCGAAGCCTTCCAC	Forward primer to amplify hypothetical aldehyde dehydrogenase (IAA-producing) protein-encoding RNA
Bd2266RT-R	CGAAGCTTAGCAGCTCCTGT	Reverse primer for the above

2.6.3 **Experimental strategy and choice of soil and wheat cultivars.**

Table 3: Overview timeline of wheat sowing, inoculations, soil sampling, and plant measuring for each of the 3 pot experiments, 1 carried out in 2012-2013 and 2 in 2013-2014. Calendar dates and days of experiment (from wheat seed sowing) are given. Treatments and general plant upkeep e.g. Osmocote fertiliser addition (detailed in Section 2.6.11) are shown in dark grey, soil sampling and measurements are shown in light grey, and white shows no action taken. All pots were incubated outside in an open, sunlit netted tunnel and only watered by natural precipitation/by hand in dry conditions.

		Pot Experiment 1a									
2012-2013	Date	24/10/2012	17/04/2013	23/04/2013	25/04/2013	26/04/2013	29/04/2013	01/05/2013	28/05/2013	18/06/2013	12/08/2013
	Day of Experiment	1	176	182	184	185	188	190	217	238	293
	Soil = Delafield Event	Pots sown with wheat seed	1st soil samples	First Bdellovibrio inoculation	2nd soil samples	Take-all inoculation	Second Bdellovibrio Inoculation	3rd soil samples	4th soil samples, representative plant heights measured, osmocote fertiliser added	Plant heights measured	Full growth: 5th Soil samples, full set of plant measurements taken
		Pot Experiment 2									
2013-2014	Date	28/10/2013	08/04/2014	08/04/2014	15/04/2014	15/04/2014	16/04/2014	16/05/2014	21/05/2014	20/06/2014	14/07/2014
	Day of Experiment	1	163	163	170	170	171	201	206	236	260
	Soil = Delafield + Great Harpenden-1 Event	Pots sown with wheat seed	1st soil samples	First Bdellovibrio inoculation	2nd soil samples	Second Bdellovibrio inoculation	3rd soil samples	4th soil samples, plant heights measured	Osmocote fertiliser added	Plant heights and flag leaf senescence measured	Full growth: 5th soil samples, full set of plant measurements, and rhizosphere samples taken
		Pot Experiment 1b									
	Soil = Delafield Event	Pots sown with wheat seed	No soil samples	No addition	No soil samples	No addition	No soil samples	No soil samples	Osmocote fertiliser added	Plant heights and flag leaf senescence measured	Full growth: full set of plant measurements and rhizosphere soil samples taken

This experiment was initially designed in 2012-2013 to test the effect of *Bdellovibrio* treatment of the soil on take-all fungal infection and resultant wheat plant growth in two different wheat cultivars. Take-all fungus is known to be suppressed in soil by *Pseudomonas* PGPR species, and in turn *Bdellovibrio* is able to prey upon some species of *Pseudomonas*; therefore the hypothesis was that *Bdellovibrio* addition to the wheat rhizosphere soil may decrease the level of PGPRs, resulting in more severe take-all infection and a reduction in wheat growth and yield. However, as seen in Section 5.4.2.6, *Bdellovibrio* had a positive effect on wheat plant growth and yield, but our experimental take-all treatment had no significant effect on wheat growth and therefore the effects of *Bdellovibrio* on wheat take-all infection level could not be measured. Therefore, a second pot experiment was designed for 2013-2014 in consultation with Dr Vanessa McMillan (RRes) to test the effect of the live *Bdellovibrio* treatment on the growth of one wheat cultivar against a buffer control and a heat-killed *Bdellovibrio* control, in two different soils, to account for any potential effects of nutrient addition to the soil through the death of *Bdellovibrio* cells, releasing nutrients.

In **Pot Experiment 1a**, Two varieties of winter wheat, Cadenza and Hereward, (previous recommended commercial varieties developed by the Plant Breeding Institute in Cambridge in 1991); these two varieties are differently susceptible to take-all infection, described as low (Cadenza) or high (Hereward) Take-All Building (TAB) strains [65], and therefore any *Bdellovibrio* predation of PGPRs in the soil that suppress take-all infection of wheat may affect these varieties differently. The soil in the pots was collected from the Rothamsted field Delafield, which is a Batcombe-Carstens silty clay loam type soil with sandy inclusions [67]; loam-type soils with some sand are considered the best texture for growing a variety of crops, producing a higher yield [68], so Delafield soil is a good representative of agricultural soils in general. Winter beans were sown in Delafield on 24th October 2011 and harvested on 1st September 2012; winter beans are not susceptible to take-all disease, and so no build-up of take-all could occur in the soil prior to the experiment, to minimise the confounding effects of any residual take-all in the soil on wheat growth. Additionally, bean residues replenish the nutrient

content of the soil, typically adding 20-80 kg/Ha nitrogen [69, 70]. Triple Super Phosphate (TSP) fertiliser [71] was added at 383 kg/Ha on 12th September 2012 and Muriate of Potash (MOP) fertiliser [71] at 222 kg/Ha on 15th September 2012 to ensure adequate potassium and Phosphate levels, before the soil was collected for this experiment on the 27th September 2012. The soil was sieved to remove large stones and mixed thoroughly using a fork and spade before putting in the pots, so that the soil was uniform in each pot.

2.6.4 **Testing the effect of calcium HEPES buffer on wheat plant growth**

Bdellovibrio is routinely grown in calcium HEPES buffer (2 mM CaCl2 25 mM HEPES, pH 7.6). This buffer was therefore to be used as a control addition to pots that did not receive any live or heat-killed *Bdellovibrio* in **Pot Experiments 1a and 2**. Therefore, a pilot experiment was set up to test whether calcium HEPES buffer had any adverse effects on the health or growth of wheat plants. 10 pots each (round-rimmed, 20 cm circumference x 30 cm height) were planted with 5 Hereward wheat seeds each in commercially produced, nutrient-rich soil, and kept in an outdoor glasshouse at 15ºC with 250 lux light for 16 hours and 10ºC for 8 hours each day to mimic typical, natural growing conditions, and watered when dry. Once the seeds had germinated and seedlings appeared (after 10 days), all but 1 plant from each pot was removed. Once the plants had reached the 2-leaf stage (Feeke's stage 2, Zadok's stage 22), 150 ml calcium HEPES buffer was added to the soil immediately around the wheat roots in 5 pots, and a water control was added similarly to the remaining 5 pots. At 3, 10, 28 and 51 days after this treatment, the plants were assessed for any signs of a toxic response (grey/brown, necrotic tissue lesions) or nutrient deficiency (yellowing of leaves or stem) as well as stunted growth, symptoms described in a 1991 International Maize and Wheat Improvement Centre guide to wheat toxicity and nutrient deficiency [72], and photographs of each plant were taken to document their health and growth at each of these 4 assessment points.

2.6.5 **Preparing and planting the wheat**

Pot Experiment 1a (2012-2013): On day 1, 24/10/2012 (Table 3), 160 each of Hereward and Cadenza variety wheat grains were pre-germinated for 4 days on water-moistened Wypall* laboratory tissue, in a lidded plastic container on an indoor work bench, exposed to moderate sunlight. 320 kg of Delafield soil was sieved to remove large stones and clumps, and 8 kg of this processed soil was added to each of 40 black plastic square-rimmed pots with drainage holes (height 20 cm x width 21 cm x depth 30 cm). Eight 2 cm-deep indents were made in the soil of each pot in a 3 x 2 x 3 row pattern from left to right. One pre-germinated grain was planted in each indent; 20 pots received Hereward grain and 20 Cadenza. Soil from each pot was used to cover over the planted grains. The pots were arranged in 5 blocks of 8 pots, and each block was randomised using GenStat [73] with regards to wheat variety (Hereward or Cadenza), *B. bacteriovorus* HD100 treatment or a calcium HEPES buffer control, and inoculation with *G. graminis* var. *tritici* or a water control (Table 4).Each pot was placed on a plastic saucer. The plants were grown in a netted tunnel to protect them from predators, while exposing them to natural weather conditions, and watered only when visibly dry until April 2013.

Table 4 . Detail and timing of *Bdellovibrio*/calcium HEPES control and Take-all/water control treatment combinations administered to the pots in Pot Experiment 1a (2012-2013)

Treatment	Wheat Variety	No. pots	1st Inoculation (400 ml)	2nd Inoculation +72 h (200 ml)	3rd Inoculation +144 h (400 ml)
Bdellovibrio + take-all	Hereward	5	8.7×10^{10} Live *Bdellovibrio*	Take-all	1.4×10^{11} Live *Bdellovibrio*
Bdellovibrio + take-all	Cadenza	5	8.7×10^{10} Live *Bdellovibrio*	Take-all	1.4×10^{11} Live *Bdellovibrio*
Take-all alone	Hereward	5	calcium HEPES buffer	Take-all	calcium HEPES buffer
Take-all alone	Cadenza	5	calcium HEPES buffer	Take-all	calcium HEPES buffer
Bdellovibrio alone	Hereward	5	8.7×10^{10} Live *Bdellovibrio*	Water	1.4×10^{11} Live *Bdellovibrio*
Bdellovibrio alone	Cadenza	5	8.7×10^{10} Live *Bdellovibrio*	Water	1.4×10^{11} Live *Bdellovibrio*
Buffer control	Hereward	5	calcium HEPES buffer	Water	calcium HEPES buffer
Buffer control	Cadenza	5	calcium HEPES buffer	Water	calcium HEPES buffer

Pot Experiment 1b (2013-2014): Using the post-harvest soil in pots from **Pot Experiment 1a**, this experiment tested for any longevity that the *Bdellovibrio* treatment might have on take-all infection and wheat plant growth. The post-harvest pots of soil from Experiment 1a were over-sewn with Conqueror wheat grains (a currently farmed commercial variety, recommended by the Home-Grown Cereals Authority, HGCA [74]), pre-germinated as above, left in their randomised order in the netted tunnel, and given no further treatment.

Pot experiment 2 (2013-2014) was also run in an outdoor netted tunnel, but the climactic conditions were very different in year 2, when this experiment was run, than year 1, when **Pot Experiment 1a** was run (see Appendix CD: Rothamsted weather reports 2012-2014.xlsx). Pots were prepared and sown as above with the following differences: 30 pots were included in total, 15 containing soil from the Rothamsted field site Delafield (as above) and 15 from Great Harpenden-1 (GH-1, a typical Batcombe-type silt loam/silty clay loam), into which pre-germinated Hereward variety grains only were sown, as above. Pots were randomised according to soil type and B. *bacteriovorus* HD100 treatment in 5 blocks of 6 pots (Table 5).

Table 5 . Detail and timing of Live *Bdellovibrio*/Heat-Killed *Bdellovibrio* control/calcium HEPES control treatments administered to the pots in Pot Experiment 2.

Treatment	Soil Type	No. Pots	1st Inoculation (400 ml)	2nd Inoculation +144 h (400 ml)
Live *Bdellovibrio*	Delafield	5	8.3 x 10^{10} Live *Bdellovibrio*	1.4 x 10^{11} Live *Bdellovibrio*
Live *Bdellovibrio*	GH-1	5	8.3 x 10^{10} Live *Bdellovibrio*	1.4 x 10^{11} Live *Bdellovibrio*
Heat-killed *Bdellovibrio*	Delafield	5	Heat-killed *Bdellovibrio*	Heat-killed *Bdellovibrio*
Heat-killed *Bdellovibrio*	GH-1	5	Heat-killed *Bdellovibrio*	Heat-killed *Bdellovibrio*
calcium HEPES buffer	Delafield	5	calcium HEPES buffer	calcium HEPES buffer
calcium HEPES buffer	GH-1	5	calcium HEPES buffer	calcium HEPES buffer

2.6.6 **Preparing *B. bacteriovorus* HD100 inoculum (Pot Experiments 1a and 2)**

B. bacteriovorus HD100 was cultured from frozen stocks using a routine overlay plate technique on YPSC medium (0.125 g/L Magnesium Sulphate, 0.25 g/L sodium acetate, 0.5 g/L bacto peptone, 0.5 g/L yeast extract, 0.25 g/L calcium chloride dihydrate, pH 7.6). 5 ml of 55^0C YPSC agar (containing 6 g/L bacteriological agar) was mixed with 150 µl E.coli S17-1 prey (containing 1.5 x 10^7 live cells) and poured over a base layer of YPSC agar medium (containing 10 g/L bacteriological agar) in a petri dish and left to set. 150 µl defrosted *Bdellovibrio* containing (3.7 x 10^7 live cells) was then spotted on to the surface, spread by tilting the plate, and left to dry in a class II sterile cabinet. The plate was incubated for 72 h at 29^0C, at which point clear plaques containing predatory *Bdellovibrio* had formed. A plug of agar taken from this plate using a 1 ml pipette tip was used to inoculate 2 ml 2mM calcium HEPES buffer, pH 7.6, containing 150 µl *E. coli* S17-1 prey (1.5 x 10^7 live prey cells). This small liquid culture was incubated with 200 rpm shaking at 29^0C for 48 h, at which point *Bdellovibrio* had lysed the *E. coli* prey.

P. putida was grown in YT medium broth (5 g/L sodium chloride, 5 g/L peptone, 8 g/L tryptone, pH 7.5), from single colonies on YT medium agar, at 29^0C for 15 hours with 200 rpm shaking as prey for culturing *B. bacteriovorus*. This prey was chosen as it commonly inhabits soil, and therefore should not alter the microbial community of the soil as much as the *E. coli* prey usually used in this laboratory for culturing *Bdellovibrio*: *E. coli* K-12, from which this prey strain is derived, was originally a faecal contaminant [75].

For each of the 2 *Bdellovibrio* inoculations, 10 x 50 ml *B. bacteriovorus* HD100 cultures were prepared by adding 1 ml *Bdellovibrio* (containing 2.5 x 10^8 live *Bdellovibrio* cells) to 3 ml *P. putida* prey (containing 2.0 x 10^9 live prey cells) in 50 ml 2mM calcium HEPES buffer pH7.6. 10 x 1 L *B. bacteriovorus* HD100 cultures were prepared in 10 x 2 L conical flasks, each containing 1 L 2mM calcium HEPES buffer pH 7.6, 65 ml *P. putida*, grown as above (containing 4.3 x 10^{10} *P. putida* cells) and 50 ml *B. bacteriovorus* HD100

containing 1.2 x 10^{10} live *Bdellovibrio* cells. These 1 L cultures were prepared the day before inoculation and were incubated at 29˚C for with 200 rpm shaking for 20 hours. This was sufficient time for *B. bacteriovorus* HD100 to clear the *P. putida* prey, verified by light microscopy.

800 ml of each cleared 'prey lysate' was divided between 2 x 1L screw-top duran bottles, a total of 20 x 400 ml cultures for each set of *B. bacteriovorus* inoculations into pots (each containing 8.7 x 10^{10} /1.4 x 10^{11} live *Bdellovibrio* cells and 1.1 x 10^{5} / 8.5 x 10^{5} residual *P. putida* prey cells in inoculation 1 / 2 in experiment 1a; and 8.3 x 10^{10} /1.4 x 10^{11} live *Bdellovibrio* cells and 7.6 x 10^{6}/1.2 x 10^{7} residual *P. putida* prey cells in inoculation 1 / 2 in experiment 2). This left a head space of approximately 400ml per bottle to ensure aerobicity during transport to Rothamsted Research. All 20 cultures were left live for **Pot Experiment 1a**.

For **Pot Experiment 2**, 10 of these bottled cultures were microwaved on full power. Cultures were seen to be visibly boiling during this treatment, which was carried out for 10 minutes for each bottle, until no swimming *Bdellovibrio* cells were observable by light microscopy. This was taken as a rapid proxy for viability as quick transportation of cultures was needed.

There were no previous studies of *Bdellovibrio* addition to soil by which to estimate an appropriate *Bdellovibrio* cell density in the inoculum- enough so that any effect would be observable, but not too much so as to risk the cells becoming anaerobic during transport- so this was estimated.

Although there were some remaining *P. putida* prey cells in the inoculum, the number left were similar to previous residual prey counts by others [76]; as the volume of inoculum was so large, it could not feasibly be filtered to remove prey due to a long processing time, leading to static time in pots and a drop in respiration and good condition of predators. This could result in reduction of *Bdellovibrio* motility through soil, reducing their spread around the wheat rhizosphere, which may have dampened any effects observed on their addition to the soil.

All *Bdellovibrio* cultures were transported at room temperature (25^0C) by car from the University of Nottingham to Rothamsted Research (journey time: 2 hours) on the morning of each *B. bacteriovorus* inoculation date.

2.6.7 Preparing *Gaeumannomyces graminis* var *tritici* (take-all) inoculum (Pot experiment 1a only)

The *G. graminis* inoculum was prepared by Dr Vanessa McMillan (RRes), 100 g silver sand, 3 g maizemeal and 10 ml of distilled water were put in conical flasks which were autoclaved twice, with 48 hours between autoclaves. The flasks were inoculated with agar discs from *G. graminis* var. *tritici* cultures grown on Potato Dextrose Agar (3 disks per flask). The *G. graminis* var. *tritici* isolate used was a new isolate (No. 66) recovered by Gail Canning in Autumn 2012 from the Rothamsted field, New Zealand. The sand/maizemeal cultures were then kept at 19^0C (room temperature) for 5 weeks, with shaking once a week (for even colonisation), and kept at 4^0C for inoculation on 24th April 2013 (Table 3).

A 'soil calibration' test was carried out by Dr Vanessa McMillan (RRes) before the *G. graminis* var. *tritici* inoculum was added to the soil. The *G. graminis* var. *tritici* maizemeal-sand culture was diluted with silver sand (in a ratio of 1:50, 1:100, 1:150, 1:200, 1:250, 1:300 and 1:350) and 50 g of each dilution was mixed with 250 g take-all free soil and added to plastic drinking cups, each containing 50 cm^3 damp sand in the bottom over four drilled holes for water drainage. Ten Hereward variety wheat seeds were placed on the surface and covered over with horticultural gravel. 5 replicates per dilution were set up, and were watered and kept under controlled conditions (16 hour day, 70% RH, day/night temperatures 15/10°C, twice weekly watering to simulate natural growth conditions) in a randomised design. After 5 weeks, the plants were removed, their roots washed to remove soil, and the percentage of infected/healthy plants and roots were calculated. The aim was to determine a dilution at which 50% of plant roots were infected, which would be used in the pot tests. For each inoculation, 5 g of the *G. graminis* sand/maizemeal culture was added to 200 ml water.

This method of *G. graminis* inoculum preparation was previously used in a published study by McMillan and coworkers, 2014 [77].

2.6.8 **Inoculating the pots**

In pot experiment 1a, 2.3 x 10^{11} *Bdellovibrio* (and 9.6 x 10^{5} residual *P. putida* prey cells) were added to each pot in total from the 2 x 400 ml inoculations made 144 h apart, with control pots receiving only 2 x 400 ml calcium HEPES buffer, as detailed in Table 4; the take-all inoculations were made in 1 inoculation of 200 ml, with control pots receiving 200 ml water. Each inoculation was poured into the pot, intermittently and slowly (3-5 minutes) so that the liquid could soak in and not run off, and close to the soil to avoid transferring *B. bacteriovorus* HD100 or *G. graminis* var *tritici* into control pots through splashing (Figure 4). Plastic dishes were placed beneath each pot to contain any liquid that drained through the soil to the bottom; the volume of liquid was previously optimised by Dr Vanessa McMillan (RRes) to be appropriate to the soil volume and therefore minimise this run-off from the pots.

Figure 4 . Young, healthy wheat plants being inoculated with *Bdellovibrio* in experiment 1a on day 182 (23/4/2013).

In Pot Experiment 2, A total of 2.2 x 10^{11} *Bdellovibrio* cells (and 2.0 x 10^{7} residual *P. putida* prey cells) were added to each pot in the 2 x 400 ml live *Bdellovibrio* inoculations, again 144 hours apart, as detailed in Table 5; in the heat-killed *Bdellovibrio* additions, a total of 1.0 x 10^{11} killed *Bdellovibrio* cells

and 1.5 x 10^{11} residual, killed *P. putida* cells were added to each pot in 2 x 400 ml; control pots received 400 ml calcium HEPES buffer, and all inoculations were made into the pot soil as above.

The first inoculation in **Pot Experiment 2** contained a contaminant, discovered post-inoculum-preparation by light microscopy (Figure 28). One culture had visibly more contaminant cells in it than the rest, so this one was discarded, and only the remaining 9 litre lysates, which had fewer contaminants, were used for the inoculation. The contaminants were enumerated by dilution of the inoculum and spreading on King's medium B agar, in order to distinguish between the contaminant and the residual *P. putida* prey cells (as only *Pseudomonads* produce green siderophores on King's medium B, and the morphology of the contaminant suggested it was not a *Pseudomonad.*

Genomic DNA was extracted from a liquid culture of this contaminant, grown in 10 ml YT medium broth at 29ºC for 15 h with shaking at 200 rpm, using a Sigma Genomic DNA Extraction kit (Cat No. NA2110). The 16s ribosomal DNA was amplified using a PCR reaction using the 8_F and 1492_Rev primers (see Section 2.6.2), using the same PCR programme described in Section 2.5.8**.** The resulting 16s rDNA fragment was Sanger sequenced by Source Bioscience, using the same primers as above. The BLAST online search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to determine the identity of the contaminant by comparison with other bacterial 16s rDNA sequences.

The second inoculum of *Bdellovibrio* was normal, containing highly motile *Bdellovibrio* cells as expected with no contaminant cells present.

2.6.9 End point analysis of *Bdellovibrio* survival by plaque assay of enrichments from the wheat pot rhizosphere soil

To test for any remaining live, predatory *Bdellovibrio bacteriovorus* HD100 from the soil in Pot Experiment 1a and confirm their survival until full wheat growth, an enrichment method was used in case *Bdellovibrio* numbers were low, before the sequencing results were known. 2 g soil from each pot

(collected in the same way as for DNA extractions above) was resuspended by vortex mixing in 5 ml calcium HEPES buffer (2 mM $CaCl_2$ 25 mM HEPES, pH 7.6), and left to settle for 24 hours to allow soil sediment and supernatant to separate. To give the best chance for *B. bacteriovorus* HD100 detection vs. other environmental predatory bacteria at low levels, two prey were offered: 2 x 1 ml supernatant from each sample were each enriched with 100 µl *E. coli* S17-1 prey (usual laboratory prey for *B. bacteriovorus* HD100, containing 1.08 x 10^7 cells per 100 µl, pre-grown in 50 ml YT broth at 37^0C with 200 rpm shaking for 15 h) or *P. putida* prey (used for the soil inoculum, containing 6.65 x 10^7 cells per 100 µl, pre-grown in 50 ml YT medium broth at 29^0C, 200 rpm shaking, for 15 h), and the cultures were incubated at 29^0C for 48 h. A further 2 x 1ml from each sample was filtered twice through a 0.45 µm millipore filter before prey-enrichment to purify any residual *Bdellovibrio* away from larger prey as above. The remaining original sample was stored at 5^0C.

All filtered and unfiltered, original and enriched samples prepared above were tested for predation by spotting at 5 µl/spot onto an agar lawn containing 9.98 x 10^7 *P. putida* prey on YPSC medium agar overlay plates. Additionally, the following samples were serially diluted 1 in 10, to a 10^{-5} dilution, and plated on individual agar plates in a *P. putida* prey lawn at 10^{-2} to 10^{-5}: 3 filtered and 3 unfiltered enrichments on *P. putida* prey of samples from *Bdellovibrio*-added pots (Pot no. 3, 4, and 8), 2 filtered and unfiltered enrichments on *P. putida* prey of samples from non-*Bdellovibrio* added pots, and two original unenriched samples, one from *Bdellovibrio*-added (Pot no. 3) and one non-*Bdellovibrio* added (Pot no. 12) soil. Plates were incubated at 29^0C for 5 days, at which point *Bdellovibrio* plaques had appeared. Each prey-enriched sample was also checked by light microscopy for the presence/absence of live, motile *Bdellovibrio* cells, and those samples in which *Bdellovibrio* were observed also produced plaques on the YPSC overlay plates.

Bdellovibrio were cultured from these plaques on *P. putida* prey, according to standard *Bdellovibrio* culturing methods (see Section 2.4.2). DNA extractions were carried out on *Bdellovibrio* cultured from Pot no. 3, 4, and 8, using a

Sigma Genomic DNA Extraction Kit (Cat No. NA2110), 16s rDNA sequences were amplified by PCR using the primers 8_F and 1492_Rev (Section 2.6.2), and Sanger sequenced by Source Bioscience sequencing.

To determine whether *Bdellovibrio* were found in soil proximal to the wheat root in Pot Experiments 1b and 2 at wheat harvest on day 260, the soil attached to root system of one representative mature wheat plant from each pot was washed off with and resuspended in 100 ml analar water, as shown in Figure 5, and the sample was allowed to settle and separate as above. Filtered and unfiltered enrichments of each sample were made as above, and the original samples were retained at 5ºC. Enrichments were incubated at 29ºC for 72 h. All original and enriched, filtered and unfiltered samples were diluted 1 in 10 from 10^0 to 10^{-2}, and plated as above on YPSC overlay agar plates containing 150 µl *P. putida* prey as well as on plates containing *E. coli* S17-1 prey, and incubated for 14 days.

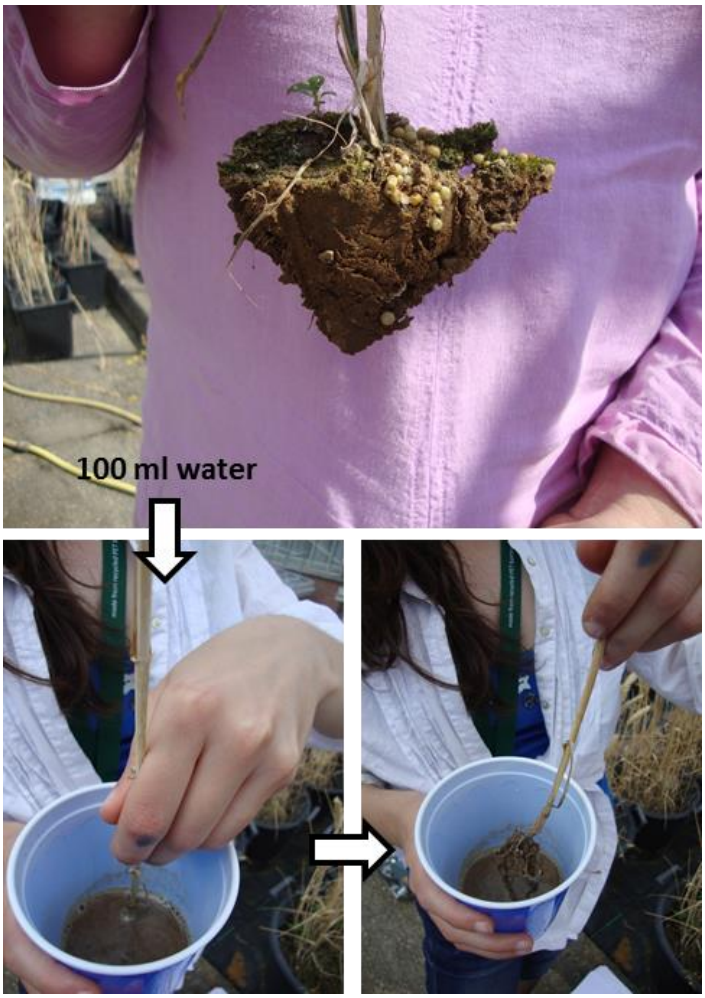


Figure 5 . Resuspending root-proximal soil in analar water for determining the presence of *Bdellovibrio.*

2.6.10 **Assessing protein and nitrogen, Carbon and Oxygen content of live *B. bacteriovorus* HD100 cultures**

In case the live *Bdellovibrio,* when added to the pots, did not survive, and therefore contributed to the pool of important chemical elements in the soil that wheat plants can use to grow, a Lowry assay [78] was carried out on a sample of the *Bdellovibrio* inoculum from Pot Experiments 1a and 2 to determine how much protein and therefore nitrogen was added to the pots. 130 ml of the *Bdellovibrio* liquid culture, grown as an extra litre lysate but superfluous to the required amount for the inoculations, was harvested in a Sigma Ultracentrifuge for 20 min, at 5100 rpm and 29^0C. The supernatant was discarded, and the pellet resuspended in 1.3 ml SDW. 3 x 20, 30 and 50 µl samples of this and a set of Bovine Serum Albumin (BSA, Sigma)

standards (0, 20, 40, 60, 80 and 100 µg) were used in the Lowry assay Samples and standards were put into cuvettes and made up to 500 µl with SDW. 1.5 ml Lowry solution was added to each cuvette, and incubated for 20 min in the dark at 21^0C. 150 µl Folin's reagent was added to each sample, mixed, and incubated for 45 min in the dark at 21^0C, after which the $OD750_{nm}$ of each sample was measured, using the 0 µg BSA sample as a blank.

2.6.11 **Fertilising the wheat pots with Osmocote® fertiliser**

217 days after wheat planting (Table 3), 2 x 10 g pellets of Osmocote® Exact Tablet Patterned Release Fertiliser were pushed into the soil on opposite sides of each pot and lightly covered over with surface soil. The fertiliser contained 1.4 g N (0.65 g of which nitrate NH_4, 0.75 g ammonial NH_3-N), 0.35 g phosphorus (0.3 g of which water soluble), 0.91 g K, 0.12 g Mg (0.06 g of which water soluble), 0.002 g Boron (water soluble), 0.004 g Iron, 0.005 g Manganese (water soluble), 0.002 g Molybdenum (0.0014 g of which water soluble), and 0.0015 g Zinc (0.001 g water soluble). The fertiliser releases these nutrients over 5-6 months in the soil at 21^0C, which more than covered the remaining 2-3 months it took the wheat to reach full growth, post-fertilisation, in all experiments.

2.6.12 **Soil sampling for DNA extractions, 16s metagenomic sequencing and analysis (Pot Experiment 1a)**

The wheat pot soil was sampled at 5 time points over the course of the wheat growth (Table 3). 5-10 g soil was taken from each pot using a 10 mm cork borer pushed into the soil near the plant roots, taking care to maintain the integrity of all plants. Samples were sieved through 2 mm metal mesh and stored immediately at -20^0C in small zip-locked plastic bags. The cork borer and sieve were washed with water between processing samples that received different combinations of *Bdellovibrio* and take-all. Frozen samples were stored for 4-8 months before DNA extractions were prepared, as the samples were taken over the course of 4 months during wheat growth (Table 3).

When all samples had been collected, the total bacterial DNA was extracted from 0.25 g of each sample (±0.005 g) using a MoBio PowerSoil® DNA Isolation Kit (Cat. No. 12888-100) according to the standard accompanying protocol (provided and explained by Dr Ian Clark at Rothamsted Research), using an MP Biomedicals FastPrep®-24 bead beater for the cell lysis step. Care was taken to keep the samples on dry ice before and after weighing while other samples were being weighed before the extraction was carried out. DNA preparations were carried out in a laboratory at Rothamsted Research and not in the Nottingham University *Bdellovibrio* research laboratory so there was no chance of *Bdellovibrio* contamination. The concentration (ng/µl) of extracted DNA in each sample was measured using a NanoDrop spectrophotometer and each sample was stored at -80^0C.

A sample of each DNA extraction, containing 10 ng DNA, was transferred to one well of a 96-well Eppendorf PCR plate. 2 plates were required to contain all 100 samples. The liquid samples were dried in a SpeedVac vacuum concentrator until no liquid remained (30 min), sealed with foil seals, and sent via FedEx courier service to Argonne National Laboratories (ANL, Illinois, USA). ANL carried out paired-end 16s metagenomic sequencing on all samples using an Illumina MiSeq sequencer. The principle of this sequencing method [79] is that a template library of fragments from the V3-V4 variable regions of 16s rRNA from each metagenomic soil bacterial/archaeal DNA sample is prepared, and indexed barcodes and adapters added, using limited cycle PCR with the 515f and 806r primers for 16s V4 region amplification for soil metagenomic DNA analysis listed in Section 2.6.2, and these adapted fragments are hybridised to a flow cell. The forward and reverse ends of these fragments are sequenced separately, creating a complementary, paired-end set of reads. The reads from all samples are pooled (as they are barcoded dependent on their sample of origin). DNA sequencing was not available until well after the pot experiment had been completed.

The output is a series of one forward and one reverse set of paired-end sequence reads of 151 nucleotides each, with a 49 bp overlap to make a single set of 253-nucleotide sequences. The reads were barcoded to

distinguish between reads belonging to the 100 different samples. This typically produced 100,000 reads per sample.

2.6.13 **Assessing *B. bacteriovorus* survival in soil after inoculation**

A separate pot experiment was conducted to estimate the number of *B. bacteriovorus* HD100 that survived in the pots in Pot Experiment 1a and 2.This was carried out by Dr Carey Lambert (University of Nottingham) in light of the results from the metagenomic analysis from Pot Experiment 1a, in which the number of *Bdellovibrio* per g soil was calculated from the number of *Bdellovibrio* 16s rDNA sequence reads in each sample (Section 5.4.3.3). By recovering and enumerating *B. bacteriovorus* from *Bdellovibrio*-treated pot soil, I could then estimate the difference between the numbers of *Bdellovibrio* reported in the metagenomic analysis and the number recovered and enumerated from the pot soil. This experiment was conducted at the end of my Ph.D. project, in April 2015, after all pot experiments were complete.

One pot of soil was set up according to the same method used in all other pot experiments (described in Section 2.6.5), with the exception that no wheat plants were planted; the soil that was used in this experiment was from the Rothamsted field site Bones Close, which is a Silty Clay Loam type soil similar to Delafield. This was suggested by Dr Vanessa McMillan (RRes), as all Delafield soil that was collected for my study had been used, and the field itself was in use by April 2015 by other researchers growing crops *in situ,* precluding the collection of any additional soil.

A liquid culture of *B. bacteriovorus* HD100, grown on *P. putida* prey and containing 1 x 10^{11} *B. bacteriovorus* HD100, was prepared and added to the pot, using the same methods as for Pot Experiment 1a and 2, described in Section 2.6.6 and 2.6.8. The pot was kept outside under natural weather conditions in Nottingham. The soil was sampled as described in Section 2.6.12, from the middle and towards the outside rim of the pot to 10 cm depth 48 hours after *B. bacteriovorus* HD100 addition. These soil samples were immediately resuspended in calcium HEPES buffer, filtered through an 0.45 µm filter, serially diluted 1 in 10 to 10^{-7}, and enumerated on a lawn of *P.*

putida prey in YPSC medium after incubation at 29°C, as described in Section 2.6.9.

2.6.14 **Measuring and assessing the wheat plants**

The plants were monitored closely throughout growth for signs of nutrient and water deficiency. At full growth (Table 3), the following measurements (as advised by Vanessa Macmillan, RRes) were taken from each plant in each pot: main shoot height (cm ±0.25, from soil to ear tip), number of side tillers, side tiller height (cm ±0.25, from soil to ear tip), length of flag leaf (mm ± 0.5, closest to primary ear on main shoot- excluded from measurements in Experiment 2), and flag leaf senescence (on a scale of 1-10 least to most senescent, shown in Appendix 2), length of primary ear (on the main shoot, cm ±0.25), length of all ears (on main shoot and side tillers, cm ± 0.25), and number of additional ears. The ears from all plants in each pot were harvested and grain from the ripe ears was threshed using a threshing board to remove the chaff. Grains were dried at 105ºC for 16 h to remove all moisture, and then weighed in single-pot batches (g ±0.05, carried out by Chris Hall as instructed by Dr Vanessa McMillan, both RRes).

2.6.15 **Assessing *G. graminis* var *tritici* (take-all) infection of the mature wheat plants (Experiments 1a, 1b and 2).**

In Experiments 1a, 1b and 2 (to test the original hypothesis in all experiments), the mature wheat plants were removed from the pots after their measurement as above and washed to remove the soil, and the roots were examined for symptoms of take-all disease. The plants from each pot were assessed by eye and each was scored from 0-5 according to estimated percentage of primary roots (descending directly from the stem) infected with take-all: 1-10% = 1, 11-25% = 2, 26-50% = 3, 51-75% = 4, or 76-100% = 5. The Take-All Index (TAI) for plants in each pot was calculated by multiplying the percentage of plants in each of the take-all severity categories by the corresponding score value, adding these together, and dividing by 5 (max score = 100). This analysis method has been used in previous studies [80,

81].These data were given to Rodger White (RRes), who calculated significance of differences between the treatment groups in each of the three studies using Analysis of Variance (ANOVA).

2.6.16 **Assessing residual *G. graminis* var *tritici* (take-all) inoculum in the pot soil from Pot Experiments 1a, 1b and 2**

Take-all inoculum builds up in the soil over successive growing years and thus may not be captured by analysis of the mature plants as above; so to measure the take-all infectivity of the soil in **Pot Experiment 1a**, a soil core bioassay was used [65, 81]. 5 x 250 ml soil cores were taken from each big pot in plastic drinking cups after wheat plants were removed. 10 Hereward variety wheat seeds were planted in the top of each soil core, covered over with horticultural gravel, and incubated in a randomised order on a growth room shelf for 5 weeks (16 hour day, 70% relative humidity, day/night temperatures 15/10°C, twice weekly watering to simulate natural outdoor conditions).

After 5 weeks, the plants were removed from pots and washed to remove soil, before being examined for take-all symptoms: the total number of plants and roots, and the number of plants and roots infected with take-all, were counted and the percentage of plants and roots infected with take-all was calculated as a measure of take-all infectivity. These data were logit-transformed and Analysis of Variance was carried out to determine any significant differences in the take-all infectivity of the soil between the treatment groups, as was carried out in previous studies of take-all infection [65, 81]. The purpose of the logit transformation is to fit the take-all infection data to a linear, logistic regression model, which was first introduced as a way of analysing bioassay data such as this by Joseph Berkson in 1944 [82].

2.6.17 **Chemical analysis of the soil**

As our initial hypothesis had not anticipated positive crop yield changes with *Bdellovibrio* addition, we had to analyse soil chemical contents from the samples we had. These samples were taken to 10 cm depth close to the

roots of the wheat plants from each pot using an 8 mm cork borer, and typically yielded 5-10 g of soil (wet weight) per sample. Additionally, all Hereward soil samples from **Pot Experiment 1a** had already been used for DNA extractions for the metagenomic study; therefore, for the chemical analyses which required up to 50 g soil, the samples had to be pooled together, detailed in the paragraphs that follow.

The pH, Nitrogen (N), Phosphorus (P) and Potassium (K) levels were measured in 3 samples from Pot Experiment 1a (frozen soil). These were: 1. Pooled samples from all pots planted in Delafield soil with Cadenza wheat before inoculation, 2. Pooled samples from all Cadenza pots treated with *Bdellovibrio* and *Bdellovibrio* + take-all, after both 1st and 2nd inoculations, 3. Pooled samples from all Cadenza pots treated with *Bdellovibrio* and *Bdellovibrio* + take-all, at both 4 and 15 weeks after all inoculations. All soils were sieved through a 2 mm mesh sieve.

The preparation of soil for the N, P and K analyses, and the entire pH analysis procedure, was carried out by me with supervision from Adrian Crosland at the Analytical Chemistry Unit (RRes).

For pH analysis, 0.5 g of each of the three pooled samples detailed above was mixed vigorously with 5 ml water, and Whatman® pH indicator paper (Cat. No. 2614 991, range = pH 4.5-10) was submerged in the sample for 1 minute, until indicator colours stopped changing, before reading.

For the K and P analysis, samples were air-dried in a drying cabinet for 48 h. 50 g of each sample was given to Adrian Crosland (RRes) to carry out extractions and analyses of these two elements in the soil samples. For the P analysis, an Olsen extraction in sodium bicarbonate ($NaHCO_3$) was carried out (method described in Olsen *et al*., 1954 [83]). This maximises the solubility of P by displacing PO_4^{3-} ions from exchange sites on soil particles; thus, more available P is detected. for the K analysis, an ammonium nitrate extraction was carried out (method described in “Analysis of Agricultural Materials (methods handbook) from the Ministry of Agriculture, Fisheries and Food [84]), which maximises K solubility in a similar way to the Olsen extraction for P. The level of K and P in the extract was measured by mass

spectrometry using the Agilent 7500i ICP-MS system and the the Skalar SAN^{PLUS} Automated Wet Chemistry Analyser system respectively (as used in previous studies, e.g. *Dube et al*., 2014 [85]).

A potassium chloride extraction (As described in "Analysis of Agricultural Materials (methods handbook) from the Ministry of Agriculture, Fisheries and Food [84]) was carried out by me to measure the levels of nitrate (NO_3^-) and ammonium (NH_4^+) available N in the soils. The basic principle of this extraction is that the NO_3^- ions are soluble in any water-based solution, and the NH_4^+ ions present on soil particles are displaced by the K^+ ions in the potassium chloride solution. 50 g (± 0.05 g) of each soil sample was weighed into a wide-necked 200 ml polythene bottle, to which 100 ml (± 0.5 ml) 2M potassium chloride (pH 5.65) was added and the bottle sealed. This was carried out in duplicate for each sample. All samples were shaken in a reciprocating shaker at 120 strokes per minute for 120 min and allowed to stand and settle for 30 min. The supernatant was decanted through a fluted Whatman No. 1 filter paper, discarding the first 10 ml filtrate. The samples were stored at 5^0C until analysis by mass spectrometry (carried out by Adrian Crosland, RRes) using the Skalar SAN^{PLUS} Automated Wet Chemistry Analyser system (as used in previous similar studies e.g. Trinsoutrot *et al*., 2000 [86]).

The amounts of each element (or ion, in the case of the N analysis) measured in each sample were sent back to me by Adrian Crosland, and units were expressed in mg element/kg of soil.

2.6.18 **Statistical analysis of wheat plant physiology and grain production.**

Analysis of Variance (ANOVA) testing was used to assess the effect of each variable (*Bdellovibrio* treatment, take-all addition, and variety) on the measurements taken above (Section 2.6.14), as indicators of wheat plant growth. Where data were unbalanced due to extremely different numbers of data points reported in each treatment group, Restricted Maximum Likelihood Analysis (REML) was carried out instead. The analyses were carried out by

statistician Rodger White (RRes) using Genstat analysis software [73] This software was developed at Rothamsted Research and this method is standard procedure for crop analysis (e.g. [65]).

These statistical tests calculated the significance (probability, p) of any differences in each measurement between pot treatments, assessing their effects individually (e.g. *Bdellovibrio* treatment is a comparison of all *Bdellovibrio*-treated pots with non-*Bdellovibrio* treated pots, regardless of take-all treatment or wheat variety), and also any interactions between the treatments in combination with each other (e.g. the interaction between *Bdellovibrio* and take-all treatments is a comparison between pots treated with *Bdellovibrio* and take-all, take-all alone, *Bdellovibrio* alone, and control pots, again regardless of wheat variety). Any missing measurements, e.g. where one plant out of the eight in each pot did not grow, were estimated automatically by GenStat in the ANOVA analysis; these values had no effect on the sum-of-squares (the measurement of variability of the data set around the mean, used to calculate the probability of significance).

2.6.19 **Statistical analysis of metagenomic data from Pot Experiment 1.**

QIIME (Quantitive Insights Into Microbial Ecology, LINUX version 1.8.0, [87]) was used by myself with advice from Martin Blythe for all statistical analysis of the soil metagenomic data (in Section 5.4.3) returned from the soil samples. The following commands were used, in order, with no changes to their default settings unless stated otherwise:

Command	**Function**	**Alterations to default settings**
Join_paired_ends.py	Joins the paired 151 bp sequences to create single 253 bp sequences, each representing 1 individual bacterial cell from one of the samples	Minimum overlap – 40 bp, % mismatch- 25
Split_libraries_fastq.py	Assigns sequences to a single sample using the barcodes on each sequence and a mapping file containing sample IDs and their corresponding barcodes.	Minimum Phred quality score = 20 (base call accuracy = 99%)

	Simultaneously filters sequences for quality and removes barcodes	
Pick_de_novo_otus.py	Clusters sequences of >97% similarity into operational taxonomic units (OTUS) and assigns taxonomic classification to the OTUS using UCLUST, producing an OTU table	
Filter_taxa_from_otu_table.py	Filters specific OTUs from the OTU table	Genus “Phormidium” and Class “Chloroplast” filtered to remove any chloroplast sequences (a major source of between-sample variation, possibly due to algae from some pots)
Sort_otu_table.py	Rearranges the OTU table into order of sequences in the mapping file for ease of sample comparison	
biom summarize-table	Summarises the number and length of sequences in the OTU table, including total number of samples, and minimum, maximum, median, mean, and standard deviation of length	
Make_otu_heatmap_html.py	Creates a searchable index of the number of each bacterial taxa present in the samples, colour-coded dependent on the percentage of the total sample each taxon represents	
Summarize_taxa.py	Creates a summary table where taxonomic groups are in rows and sample IDs are in columns in order to use the plot_taxa_summary.py function	
Plot_taxa_summary.py	Creates bar charts at the phylum, class, order, family, and genus level for each sample, showing the percentage of each taxon in each sample	
Beta_diversity.py	Compares OTU abundance between samples in pairwise comparisons. Output is a table of dissimilarity	

	values between OTUs in all combinations of samples.	
Principal_coordinates.py	Computes a set of 3 values for each sample, using the beta_diversity.py output, based on their dissimilarity to one another based on three principal co-ordinates (PCs, which explain the most variation in bacterial/archaeal community composition).	Used 'weighted unifrac' option to take into account abundance as well as presence of each taxon in a sample
Make_3d_plots.py	Plots the values created in principal_coordinates.py using the 3 PCs as axes on a 3-dimensional graph to visualise differences between samples in bacterial/archaeal community composition.	Used additional 'biplots' option to produce a list of taxa contributing most to the variation between samples.

2.6.20 **Assessing the expression of putative phytohormone genes throughout the *B. bacteriovorus* HD100 predatory cycle, and in Host-Independent (HI) strains**

To determine whether *B. bacteriovorus* HD100 could affect plant growth directly, rather than by preying upon other Gram-negative species in the soil, I conducted BLAST-P searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) in the *B. bacteriovorus* HD100 genome to look for hypothetical proteins with Amino Acid (AA) sequence homology to known phytohormone genes in other bacterial species.

To determine whether the hypothetical proteins of interest were expressed in *B. bacteriovorus,* and were therefore likely functional, I carried out Reverse Transcription Polymerase Chain Reactions (RT-PCRs) on 0.5 µl RNA isolated from synchronous, prey-dependent cultures of *B. bacteriovorus* HD100, grown on *E. coli* S17-1 prey, in Attack Phase (AP) and at 15 min, 30 min, 45 min, 1 h, 2 h, 3 h, and 4 h during 4-hour predatory cycle (isolated by Dr Carey Lambert, a post-doc in my laboratory at the University of Nottingham).

In these RT-PCRs I used primers designed by Dr Carey Lambert to hybridise to the RNA sequences encoding the hypothetical proteins under investigation. All primer sequences are given in Section 2.6.2**;** to ensure that the primers did not hybridise to the prey *E. coli* S17-1 RNA, which would give a false-positive result in the RT-PCRs, I used *E. coli* S17-1 RNA alone as a control, along with a positive control of attack-phase *B. bacteriovorus* HD100 DNA, and a negative control of no RNA/DNA template. The expression of these proteins of interest was also investigated in 4 Host Independent (HI) strains, originally derived from *B. bacteriovorus* HD100 by Dr Carey Lambert (HI2, 13, 22, and 26); 1 µl RNA was used for these HI strains, and so an additional AP *B. bacteriovorus* HD100 RNA control was used, also at 1 µl RNA. For these RT-PCRs I used a QIAGEN RT-PCR kit (Cat. No. 210212) according to the manufacturer's protocol.

The RT-PCR programme used was as follows:

Temperature (ºC)	Time (min:sec)	No. of Cycles
94	15:00	1
94	01:00	27-32
50	01:00	
72	01:00	
72	10:00	1

On completion of the RT-PCR, electrophoresis of all samples on a 2% Agarose gel was carried out at 100 v for 30 min. The number of cycles used in the RT-PCR was optimised on an individual basis for each protein under investigation, so that their patterns of expression throughout the predatory cycle in *B.bacteriovorus* HD100 could be easily observed. Levels of expression could therefore not be compared quantitatively between hypothetical proteins, but the expression level of one hypothetical protein

could be compared across all different time points throughout the HD predatory cycle.

3 *Bdellovibrio* Predation of Diverse Plant Pathogens

3.1 Chapter Introduction

In this chapter, a range of Gram-negative (mostly *Pseudomonas*) bacterial plant pathogens that infect agriculturally important plants, fungi, and trees, and some Plant Growth-Promoting Rhizobacteria species (PGPRs), were screened *in vitro* for susceptibility to predation by *B. bacteriovorus* HD100*,* using a high-throughput optical density measuring assay. The range of species tested were selected and supplied by Dr Rob Jackson (co-supervisor, University of Reading). This work led on to Chapter 4 and informed Chapter 5. The model plant *Nicotiana benthamiana* was also tested as a host in which to assess predatory killing of *Pseudomonas* plant pathogens by *B. bacteriovorus,* but the use of *N. benthamiana* was not continued beyond this preliminary study.

3.1.1 Considering *Bdellovibrio* predatory interactions with crop pathogenic species

Crop pathogenic *Pseudomonas* species produce and secrete a number of extracellular polysaccharides and surface structures, toxins, enzymes and siderophores that mediate survival on the surface of the host crop, invasion of host tissue, pathogen population growth, and survival within the host. Different species produce different repertoires of pathogenic effectors, which reflect their adaptation to different host plants [88-91]. Some effectors, however, have detrimental effects on other bacterial species to avoid competition for resources [88]. This may affect the efficacy of *Bdellovibrio* predation against the different strains, as some effectors may directly kill *Bdellovibrio* or otherwise limit its predatory activity. These are discussed in Sections 3.1.1.2 to 3.1.1.4.

Crucially, the timing of effector production is controlled in many plant pathogenic species through Quorum Sensing (QS) so that resources are only invested in producing effectors when pathogen numbers are high, e.g. on the surface of a host plant, discussed in Section 3.1.1.5. Individual effectors are

also either secreted into bacterium-host intercellular spaces or directly into the plant host, via different transport systems, discussed in Section 3.1.1.6. This temporal and spatial control of effectors could also determine whether *B. bacteriovorus* is able to prey on species *in vitro* compared with *in vivo,* and is thus an important consideration in these preliminary *in vitro* assays that I carried out.

3.1.1.1 *Exopolysaccharides and surface layers*

Plant pathogenic and commensal *Pseudomonas* species are able to survive on the external surfaces of their host plants, e.g. plant leaves, roots and mushroom caps. Some species also have an 'epiphytic' stage, in which they multiply on the surface of the host plant before entering and establishing in the tissue of the host, or causing any symptoms of disease [92, 93]. To withstand harsh environmental conditions in this epiphytic stage, such as damage by exposure to ultraviolet (UV) light or dessication in dry conditions, some *Pseudomonas* species form 'aggregates': groups of bacterial cells on the host plant surface, which are often surrounded by exopolysaccharides produced by the bacterial cells. QS communication (discussed in Section 3.1.1.5) between the cells plays a role in aggregate formation and affects the aggregate size [93]. *Bdellovibrio* predation may be less efficient against aggregates than free-swimming *Pseudomonas*; previously, *Bdellovibrio* predation has been shown to be less efficient on reducing bacterial populations in 'biofilms', where they are attached to a surface in layers, than on free-swimming cells [94]. Biofilms have some similar properties to epiphytic aggregates, such as the exopolysaccharide component, although biofilms have a more highly structured extracellular matrix [95].

Similarly, The surface layer (S-layer), an extracellular component of the outer membrane composed of glycoproteins, are present on the phylogenetically diverse bacterial species, including *Pseudomonas putida* and other *Pseudomonas* spp. [96], and it is suggested that that the S-layer-encoding genes are transferred horizontally between species [97]. *B. bacteriovorus* predation is known to be inhibited by the presence of an S-layer [98]; If resistance to *B. bacteriovorus* predation by some of the species in my *in vitro* studies occurs and does not affect closely related *Pseudomonas* species,

this may therefore indicate resistance due to the presence of an S-layer, or other horizontally transferred trait (though it may also be due to unrelated, species-specific mechanisms).

This possible effect on predation efficiency is an important consideration in potentially using *Bdellovibrio* as a control agent for *Pseudomonas* infections by application to the surfaces of plants, as proposed in my Ph.D. project. In the OD measuring assays, *Bdellovibrio* predation of the pathogen and commensal species was tested in a liquid buffer/medium mixture with temperature regulation and regular shaking; predatory activity of *Bdellovibrio* against any of these species discovered at this stage may not necessarily translate to predation in their natural environment on the biotic surfaces that they colonise, in part due to the formation of biofilms or aggregates, however the OD measuring assay provided a rapid first screen for interactions.

3.1.1.2 *Toxins*

Phytotoxins are agents that promote disease progression and cause disease symptoms in plant hosts by damaging host cells and tissues [89]. Their functions are diverse and include inducing chlorosis (coronatine, phaseolotoxin and tagetotoxin), necrosis of host plant tissue and other bacterial and fungal species (syringomycin, syringotoxin, syringostatin and pseudomycin), suppression of host immune responses (coronatine), and interference with toxic compound breakdown in the host (tabtoxin) [88, 89, 91].

Some phytotoxins are common to several pathovars. Coronatine, which promotes the opening of stomata, is produced by pathovars of *Pseudomonas syringae* that infect herbaceous plants, such as *P. syringae pvs. tomato*, *maculicola* and *glycinea* [89, 91]; coronatine thus facilitates entry into herbaceous plant tissue and is therefore particularly useful to pathogens of these plants. Conversely, some phytotoxins are particular to a single species: tolaasin, for example, is produced only by *P. tolaasii*, a natural pathogen of mushrooms. Tolaasin is a lipodepsipeptide that makes pores in mushroom cell membranes, degrading cell and tissue structure to form the sunken, brown ‘blotches’ characteristic of *P. tolaasii* infection [99]. Other

lipodepsipeptides are similarly specific to single *Pseudomonas* pathovars, reflecting their adaptation to different hosts [89].

A few phytotoxins also have antimicrobial activity: syringopeptin has activity against Gram-positive and fungal species; syringomycin is also active against fungal species; and phaseolotoxin inhibits the growth of the Gram-negative bacterial species *E. coli* and *Salmonella typhimurium* [89, 100]. It is feasible that some phytotoxins may affect *Bdellovibrio* survival and predation; however, as the Gram-negative *Bdellovibrio* only grows and replicates inside prey cells, and not extracellularly, the effects of toxins that inhibit growth like phaseolotoxin are not likely to affect *Bdellovibrio.*

3.1.1.3 *Extracellular Enzymes*

Proteases, lipases, and carbohydrases (particularly pectinases, cellulases and chitinases) have all been identified as important effectors in *Pseudomonas* plant diseases, and especially in soft-rot pathogens such as *P. atrosepticum* and *P. marginalis*, in which they are produced in large quantities [101, 102]. Proteases and lipases are produced by most *Pseudomonas* and other plant pathogenic bacterial species, and are directly involved in disease progression, though their specific actions on the host have not been well studied [101, 103]. The role of carbohydrases are better characterised, particularly Plant Cell Wall Degrading Enzymes (PCWDEs) such as pectin and pectate lyases and cellulases [104]. These enzymes are induced in *Pseudomonas* pathovars during infection, such as in *P. syringae* pv. *tomato* DC3000, which causes soft rot in tomato, and in *P. syringae* B728a, which causes brown spot in bean [105]. The degradation of pectin, which cements cells together in plant tissue, and cell wall components, which provide cell structure, cause plant tissue to turn soft and water-soaked, a symptom of diverse plant diseases such as soft rots, blights and leaf spots [104]. Some of these enzymes important to pathogenicity are absent in non-pathogenic strains of *Pseudomonas*, such as the plant growth promoter *P. putida*, highlighting their role in causing infection [105]. As secreted enzymes have a more broad-range activity than toxins, they are more likely to have direct effects on *Bdellovibrio* survival and predation.

3.1.1.4 *Siderophores*

Siderophores are Fe (III)-chelating molecules that facilitate the uptake of iron by bacterial cells. The major type of siderophores in fluorescent *Pseudomonas* species (such as those in this chapter) are the pyoverdins, which fluoresce under UV light and are responsible for the green colour of colonies grown on King B medium agar [90]. As pyoverdins facilitate iron uptake from the host, this may limit the growth of other microorganisms on the host that require iron uptake to survive. However, as host-dependent *Bdellovibrio* do not grow or replicate outside of their Gram-negative bacterial host, a lack of iron in the plant environment is not predicted to affect their survival. Pyoverdins produced by some pathovars are directly required for pathogenicity to their host, such as in *P. syringae pv. tabaci*, but in other pathovars pyoverdins do not have a direct pathogenic role, as in *P. syringae pv. tomato* DC3000 [106, 107]. *Bdellovibrio* itself has enterobactin-like siderophores, complexes formed from the gene products of *bd1576*, *bd1392* and *bd1574* (*iucA*, *B* and *C* respectively).

3.1.1.5 *Temporal control of pathogenic effector production by Quorum Sensing (QS)*

Quorum Sensing (QS) signal-receptor systems allow many bacterial species to detect the presence of neighbouring cells in their population. The most common and well-characterised quorum sensing system in Gram-negative species involves the *N*-Acyl Homoserine Lactone (AHL) signalling molecule, encoded by *luxI* gene homologues [108]. The concentration of AHL increases with growth of the bacterial population, until it reaches a threshold level, at which point it induces a response, mediated by AHL binding to the LuxR receptor homologue. LuxR regulates the transcription of selected genes, and thus alters gene expression patterns[108]. This system regulates processes involved in pathogenicity in several plant pathogenic species: for example, in the soft-rot pathogens *P. atrosepticum* and *P. marginalis,* both of which were included in my initial *in vitro* predation tests, QS controls the production of PCWDEs, co-ordinating a pathogenic attack when cells multiply rapidly on the surface of their host crop plant [101, 102]. The AHL QS system also regulates biofilm formation *in vitro* by *P. fluorescens [109],* and AHL has been

detected in biofilms formed in natural habitats such as wetland ponds [110]. PCWDEs and biofilm formation may prevent or slow predation as discussed in Sections 3.1.1.1 and 3.1.1.3, and thus QS-mediated pathogenicity in the plant pathogenic species that I tested could inhibit *B. bacteriovorus* predation *in vitro* or *in vivo,* if cell numbers reach the required threshold.

Another QS system controlling the expression of virulence factors is also present in *Pseudomonas spp.,* such as those tested in this study: The 4-hydroxy-2-alkylquinoline PQS [111]. In addition to regulating virulence factor expression, PQS is involved in sensing iron in the environment: PQS binds to iron, trapping it near to the cell and promoting the expression of genes involved in iron scavenging, such as siderophores. Conversely, *P. aeruginosa* QS is activated under low-iron conditions [111]. Thus, QS and environmental sensing are linked; this is an important consideration in my study, as King's Medium B present in the buffer-medium mixture that I used in the *in vitro* $OD600_{nm}$ assays promotes the production of iron-scavenging siderophores. Thus pathogen production of potentially bacterially toxic factors (discussed in Section 3.1.1.2 and 3.1.1.3) that are regulated by QS may differ between *in vitro* and *in vivo* conditions, and so susceptibility to *B. bacteriovorus* predation *in vitro* may not necessarily translate as susceptibility *in vivo.*

3.1.1.6 *Modes of pathogenic effector secretion*

There are several systems by which plant pathogens secrete the extracellular pathogenic effectors described in Sections 3.1.1.2- 3.1.1.4, which affects the compartment into which the effectors are delivered [112]. The Type III Secretion System (T3SS) and the Type VI Secretion System (T6SS) require contact of a needle-like component of the secretory apparatus with host crop cells, and inject pathogenic effectors directly into them [113]. Effectors that are delivered via this system, most notably those encoded by the T3SS hypersensitive response and pathogenicity (hrp) genes in *P. syringae pv phaseolicola* [8], are thus not present in the intercellular space between host and pathogen. *B. bacteriovorus* would therefore not come into contact with these effectors, and so I will not review them in detail here.

However, the T6SS also plays a role in competition between bacterial species; for example, *Pseudomonas syringae pv. tomato* DC3000 produces the T6SS effector Hcp2, which is not required for pathogenicity in the host tomato plant (demonstrated in the virulence of a *hcp2* deletion mutant strain [114]), but inhibits the growth of enterobacterial strains such as *E. coli* on agar plates *in vitro* [114]. Similarly, *Pseudomonas aeruginosa* produces T6SS effectors Tse1 and Tse3, which are injected into the periplasm of other Gram-negative bacteria such as *E. coli,* where they degrade peptidoglycan, thus disrupting the bacterial cell wall [115]. Thus, T6SS may play a role in direct resistance to *B. bacteriovorus* predation resulting from cellular damage, and is an important consideration in this study.

Other secretion systems deliver effectors into the plant host-pathogen intercellular space, and thus would come into contact with *B. bacteriovorus* cells in my *in vitro* tests. The Type I and Type II secretion systems (T1SS and T2SS) are involved in the secretion of PCWDEs, for example by *Pectobacterium atrosepticum* [101], one of the strains included in this study. Therefore, the production and secretion of enzymes may be an important factor in any resistance to *B. bacteriovorus* HD100 that is observed.

Similarly, although little is known about the secretion of the specific toxins produced by plant pathogenic bacteria, a mutation study in *P. syringae pv. phaseolicola* suggested that *syrD,* a gene encoding a protein with sequence homology to members of the ATP Binding Cassette (ABC) transporter superfamily, is required for the efficient production and secretion of syringomycin (SyrB) [116]. Thus syringomycin is also likely secreted into the intercellular space, where it may come into contact with *B. bacteriovorus,* though this may not be the case for other toxins produced by other strains.

3.1.2 **Characterising the prey range of *Bdellovibrio***

B. bacteriovorus is able to prey upon and kill diverse species of Gram-negative bacteria from the soil and other agriculturally important locations: this includes *E. coli*, on which *B. bacteriovorus* HD100 (the strain used in this study) is routinely cultured; *Salmonella* bacteria that colonise the guts of

young chicks [25]; naturally soil-dwelling *Erwinia* and *Agrobacterium* [21]; and some *Pseudomonas* spp. including *P. glycinea,* which causes bacterial blight of soybean (as described in Section 1.1.4) [23], and the soil-dwelling *P. syringae* [21]. Therefore, *Bdellovibrio* has been tested against and found to prey upon and kill a wide range of Gram-negative genera, but in this study I tested the *in vitro* predatory activity of *Bdellovibrio* against a wider range of species from one single genus, *Pseudomonas,* which is known to include diverse pathogens of a wide variety of plants, as well as PGPRs (and some species from other genera were also tested).

3.2 Specific Research Aims

The aim of this preliminary chapter was to assess the susceptibility of a range of agriculturally important, Gram-negative, bacterial crop pathogens/PGPR species to *B. bacteriovorus* predation *in vitro*, with a view to testing *B. bacteriovorus* as a 'food security agent' against bacterial diseases of crop plants *in vivo.* Therefore my specific research aims in this chapter were:

- To test a range of 16 *Pseudomonas* crop pathogen/PGPR species (and 4 species from other genera) for susceptibility to *Bdellovibrio* predatory killing *in vitro,* over 24 hours*,* using an Optical Density ($OD600_{nm}$) measuring assay in a BMG plate reader as an indicator of test strain growth or suppression

- To visualise mixtures of *Bdellovibrio* and any test strain identified as susceptible to *Bdellovibrio* predatory killing *in vitro*, using Transmission Electron Microscopy (TEM), to confirm whether any predatory interactions were observed

- To assess the suitability of *Nicotiana benthamiana* as a living, model host plant in which to investigate *B. bacteriovorus* predatory killing of any susceptible crop-pathogenic strains *in vivo*, by inoculating each test prey strain or *B. bacteriovorus* into the leaves of whole, live *N.*

benthamiana plants, visually monitoring the leaves for disease symptoms, and enumerating any test strain/*B. bacteriovorus* cells that can be recovered after 4 days

3.3 Hypothesis

I predicted that:

- *B. bacteriovorus* would suppress the growth of some of the 20 diverse test strains *in vitro,* likely due to predatory killing; other test strains may grow in the presence of *B. bacteriovorus*, indicating resistance to predatory killing

- the level of susceptibility/resistance to *B. bacteriovorus* predatory killing may vary between strains, and this variation may be due to the adaptation of the strains, which inhabit a wide range of plant hosts or soil environments

- TEM visualisation would show predatory interactions, such as attachment and bdelloplast formation, between *B. bacteriovorus* and susceptible test strains, confirming predatory invasion as the cause of test strain growth suppression *in vitro* by *B. bacteriovorus*

- Plant-pathogenic test strains would increase in number over time after inoculation into *N. benthamiana* leaves, while pathogens of non-plant crops (e.g. mushroom) may not increase in number or cause disease symptoms, as these strains produce different host-specific, disease-causing effectors

- Host-dependent *B. bacteriovorus* would not cause disease symptoms in *N. benthamiana* tissue, or increase in number over time in the leaf tissue in the absence of prey bacteria

3.4 Results

3.4.1 Measuring the effect of *Bdellovibrio* predation on the growth of *Pseudomonas* pathogen/commensal strains.

The aim of this experimental series was to determine the effect of *B. bacteriovorus* HD100 addition, *in vitro,* on the growth of 15 crop or soil-associated *Pseudomonas* strains (and 4 strains from other genera), hereafter referred to as 'test strains', and thus identify which were killed particularly well by *Bdellovibrio* for further *in planta* tests. This selection of test strains, made by my supervisor Rob Jackson (University of Reading), included both pathogens of a wide variety of crops and commensals (Plant Growth-Promoting Rhizobacteria, PGPR).

Briefly, samples of each test strain (that had been cultured in King's medium B for 15 h at 29ºC with 200 rpm shaking) were added to a 50/50 calcium HEPES buffer/King's medium B mixture, in both the absence and presence of live *Bdellovibrio* at 4 x 10^6 cells or 1.6 x 10^7 cells. The buffer/medium mixture allowed slow growth of the test strain; the $OD600_{nm}$ of these samples (an indicator of test strain growth) was measured every 30 min for 24 hours. These values are plotted separately for each test strain on the graphs that follow in Figure 6-Figure 9**,** and are all plotted on the same scale for ease of comparison; the graphs are grouped by susceptibility of the test strains to *Bdellovibrio* predation. An increase in the $OD600_{nm}$ of the sample in the absence of *B. bacteriovorus* indicates growth of the test strain, while no increase in the presence of live, predatory *B. bacteriovorus* indicates suppression of test strain growth, likely due to predation by *Bdellovibrio.* By enumerating the test strain samples that were added to the mixtures, I could calculate how many cells were present in the samples to start with; I could therefore use the $OD600_{nm}$ values of each sample at the start and end of the assay to calculate the number of test strain cells present after 24 hours under each condition. For ease of comparison, these are shown in the tables accompanying each set of graphs.

As shown in Figure 6, the $OD600_{nm}$ of *P. avellanae* 48 (which causes hazelnut canker), *P. syringae pv. phaseolicola* (halo blight in bean), *P. agarici* 2289 (drippy gill in mushrooms) and *P. tolaasii* 2192^T (brown blotch in mushrooms) increased in the absence of any live *B. bacteriovorus* (by 0.38, 0.61, 0.47, and 0.84, corresponding to 3.43×10^7, 2.93×10^9, 8.55×10^7 and 8.67×10^7, cells, respectively, detailed in Table 6), but did not increase to any significant extent in the presence of either concentration of 4×10^6 or 1.6×10^7 live *B. bacteriovorus* cells. This indicated that the population growth of these *Pseudomonas* strains was completely suppressed by *Bdellovibrio* (the population number of each strain at 0 and 24 hours after the beginning of the assay is shown in Table 6), which therefore was likely able to easily prey upon these strains. The growth of *P. tolaasii* and *P. syringae pv. phaseolicola* was more variable in the absence of *Bdellovibrio* compared with that of *P. avellanae* and *P. agarici,* indicated by the large 95% confidence intervals (CI, n = 4, shown as error bars on the graphs); this variability in cell numbers has previously been documented in *P. tolaasii* [9].

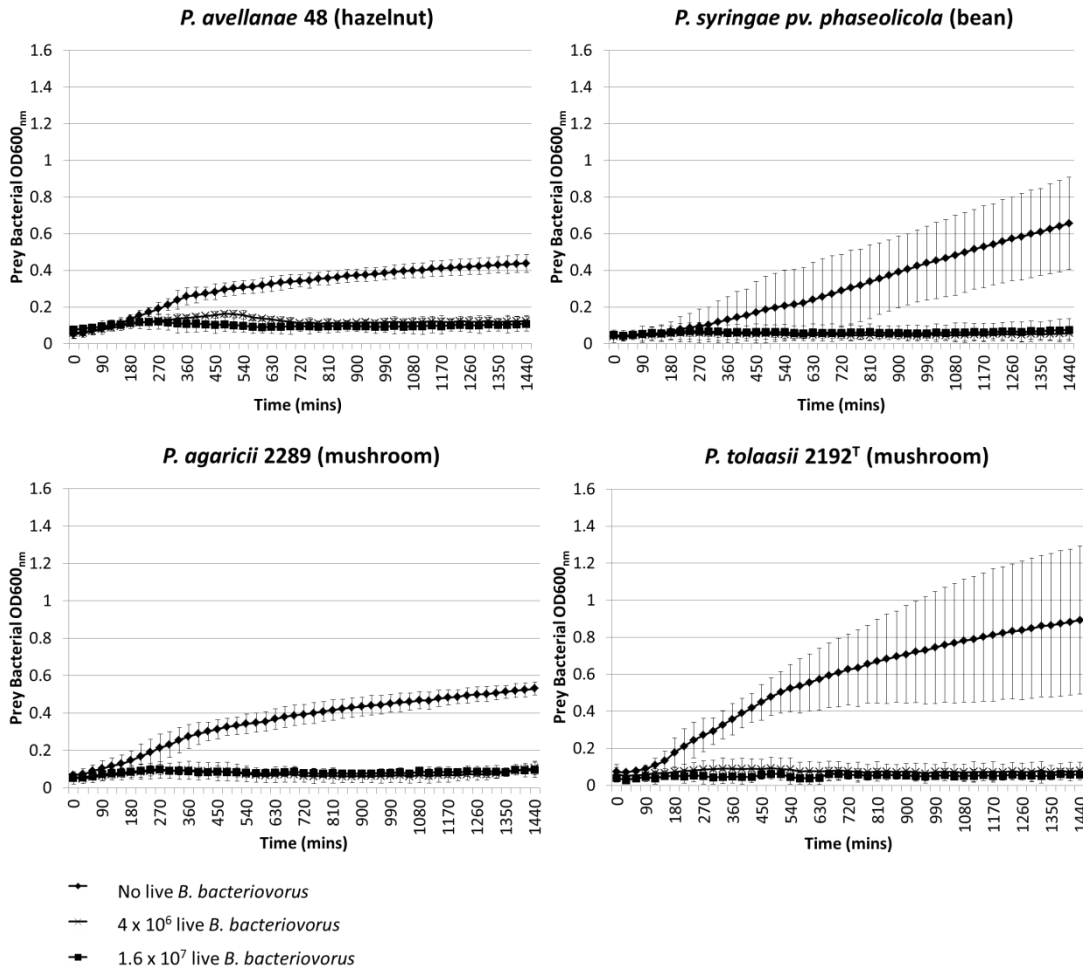


Figure 6 . $OD600_{nm}$ over 24 hours, *in vitro*, of 4 *Pseudomonas* strains in the absence and presence of *Bdellovibrio bacteriovorus*. The mean $OD600_{nm}$ of samples is shown, in the absence or presence of live *B. bacteriovorus* HD100 added at 4 x 10^6 or 1.6 x 10^7 Plaque Forming Units (PFU) (n = 4). The increase in $OD600_{nm}$ in the absence of *Bdellovibrio* indicates *Pseudomonas* strain growth, while no increase in the presence of 4 x 10^6 or 1.6 x 10^7 *B. bacteriovorus* HD100 indicates inhibition of *Pseudomonas* strain growth. Error bars indicate 95% Confidence Intervals for each $OD600_{nm}$ value (n = 3 biological repeats). The plant host/Plant Growth-Promoting Rhizobacteria (PGPR) identity is given in brackets after the test strain name.

Table 6 . Mean $OD600_{nm}$ (left hand columns) and population number (right-hand columns) of the 4 *Pseudomonas* strains shown in Figure 6 in samples at the start of the $OD600_{nm}$ assay (T = 0) and at the end of the assay in the absence of *B. bacterovorus* HD100 or in the presence of 4×10^6 or 1.6×10^7 live, predatory *B. bacteriovorus* HD100 cells (T = 24). Colony Forming Units (CFU) of each strain sample was enumerated on King's medium B agar at the beginning of the assay; this number and the starting $OD600_{nm}$ value was used to calculate the final number of each strain in the samples, using the corresponding mean final $OD600_{nm}$ values at 24 hours. Each value is the mean of n = 4 repeats for each strain.

	$OD600_{nm}$ value				Equivalent cell number of test strain			
	T = 0	T=24 h			T = 0	T = 24 h		
Number of live *B. bacteriovorus* HD100 cells		0	4×10^6	1×10^7		0	4×10^6	1.6×10^7
Pseudomonas avellanae 48	0.06	0.44	0.12	0.11	5.68×10^6	4.00×10^7	1.12×10^7	9.91×10^6
Pseudomonas syringae pv. *phaseolicola*	0.04	0.66	0.06	0.07	2.04×10^8	3.14×10^9	2.79×10^8	3.58×10^8
Pseudomonas agarici 2289	0.06	0.53	0.11	0.10	1.08×10^7	9.63×10^7	1.94×10^7	1.74×10^7
Pseudomonas tolaasii 2192^T	0.06	0.89	0.08	0.06	5.88×10^6	9.25×10^7	8.16×10^6	6.16×10^6

Conversely, the $OD600_{nm}$ of *B. vietnamiensis* G4 (a soil-dwelling PGPR), *P. marginalis* 667 (which causes soft rot in a diverse range of organisms) and *P. atrosepticum* SCRI143 (which causes blackleg in potatoes), as shown in Figure 7, was increased over 24 hours in the absence of *Bdellovibrio* (by 0.32, 0.58, and 0.41, corresponding to 3.48×10^7, 6.45×10^7 and 1.85×10^7 cells, respectively, detailed in Table 7); similar increases in $OD600_{nm}$ and therefore population number was also observed in the presence of 4×10^6 *B. bacteriovorus* HD100 (where $OD600_{nm}$ increased by 0.35, 0.51 and 0.36, corresponding to 3.73×10^7, 5.62×10^7 and 1.63×10^7 cells, respectively), and in the presence of 1.6×10^7 *B. bacteriovorus* HD100 cells (where $OD600_{nm}$ increased by 0.38, 0.48 and 0.31, corresponding to 4.14×10^7, 5.37×10^7, and 1.37×10^7 cells, respectively). As no significant difference was observed in strain growth between samples in the absence or presence of predatory *B. bacteriovorus* HD100, *B. bacteriovorus* HD100 was likely unable to prey upon these strains, unlike those shown in Figure 6**.**

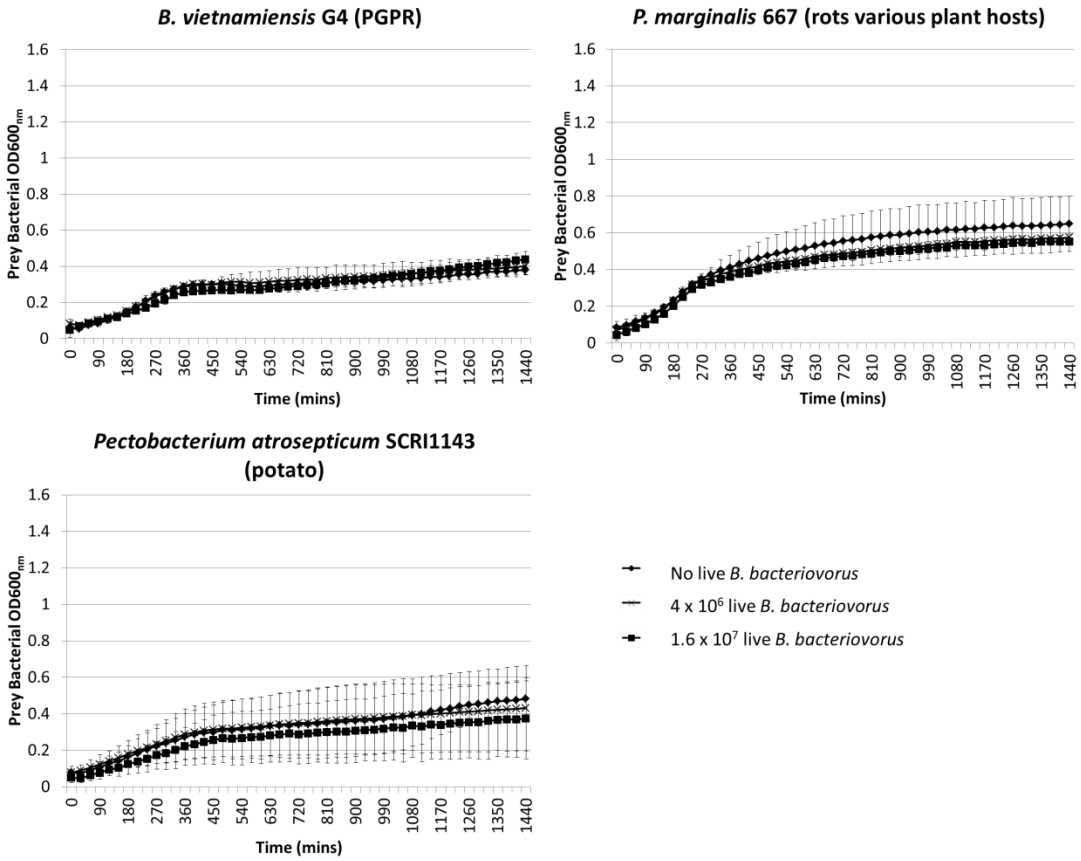
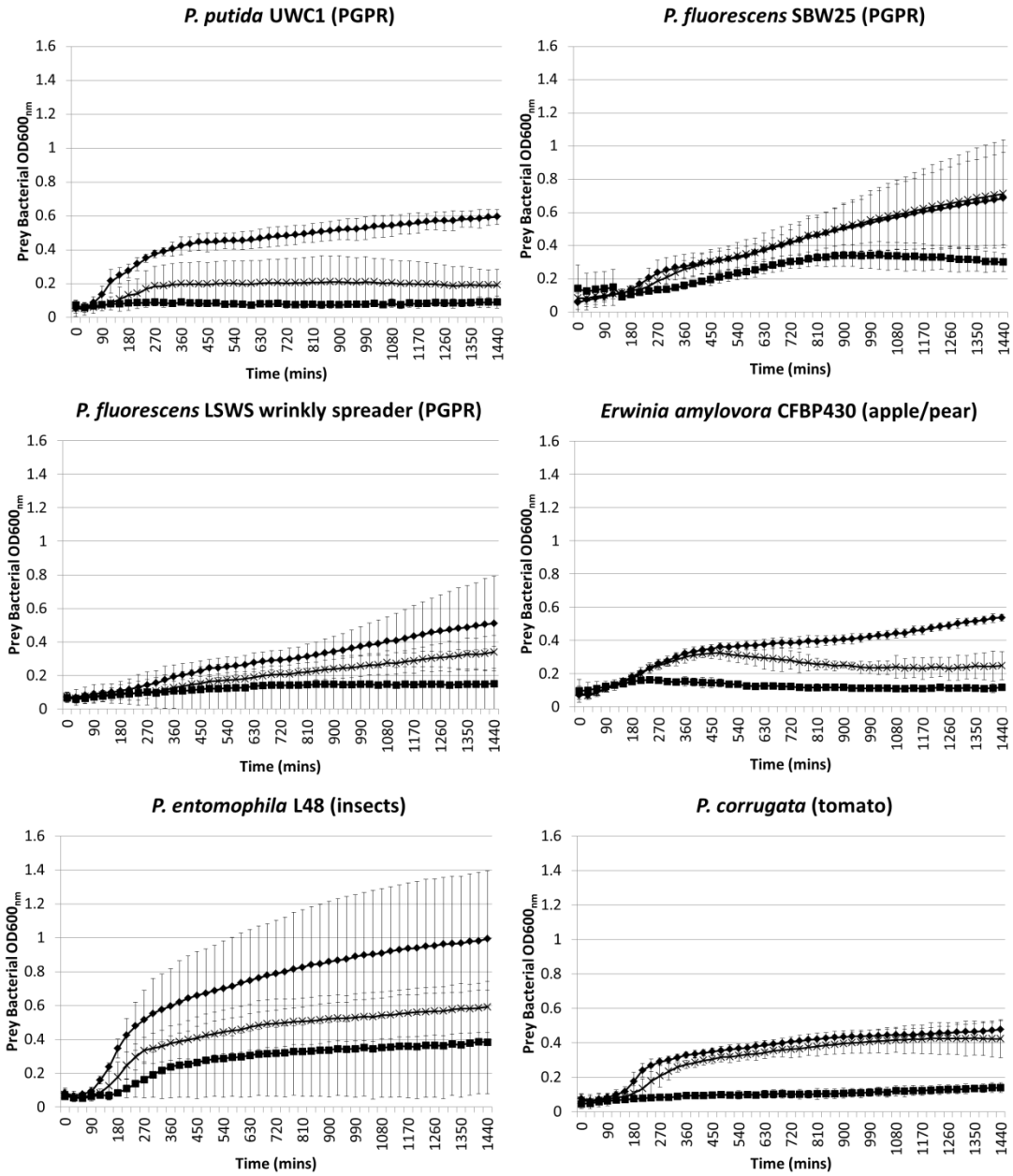


Figure 7 . $OD600_{nm}$ over 24 hours, *in vitro*, of 3 bacterial strains in the absence and presence of *Bdellovibrio bacteriovorus*. The mean $OD600_{nm}$ of samples is shown in the absence or presence of live *B. bacteriovorus* HD100 added at 4 x 10^6 or 1.6 x 10^7 Plaque Forming Units (PFU) (n = 4). The increase in $OD600_{nm}$ in the absence and presence of *Bdellovibrio* at both concentrations indicates bacterial strain growth. Error bars indicate 95% Confidence Intervals for each $OD600_{nm}$ value (n = 3 biological repeats). The plant host/Plant Growth Promoting Rhizobacteria (PGPR) identity is given in brackets after the test strain name.

Table 7 . Mean $OD600_{nm}$ (left hand columns) and population number (right-hand columns) of the 3 *Pseudomonas* strains shown in Figure 7 in samples at the start of the $OD600_{nm}$-measuring assay (T = 0) and at the end of the assay in the absence of *B. bacterovorus* HD100 or in the presence of 4×10^6 or 1.6×10^7 live, predatory *B. bacteriovorus* HD100 cells (T = 24). Colony Forming Units (CFU) of each strain sample was enumerated on King's medium B agar at the beginning of the assay; this number and the starting $OD600_{nm}$ value was used to calculate the final number of each strain in the samples, using the corresponding mean final $OD600_{nm}$ values at 24 hours. Each value is the mean of n = 4 repeats for each strain.

	$OD600_{nm}$ value				Equivalent cell number of test strain			
	T = 0	T=24 h			T = 0	T = 24 h		
No. of live *B. bacteriovorus* HD100 cells		0	4×10^6	1×10^7		0	4×10^6	1.6×10^7
Burkholderia vietnamiensis G4	0.06	0.38	0.40	0.44	7.00×10^6	4.18×10^7	4.43×10^7	4.84×10^7
Pseudomonas marginalis 667	0.07	0.65	0.57	0.55	7.40×10^6	7.19×10^7	6.36×10^7	6.11×10^7
Pectobacterium atrosepticum SCRI1043	0.07	0.48	0.43	0.37	3.09×10^6	2.16×10^7	1.94×10^7	1.68×10^7

For 10 of the remaining strains, shown in Figure 8, the $OD600_{nm}$ (and therefore test strain population number, detailed in Table 8) increased over 24 hours in the absence of *B. bacteriovorus* HD100; increased to a lesser extent, reaching a lower final $OD600_{nm}$/population number, in the presence of 4×10^6 live *B. bacteriovorus* HD100 cells; and increased to an even lesser extent (in the case of *P. fluorescens* SBW25, *P. entemophila* L49*, P. corrugata* and *P. marginalis pv. pastinaceae*) or there was no significant net increase (in the case of all other strains shown in Figure 8) in the $OD600_{nm}$/population number in the presence of 1.6×10^7 *B. bacteriovorus* HD100 cells. This indicates a dose-response relationship between the number of *B. bacteriovorus* HD100 added and the test strain population number, suggesting that *B. bacteriovorus* is able to prey upon these strains, but to a lesser extent than the well-preyed upon strains in Figure 6**,** so the test strains in this case have some mechanism that impedes but does not abolish *B. bacteriovorus* predation.


P. putida UWC1 (PGPR)
P. fluorescens SBW25 (PGPR)
P. fluorescens LSWS wrinkly spreader (PGPR)
Erwinia amylovora CFBP430 (apple/pear)
P. entomophila L48 (insects)
P. corrugata (tomato)
Prey Bacterial OD600nm
Time (mins)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
0
90
180
270
360
450
540
630
720
810
900
990
1080
1170
1260
1350
1440

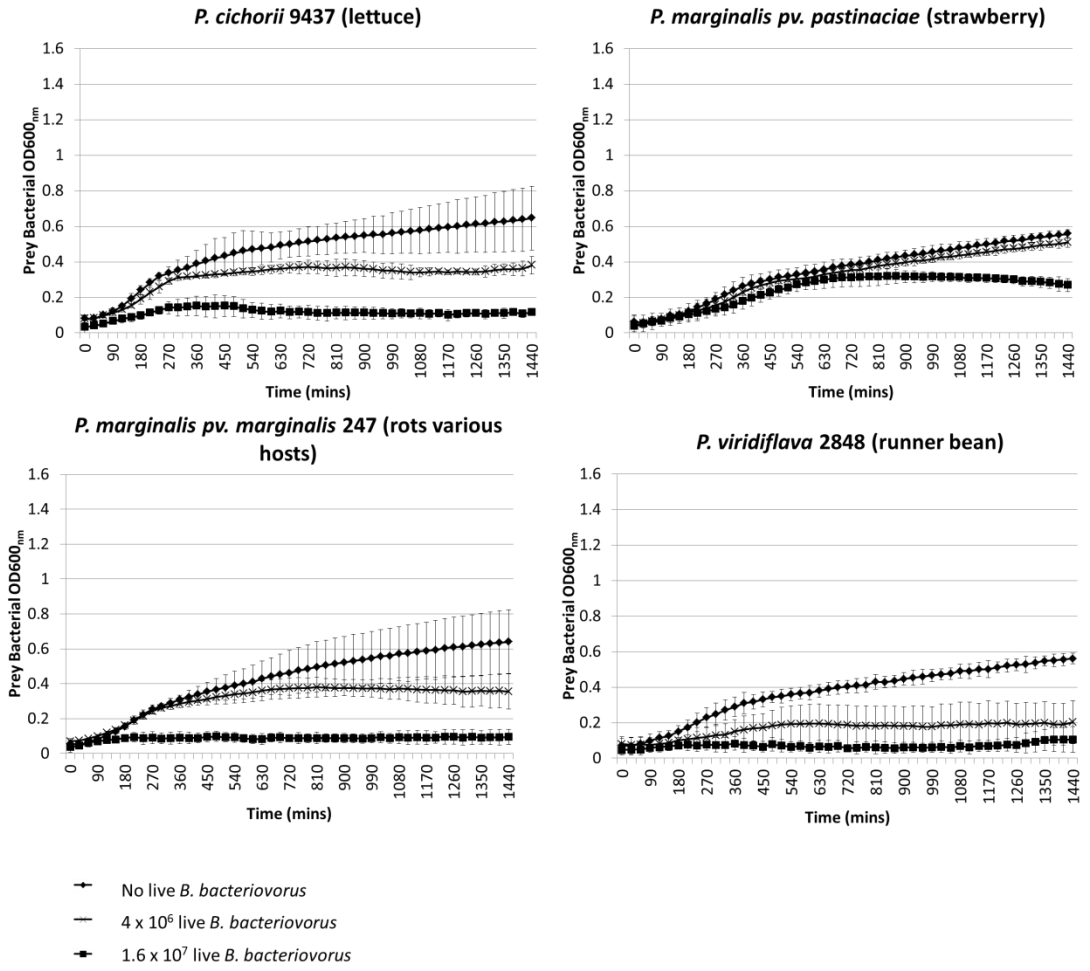


Figure 8 . $OD600_{nm}$ over 24 hours, *in vitro*, of 3 *Pseudomonas* strains in the absence and presence of *Bdellovibrio bacteriovorus*. The mean $OD600_{nm}$ of samples is shown, in the absence or presence of live *B. bacteriovorus* HD100 added at 4 x 10^6 or 1.6 x 10^7 Plaque Forming Units (PFU) (n = 4). The increase in $OD600_{nm}$ in the absence of *Bdellovibrio* indicates *Pseudomonas* strain growth, which is reduced with the addition of 4 x 10^6 *B. bacteriovorus* HD100, and reduced further or suppressed completely with the addition of 1.6 x 10^7 *B. bacteriovorus* HD100. Error bars indicate 95% Confidence Intervals for each $OD600_{nm}$ value (n = 3 biological repeats). The plant host/Plant Growth-Promoting Rhizobacterial (PGPR) identity is given in brackets after the test strain name.

Table 8 . Mean $OD600_{nm}$ (left hand columns) and population number (right-hand columns) of the 10 *Pseudomonas* strains shown in Figure 8 in samples at the start of the $OD600_{nm}$-measuring assay (T = 0) and at the end of the assay in the absence of *B. bacterovorus* HD100 or in the presence of 4 x 10^6 or 1.6 x 10^7 live, predatory *B. bacteriovorus* HD100 cells (T = 24). Colony Forming Units (CFU) of each strain sample was enumerated on King's medium B agar at the beginning of the assay; this number and the starting $OD600_{nm}$ value was used to calculate the final number of each strain in the samples, using the corresponding mean final $OD600_{nm}$ values at 24 hours. Each value is the mean of n = 4 repeats for each strain.

	$OD600_{nm}$ value				Equivalent cell number of test strain			
	T = 0	T=24 h			T = 0	T = 24 h		
No. of live *B. bacteriovorus* HD100 cells		0	4 x 10^6	1 x 10^7		0	4 x 10^6	1 x 10^7
Pseudomonas putida UWC1	0.06	0.59	0.19	0.09	5.32 x 10^6	5.40 x 10^7	1.73 x 10^7	8.18 x 10^6
Pseudomonas fluorescens SBW25	0.09	0.68	0.71	0.30	1.08 x 10^7	7.92 x 10^7	8.24 x 10^7	3.46 x 10^7
Pseudomonas fluorescens LSWS	0.07	0.51	0.34	0.15	2.54 x 10^7	1.82 x 10^8	1.21 x 10^8	5.40 x 10^7
Erwinia amylovora CFBP430	0.08	0.54	0.25	0.12	1.50 x 10^7	9.70 x 10^7	4.46 x 10^7	2.09 x 10^7
Pseudomonas entomophila L48	0.07	0.99	0.59	0.38	2.64 x 10^6	3.54 x 10^7	2.11 x 10^7	1.37 x 10^7
Pseudomonas corrugata	0.06	0.48	0.42	0.14	1.29 x 10^7	9.68 x 10^7	8.57 x 10^7	2.84 x 10^7
Pseudomonas cichorii 9437	0.06	0.65	0.38	0.11	4.52 x 10^6	4.62 x 10^7	2.71 x 10^7	8.17 x 10^6
Pseudomonas marginalis pv. *pastinaciae*	0.05	0.56	0.51	0.27	3.48 x 10^6	4.00 x 10^7	3.65 x 10^7	1.93 x 10^7
Pseudomonas marginalis pv. *marginalis* 247	0.06	0.64	0.36	0.10	2.14 x 10^6	2.49 x 10^7	1.38 x 10^7	3.69 x 10^6
Pseudomonas viridiflava 2848	0.07	0.56	0.20	0.10	9.52 x 10^5	8.18 x 10^6	2.95 x 10^6	1.52 x 10^6

The titre of *Bdellovibrio* required to suppress growth is also variable between strains: in some cases, the final $OD600_{nm}$ values/population numbers in the presence of 4 x 10^6 *B. bacteriovorus* HD100 was more similar to the final values in the absence of *B. bacteriovorus* HD100, e.g. for *P. marginalis pv. Pastinaceae*, where the $OD600_{nm}$ reached 0.51 (corresponding to 3.65 x 10^7 cells) after 24 hours in the presence of 4 x 10^6 *B. bacteriovorus,* which is closer to the value in the absence of any live *B. bacteriovorus* (0.56, corresponding to 4.00 x 10^7 cells) than in the presence of 1.6 x 10^7 *B. bacteriovorus* cells (OD600nm = 0.27, corresponding to 1.93 x 10^7 cells). Conversely, in other strains the final $OD600_{nm}$ /population number in the presence of 4 x 10^6 *B. bacteriovorus* HD100 was more similar to the value in the presence of 1.6 x 10^7 *B. bacteriovorus* HD100, e.g. for *P. putida,* where the final $OD600_{nm}$ value in the presence of 4 x 10^6 *B. bacteriovorus* was 0.19 (corresponding to 1.73 x 10^7 test strain cells) which is closer to the value in

the presence of 1.6 x 10^7 *B. bacteriovorus* cells ($OD600_{nm}$ = 0.09, corresponding to 8.18 x 10^6 test strain cells) than to that in the absence of any live *B. bacteriovorus* ($OD600_{nm}$ = 0.59, corresponding to 5.40 x 10^7 test strain cells).

The remaining two strains have more unique growth patterns: the $OD600_{nm}$ of *P. syringae pv. tomato DC3000* NEW increased in the absence and presence of 4 x 10^6 and 1.6 x 10^7 *Bdellovibrio* cells before decreasing and increasing again in the presence of 4 x 10^6 and 1.6 x 10^7 *Bdellovibrio* cells (Figure 9, Table 9). This could indicate a fluctuation in growth in response to *Bdellovibrio,* but could equally be due to a quorum sensing mechanism or a prophage bursting from the strain. An increase in $OD600_{nm}$ in the presence of 4 x 10^6 and 1.6 x 10^7 *Bdellovibrio* cells was observed in *Xanthomonas campestris pv. campestris* 8004 that reached a lower final $OD600_{nm}$ than in the absence of *Bdellovibrio* (Figure 9, Table 9). This indicates that its growth is slowed, but not inhibited completely, by the addition of *Bdellovibrio*.

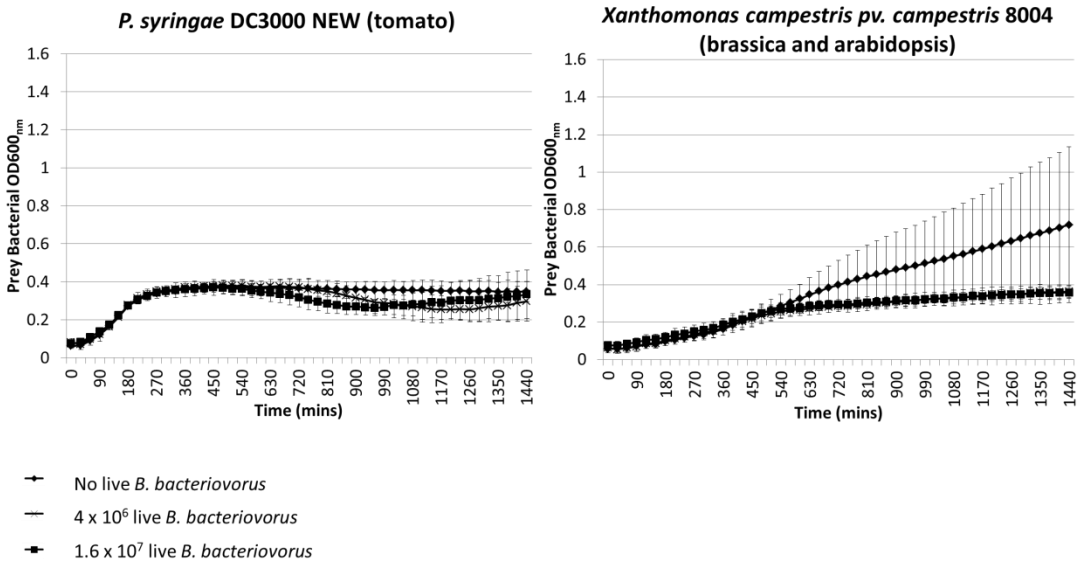


Figure 9 . $OD600_{nm}$ over 24 hours, *in vitro*, of 2 pathogenic strains in the absence and presence of *Bdellovibrio bacteriovorus*. The mean $OD600_{nm}$ of samples is shown, in the absence or presence of live *B. bacteriovorus* HD100 added at 4×10^6 or 1.6×10^7 Plaque Forming Units (PFU) (n = 4). The increase in $OD600_{nm}$ in the absence of *Bdellovibrio* indicates *Pseudomonas* strain growth, which is reduced to different extents with the addition of 4×10^6 or 1.6×10^7 *B. bacteriovorus* HD100. Error bars indicate 95% Confidence Intervals for each $OD600_{nm}$ value (n = 3 biological repeats). The plant host/Plant Growth-Promoting Rhizobacterial (PGPR) identity is given in brackets after the test strain name.

Table 9 . Mean $OD600_{nm}$ (left hand columns) and population number (right-hand columns) of the 10 *Pseudomonas* strains shown in Figure 9 in samples at the start of the $OD600_{nm}$-measuring assay (T = 0) and at the end of the assay in the absence of *B. bacteriovorus* HD100 or in the presence of 4×10^6 or 1.6×10^7 live, predatory *B. bacteriovorus* HD100 cells (T = 24). Colony Forming Units (CFU) of each strain sample was enumerated on King's medium B agar at the beginning of the assay; this number and the starting $OD600_{nm}$ value was used to calculate the final number of each strain in the samples, using the corresponding mean final $OD600_{nm}$ values at 24 hours. Each value is the mean of n = 4 repeats for each strain.

	$OD600_{nm}$ value				Equivalent cell number of test strain			
	T = 0	T=24 h			T = 0	T = 24 h		
No. of live *B. bacteriovorus* HD100 cells		0	4×10^6	1×10^7		0	4×10^6	1×10^7
Pseudomonas syringae DC3000 NEW	0.07	0.35	0.30	0.34	6.76×10^6	3.22×10^7	2.74×10^7	3.10×10^7
Xanthomonas campestris pv. *campestris* 8004	0.07	0.72	0.36	0.36	6.31×10^6	6.84×10^7	3.45×10^7	3.41×10^7

It is important to note that pathogens from a variety of hosts, as well as PGPRs, have similar susceptibilities to *Bdellovibrio* predation, as indicated by the diversity of pathogens/PGPRs shown in Figure 6 - Figure 9**.** For example, the strains that were well preyed upon by *Bdellovibrio* include two pathogens of mushrooms, one of hazelnut (a woody plant host) and one of bean (a herbaceous plant host, Figure 6). Similarly, the strains that are not preyed upon by *B. bacteriovorus* include one PGPR, a pathogen that causes rot in various hosts, and a potato pathogen. This suggests that adaptation to a particular kind of crop host, such as herbaceous plants, woody plants, or fungi, does not necessarily contribute to susceptibility or resistance to *B. bacteriovorus*. Instead, the strains may have something else in common or different that determines resistance to *B. bacteriovorus*, which may result for example from phylogenetic relatedness; however, it is important to note that the S-layer (one possible mechanism of resistance to predation [117], discussed in Section 3.1.1.1) is present in species from phylogenetically diverse groups of bacteria [118], suggesting horizontal gene transfer also plays a role in the acquisition of resistance to *B. bacteriovorus* predation.

However, to determine whether any phylogenetic pattern could be observed in *B. bacteriovorus* predation-resistant/susceptible strains, I plotted the *Pseudomonas* pathogens/PGPRs that I used in my study onto a pre-existing *Pseudomonas* phylogeny constructed by Mulet et al. in 2010 [119]. The authors used concatenated sequences of four ‘housekeeping’ genes (16S rRNA, *gyrB*, *rpoB* and *rpoD*) from 107 *Pseudomonas* strains, and used the neighbour-joining tree construction method, which sequentially clusters pairs of sequences according to similarity. The phylogeny with strains mapped from my study is shown in Figure 10. *P. entemophila,* which was used in my study, was not represented in this phylogeny, so I was not able to include it in this analysis; however, all other strains in my study were represented. *P. syringae* DC3000 NEW, which had a unique growth pattern in the presence of *B. bacteriovorus* (as shown above in Figure 9) was categorised as low susceptibility to *Bdellovibrio* predation along with *P. marginalis* 667, as there was no net reduction in *P. syringae* DC3000 NEW growth over 24 hours where live *B. bacteriovorus* was present.

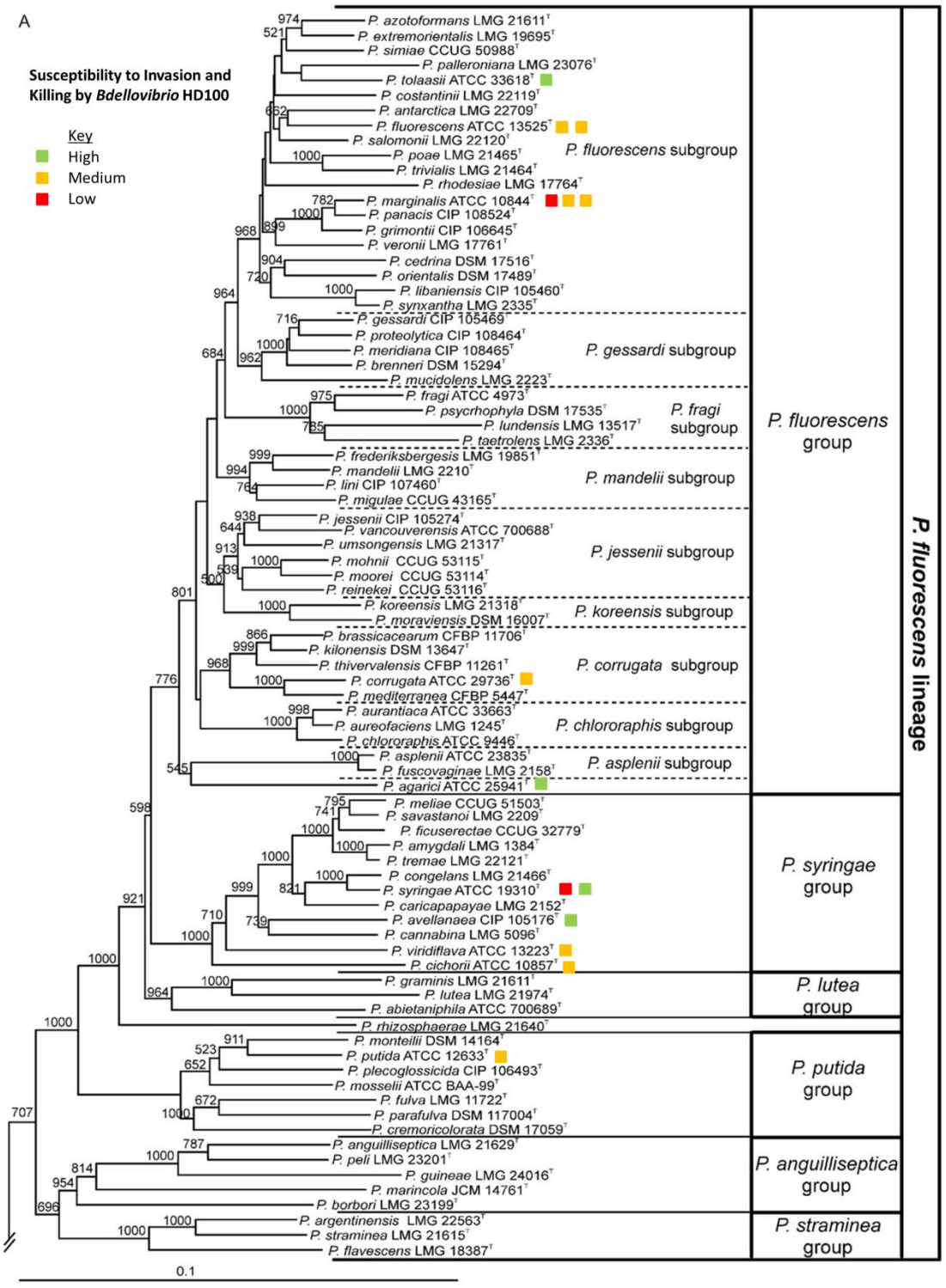


Figure 10 . The phylogenetic relationship between *Pseudomonas* strains used in my study that were preyed upon and killed by *Bdellovibrio bacteriovorus* HD100 to a high, medium, and low extent; phylogeny taken from Mulet and coworkers (2010) [119].

The majority of strains in my predation study were members of the *P. fluorescens* subgroup or the *P. syringae* subgroup, with other single strains mapped to the *P. corrugata* and *P. putida* subgroups; *P. agarici* was placed

in this phylogeny in a subgroup on its own. The two *Pseudomonas* strains that were not well preyed upon and killed by *B. bacteriovorus* (*P. syringae* DC3000 NEW and *P. marginalis* 667) are in different subgroups (*P. syringae* and *P. fluorescens*, respectively) and do not cluster together; they are therefore not closely related in the context of the *Pseudomonads*. Similarly, the 4 strains that were well preyed upon and killed by *B. bacteriovorus* cluster in 3 different subgroups, indicating a similar lack of relatedness between these strains within the *Pseudomonads*. *P. tolaasii* 2192^T and *P. agarici* 2289*,* both mushroom pathogens, are also not closely related, which suggests that their pathogenic ability is probably not due to co-evolved factors, and the mechanisms by which they survive on their mushroom host may be quite different.

Taken together, these observations suggest that the ability of *B. bacteriovorus* to prey upon and kill these *Pseudomonas* strains is not limited to closely related strains, for example due to similar phenotypic characteristics e.g. the toxins, enzymes or siderophores that they produce. Instead, the prevention of predatory activity by the strains with low susceptibility may be due to strain-specific factors, such as toxins, or mechanisms, such as an S-layer, and these may be quite different between the strains. Conversely, the characteristics of strains that are permissive to predation and killing by *B. bacteriovorus,* such as a relative lack of destructive metabolites such as proteases, or a lack of any protective S-layer, may also be different. These possibilities are discussed in Section 3.5.

3.4.2 Visualising *Bdellovibrio/Pseudomonas* pathogen mixtures using Transmission Electron Microscopy

As the growth of *P. avellanae, P. syringae pv. Phaseolicola, P. agarici* 2289 and *P. tolaasii* 2192^T was inhibited well by *B. bacteriovorus* HD100 (Figure 6), Transmission Electron Microscopy (TEM) was used to visualise these strains in the presence of *Bdellovibrio*. In all cases, images showed *Bdellovibrio* attachment to *Pseudomonas* (Figure 11), with *P. agarici* 2289 beginning to form a Bdelloplast. Attachment is an important first step in the

predatory cycle (Figure 2), which indicates that growth inhibition in these two strains shown in the “flo” assay results is due to predation by *Bdellovibrio*.

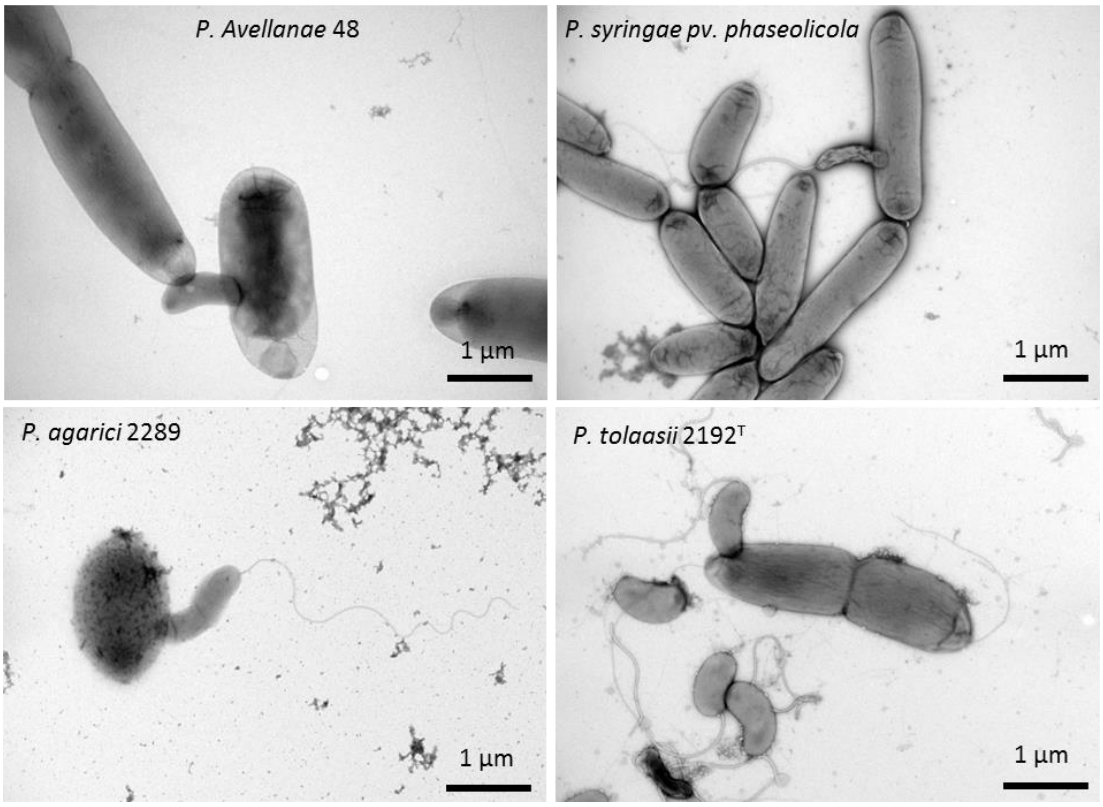


Figure 11 . Transmission Electron Microscope (TEM) pictures of *B. bacteriovorus* HD100 attachment to *P. avellanae* 48, *P. syringae pv. phaseolicola, P. agaricii* 2289 (showing Bdelloplast rounding of the cell) and *Pseudomonas tolaasii* 2192^{T}. Samples were stained with 0.5% w/v uranyl acetate in dH_2O. Pictures were taken at a magnification of 20000 x with a JEOL 1200Ex electron microscope.

3.4.3 Assessing *N. benthamiana* (tobacco) leaves as a model for testing *in vitro Bdellovibrio/Pseudomonas* predatory interactions *in vivo*

In this assay, I aimed to gauge the symptoms in *N. benthamiana* (tobacco) leaves resulting from inoculation with *B. bacteriovorus* HD100, *P. avellanae* 48, *P. syringae pv. phaseolicola, P. syringae pv. tabaci* 11528, *P. agarici* 2289, and *P. tolaasii* 2192^{T}, with a view to using tobacco as a model host for *in vivo* tests of *Bdellovibrio* predation of these *Pseudomonas* pathovars. I chose these *Pseudomonas* strains for this preliminary *in planta* test because they had been well preyed-upon by *Bdellovibrio* in the *in vitro* tests

(described in Section 3.4.1), and so testing these strains in particular was my priority. I also aimed to assess the survival or death of each *Pseudomonas* pathogen and *B. bacteriovorus* HD100 after inoculation in the *N. benthamiana* leaf tissue by enumerating any live bacteria that could be recovered from the inoculated tissue both immediately after inoculation (T = 0), and 4 days after inoculation (T = 4 days, when disease symptoms appeared), to ensure that the *Pseudomonas* pathogens were able to survive and replicate well in the *N. benthamiana* leaves, with a view to testing the effect of *Bdellovibrio* predation on *Pseudomonas* pathogen numbers in this model plant host.

At T = 4 days, tobacco leaf tissue inoculated with *P. avellanae* 48 and *P. syringae pv. phaseolicola* alone had lost its green colour and turned opaque, clearly indicating tissue death due to infection by these strains (Figure 12 b and c) when compared with control leaf tissue (Figure 12 a). Conversely, tobacco leaf tissue inoculated with *P. tolaasii* 2192^{T} (a mushroom pathogen) alone and *B. bacteriovorus* HD100 alone showed no such symptoms of tissue death, indicating that these strains had no adverse effects on tobacco leaf tissue (Figure 12 e and f). Tobacco leaf tissue inoculated with *P. agarici* 2289 (a mushroom pathogen) developed mild symptoms of infection, but not to the same extent as other strains (Figure 12 e).

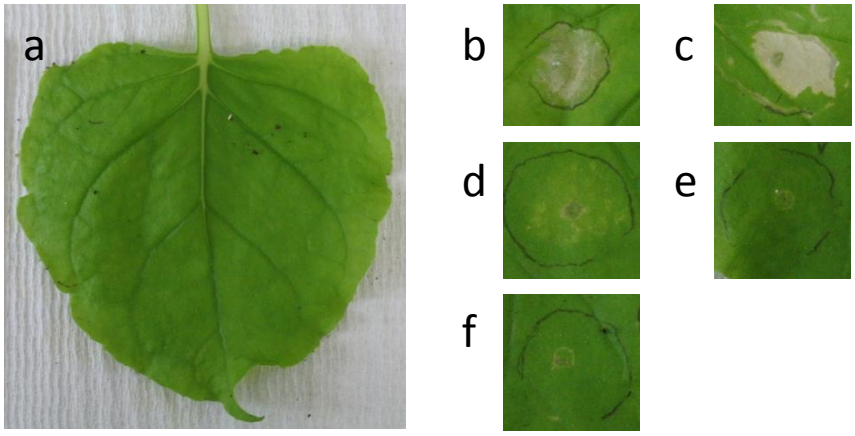


Figure 12 . Inoculated leaf tissue of (b) *P. avellanae* 48, (c) *P. syringae pv. phaseolicola*, (d) *P. agarici* 2289, (e) *P. tolaasii* 2192^{T} and (f) *B. bacteriovorus* HD100, as compared to a healthy, uninoculated leaf in (a).

I then tested whether any *Pseudomonas* pathogen and *B. bacteriovorus* HD100 cells could be recovered and enumerated from inoculated leaf tissue both immediately after inoculation and then 4 days after inoculation, when the symptoms of infection as shown in Figure 12 had appeared. This was an important first test to conduct in view of using *N. benthamiana* as a model organism in which to test *Bdellovibrio* predation of *Pseudomonas* pathogens *in planta*, as the relative predator-pathogen numbers would indicate whether *Bdellovibrio* are able to survive and prey upon these *Pseudomonas* pathogens, killing them *in vivo* as well as *in* vitro as shown in Section 3.4.1. The number of Colony Forming Units (CFU) of each *Pseudomonas* pathovar recovered from the inoculated *N. benthamiana* tissue is shown in the graph in Figure 13**.**

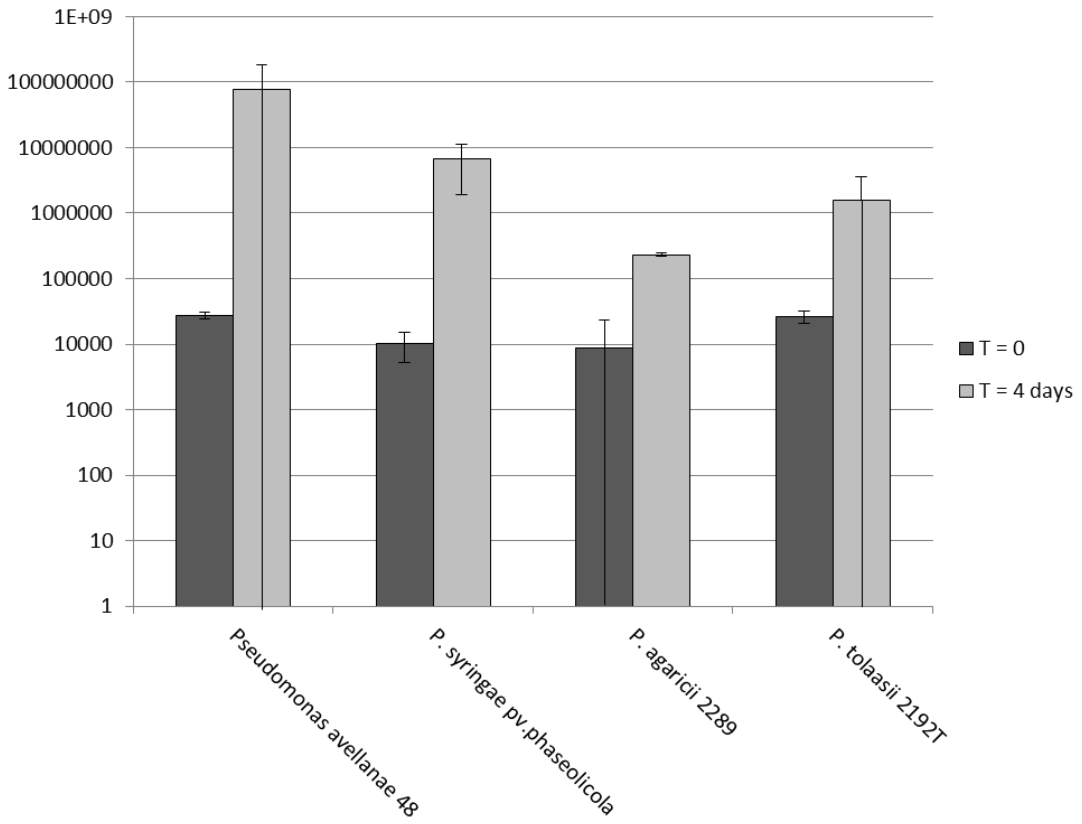


Figure 13 . Number (CFU) of each *Pseudomonas* pathovar recovered from inoculated *Nicotiana benthamiana* (tobacco) leaf tissue directly after inoculation (T = 0, dark grey) and 4 days after inoculation (T = 4 days, light grey), when symptoms of disease had appeared in some inoculated tissue. Error bars show +/- 1 standard deviation from three dilution series made separately from the same original sample of inoculated leaf tissue resuspended in calcium HEPES buffer.

The number of live cells of each *Pseudomonas* pathovar recovered from the inoculated tissue was similar for each isolate, between 8.73 x 10^3 (*P. agaricii*) and 2.73 x 10^4 (*P. avellanae*). This was considerably lower than the number of cells in each 0.25 ml inoculation, which ranged from 1.78 x 10^7 to 5.63 x 10^8, indicating that a 10^4-10^5 –fold drop in numbers occurred during the inoculation process. This may have been due to cell death resulting from the inoculation procedure itself (which involved high-pressure inoculation of the liquid cultures into the leaf tissue through a small opening) or due to loss of the inoculum through leaking or dripping (although care was taken to minimise leakage of the inoculum on to the surface of the leaf instead of into the leaf tissue, small leaks still occurred).

Although some of the bacterial counts were highly variable which may have been due to insufficiently homogenised samples in this pilot test (as indicated by the error bars showing standard deviation on the graph), the number of each *Pseudomonas* pathovar increased from T = 0 to T = 4 days after inoculation, when disease symptoms had appeared; this increase was greatest in *P. avellanae* (from 2.73×10^4 to 7.58×10^7) and *P. syringae* pv. *phaseolicola* (from 1.03×10^4 to 6.65×10^6). These two pathovars infect Hazelnut and bean plants, respectively, and so the *N. benthamiana* tissue is similar to that of the host that they were originally isolated from; the number of the two mushroom pathogens *P. agarici and P. tolaasii,* on the other hand, did not increase in number as much (from 8.72×10^3 to 2.3×10^5 and from 2.67×10^4 to 1.55×10^6, respectively). This is likely because they are pathogens of mushrooms and so they are not well adapted to living in the contrasting environment of plant leaf tissue. These results correlate with the disease symptoms that were clearly visible in the plant tissue inoculated with *P. avellanae* and *P. syringae pv. phaseolicola,* but not in *P. agarici* or *P. tolaasii,* shown in Figure 12**.**

I was unable to recover any live *B. bacteriovorus* from the plant leaf tissue on YPSC overlay plates using *E. coli* prey, which is routinely used in the laboratory to culture *Bdellovibrio,* from both samples taken at T = 0 and at T = 4 days. *B. bacteriovorus* HD100 can survive without prey for short amounts of time without dying, and so I expected to recover some live, predatory *Bdellovibrio* from the inoculated leaf tissue at T = 0 days. However, as this was not the case, it is possible that the *Bdellovibrio* were either rapidly killed by a substance produced by the *N. benthamiana* plant present in the intercellular leaf space, or that the treatment of the overlay plates was not optimal for *B. bacteriovorus* growth, despite the fact that the overlay plate technique is routinely used to culture *B. bacteriovorus* in the laboratory. Due to the long (12-week) generation time of the *N. benthamiana* plants, in combination with the difficulties encountered with recovering *B. bacteriovorus* from the *N. benthamiana* leaf tissue, I decided not to pursue the use of *N. benthamiana* as a model host for these *in vivo* studies of predation, as optimising and then conducting the tests would have taken a relatively long

time. Instead, I opted for the more easily manipulated post-harvest mushroom *Agaricus bisporus*, which I could acquire in large numbers from the supermarket, in which to test *in vivo* predation of *P. tolaasii* in Chapter 4.

3.5 Discussion

3.5.1 *B. bacteriovorus* HD100 preys upon and kills some plant pathogenic/PGPR species *in vitro,* but not others

The population growth of 4 out of the 20 pathogenic strains that I tested (*P. avellanae* 48: hazelnut canker, *P. syringae pv.* phaseolicola: halo blight of bean, *P. tolaasii* 2192^{T}: brown blotch in mushrooms,and *P. agarici* 2289: drippy gill in mushrooms) was completely suppressed in the presence of both 1.6 x 10 ^{7} and 4 x 10^{6} *B. bacteriovorus* HD100 cells over 24 hours, indicating predatory killing of these test strains. Conversely, no such population growth suppression was observed in the spp. *B. vietnamiensis* G4 (a PGPR), *P. marginalis* 667 (soft rot in various host plants), and *P. atrosepticum* SCRI143 (blackleg in potato), indicating that these strains are resistant to predatory killing by *B. bacteriovorus.* The remaining strains were preyed upon to a variable extent by *B. bacteriovorus*, with population growth suppression generally occurring to a greater extent in the presence of 1.6 x 10^{7} than with 4 x 10^{6} *B. bacteriovorus* cells, suggesting that they have some mechanisms that inhibit *B. bacteriovorus* predation to a certain extent.

Both of the groups that were susceptible or resistant to *B. bacteriovorus* predation and killing, respectively, were diverse, including pathogens of a range of different hosts that produce repertoires of different pathogenicity effectors, as I explained in Section 3.1.1. Additionally, when I examined the phylogenetic relationship between susceptible and resistant strains, I found that susceptibility/resistance to *B. bacteriovorus* was not associated with phylogenetic relatedness, as shown in Figure 10. These observations together suggest that the mode of resistance to *B. bacteriovorus* HD100 predation exhibited by the 3 resistant strains may be unique to each strain, or alternatively common to all 3 strains, and possibly encoded by the same, horizontally-transferred gene cassette.

A possible mechanism of resistance is through physical blocking of *Bdellovibrio* attachment, e.g. throught the production of an S-layer [117], or other extracellular/outer membrane structures. For example, *B. vietnamiensis* G4, which was not susceptible to *B. bacteriovorus* predation, produces a large (2463 AA, 229 kDa) outer membrane adhesin-like protein; mutation studies have suggested that its presence prevents *B. vietnamiensis* colonisation of plant roots, possibly due to physical blocking of smaller adhesins responsible for the molecular interaction with the root surface required for attachment [39]. Although the molecular basis of *B. bacteriovorus* attachment to Gram-negative prey cells is not yet well understood, the same blocking mechanism may also prevent predator attachment to the outer membrane of *B. vietnamiensis* G4. This could be tested by deletion mutagenesis of the gene that codes for this protein in *B. vietnamiensis* G4, followed by further *in vitro* predation assays.

P. atrosepticum pathogenicity factors are primarily Plant Cell Wall-Degrading Enzymes (PCWDEs), secreted by the Type I and Type II systems into the extracellular space, which includes pectin and cellulose-degrading enzymes and proteases [101]. The production of these PCWDEs is controlled by the well-studied quorum-sensing system involving *N*-acyl-homoserine lactone (AHL) signal molecules, where virulence increases with AHL concentration, described in Section 3.1.1.5. It was previously shown that AHL concentration on potato tubers plateaued at a concentration of between 1×10^6 and 1×10^7 *P. atrosepticum* cells per ml [101], which is lower than their starting concentration of 3.06×10^6 cells in the 264 µl sample in each well in my assay; the production of these enzymes by *P. atrosepticum* may have damaged *B. bacteriovorus* cells, resulting in prevention of *B. bacteriovorus* predation.

Like *P. atrosepticum, P. marginalis* is also a soft-rot pathogen and also produces PCWDEs [102], controlled by a quorum-sensing mechanism [120], and thus may prevent *B. bacteriovorous* predation in a similar way. In addition, *P. marginalis* produces a biosurfactant called PM factor, which degrades a wide variety of hydrocarbons [121]. Secreted biosurfactants can remain close to the cell wall of the bacterium that produces it (or be secreted

onto other surfaces), and can selectively inhibit or permit the attachment of different bacterial species [122]. Thus, it is possible that PM factor may play a role in inhibiting *B. bacteriovorus* attachment to *P. marginalis,* through a different mechanism to *B. vietnamiensis,* and could similarly be tested by deletion mutagenesis and further predation assays.

Predatory attachment, which is possibly prevented in the predation-resistant strains, was observed in all 4 pathogens that were well preyed-upon by *B. bacteriovorus*, as shown in Figure 11. These strains thus lack any apparently obvious means of resisting *B. bacteriovorus* predation, whether through the production of physical barriers or toxins.

The main virulence effectors produced by *P. syringae pv. phaseolicola* are delivered directly into host plant cells via a Type III Secretion System [8]; thus *B. bacteriovorus* would not be exposed to these eukaryotic-targeted effectors. However, *P. syringae pv. phaseolicola* also produces phaseolotoxin, a tripeptide that is likely secreted through an ABC transporter system into the extracellular space [116]. Phaseolotoxin inhibits ornithine carbamoyltransferase, a key enzyme in the urea cycle and in arginine biosynthesis in plants and bacteria [8]. As mentioned in Section 3.1.1.2, phaseolotoxin has previously been found to inhibit the growth of the Gram-negative species *E. coli* and *Salmonella typhimurium*, which take up the toxin by an oligopeptide transport system (Opp) involving oligopeptide permease [100]. The *B. bacteriovorus* HD100 genome contains two putative oligopeptide permease-encoding genes [123], at the adjacent loci *bd2709* and *bd2710*, so it is theoretically possible that *B. bacteriovorus* HD100 could take up the toxin. However, the secretion of phaseolotoxin by *P. syringae pv. phaseolicola* only occurs at a narrow temperature range, 18-20ºC, and it was not detected at 30ºC [8]. The *in vitro* predation assay was carried out at 29ºC, and so it may be that phaseolotoxin was not produced in these conditions, and thus would not affect *B. bacteriovorus* predation. Further studies would be required to confirm whether *B. bacteriovorus* would still prey upon and kill *P. syringae pv. phaseolicola* in a host plant, under conditions permissive to phaseolotoxin production, which led to my tests of *N. benthamiana* as a model in which to assess this (Section 3.4.3).

Similarly, *P. tolaasii* cells can switch between saprophytic and pathogenic lifestyles [124]; switching to a pathogenic lifestyle, which involves the production of tolaasin and other effectors, is known to occur when the number of *P. tolaasii* cells reaches a certain threshold, as after replication on the surface of its mushroom host (this is discussed in more detail in Section 4.1.2). Tolaasin has previously been shown to disrupt bacterial membranes [125], but it is unlikely that this toxin was produced under the *in vitro* buffer conditions of the assay in Section 3.4.1, where *P. tolaasii* were initially at low numbers in a buffer/medium mixture. Additionally, *P. tolaasii* also produces proteases which are likely involved in pathogenicity; one metalloprotease was previously found to be produced mainly during the exponential and stationary growth phases (as on the surface of a mushroom) [9]. *B. bacteriovorus* predation, which suppressed any *P. tolaasii* population growth under these *in vitro* conditions as shown in Figure 6, may have also prevented the production of proteases that could be harmful to *B. bacteriovorus* cells. *P. tolaasii* also produces lipases, which could disrupt the lipid *B. bacteriovorus* outer membrane; the production of these lipases may similarly occur during population growth as on the surface of a mushroom. Thus, in a similar way to *P. syringae, B. bacteriovorus* predation of *P. tolaasii* could be inhibited in its natural context; this led me to test whether *in vivo* predation of *P. tolaasii* by *B. bacteriovorus* occurred on the surface of *Agaricus bisporus* mushrooms in Chapter 4 of my thesis.

The *P. avellanae* genome is known to contain pathogenic effector genes [126]; however, little is yet known about the secretion of these disease-causing effectors during host-pathogen interactions, due to difficulties associated with manipulating woody host plants [126]. *P. avellanae* infection occurs through ‘opportunistic’ routes, e.g. rain splashing into cracks in hazelnut tree bark, or onto fresh leaf scars that allow entry into the inner trunk and branches of the tree [127]; thus initial pathogen entry into the host does not require manipulation of the host plant through the production of PCWDEs or toxins, so any that might potentially affect *B. bacteriovorus* may only be produced inside the host plant.

P. agarici, unlike most other *Pseudomonas* pathogens including those tested here, does not produce as many (if any) potentially bacterially toxic effectors *in vitro* or in its natural context on the gill tissue of mushrooms, demonstrated in previous systematic studies [88, 128]; it has been suggested that because *P. agarici* does not produce a host response in mushroom (as *P. tolaasii* does, for example), it can more easily gain access to the inner tissue of the mushroom and cause infection deeper in the tissue. Thus, *P. agarici* may be susceptible to *B. bacteriovorus* predation due to this lack of effector production.

3.5.2 *Pseudomonas* pathogen strains, but not *B. bacteriovorus,* could be recovered from inoculated *N. benthamiana* tissue, where they increased in number

With a view to testing *B. bacteriovorus* predatory killing of the susceptible pathogens I identified *in vivo* in a natural, *in planta* setting, I assessed the suitability of the model plant *Nicotiana benthamiana* (tobacco) as a host. When the predation-susceptible pathogen strains were inoculated separately into *N. benthamiana* leaf tissue, their population numbers increased over 4 days, as shown in Figure 13; this increase was greater for *P. avellanae* and *P. syringae pv. phaseolicola* than for *P. tolaasii* and *P. agarici,* likely due to the adaptation of the former strains to thrive in leafy plant hosts, and of the latter strains to fungal (mushroom) hosts. This was consistent with the formation of necrotic lesions in the leaf tissue inoculated with *P. avellanae* and *P. syringae pv. phaseolicola*, but not with *P. agarici* and *P. tolaasii* (Figure 12). Therefore, *N. benthamiana* could be used as a model plant host for infection studies with *P. avellanae* and *P. syringae pv. phaseolicola*; however, when I inoculated *B. bacteriovorus* into the *N. benthamiana* leaf tissue, no live *B. bacteriovorus* could be recovered from tissue either immediately after inoculation or at 4 days post-inoculation.

This could be due to Pathogen-Associated Molecular Pattern (PAMP) recognition of *B. bacteriovorus* by *N. benthamiana,* which triggers an initial immune response in the plant host against bacteria and other microbes [129]; flagellins are well-studied as PAMPs recognised by *N. benthamiana,* and are

present on attack-phase *B. bacteriovorus* cells for fast swimming motility and prey location, albeit under a membrane flagellar sheath in live *B. bacteriovorus* [130]. Reactions to PAMP detection in plants, via cell surface receptors, include the production of reactive oxygen species and MAP kinases[131], which in the context of this study may have resulted in *B. bacteriovorus* cell death. Another possibility is that the conditions in which I conducted the routine overlay plate method of *B. bacteriovorus* recovery on a prey cell lawn (as described in Section 2.5.3) were not optimal for recovering any live predatory cells.

3.6 Chapter conclusions

B. bacteriovorus preyed upon and killed some plant pathogenic/PGPR strains, but not others, *in vitro.* Predatory killing of susceptible strains was confirmed by visualising *B. bacteriovorus* attachment to prey. The mechanism of resistance likely varies between strains, but most likely involves physical blocking of predatory attachment; conversely, susceptibility to *B. bacteriovorus* predation is likely due to the lack of such extracellular structures, toxins, or other effectors produced by the susceptible strains, under the conditions of the *in vitro* assay.

Although some predation-susceptible pathogenic strains successfully replicated and produced pathogenic symptoms in *N. benthamiana* leaf tissue after inoculation, no live *B. bacteriovorus* inoculated into *N. benthamiana* tissue could be recovered; additionally, *N. benthamiana* has a relatively long growth period and thus *in vivo* studies in this host would be slow. Taken together, these results led me to test predatory killing of the predation-susceptible mushroom brown-blotch pathogen *P. tolaasii* 2192^{T} *in vivo,* on the surface of readily available and easily manipulated post-harvest *Agaricus bisporus* mushrooms, in Chapter 4.

4 *Bdellovibrio* as a Food Security Guard against the Mushroom Pathogen, *Pseudomonas tolaasii*

4.1 Chapter Introduction

The *in vitro* screening assay in Chapter 3 identified *P. tolaasii* 2192^T, an important spoilage pathogen of commercially produced button mushrooms, as highly preyed upon by *Bdellovibrio*. This led me to investigate the use of *Bdellovibrio* as a food security agent to prevent this post-harvest mushroom spoilage *in vivo,* on the surface of the button mushroom, *Agaricus bisporus*, with the potential to increase mushroom crop yield.

4.1.1 *Pseudomonas tolaasii* causes brown blotch disease of mushrooms

Pseudomonas tolaasii is a Gram-negative, naturally soil-dwelling bacterial pathogen that causes brown blotch disease in several varieties of cultivated mushrooms [132-134]. The disease is characterised by brown lesions on the outer layers (2-3 mm depth) of the mushroom pileus and stipe, which range from small, light brown spots to larger, dark, sunken and wet lesions, depending on disease severity. This brown discolouration results from mushroom production of melanin, which is a defence response induced in this case by *P. tolaasii* producing the toxin tolaasin. Tolaasin is an 18-amino acid lipodepsipeptide that forms ion channels and also acts as a biosurfactant to disrupt the plasma membrane of mushroom cells, allowing *P. tolaasii* access to cell-nutrients [125, 135-137]. Infection is also reported to result in slower development of the mushroom crop with a lower yield [9]. The economic impact of the disease is significant, resulting in loss of visual appeal to consumers and regular crop reductions of 5-10% in the UK [138]. The disease is found worldwide: *P. tolaasii* mushroom infection has been documented in several countries, including the USA, Spain, Serbia, the Netherlands, Japan and Korea [132, 133, 139-142].

4.1.2 **Controlling *P. tolaasii* infection in mushrooms is difficult**

A major obstacle in the control of *P. tolaasii* infection that contributes to its broad prevalence is that some of the bacterial species present in the casing soil around mushrooms, such as *Pseudomonas putida*, are necessary for promoting the initial stages of mushroom growth [143, 144]. This means that the casing soil cannot be sterile, and broad range antibiotic and antiseptic treatments cannot be used in the mushroom-growing process; consequently, *P. tolaasii* may become endemic in the casing soil and compost used in mushroom cultivation [145].

P. tolaasii survives well in nutrient-poor environments, such as the casing soil prior to mushroom growth, by altering the production of various enzymes, thus switching between pathogenic non-fluorescent (Smooth colony morphology on King's Medium B agar, S-type) and non-pathogenic fluorescent (Rough colony morphology, R-type) forms [124, 146]. *P. tolaasii* also uses flagellar-mediated chemotaxis in the wet casing soil to move towards nutrient 'signals' produced by the mushroom; once on the pileus surface, they attach and initiate disease rapidly [136, 147]. Symptoms can appear on mushrooms at all stages of development; some apparently unaffected mushrooms also develop symptoms after harvesting, making it difficult to immediately identify and target *P. tolaasii* infections [148]. Furthermore, the pathogen is spread easily on the hands of mushroom pickers, and epidemics can occur between multiple mushroom houses [9].

4.1.3 **Current methods for controlling *P. tolaasii infection***

Due to the adaptability and persistence of *P. tolaasii*, and the limitations on treatment options, there are very few effective methods for controlling *P. tolaasii* infection that are also safe to use on crops intended for human consumption. The current best methods of disease prevention are addition of chlorinated compounds such as calcium hypochlorite to irrigation water, and careful control of growth conditions; for example, the surface moisture of mushrooms and water level in the casing soil to minimize *P. tolaasii*

chemotaxis and motility; however, the success of disease prevention is highly variable, and not guaranteed [9, 142, 149].Other disinfectants and antibiotic compounds such as chloramine T and bronopol have been suggested as potential treatments [7, 142], as well as natural plant extracts from *Salvia miltiorrhiza* [150], and the White Line Inducing Principle (WLIP) produced by *Pseudomonas reactans*, which reacts with tolaasin produced by *P. tolaasii* [151]. Other Pseudomonads that are antagonistic to *P. tolaasii*, such as *Pseudomonas flourescens*, have also been investigated as biocontrol strains [152]. Most recently, the application of a *P. tolaasii*-specific bacteriophage has been proposed as a novel method of controlling *P. tolaasii* infection [153], but to our knowledge none of these alternative disease prevention methods have been tested or used commercially.

4.1.4 **Other Gram-negative mushroom-associated bacteria**

Although *P. tolaasii* is arguably the most problematic pathogen of cultivated mushrooms, several other mushroom-dwelling, Gram-negative bacterial species have previously been identified, including both pathogens and non-pathogenic species.

4.1.4.1 ***Pathogens***

In addition to *P. tolaasii,* several other species of *Pseudomonas* cause disease in mushrooms. *P. agarici,* which I also found to be well preyed upon by *B. bacteriovorus in vitro* in Chapter 3 (shown in Figure 6), causes drippy gill of mushroom; this disease is characterised by black spots on the gill tissue of mushrooms that ooze liquid droplets, and was first identified as the causal agent in a severe outbreak of the disease in a New Zealand mushroom crop [154]. Other *Pseudomonas* species cause disease symptoms that are similar to the brown blotches caused by *P. tolaasii*: *P. gingeri*, for example, causes ‘ginger blotch’, a cap lesion disease that produces ginger-coloured blotches, instead of dark brown. The mechanism of *P. gingeri* infection is as yet unknown [155]*;* however, given that the lesion colour is notably different, the causal agent produced by *P. gingeri* is likely to

have a slightly different mechanism of action, inducing induce a slightly different response to infection by the mushroom.

Other, non-*Pseudomonas* Gram-negative bacteria that cause disease in mushrooms include *Janthinobacterium agaricidamnosum*, where infection results in a rapid blackening and rotting of mushroom tissue, mediated by the recently discovered causal agent jagaricin (a lipopeptide with an as-yet unknown function) [156, 157] *Ewingella americana*, which causes browning and moderate degradation of the internal stipe tissue [158]; and *Burkholderia gladioli* pv. *agaricicola*, which causes the formation of cavities in the mushroom tissue mediated by the secretion of proteases and chitinases [159].

4.1.4.2 *Commensals*

As mentioned above, some commensal species associated with *A. bisporus* have been identified such as *P. putida,* required for mushroom fruiting body formation, and *P. reactans* and *P. fluorescens,* which inhibit *P. tolaasii* infection. In addition to this, other studies have reported a diverse range of bacterial species isolated from healthy mushroom tissue, including Fluorescent *Pseudomonas, Flavobacterium, Chryseobacterium, Coryneform, Lactobacillus and Pediococcus* sp., and the total number of native bacteria present on the mushroom surface is previously reported in the range of 1 x 10^6 to 1 x 10^7 [160].

In this study I hypothesised that *B. bacteriovorus* inoculated on the surface of *Agaricus bisporus* mushrooms would prevent brown blotch symptoms caused by experimental infection with *P. tolaasii*; however, diverse bacterial species are normally present on natural and cultivated mushroom surfaces, shown in the studies above. I therefore considered the possibility that *B. bacteriovorus* treatment might interact with and affect the levels of endogenous species present on commercially produced and natural mushroom surfaces (as shown in Section 4.4.4 and 4.4.5). Bacterial-fungal interactions such as those described in this chapter have been the subject of recent reviews [155] as they involve interesting cross kingdom biology, but also affect crop productivity and thus global food security.

4.2 Specific Research Aims

- To assess the potential of *B. bacteriovorus* HD100 to reduce the symptoms of *P. tolaasii* infection on the surface of post-harvest *Agaricus bisporus* mushrooms, by measuring the intensity of brown-blotch lesions and number of *P. tolaasii* CFU recovered from experimentally infected mushrooms, with and without *B. bacteriovorus* treatment
- To visualise *B. bacteriovorus* and *P. tolaasii* on the surface of *A. bisporus* mushrooms using Scanning Electron Microscopy (SEM), to assess *Bdellovibrio* survival and predatory activity against *P. tolaasii* on this natural, biotic surface
- To assess the impact of *B. bacteriovorus* treatment on any other bacterial species naturally inhabiting the surface of both commercially produced and organic, garden mushrooms, by isolating and enumerating them from mushroom cap tissue, identifying them using 16s rDNA sequencing, and using an Optical Density ($OD600_{nm}$) assay to determine *B. bacteriovorus* predatory killing of each isolate *in vitro*

4.3 Hypothesis

- *B. bacteriovorus* HD100 will prey upon and kill the mushroom pathogen *P. tolaasii* 2192^{T} on the surface of *A. bisporus* mushrooms, lowering their numbers and therefore reducing the brown-blotch symptoms resulting from *P. tolaasii* infection
- *B. bacteriovorus* will survive on the surface of *A. bisporus* caps, and predatory attachment, invasion and killing of experimentally-inoculated *P. tolaasii* cells by *B. bacteriovorus* on the surface of the mushroom will be observed in SEM images
- Other diverse, commensal/pathogenic bacterial strains will be present on the surface of both commercially produced and organic, garden mushrooms

- *B. bacterivorous* will be able to prey on some of the mushroom-isolated strains *in vitro*, suppressing their population growth and thus preventing a rise in $OD600_{nm}$ in the OD assay

4.4 Results

In Chapter 3, I found that *B. bacteriovorus* suppressed *P. tolaasii* 2192^T growth *in vitro*; thus, I reasoned that this effect might be replicated in a more natural environment; thus, in this chapter, I investigated the effect of *B. bacteriovorus* HD100 treatment on the pathogenic brown blotch lesion symptoms in post-harvest mushrooms resulting from infection with *P. tolaasii.*

4.4.1 Brown blotch lesion intensity was reduced by *Bdellovibrio* application onto mushrooms.

To determine whether symptoms of *P. tolaasii* infection, a function of bacterial metabolism and growth, were reduced with *Bdellovibrio* treatment in a natural context, the intensity of lesions formed by *P. tolaasii* 2192^T on the post-harvest pileus surface of the cultivated button mushroom *Agaricus bisporus* was measured in the presence and absence of *B. bacteriovorus* HD100, as shown in Figure 14. Mushroom pilei inoculated with *P. tolaasii* 2192^T alone, in the absence of any treatment with *B. bacteriovorus* HD100, formed dark, wet surface lesions (pictured in Figure 16), the primary symptom of brown blotch disease, after 48 hours at 29^0C (mean intensity = 0.019 ^{1}/PV ± 0.0005, n = 30). In contrast, pilei treated with a King's Medium B control (the preferred growth medium of *P. tolaasii*) did not form these dark lesions (mean intensity = 0.012 ^{1}/PV ± 0.0005, n = 30); similarly, those treated with *B. bacteriovorus* HD100 alone, and not inoculated with *P. tolaasii* 2192^T, also did not form dark lesions (mean intensity = 0.010 ^{1}/PV ± 0.0005, n = 30), so *Bdellovibrio* application itself did not have a significant adverse effect on the appearance of mushroom pilei.

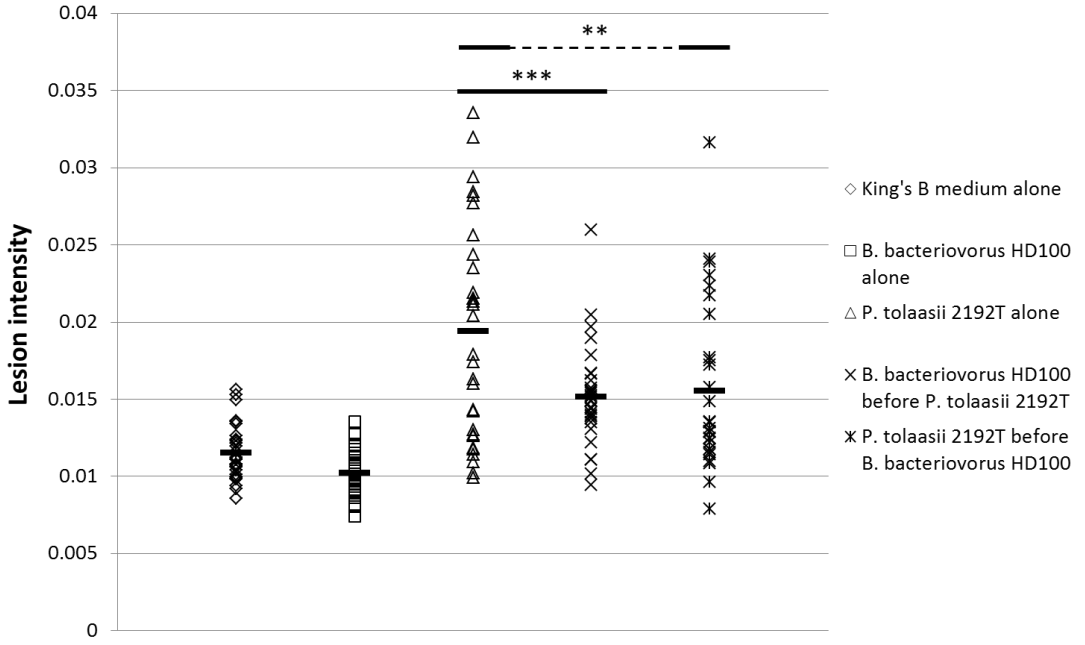


Figure 14 . Lesion intensity on *P. tolaasii*-inoculated mushrooms in the presence and absence of *Bdellovibrio*. Lesion intensities on mushroom pilei under 5 different treatment conditions, detailed to the right of the graph. Each *P tolaasii* 2192^T inoculation contained 1.7 x 10^6 CFU, and each *B. bacteriovorus* HD100 inoculation contained 2.9 x 10^6 PFU. Higher lesion intensity indicates a greater level of brown blotch disease symptoms and therefore a higher level of *P. tolaasii* infection. Horizontal black bars indicate the mean lesion intensity value for each treatment group. Separate Student's t-tests of significance were conducted between mushrooms inoculated with *P. tolaasii* 2192^T alone and mushrooms treated with *B. bacteriovorus* HD100 before *P. tolaasii* 2192^T inoculation, and mushrooms inoculated with *P. tolaasii* 2192^T alone and mushrooms treated with *B. bacteriovorus* HD100 after *P. tolaasii* inoculation. T-test comparisons are shown on the graph as horizontal lines above the treatment groups, with t-test significance values above them if they are significant at the 95% confidence level: ** $p < 0.01$, *** $p < 0.001$.

Post-harvest mushrooms treated with *B. bacteriovorus* HD100 either 30 minutes before or 30 minutes after *P. tolaasii* 2192^T inoculation developed significantly lighter lesions than those inoculated with *P. tolaasii* 2192^T alone (average intensity = 0.015 and 0.016 1/PV ± 0.0005 respectively, n = 30 in both cases, vs. 0.019 1/PV ± 0.0005 for mushrooms inoculated with *P. tolaasii* 2192^T alone). The significance of the difference in lesion intensities between *B. bacteriovorus* HD100 treated and untreated, *P. tolaasii* 2192^T inoculated mushrooms was greater when *Bdellovibrio* was added before *P. tolaasii* 2192^T than when added after (Student's t-test $p<0.001$ for *B. bacteriovorus* HD100 added before *P. tolaasii* 2192^T vs. *P. tolaasii* 2192^T

alone, p<0.01 for *B. bacteriovorus* HD100 added after *P. tolaasii* 2192^T vs. *P. tolaasii* 2192^T alone). However, lesion intensities on mushrooms treated with *B. bacteriovorus* HD100 both before and after *P. tolaasii* 2192^T inoculation were also significantly different from lesions on King's medium B control mushrooms (average intensity 0.015 and 0.016 1/PV vs. 0.012 1/PV, p <0.001), suggesting that lesion formation by *P. tolaasii* was not completely abolished by *B. bacteriovorus* treatment.

4.4.2 Scanning Electron Microscope images show *B. bacteriovorus* attachment and bdelloplast formation in *P. tolaasii* cells *in funga*

To confirm whether the reduction in *P. tolaasii* 2192^T numbers and brown blotch lesion intensity was due to *B. bacteriovorus* HD100 predation *in funga* or another competition for resources, the interaction between *P. tolaasii* and *Bdellovibrio* was monitored in samples from the surface of the post-harvest *A. bisporus* mushroom (shown untreated in Figure 15a), 48 hours after mushroom treatments, using Scanning Electron Microscopy (SEM). *P. tolaasii* 2192^T cells added alone to the mushroom pileus accumulated together, in the pits present between chitin fibres, arranged parallel to the pileus surface (Figure 15b). Fibrillar structures attached to the *P. tolaasii* 2192^T cells were frequently observed, which have also been documented in previous microscopic studies of this crop pathogen [161]. These resemble pili, with extracellular polymeric substances laid down on them, and may allow *P. tolaasii* to adhere tightly to the mushroom surface and to each other in a biofilm, to rapidly initiate disease (Figure 15b [162]). *B. bacteriovorus* HD100 added alone to the mushroom surface survived after 48 hours and also accumulated in the small pits between chitin fibres (Figure 15c).

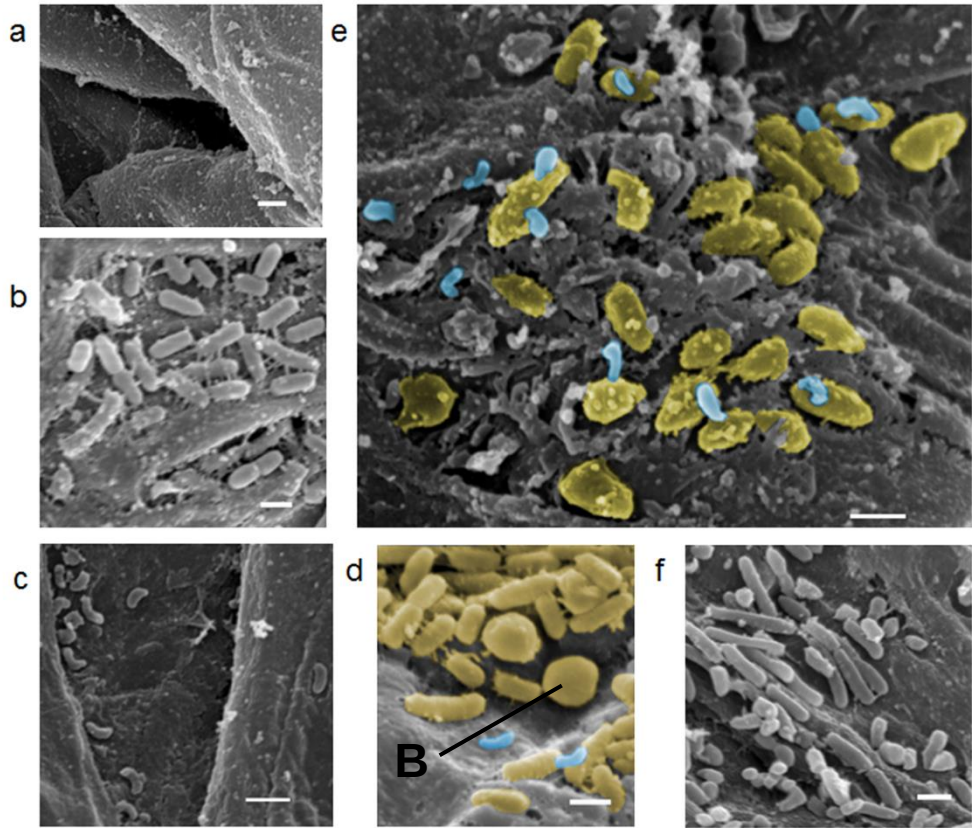


Figure 15 . Predatory interactions between *Bdellovibrio* and *P. tolaasii* "*in funga*" on the mushroom pileus surface. Scanning Electron Microscope images (with assistance from Mr. Tim Smith, University of Nottingham) showing the mushroom pileus surface 48 hours after the following treatments: **(a)** untreated mushroom pileus surface **(b)** inoculation of *P. tolaasii* 2192^T alone **(c)** Inoculation of *B. bacteriovorus* HD100 alone **(d)** and **(e)** Co-inoculation of *P. tolaasii* 2192^T and *B. bacteriovorus* HD100 and **(f)** Application of King's medium B alone. In image **(e),** *B. bacteriovorus* HD100 (blue) are shown attached at one pole to *P. tolaasii* 2192^T (yellow), a crucial first step in the predatory process. Images **(d)** and **(e)** both show rounded *P. tolaasii* 2192^T cells (labelled **B**) as well as usual rod-shaped cells. This rounding is characteristic of the bdelloplast structures formed after *Bdellovibrio* invades the host cell and begins replication. 1 µm scale bar shown.

Where *B. bacteriovorus* HD100 was added to the mushroom surface both before (Figure 15e) and after *P. tolaasii* 2192^T (Figure 15d), *B. bacteriovorus* HD100 attachment to *P. tolaasii* 2192^T cells was observed: a crucial first step in the predatory process. In addition, bdelloplasts, the rounded, dead *P. tolaasii* structures shown in Figure 15d in which *Bdellovibrio* establish, grow and replicate after attachment and invasion, were also observed where *B. bacteriovorus* HD100 was added before or after *P. tolaasii* 2192^T. Although a valid statistical survey was not possible in these SEM samples, bdelloplasts

were most clearly visible on the mushroom surface where *B. bacteriovorus* HD100 was added before *P. tolaasii* 2192^{T} **(**Figure 15d).

A King's Medium B control addition to the pileus resulted in the growth of different types of bacterial cells, with different morphologies that were distinct from that of *P. tolaasii* 2192^{T} & *B. bacteriovorus* HD100 (Figure 15f); however, typically, no bacterial cells were observed on untreated mushroom tissue (Figure 15a). This indicates that the supermarket mushrooms carry a small, indigenous bacterial microflora that replicates readily in added growth medium, which may impact upon *P. tolaasii* CFU numbers recovered from experimentally inoculated tissue, as described below.

4.4.3 **Application of *Bdellovibrio* before inoculation with *P. tolaasii* reduced the number of *P. tolaasii* in infected mushroom tissue.**

To determine whether the reduction in lesion intensity after treatment with *B. bacteriovorus* HD100 correlated with a reduction in *P. tolaasii* 2192^{T} cell numbers, CFU were recovered and enumerated from mushroom tissue that had been inoculated with *P. tolaasii* 2192^{T} and pre-treated with *B. bacteriovorus* HD100*,* compared with a *P. tolaasii* 2192^{T} inoculated, non-*B. bacteriovorus* HD100 treated control (Figure 16).

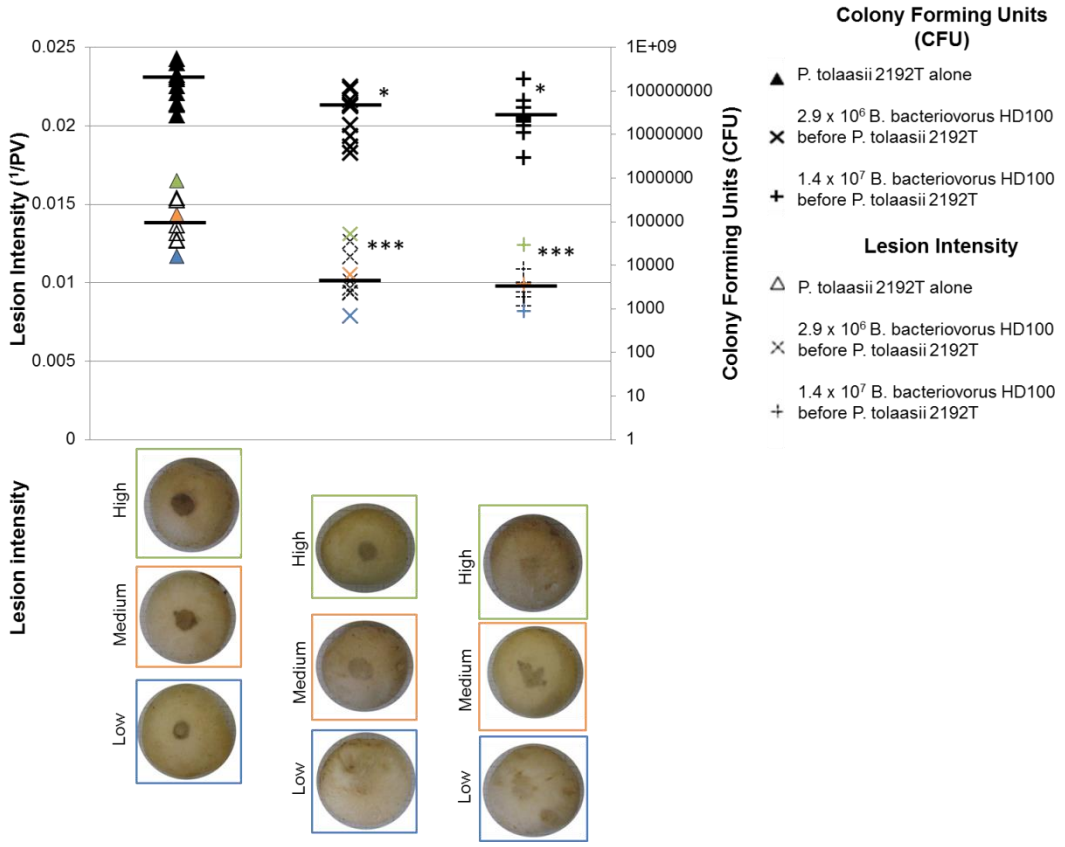


Figure 16 . Bacterial CFU numbers recovered from *P. tolaasii*-inoculated mushrooms in the presence and absence of *B. bacteriovorus* HD100. Lesion intensities and number of bacterial colony forming units (CFU) recovered from mushroom pilei subject to three different treatments detailed to the right. Each *P. tolaasii* 2192^{T} inoculation contained 1.7 x 10^{6} CFU. Images of mushrooms with typical: high, mean, and low intensity lesions in each group are shown below the graph. Horizontal black bars indicate the mean values for lesion intensity/CFU count in each treatment group. Student's t-test of significance between *B .bacteriovorus*-treated and non-treated mushrooms inoculated with *P. tolaasii* 2192^{T}: * p <0.05, *** p <0.001.

A mean number of 4.5 x 10^{7}/3.9 x 10^{7} CFU were recovered from mushrooms pre-treated with 2.9 x 10^{6}/1.4 x 10^{7} PFU live *B. bacteriovorus* HD100, respectively, which were both significantly lower than the mean 1.9 x 10^{8} CFU recovered from mushrooms inoculated with *P. tolaasii* 2192^{T} alone (Student's t-test of difference p<0.05); these observations correlated with a significant reduction in lesion intensity (p<0.001) on mushrooms treated with 2.9 x 10^{6} or 1.4 x 10^{7} PFU *B. bacteriovorus* HD100 (mean = 0.010 ^{1}/PV in

both cases) compared with mushrooms inoculated with *P. tolaasii* 2192^{T} alone (mean = 0.014 ^{1}/PV). Despite this significant reduction in lesion intensity, the total number of CFU recovered from *B. bacteriovorus* HD100 treated mushrooms onto King's Medium B was high, suggesting that the bacteria recovered and counted from seemingly similar, beige-coloured colonies on the King's Medium B plates were not solely pathogenic *P. tolaasii* 2192^{T}, but might include other species indigenous to the mushroom pileus surface that are not well preyed upon by *B. bacteriovorus* HD100, as observed in SEM images of mushroom tissue to which King's medium B broth was added alone (as shown in Figure 15f).

4.4.4 *Enterobacter* species are present on the surface of some commercially produced supermarket mushrooms.

Due to the discovery in *Section 4.4.3*, to confirm the identity of the bacteria seen in **Figure 15 d and e** and recovered from supermarket mushroom tissue pre-treated with *B. bacteriovorus* HD100 before *P. tolaasii* 2192^{T} at both 2.9 x 10^{6} and 1.4 x 10^{7} PFU ml^{-1}, 20 colonies taken from the King's medium B agar plates used to enumerate bacterial CFU, recovered from the treated mushroom tissue of two mushrooms in each group, were grown on Coliform Chromogenic agar (oxoid). This agar contains two chromogenic substrates that turn purple when cleaved by the enzymes glucorinidase and galactosidase, which are both present in coliforms such as *E. coli*, and absent from Pseudomonads (including *P. tolaasii*); all 20 colonies recovered from the *B. bacteriovorus*-treated mushroom tissue and grown on Coliform Chromogenic Agar were pigmented purple indicating them as coliform, closely related to *E. coli*, and therefore as indigenous species to the mushroom pileus, and distinctly different to *P. tolaasii* 2192^{T}, which produced straw coloured colonies when directly inoculated on the chromogenic agar. Three of these coliform isolates were identified by 16s rDNA sequencing as members of the *Enterobacter* genus using the BLAST online tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi), which showed that all three isolates were most closely related to *Enterobacter* sp. LB9 (GenBank: JQ864377.1) matching 99% identity, and are likely the same species. *Enterobacter* are

commonly found in soil, and were thus likely present in the casing soil, or in soil particles attached to the mushroom hyphae used as spawn. Figure 17 shows the phylogenetic relationship of Supermarket Mushroom Isolate 1 with other soil-dwelling *Enterobacteraceae* Genera.

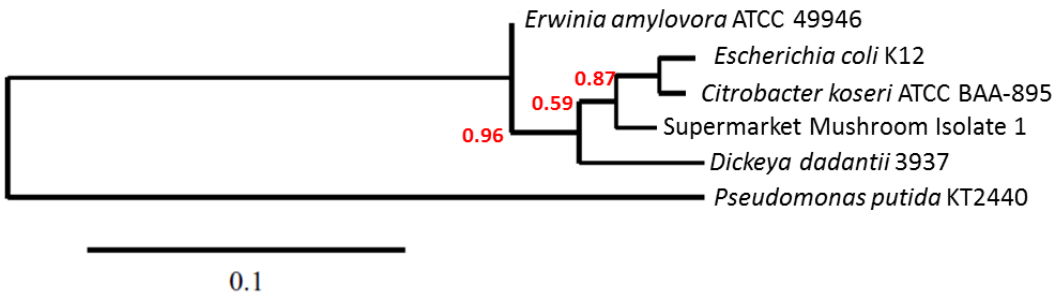


Figure 17 . Maximum-Likelihood (ML) phylogenetic tree showing the relationship between Supermarket Mushroom Isolate 1, Identified by BLAST searching to be an strain of *Enterobacteria*, with other soil-dwelling members of the *Enterobacteraceae* family, based on their 16s rDNA sequences. *Pseudomonas putida,* a member of the *Pseudomonadaceae* family, was used as an outgroup. Bootstrap values are shown in red next to the branches that they support. The programme I used is found at http://phylogeny.lirmm.fr/phylo_cgi/index.cgi).

The recovery of *Enterobacter* species explains the relatively high number of total bacterial colonies recovered on King's medium B agar from mushroom tissue treated with *Bdellovibrio*, despite the reduction in the dark lesions characteristic of *P. tolaasii* infection: *Bdellovibrio* predation rapidly reduces *P. tolaasii* population numbers on the mushroom surface, but does not necessarily reduce those of other non-disease causing, likely mushroom-indigenous species, such as the *Enterobacter* isolated in this study. The King's Medium B in which *P. tolaasii* 2192^{T} and *B. bacteriovorus* HD100 were added to the surface of the mushroom during test inoculations, and the cell-lysate debris left behind after *P. tolaasii* death due to predation, may then allow these indigenous *Enterobacter* to occupy the niche caused by *Bdellovibrio* predation of *P. tolaasii*.

To determine whether *Bdellovibrio* could prey upon these *Enterobacter* species, hereafter referred to as Supermarket Mushroom Isolates (SMIs), I

carried out Optical Density ($OD600_{nm}$) assays, as used in **Chapter 3**, to test *Bdellovibrio* predation in a range of plant pathogenic/commensal species. The results are shown in the graphs in Figure 18 (n = 3 for each isolate). The pattern of growth in both the absence and presence of *Bdellovibrio* at the two different concentrations was very similar for all three isolates, suggesting they may be isolates of the same species, as the BLAST data above suggests.

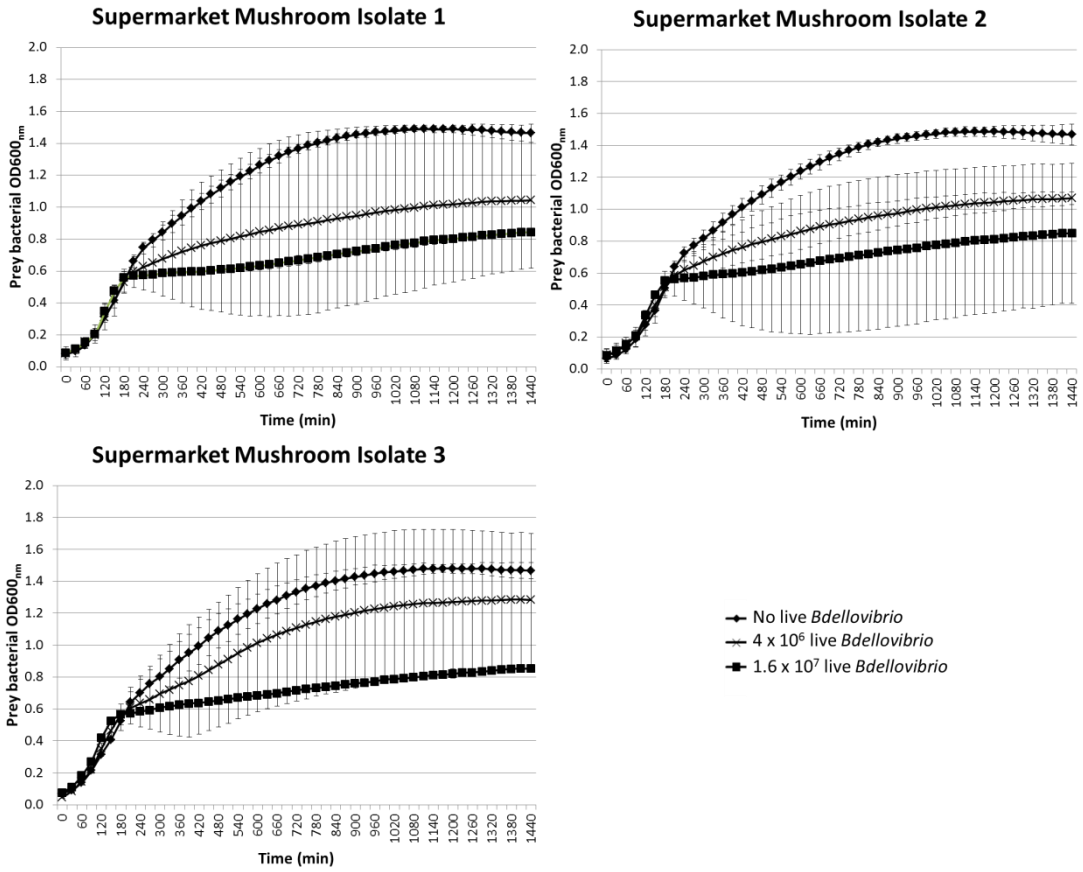


Figure 18 . Growth of *Enterobacter* isolates from supermarket mushroom tissue, *in vitro* over 24 hours, in the presence and absence of *Bdellovibrio bacteriovorus*. These experiments were conducted in buffered King's medium B, as for the $OD600_{nm}$ assays in section 3.4.1. Mean $OD600_{nm}$ of *Enterobacter* samples in the absence or presence of live *B. bacteriovorus* HD100 added at 4 x 10^6 or 1.6 x 10^7 Plaque Forming Units (PFU) (n = 4). The increase in $OD600_{nm}$ in the absence of *Bdellovibrio* indicates *Enterobacter* growth, which is variable and only slightly reduced in the presence of live *Bdellovibrio* at both concentrations. Error bars indicate 95% Confidence Intervals for each $OD600_{nm}$ value.

In the absence of live *Bdellovibrio,* all three SMIs grew from an initial $OD600_{nm}$ of 0.1 (corresponding to 5.6 x 10^7 SMI cells) to an $OD600_{nm}$ of 1.4

(7.84 x 10^8 SMI cells). In all three strains the mean final $OD600_{nm}$ of samples containing 1.6 x 10^7 *Bdellovibrio* cells ($OD600_{nm}$ = 0.8, corresponding to 4.48 x 10^8 SMI cells) and 4 x 10^6 *Bdellovibrio* cells ($OD600_{nm}$ = 1-1.2, corresponding to 5.6 x 10^8 – 6.72 x 10^8 SMI cells) was lower than in the absence of any live *Bdellovibrio*, despite the high variability in SMI growth under these conditions, indicated by the high 95% CI values. However, despite this reduction, the presence of *Bdellovibrio* does not prevent the SMI isolate population from increasing, which suggests that *Bdellovibrio* are not able to prey upon the SMIs as efficiently as on *P. tolaasii* (Figure 6). This provides further support to the theory that the SMI *Enterobacter* isolates grew in the niche vacated by *Bdellovibrio* predatory killing of *P. tolaasii,* as the SMIs were not as easily preyed upon. In light of these results, I went on to characterise the predatory activity of *B. bacteriovorus* against **other** mushroom-associated bacteria, from organic, **garden** mushroom cap tissue in the following section.

4.4.5 *B. bacteriovorus* HD100 also preys upon and kills some other Gram-negative bacteria isolated from garden mushroom cap tissue

To test the range of *Bdellovibrio* predation of mushroom-associated pathogens and commensals, I isolated 5 bacterial strains from the cap tissue of a garden-grown, organic mushroom with grey lesions on the surface, symptomatic of a bacterial infection (hereafter the Garden Mushroom Isolates are referred to as GMIs). Of these 5 bacterial strains, 2 were not used in further tests described here because one looked identical to another GMI isolated on a different agar (LB and King's medium B agar), and the other had green fluorescent colony colour on King's medium B typical of a *Pseudomonas* strain such as those that I previously tested in Chapter 3: as my aim here was to test the range of predation against mushroom-associated bacteria, I wanted to avoid selecting any similar strains if possible.

As with the SMIs in Section 4.4.4, I isolated the 16s rDNA of these three GMIs, conducted BLAST searches, and created phylogenies to determine their likely identity. The phylogenies are shown in Figure 19 and Figure 20**.**

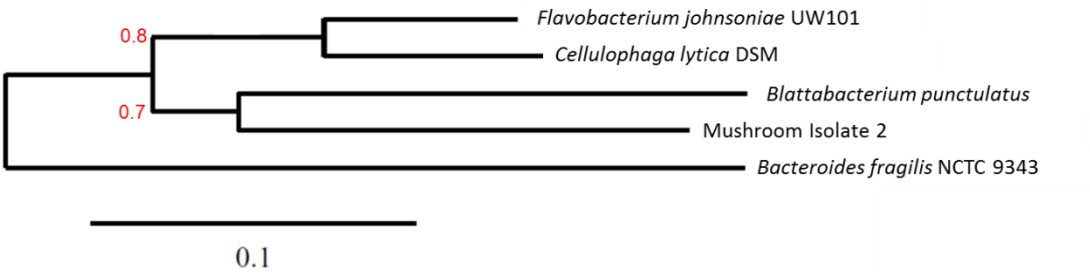


Figure 19 . Maximum-Likelihood (ML) phylogenetic tree based on 16s rDNA sequences, showing the relationship between Garden Mushroom Isolate 2, Identified by BLAST searching to be an strain of *Chryseobacterium*, with other soil-dwelling members of the *Flavobacteraceae* order. *Bacteroides fragilis,* a member of the *Bacteroidales* family, was used as an outgroup. Bootstrap values are shown in red next to the branches that they support. The programme I used is found at http://phylogeny.lirmm.fr/phylo_cgi/index.cgi).

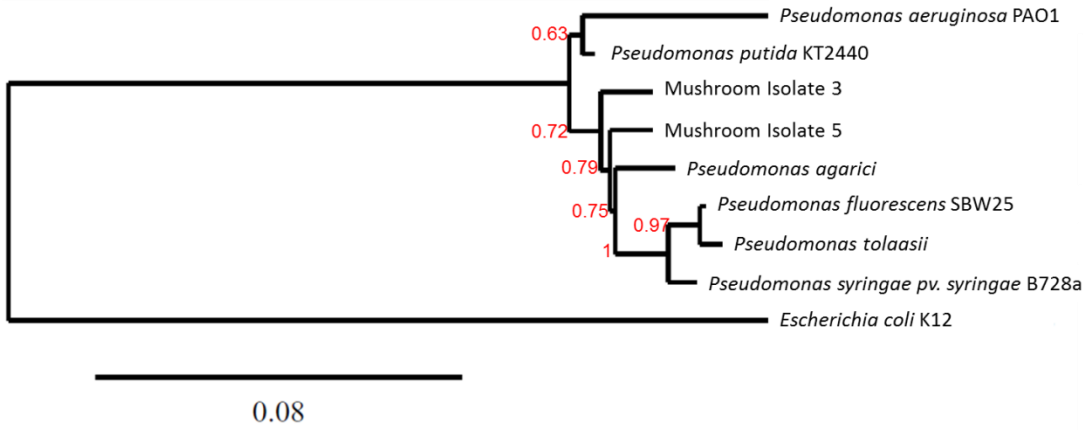


Figure 20 . Maximum-Likelihood (ML) phylogenetic tree based on 16s rDNA sequences, showing the relationship between Garden Mushroom Isolates 3 and 5, Identified by BLAST searching to be *Pseudomonas* species, with other soil-dwelling members of the *Pseudomonadaceae* family. *Escherichia coli,* a member of the *Enterobacteriaceae* order, was used as an outgroup. Bootstrap values are shown in red next to the branches that they support. The programme I used is found at http://phylogeny.lirmm.fr/phylo_cgi/index.cgi).

GMI 2 was identified as most closely related to *Chryseobacterium jejuense* strain JDG189 (GenBank: JX035956.1). *C. jejuense*, which was first isolated from soil in Korea, grows as yellow-pigmented colonies on agar. This yellow colony characteristic was also observed in GMI 2, thus supporting these BLAST results [163].

Even though they did not grow as green fluorescent colonies on King's medium B, GMI3 and 5 were identified in BLAST searches to be *Pseudomonas* species, though the most closely related sequences were from isolates of *Pseudomonas* which did not give a species name (Isolate 3: Uncultured *Pseudomonas* sp. clone BJP8S22-c01, GenBank: KF851144.1; Isolate 5: sp. A-13, GenBank: AY556391.1). As I previously explained in Chapter 1 and Chapter 3, *Pseudomonas* isolates are diverse and can be PGPRs, such as *P. fluorescens,* or pathogens that live on a wide variety of plant species. Thus, the phylogenetic tree that I constructed in Figure 20 gives more information about the potential identity of these isolates. The phylogeny shows that the two GMIs are distinctly separate species (this clade has a bootstrap support value of 0.72; a value higher than 0.7 is generally accepted as strong support [164]). The GMIs cluster between *Pseudomonas putida*, a PGPR, and *Pseudomonas agarici,* a mushroom pathogen that causes drippy gill disease (gill tissue rot), suggesting that GMI 3 and 5 may be most closely related to these species. However, as explained in Chapter 3, some closely related *Pseudomonas* species can have extremely different PGPR/pathogen characteristics, and so PGPR or pathogen status of GMI 3 and GMI 5 is not possible to infer from this phylogenetic analysis alone.

To characterise the three GMIs that I isolated further, I conducted a test of Koch's postulates for each of the GMIs, inoculating them on commercially grown supermarket mushroom caps, to determine whether any of the isolates formed pathogenic lesions and could therefore be identified as the causal agent of the grey lesion they were isolated from. I did not carry this test out for the SMIs, as they were likely commensals, which were present naturally on the surface of the 'healthy' pre-inoculation supermarket mushrooms, without causing disease symptoms. Each GMI was inoculated on to the surface of the mushroom, according to the same method as for the '*P. tolaasii* alone' inoculation administered to mushrooms in Section 2.5.5 (n = 8 mushrooms for each GMI) at 2.01×10^7, 4.19×10^6, and 2.35×10^7 cells per 15 µl inoculation of GMI 2, 3 and 5 respectively. LB medium and King's medium B control inoculations were also made for comparison (n = 4

mushrooms for each buffer). The intensities of the inoculated tissue in each treatment group, as measured after static incubation for 48 hours at 29ºC, are displayed graphically in Figure 21, along with close-up, typical examples of the lesions.

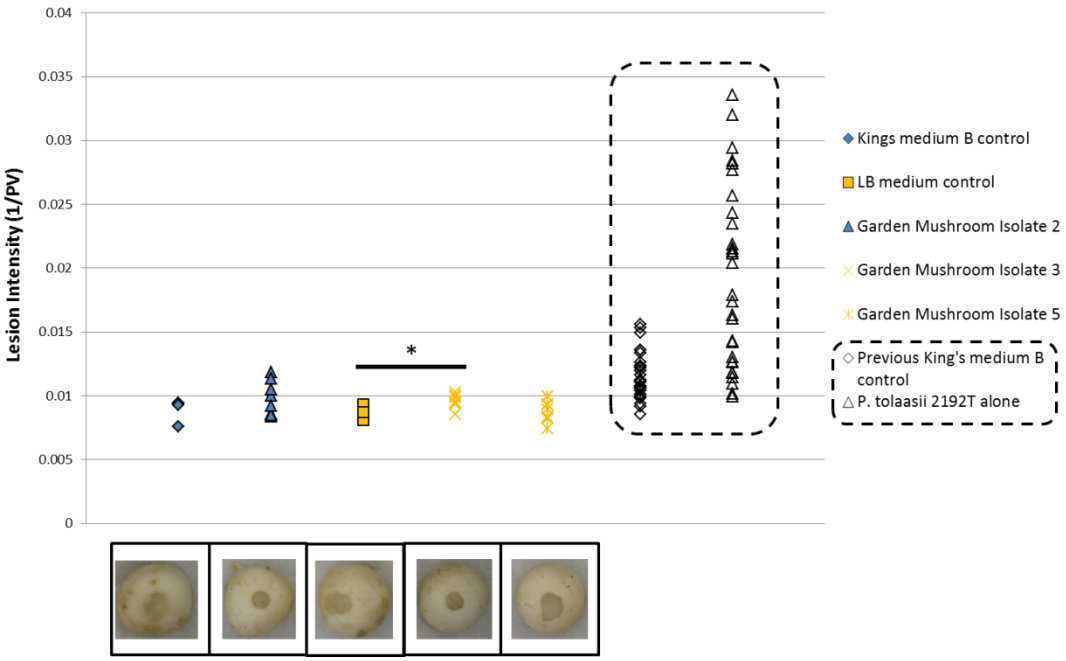


Figure 21 . Lesion intensity on mushroom cap tissue inoculated with bacterial isolates from garden mushroom tissue (Garden Mushroom Isolates 2, 3 and 5), compared with an King's medium B and an LB medium control. The King's B medium control corresponds to Garden Mushroom Isolate 2 (GMI2), both shown on the graph in blue, and the L B medium control corresponds to GMI3 and 5, shown in yellow, as these are the media in which they were grown. Also included on the graph in black are the lesions formed on mushrooms treated with King's medium B control and *Pseudomonas tolaasii* 2192^{T} alone in my previous lesion intensity assay, also shown in **Figure 14,** for comparison. Student's t-test of significant differences: * $p < 0.05$, between groups connected by the horizontal black bar.

These intensity of the spots formed on the mushroom surface inoculated with GMI2, 3 and 5 (mean intensity ^{1}/PV = 0.0098, 0.0096 and 0.0089, respectively) were similar to the intensities of the King's medium B and LB control spots (^{1}/PV = 0.0089 and 0.0087, respectively), and these lesions did not look very different, as shown in Figure 21. These values are all in the range of the King's medium B buffer control addition (^{1}/PV = 0.012), and not the *P. tolaasii* inoculation that caused a dark, pathogenic lesion (^{1}/PV = 0.019) in my original mushroom inoculation study, shown on the graph in

Figure 21 for comparison; thus the GMIs did not form the same dark lesions on mushrooms. However, the average lesion intensity for mushrooms inoculated with GMI3 was significantly higher than that of the corresponding LB medium control (^{1}/PV = 0.0096 vs. 0.0088, student's t-test of significance p-value = 0.037). Although non-significant, the difference between the mean lesion intensity in the GMI2-inoculated mushrooms was also higher than in the corresponding King's medium B control (0.0098 vs. 0.0089). The lack of significance here may be due to a small replicate number for both of the groups (n = 4 mushrooms in the control, n = 8 mushrooms in the GMI 2-inoculated group), and the greater variation in intensity of spots formed on the King's medium B control compared with the LB medium control; due to time constraints at the end of my Ph.D., I could not carry out any further repeats of this inoculation study. The average intensity of spots formed on the mushroom inoculated with GMI 5 was not much higher than the LB medium control (0.0089 vs. 0.0087), and the difference was non-significant. These data suggest that GMI 3 may be a lesion-forming pathogenic agent, and therefore possibly the cause of the grey lesion that they were isolated from, but that GMIs 2 and 5 may be non-pathogenic, potentially commensal species co-isolated from the infected mushroom tissue.

I then conducted *in vitro* $OD600_{nm}$ assays, as for the *Pseudomonas* isolates in Chapter 3 and for the SMIs in Section 4.4.4**,** to determine population growth for each of the GMIs in the absence and presence of predatory *B. bacteriovorus* HD100. The resulting data are displayed in the graphs in Figure 22**.**

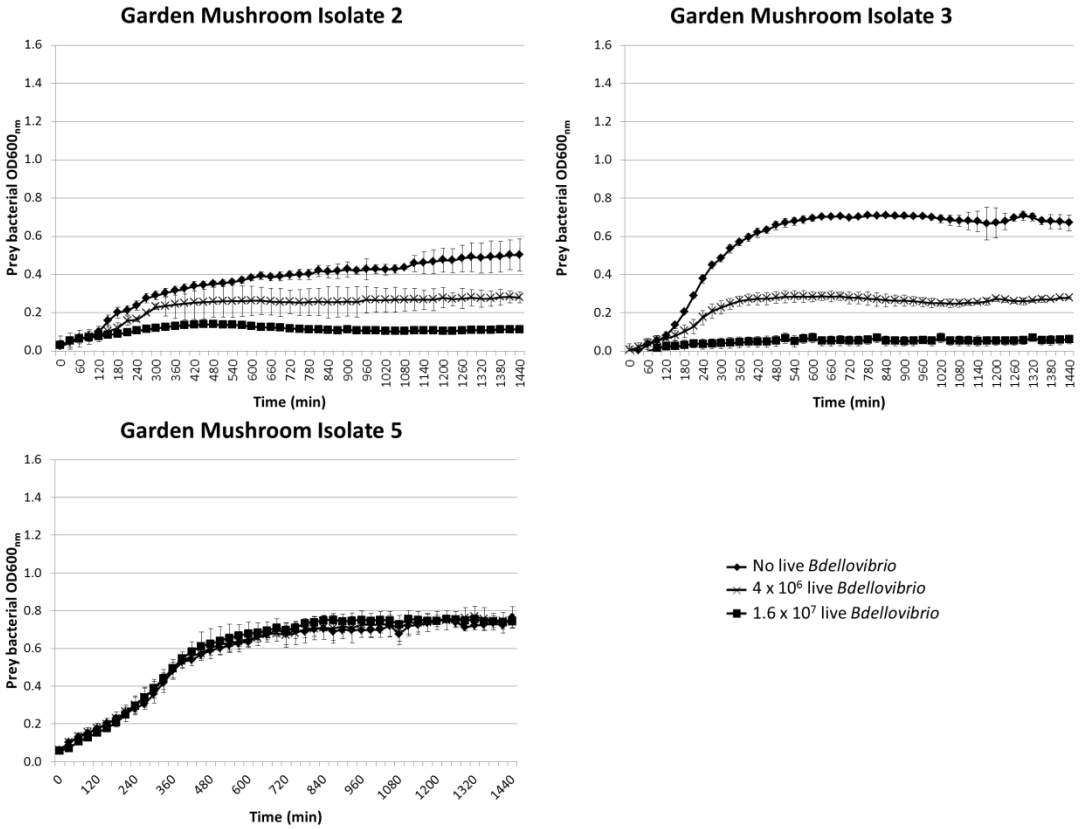


Figure 22 . Growth of Garden Mushroom Isolates (GMIs) *in vitro* over 24 hours, in the presence and absence of *Bdellovibrio bacteriovorus*, Mean $OD600_{nm}$ of GMI samples in the absence or presence of live *B. bacteriovorus* HD100 added at 4 x 10^6 or 1.6 x 10^7 Plaque Forming Units (PFU) (n = 4). The increase in $OD600_{nm}$ in the absence of *Bdellovibrio* indicates GMI growth; smaller or no increase in the presence of *Bdellovibrio* at both concentrations, in the case of GMI 2 and 3, indicates predatory killing by *Bdellovibrio.* Error bars indicate 95% Confidence Intervals for each $OD600_{nm}$ value.

The population growth of all GMI strains increased over 24 hours in the absence of live *B. bacteriovorus*, indicated by an increase in $OD600_{nm}$ (and therefore GMI cell number) in all strains (from $OD600_{nm}$ = 0.02 to 0.5 in GMI 2, corresponding to an increase from 1.07 x 10^8 to 2.68 x 10^9 cells; from $OD600_{nm}$ = 0.01 to 0.68 in GMI 3, an increase from 2.23 x 10^7 to 1.52 x 10^9 cells; and from $OD600_{nm}$ = 0.05 to 0.75 in GMI 5, an increase from 1.26 x 10^8 to 1.89 x 10^9 cells). However, this population growth was reduced in the cases of GMI 2 and 3 in the presence of 4 x 10^6 live *B. bacteriovorus* HD100 cells, reaching a final $OD600_{nm}$ of 0.3 in both strains (corresponding to a prey cell number of 1.61 x 10^9 and 6.69 x 10^8, for GMI 2 and 3 respectively); whereas growth was almost completely inhibited in both strains in the

presence of 1.6 x 10^7 live *B. bacteriovorus* HD100 cells, with only slight, initial increases from the starting GMI population ($OD600_{nm}$ = 0.02 to 0.1, corresponding to an increase from 1.07 x 10^8 to 5.35 x 10^8 cells, for GMI 2; $OD600_{nm}$ = 0.01 to 0.07, an increase from 2.23 x 10^7 to 1.56 x 10^8 cells, for GMI 3). This indicates that the growth of GMI 2 and 3 were suppressed in the presence of *Bdellovibrio*, likely due to predation. However, the $OD600_{nm}$ (and therefore population growth) of GMI 5 increased to the same extent in the presence of both concentrations of *B. bacteriovorus* as it did in the absence of any live *Bdellovibrio*; this indicates that *Bdellovibrio* is unable to prey upon GMI 5.

4.5 Discussion

4.5.1 *Bdellovibrio* is a potential 'food security' agent that decreases post-harvest spoilage in mushrooms

Building on the results I obtained in Chapter 3, which showed that *B. bacteriovorus* HD100 preyed upon and killed *P. tolaasii* 2192^T *in vitro, I* showed in this chapter that this predatory killing also occurs *in vivo* (*in funga*), suppressing population growth of the pathogen on post-harvest mushrooms (Figure 15 & Figure 16). *Bdellovibrio* effectively reduces the dark lesions of brown blotch disease caused by *P. tolaasii*, and this reduction is slightly greater and more significant where *Bdellovibrio* is added before *P. tolaasii* (Figure 15 & Figure 16). *Bdellovibrio* application may therefore be more effective as a preventative measure to protect mushrooms against brown blotch disease, rather than a treatment for an already infected mushroom crop, and could be explored as a background addition to mushroom compost or casing layers to maintain "health".

P. tolaasii is a difficult pathogen to control in mushroom grow-houses due to its ability to persist in nutrient-poor soils and the ease with which it spreads through mushroom compost, through flagellar swimming, and via the hands of pickers during the manual harvesting process [9]. Furthermore, commensal species in the mushroom casing soil play a key role in mushroom growth initiation, and therefore any treatment to prevent or treat *P. tolaasii*

infection must not result in a completely sterile growth environment, which may result from broad antibiotic or antiseptic treatment. Thus it is beneficial to explore post-harvest treatments, such as this study with *B. bacteriovorus.*

4.5.2 *Bdellovibrio* survives on the post-harvest mushroom surface, reducing *P. tolaasii* numbers and brown blotch disease symptoms

The SEM images confirmed that *B. bacteriovorus* HD100 survived on the post-harvest supermarket mushroom surfaces after 48 hours, and was therefore unaffected by any pre-treatment of those mushrooms for commercial purposes to promote growth and extend shelf-life in the film-covered plastic trays they were sold in (Figure 15c). *B. bacteriovorus* is therefore a viable treatment for bacterial diseases of mushrooms, such as brown blotch disease. Previous studies of mushroom infections have found that a 'threshold' number of *P. tolaasii* cells are required for the initiation of infection, which includes production of tolaasin, the chemical mediator of the brown blotch symptom development [9]. We found that when *B. bacteriovorus* HD100 was applied to the surface of post-harvest, commercially grown mushrooms before or after inoculation with *P. tolaasii*, both the intensity of the brown blotch symptoms of disease and the number of *P. tolaasii* 2192^T present the mushroom surface were significantly reduced (Figure 14 and Figure 16), supporting the threshold hypothesis.

The SEM images also showed that *B. bacteriovorus* HD100 attached to, invaded and killed *P. tolaasii* 2192^T cells by forming bdelloplasts on the pileus surface, when added both before or after *P. tolaasii* 2192^T inoculation (Figure 15 d and e); thus, reduction in *P. tolaasii* 2192^T numbers and disease symptoms was due to predatory activity by *B. bacteriovorus* HD100. These rounded bdelloplast structures were more clearly visible where *B. bacteriovorus* was added to the mushroom surface before *P. tolaasii* 2192^T (Figure 15d). This correlates with the greater reduction in lesion intensity measurements on mushrooms where *B. bacteriovorus* was added before rather than after *P. tolaasii* (Figure 14). This suggests that *Bdellovibrio* attachment to prey and subsequent bdelloplast formation may be easier, and

occur more rapidly, where *P. tolaasii* cells have not had time to accumulate, adapt and adhere to the mushroom surface, preventing *P. tolaasii* from producing as much tolaasin, and thus reducing the extent of the characteristic brown blotch symptoms.

As the consumer preference is for white, clean-looking mushrooms with minimal surface damage, the reduction in brown blotch tissue damage by *B. bacteriovorus* application could increase the yield and possibly the shelf life of high-quality, marketable mushrooms. This study investigated the survival of *B. bacteriovorus* HD100 and its predatory activity against *P. tolaasii* on the surface of post-harvest mushrooms up to 48 hours, sufficient time for brown blotch disease to develop on untreated mushrooms. Thus studies over longer time points, covering time from transportation to the sell-by date, would need to be investigated, in future work, if *Bdellovibrio* was to be applied as a treatment to extend shelf-life.

4.5.3 *Bdellovibrio* could be used as a casing soil treatment to prevent *P. tolaasii* spread between mushrooms

In addition to reducing the population of *P. tolaasii* on the mushroom surface, *Bdellovibrio* are natural soil dwellers and so their application to casing soil could also prevent spread of brown blotch between mushrooms in the growth environment and between grow houses. In this way, the fast swimming motility of *Bdellovibrio* [165] would allow efficient location of *P. tolaasii* prey, using chemotaxis, in the wet casing soil prior to mushroom growth initiation, and translocation by gliding along the mushroom pileus surface after mushroom fruiting bodies have formed, preventing *P. tolaasii* infection establishment at multiple stages of mushroom growth; previously, the possibility of infection throughout the mushroom growth period has been an obstacle in brown blotch disease control. Further pre-harvest studies could investigate the longevity and protective effect of *Bdellovibrio* inoculated into the casing soil around mushroom mycelium, before and after fruiting body initiation, on growing *A. bisporus*. As *Bdellovibrio* preys efficiently upon some, but not all, species of *Pseudomonas* (as shown in Section 3.4.1), and some Pseudomonads in the casing soil such as *P. putida* are important in

fruiting body initiation, further studies would additionally investigate the predatory activity of *B. bacteriovorus* HD100 against these known commensal strains *in vitro* and in the casing soil to ensure that there are no effects that would have an adverse impact on mushroom fruiting body production.

4.5.4 *Bdellovibrio* is a potentially self-limiting brown blotch disease treatment

As host-dependent *Bdellovibrio* require prey cells to survive, the post-harvest treatment could also be self-limiting, as *Bdellovibrio* would die once *P. tolaasii* prey had been eradicated; further studies could quantify this. Furthermore, the *in vitro* and *in vivo* predation studies in Chapter 3 and this chapter, respectively, demonstrate that *B. bacteriovorus* may be able to survive the action of the toxins produced by *P. tolaasii* and other members of the *Pseudomonas* genus, including tolaasin and other lipases and peptidases, which cause serious damage to the mushroom pileus [166]. This suggests that *Bdellovibrio* species may be effective against other crop pathogenic bacterial species, even if they produce biologically active secreted compounds. This could be followed up with studies of the pure compounds themselves versus *B. bacteriovorus.*

4.5.5 *Enterobacter* isolated from the mushroom surface were likely commensal and were not susceptible to predation

I infrequently isolated *Enterobacter* species in our experiments from supermarket mushrooms, likely being commensals growing in number after pre-treatment with *B. bacteriovorus* HD100, suggesting that these *Enterobacter* isolates are not susceptible to *Bdellovibrio* predation. Further *in vitro,* OD assays of *B. bacteriovorus* HD100 predation of these isolates confirmed that they were not well preyed-upon by *B. bacteriovorus* HD100, as their population number increased in the presence of 4×10^6 and 1.6×10^7 *B. bacteriovorus* cells, though these increases were smaller than in the absence of any live *B. bacteriovorus* (Figure 18).

A plant growth-promoting *Enterobacter* species, *Enterobacter cloacae,* has been described previously, which colonises rice root surfaces and competes with other species in the soil microbiota for nutrients [167]. Enterobacter species have also previously been isolated from spent mushroom compost [168], where they might associate with the mushroom surface in a similar way, competing with other mushroom-indigenous bacteria as commensal species. As *Bdellovibrio* has previously been shown to prey upon diverse *Enterobacter* species [169], it was unexpected that numbers seemed unaffected by *Bdellovibrio* predation; however, *E. cloacae subsp. cloacae* was recently shown to compete with and reduce the numbers of a *R. solanacearum* population in a 1:1 *in vitro* mixture of *E. cloacae:R. solanacearum* cells, determined by enumeration [170]. This was possibly due to bacterioicin production via a putative T6SS, whose encoding genes were identified in an *in silico* analysis of the *E. cloacae* genome [170]; this could be why *B. bacteriovorus* HD100 was less able to prey upon and kill the *Enterobacter* isolates in this study. As they were isolated from *Bdellovibrio*-treated mushroom tissue, unaffected by any brown blotch disease symptoms, these isolates are unlikely to be pathogenic, and may be commensals. It could therefore be beneficial that *Bdellovibrio* are less able to prey upon the *Enterobacter* species isolated in this study, preserving any beneficial commensal effect they might have, while still protecting against *P. tolaasii* infection.

4.5.6 ***Bdellovibrio*** **could potentially be used to treat other Gram-negative, mushroom disease-causing bacteria**

I also isolated 3 different, Gram-negative bacterial species from grey lesion tissue on the cap of an organic, garden-grown mushroom; I identified these by 16s rDNA sequencing as a *Chryseobacterium* sp. (GMI2) and two *Pseudomonas* spp. (GMI3 and GMI5). When inoculated on to the surface of commercially-produced supermarket mushrooms, as in the *P. tolaasii* inoculation assay, none of the isolates produced lesions comparable to those produced by *P. tolaasii:* the range of intensity values of spots produced by the GMIs on the mushroom cap surface were similar to the values of those

on King's medium B control-inoculated mushrooms, rather than the values of the dark, pathogenic lesions formed on *P. tolaasii*-inoculated mushrooms in my original study. However, GMI3 produced spots that were slightly but significantly darker than the King's medium B buffer control spots in this inoculation assay, while GMI2 and GMI5 produced spots that were not significantly different to the buffer control (Figure 21). This suggests that GMI3 may have been the causal agent of the grey lesion these three strains were isolated from, while GMI2 and GMI5 may have been beneficial, commensal strains.

The low number of replicates in each group treated with the different GMIs (n = 8) may have resulted in the non-significant results, particularly for GMI2, where the mean intensity of the spots was also slightly higher than the buffer control; further repeats would be needed to confirm this, but time limitations in my Ph.D. prevented me from carrying this out. Some species of *Chryseobacterium* (the closest relative of GMI2) have been isolated from rotten lily plant tissue, as well as from necrotic leaf tissue of chayote (a south-American vegetable resembling a pear) [171]; interestingly some are proposed to protect some plants against fungal diseases by producing fungus-degrading proteases [172, 173], so some *Chryseobacterium* spp. may have general antifungal properties. However, some *Chryseobacterium* spp. have also been isolated from healthy mushroom tissue, so it is uncertain whether GMI2 was a pathogen or commensal.

GMI3 and GMI5 are closely related to one another, however GMI3 produced slightly but significantly darker spots on the mushroom surface, and was thus potentially the causal agent of the grey lesion on the garden mushroom that it was isolated from, but GI5 did not, and was thus likely a commensal. Pathogenic and commensal *Pseudomonas* spp. can be closely related, such as *P. tolaasii* and *P. fluorescens* (as shown in Chapter 3, in Figure 10). Conversely, two pathogenic *Pseudomonas* species that live on the same host crop, such as *P. tolaasii* and *P. agarici,* can be more distantly related; despite their close phylogenetic relatedness, this potential difference in pathogenicity is still consistent with previous studies. Further studies in which the metabolites produced by these species are characterised (with a

particular focus on chitinases or toxins with antifungal activity), for example, could be carried out to confirm whether these isolates are pathogenic or commensal [174].

Using the *in vitro* predation tests with *B. bacteriovorus* HD100, I showed that the putative mushroom pathogens GMI2 and GMI3 were preyed upon and killed by *B. bacteriovorus* HD100: the population growth of these strains in the absence of *B. bacteriovorus* was completely suppressed in the presence of 1.6×10^7 live *B. bacteriovorus* cells, and partially suppressed with 4×10^6 *B. bacteriovorus* cells. However, the growth of GI5 was not suppressed at all by *B. bacteriovorus* (Figure 22)*. B. bacteriovorus* thus preys on the potentially pathogenic GMI3, but not the likely commensal GMI5, which was also the case with *B. bacteriovorus* predation of the pathogenic *P. tolaasii* but not the commensal *Enterobacter* in commercially produced *A. bisporus* mushrooms*.* Thus, *B. bacteriovorus* is suitable for use as a post-harvest spoilage control agent in mushrooms, as the likely commensal species I tested, which may have mushroom growth-promoting effects, were not well preyed-upon and killed.

4.6 Chapter conclusions

Bdellovibrio bacteriovorus HD100 shows natural control of *Pseudomonas tolaasii,* a spoilage pathogen of mushroom crops, on the non-sterile, biotic surface of the mushroom pileus. *B. bacteriovorus* also shows control of other mushroom pathogens, but not commensal species *in vitro*; these predatory bacteria therefore have a natural ability to act as “food security guards” against Gram-negative crop pathogens.

5 The Effect of *Bdellovibrio* on Wheat Growth and the Rhizosphere Soil Bacterial Community in a Mesocosm

5.1 Chapter Introduction

In this chapter, I tested *Bdellovibrio* for its effects on factors that affect wheat yield, originally in relation to the common wheat-yield reducing fungal pathogen *Gaeumannomyces graminis* var *tritici* (take-all). I measured the effect of adding wild-type *Bdellovibrio* into the soil around wheat plant roots, grown in an outdoor pot mesocosm, on the growth of wheat plants and their susceptibility to take-all, which is known to be reduced by anti-fungal, Gram-negative plant growth promoting bacterial species, which are potential prey for *Bdellovibrio*. Unexpectedly, I found that *Bdellovibrio* increased wheat yield (Section 5.4.2.6); because of this, I then investigated the impact of *Bdellovibrio* on the wheat soil bacterial/archaeal microbiome, known to affect the properties of soil conducive to wheat plant growth.

5.1.1 **Wheat and Global Food Security**

As mentioned in the main thesis introduction, the global food supply must be doubled by 2050 to meet the needs of the growing global human population, while simultaneously making up the deficit in chronic malnutrition. A major focus of these efforts is staple crop production: wheat is one of the most important, providing 19% of calories and 20% of protein consumed by the total human population, and with 217 million Hectares (Ha) planted globally in 2010, is the most widely grown crop worldwide [175]. A recent meta-analysis of the trends in wheat yield increases indicates that wheat production is projected to increase 38% by 2050 [176], which falls considerably short of the target increase in food production, estimated at 70%-110% [1, 177].

As expansion of land area used for crop production is not a viable option for increasing crop yields, other methods focussing on improving wheat yield need to be investigated to ensure food supply targets are met. These approaches can be divided broadly into three categories: 1) improving the soil input, i.e. soil moisture retention, particle and pore size, structure,

nutrient content and availability; 2) increasing wheat cultivar efficiency, i.e. the efficiency of nutrient uptake by the wheat roots and conversion of the nutrients into grain, through wheat plant breeding and genetic engineering; and 3) reducing pathogenic spoilage and crop yield reduction or loss by manipulating the pre-existing crop soil rhizosphere microbial community, removing or blocking wheat interaction with crop pathogenic organisms, and adding PGPRs or promoting their beneficial association with wheat roots.

Soil input and wheat cultivar efficiency have historically been more thoroughly investigated as yield improvement approaches than manipulating the soil microbiota, which has only recently become a more popular avenue of research in crop yield improvement, as discussed in Section 5.1.4.3 [5]: my study adds to the research in this category. Thus it was important to ensure that the soil, taken from the Rothamsted site Delafield which is a soil type regularly used for cultivating high-yield cereal crops, and the wheat cultivars initially chosen for this study, Hereward and Cadenza, which were bred for commercial use, were representative of those used in typical modern farm conditions. Additionally, the soil type was chosen carefully to avoid soil properties such as low nutrient availability that would limit wheat growth and potentially obscure any effects of the *Bdellovibrio* addition.

5.1.2 **What is known about the Hereward and Cadenza variety wheat that I used in my study?**

The two wheat cultivars used in this study were chosen to be similar enough to grow at the same time under the same environmental conditions (they are both varieties of winter wheat, which are typically planted in October and harvested in August) but sufficiently different so that the generalizability to different cultivars of any effects observed with the different treatments in my study could be assessed. A review of what is known about the characteristics of Hereward and Cadenza, and wheat growth in Delafield-type soil, follows.

5.1.2.1 ***Wheat genetic variation***

Modern *Triticum aestivum L.* wheat cultivated varieties (cultivars), including Hereward and Cadenza used in this study, have allohexaploid genomes

(genome: BBAADD), comprising six sets of chromosomes. A recent study by Marcussen and co-workers comparing the hexaploid wheat genome with five diploid relatives shows that the A, B and D genomes are most closely related to *Triticum urarto* (Genome AA), *Aegilops speltoides* (Genome BB), and the grass seed *Aegilops tauschii* (Genome DD). The comparison showed that the AA and BB genomes diverged from a common ancestor ~6.5 million years ago, and that the DD genome arose from a homoploid hybrid speciation event (hybridisation without any change in chromosome copy number) between the AA and BB genome 1 million years later. ~0.8 million years ago, a polyploid hybridisation event (speciation increasing the chromosome number) resulted in the production of the tetraploid AABB domesticated emmer wheat (*T. turgidum*) genome; finally, another polyploid hybridisation between the AABB and DD genomes occurred ~0.4 million years ago, which resulted in the hexaploid genome of the modern wheat variety, *T. aestivum* [178]. The polyploid hybridisation events were facilitated through agricultural co-cultivation of the contributing species, and resulted in adaptation to survival in different habitats, increases in the range of climactic and geographical situations in which wheat could thrive, and as a consequence wheat is one of the most common crops grown worldwide [179].

Dubcovsky and Dvorak (2007) report that wheat, like other farmed crops, has been subject to 'domestication syndrome', in which traits that make the wheat easier or more efficient to cultivate are selected for. To identify the genetic loci associated with these domesticated phenotypic traits, Peleg and coworkers (2011) [180] conducted a Quantitative Trait Loci (QTL) analysis on a population of wild emmer (*T. turgidum*) cross-bred with domesticated durum wheat (*T. turgidum* ssp. *durum*). Amongst other trait loci, they found that the loss of the ear 'shattering' phenotype in *T. aestivum*, which prevents grains from being dispersed from the ear by the wind to allow easier harvesting and higher yield, is governed by a single gene, *Br* (Brittle rachis), found on the long arm of chromosome 2A [180]; they hypothesise that the product of this gene regulates other genes that determine the ear shattering phenotype, as this trait was previously mapped to different loci [181].

Domestication syndrome is also reported to result in accelerated development: in another similar QTL study using a cross between another species of emmer (*T. dicoccoides*) and durum wheat (*T. durum*), the genetic loci involved in the timing of wheat head development (ear and grain production, also called 'flowering') were mapped onto chromosomes 2A, 4B, 5A and 6B. In this study, the wild emmer wheat parent in the genetic cross developed late, and the durum wheat cultivar parent early, attributed to genetic differences at these loci: the wild-type QTL at 5A is hypothesised to increase developmental time, while the wild-type QTLs at 2A, 4B and 6B reduce it [182]. Early development is advantageous in wheat cultivation, as this allows for the early harvest and sale of the grain and a longer period for soil restoration between successive crops.

The selection effects of wheat domestication and the speciation event combining individual *T. turgidum* and *Ae. Tauschii genomes* on only one or two occasions together result in a reduction of *T. aestivum* wheat genetic diversity, called a 'genetic bottleneck' [183]; however, the *T. aestivum* genome retains a moderate level of diversity, at around 30% of that of domesticated emmer *T. turgidum* and similar values for that of its diploid relatives [183, 184]. This is likely due to the occurrence of gene flow (by hybridisation) between these two species, which has previously been documented [179]. As a result, a wide range of wheat cultivars with different genetic profiles and phenotypic traits are being bred and grown today.

Recently, the first wheat genome was sequenced [185], which had previously been precluded by the difficulty of differentiating between the multiple chromosomal copies. Thus, whole-genome, in-depth analysis of cultivar genetic differences has yet to be carried out; however, some other methods of measuring general genetic diversity between cultivars have been used. In one such study, single-nucleotide polymorphisms (sites in the genome where one single base pair in DNA sequence between two cultivars is different) were sequenced by Next-Generation Sequencing (NGS), mapped to the wheat chromosomes, and compared between eight common wheat varieties as a measure of genetic distance [186, 187]. Between Hereward and Cadenza wheat, used in this study, a total of 9,083 SNPs were found;

compared to the lowest reported inter-varietal SNP number (355 between Cadenza and Rialto) and the highest (28,776 between Savannah and Alchemy), Hereward and Cadenza have a low to medium level of genetic dissimilarity [187]. This is consistent with the moderate differences in physiology, growth and yield, and resistance to disease between the two varieties, described below, which made Hereward and Cadenza ideal choices for this study.

5.1.2.2 *Genetic and phenotypic differences and similarities between Hereward and Cadenza*

Hereward and Cadenza are both Winter bread wheat varieties, which are sown in October for a July-August harvest the following year; they were both developed by the Plant Breeding Institute in Cambridge in 1991, but are descended from different parent lines (Hereward = Disponent x Norman, Cadenza = Axona x Tonic)[188]. Their genetic distance from each other is reflected in their physical characteristics; in the Scottish Wheat Variety Database, the classification of their physical characteristics differ as shown in Figure 23; for example Hereward wheat has thin straw walls while Cadenza has thick, and the grains inside the ear are more tightly packed together in Hereward than in Cadenza [188].

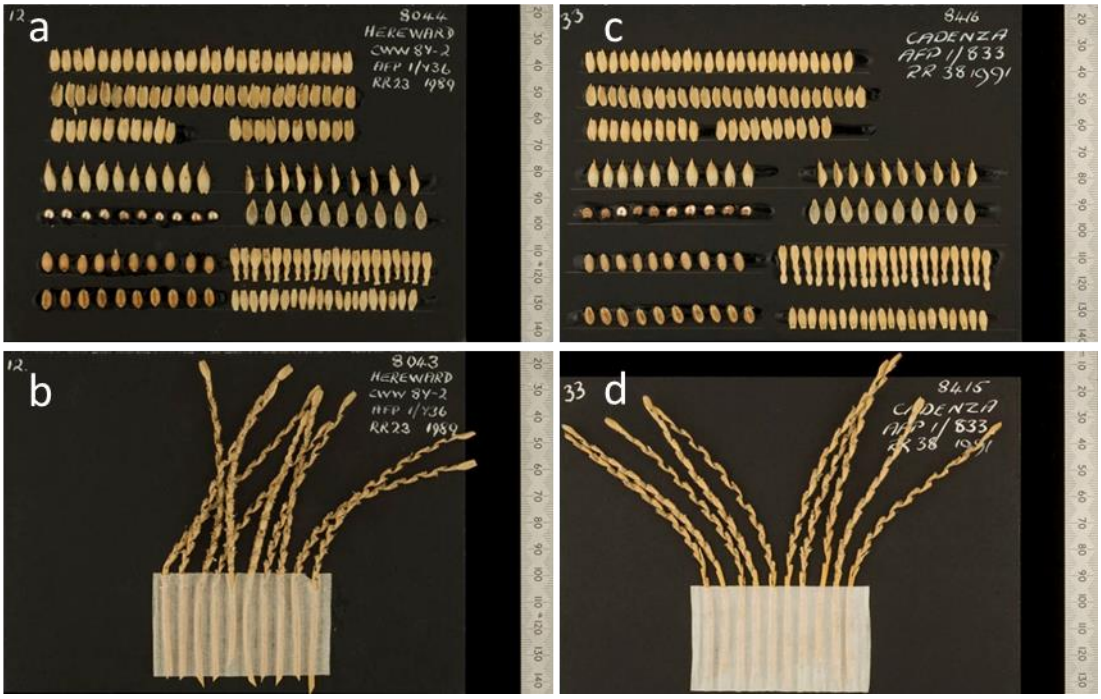


Figure 23 The grain morphology and threshed ear lengths of Hereward (a and b) and Cadenza (c and d) variety wheat [188]. The grain is larger and the threshed ear lengths shorter in Hereward, giving a more tightly packed ear morphology.

The two varieties differ in some single genetic loci where alleles or variations confer phenotypic differences that are highly observable, and which have been mapped on the genome and used in conventional plant breeding. For example, The Hereward Wheat genome contains the chromosomal translocation 1B/1R [189], where the short arm of the wheat *Triticum aestivum* choromosome 1B is replaced by the short arm of chromosome 1R from Rye (*Secaie cereaie*). This translocation is known to confer increases in grain yield, and affords wheat plants with this translocation some resistance against some fungal pathogens such as *Erysiphe graminis*, which causes powdery mildew [190-192] (though crucially for this study, it does not confer resistance to take-all disease); Cadenza, on the other hand, does not have this translocation[189], which may result in some yield and disease resistance differences between the two varieties.

The Hereward genome also contains a mutant allele of the dwarfing gene *rht-D1* (also called *rht2*, reduction in height) [189], which codes for a protein involved in the gibberellin plant hormone signalling pathway, which stimulates stem and leaf elongation. The mutation therefore results in a reduced

response to gibberellin, resulting in a dwarfed (short plant stem) phenotype [193]. This has been exploited by plant breeders, as dwarf wheat plants in general have a greater Grain Harvest Index (GHI, the weight of harvested grain as a proportion of total plant biomass). This means that a higher percentage of the nutrients taken up by the plant are used to make grain, and thus the plants are more 'efficient' as grain producers [64, 194]. Cadenza, on the other hand, has a non-mutant allele [189] and thus has a taller phenotype, so a greater proportion of nutrient resources contribute to the longer stem, and other non-harvested portions of the plant.

5.1.2.3 *Comparing nutrient uptake and efficiency between Hereward and Cadenza*

Wheat plants require several key nutrients from the soil for growth and development; nutrient supply and availability is usually the key environmental limiting factor on wheat growth, given that sunlight and water are usually plentiful. Of these nutrients, nitrogen, phosphorus, potassium and Magnesium are the most important; the close positive correlation between available nitrogen levels in the soil and wheat plant biomass and grain yield is well established [64] and has been studied in the most depth in relation to winter wheat growth and yield, nutrient cycling dynamics, soil chemistry and microbiology, for example in the Broadbalk long-term classical experiment at Rothamsted Research [195-198].

Hereward and Cadenza have previously been compared amongst a range of cultivars in terms of their nitrogen uptake and utilisation characteristics. Barraclough and coworkers (2010) conducted a 4-year field trial in which 39 commercial cultivars of winter wheat, including Hereward and Cadenza, each received 5 different amounts of ammonium nitrogen fertiliser in the soil, 0-350 kg/Ha. The study found that on average at 200 kg/Ha nitrogen, which is a comparable amount to those commonly used in commercial crop production, Hereward and Cadenza were both in the upper quartile of cultivars for total amount of nitrogen uptake, the upper-median quartile of cultivars for % grain nitrogen content, and the lower-median quartile of cultivars for total grain yield and nitrogen utilisation efficiency (the grain yield divided by the total nitrogen in all above-ground plant tissue). No other cultivars have this

specific nitrogen utilisation profile, which suggests that unlike the genotypic and phenotypic differences between Hereward and Cadenza described in Section 5.1.2.2 and the differences in their susceptibility to disease, described in Section 5.1.2.4, their nutrient uptake characteristics are relatively similar.

5.1.2.4 *Hereward and Cadenza susceptibility to the take-all fungal disease pathogen, Gaeumannomyces graminis var. tritici*

Before my project started, our collaborators at Rothamsted Research conducted a study to examine differences between *T. aestivum* wheat cultivars, including Hereward and Cadenza, in their susceptibility to infection with the yield-reducing take-all fungus, *G. graminis* var. *tritici*. This field trial, covering 4 wheat growing seasons in 5 years, measured the level of the take-all fungus in the soil after each wheat crop for a range of cultivars. This was calculated by measuring the % root infection of new wheat plants grown in samples of the soil taken after each year's wheat crop (this method is called the soil core bioassay and was subsequently used in my study, described in Section 2.6.15). The results showed that some cultivars (including Hereward, 47.8% mean root infection with take-all over 4 seasons) are consistently more susceptible than others (including Cadenza, 3.4% mean root infection with take-all); these differently infected cultivars are thus classed as high and low Take-All Building (TAB) [65]. This was a main consideration in the choice of Hereward and Cadenza cultivars for this study; we hypothesised that if *Bdellovibrio* treatment of the soil had an effect on take-all infection of wheat, then the extent of the effect might differ between the high and low TAB varieties, and may provide insight into the mechanism of take-all disease suppression or antagonism by the bacterial community in the soil.

5.1.3 **What is known about take-all disease of wheat?**

Take-all is the most agriculturally important disease of wheat worldwide: infection results in severe growth stunting, premature ripening, grain shrivelling and thus yield reduction in the host plants, resulting in substantial economic losses for wheat farmers [199]. *G. graminis* var. *tritici* is an

ascomycete fungus; its hyphae penetrate into the stele of wheat roots in the soil, where the vascular tissue is located, including the nutrient-transporting phloem, thus giving the fungus access to plant nutrients [200]. Runner hyphae also extend along the surface of wheat roots, allowing the fungus to penetrate wheat roots at several points in the same plant, and to infect nearby plants, at points where their root systems intertwine [200]. *G. graminis* var *tritici* survives saphrophytically in soil on the remnants of the previous wheat crop [199], and it can grow in a broad range of temperature (4-30ºC) and pH (3-10) conditions; although it survives best in very moist conditions, it can also infect wheat under dry conditions [200]. The fact that this pathogen thrives in contrasting climactic conditions explains the global scale of wheat yield reduction due to take-all infection.

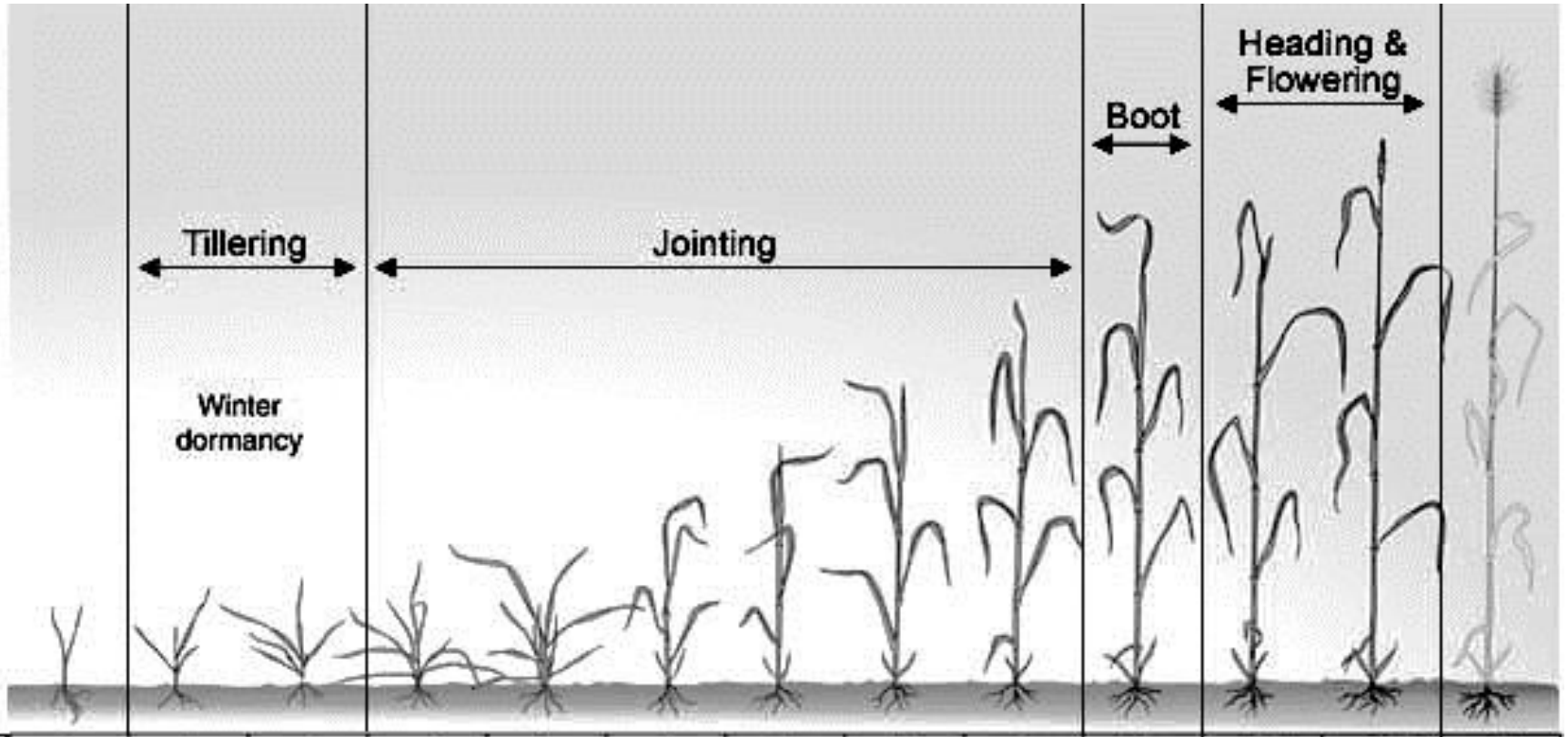
Take-all infection of wheat increases in successive wheat crops (called Take-All Build-up, TAB) before reaching a peak after 2-3 years, and then declining again in soil (called Take-All Decline, TAD) [201]. The phenomenon of TAB is consistent in different climactic conditions and soils [202], and because of it, the practice of crop rotation (growing non-cereal crops that are not susceptible to take-all, such as legumes, in years between wheat crops) is commonly used by farmers to prevent the build-up of take-all inoculum in the soil [203]. The suppressiveness of the soil towards *G. graminis* var *tritici* is associated with an increased population of *Pseudomonas fluorescens* PGPR strain bacteria in the rhizosphere [201], and previous studies provide evidence that *P. fluorescens* is the suppressive agent: *P. fluorescens* isolates from a suppressive soils inhibit *G. graminis* var *tritici* growth *in vitro*, reduce take-all disease symptoms and increase yield in wheat when applied to the wheat grain before planting [204, 205]. The molecular basis of *G. graminis* var. *tritici* suppression by *P. fluorescens* strains through their production of antibiotics and iron-scavenging siderophore molecules, and through altering the expression of *G. graminis* var *tritici* pathogenicity genes, has been extensively studied, and is discussed in more detail in Section 5.1.7.6**.**

Although the basis for the differences in take-all susceptibility between cultivars such as Hereward and Cadenza has not yet been established, McMillan and coworkers suggest that it could result from cultivar-specific

differences in rhizosphere bacteria, e.g. increased *P. fluorescens* in the rhizosphere of low TAB varieties [65]. *P. fluorescens* responds to plant root exudates [206], and when De La Fuente and coworkers inoculated an identical mixture of several *P. fluorescens* strains into the rhizospheres of both wheat and pea plants, the most effective coloniser of the root in the mixture was different between the two host crops [207]. This suggests that their root exudate profiles differ, and result in the recruitment of different *P. fluorescens* strains to the root surface; this may extend to different cultivars of the same crop, such as high and low TAB varieties of wheat. As I showed in Chapter 3, *Bdellovibrio* preys upon and kills the PGPR *P. fluorescens* SBW25, but this effect may vary between *P. fluorescens* strains, for example due to differential production of antibiotic compounds. Therefore, I hypothesised that if *Bdellovibrio* preys upon *P. fluorescens* in wheat soil and thereby reduces suppression of take-all infection, this effect may also differ between wheat cultivars, due to potential differences in the colonisation of their rhizospheres with *P. fluorescens* strains.

5.1.4 **Wheat developmental stages**

In my study, I added the *B. bacteriovorus* and *G. graminis* var. *tritici* treatments just before the main period of wheat nutrient uptake and growth, so that any effects that the treatments had on these processes could be most clearly observed. Diagrams of wheat plants at the main growth stages of winter wheat (including Hereward and Cadenza used in my study) are shown in Figure 24; there are two scales that are generally used to describe the different wheat plant growth stages, called the Feekes [208] and Zadoks [209] scales, which are indicated underneath the diagrams in this figure.



Feekes	1	2	3	4	5	6	7	8	9	10	10.1	10.5	11
Zadoks	10	21	26	30	30	31	32	37	39	45	50	60	90

Figure 24 . The Feekes and Zadok's scale for measuring wheat growth stages. [208]

The growth of the wheat plants can be divided into four main stages, indicated in Figure 24; in my pot experiments, Dr Vanessa McMillan and Prof Kim Hammond-Kosack planted the pre-germinated winter wheat seedlings in late October at Rothamsted. Once the seedlings had established in the soil, the first leaves are established on the young plant (from Feekes scale 1/Zadoks scale 10). This is followed by the first major developmental stage 'tillering', which occurs at Feekes scale 2-3/Zadoks scale 21-26: the production of (usually 1-3) extra stems, additional to the main wheat plant stem, which occurs over a period of winter dormancy (usually from October to February, dependent on the weather). After the winter dormancy period comes a period of rapid elongation of the main stem, also called 'jointing' due to the appearance of joints in the stem from which further leaves grow (Feekes scale 4-9/Zadoks scale 30-39). The final leaf to emerge is called the flag leaf, which extends vertically and in which the ear of the main stem initially develops, producing individual 'florets' (which will develop into single grains); this early development of the ear within the sheath is called 'booting', and occurs at Feekes scale 10/Zadoks scale 45. Finally, the ear emerges from the flag leaf sheath (called 'heading') and undergoes 'flowering', in which flowers emerge from the florets (Feeke's scale 10.1-10.5/Zadoks scale 50-60). This is followed by grain development, in which nutrients are transferred from the stem and other tissues in the wheat plant to fill the grain of the wheat plant, which then ripens. Once the grain is fully ripe at Feeke's scale 11/Zadoks scale 90, the wheat is considered fully mature and is harvested; this typically occurs from July-September, and is dependent on weather conditions (in hotter, dryer summer weather the wheat will reach maturity more quickly).

Dr Vanessa McMillan and I monitored the growth of the wheat in pots very carefully so that the inoculations could be made just before the main stem extension, and so that final measurements and soil samples could be taken in the middle of wheat development and also when the wheat had only just ripened fully; extra tillers can be produced by the wheat after it reaches maturity if extra rainfall occurs (Dr Vanessa McMillan, personal

communication) and so it was important to harvest the wheat as quickly as possible.

5.1.5 What is known about Delafield soil macronutrient content and cycling?

As I briefly explained in Section 5.1.7.3, nutrient uptake by wheat in the soil, which is influenced by PGPR bacterial species, is an important factor in wheat plant growth. Nitrogen (N), Phosphorus (P) and Potassium (K) are the three elements primarily required in larger amounts for adequate crop growth and a high grain yield (and are thus described as macronutrients), while other elements, including Copper, Iron, Magnesium, Manganese, and Zinc, are required in trace amounts (described as micronutrients). Figure 25 shows the main components of the N, P and K cycles in the soil, and also shows how these nutrients were added into the Delafield soil in my study (by supervisors Dr Vanessa McMillan and Prof Kim Hammond-Kosack, RRes, who advised this strategy) before the soil was collected for the pots, and also during wheat growth. This was done to avoid any confounding effects of nutrient deficiency in the wheat plants on these pot tests on testing the effect of *Bdellovibrio* addition on the wheat bacterial/archaeal microbiome and wheat plant growth.

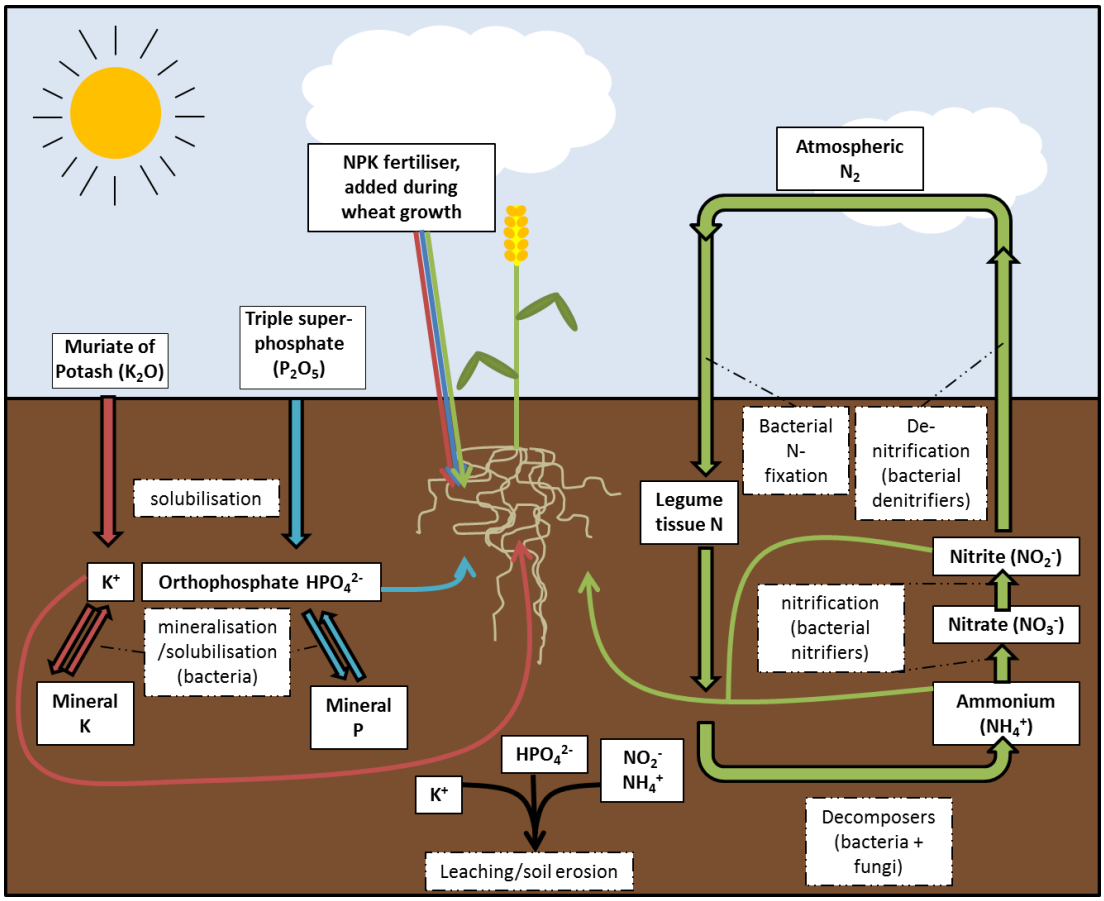


Figure 25 . The cycling of Potassium (K, red), Phosphorus (P, blue) and Nitrogen (N, green) in the Delafield soil used in my study, showing the main cause of gains and losses from the soil.

5.1.5.1 *Nitrogen*

Nitrogen is a key structural component of chlorophyll required for photosynthesis and nucleic acids, as well as amino acids, the constituents of enzymic and structural proteins. Total protein is a key commercially assayed constituent of wheat grain, and thus N is key for a high grain yield of good quality (13% protein for bread wheat varieties such as Hereward and Cadenza, used in this study) [210]. this is economically important, as the price of grain that does not meet the quality requirements is discounted or rejected for sale [210]. In this study, winter bean plants were grown in the Delafield soil, and after their harvest the bean residue (root and stem tissue) was ploughed back in to the soil in the season prior to using it in the pots for my wheat tests (Figure 25). Leguminous plants form symbiotic root associations with N-fixing bacterial species that fix N_2 from the atmosphere, and thus their tissue contains a relatively high level of N; adding it back into

the soil, as was carried out in this study, is a commonly used strategy that increases N levels in soil prior to growing cereals such as wheat [70, 210, 211]. Due to their symbiotic acquisition of nitrogen, legumes are not net users of N already present in the soil, sparing further N supplies for a following wheat crop. The HGCA estimates that the amount of N added back into the soil in bean residues is ~100 kg/Ha, under medium-rainfall winter conditions [210]. The amount of additional N required for wheat growth in the soil depends on this pre-existing soil N and the soil type; the Delafield soil initially used in my tests is a silty clay loam-type soil, which is estimated by HGCA to require 190 kg/Ha N under average UK weather conditions in addition to the bean residue [210].

To ensure an adequate N supply, Osmocote® NPK fertiliser was added to the soil by Dr Vanessa McMillan at RRes (details of which are given in Section 2.6.11), which contained an additional 175 mg/kg (700 kg/Ha) N in a slow release pellet. Recently, it has been shown that the timing of N fertiliser addition is key in optimising the growth and grain yield of wheat: Orloff and coworkers [212] tested 8 different ratios of N fertiliser (in the form of urea) added over 4 time points: before the wheat was planted, and at the start of tillering, flowering and booting. These stages are shown diagrammatically with corresponding Feeke's and Zadok's growth scale numbers in Figure 24. This was conducted over three years and at two final amounts of applied N (168 or 280 kg/Ha). They found that an 0:80:0:20 pre-plant:tillering:boot:flowering ratio produced the greatest wheat yield, with the late-season N additions improving the protein content of grain [212].

In this study, however, I planned to add the *Bdellovibrio* and take-all treatments at the tillering stage, before the main plant growth took place, so that any effects of *Bdellovibrio* addition on wheat growth would be clear. It was therefore important not to disrupt the soil environment after their addition by adding fertiliser, which may have had an effect on the microbiota in the soil, and therefore might have masked any effect of the *Bdellovibrio* addition (which I hypothesised would decrease Gram-negative bacterial levels in the soil). Instead, the Osmocote® fertiliser (which also contained P and K) was added early on in the jointing stage (as explained in methods Section 2.6.11

and shown in Figure 24) during elongation of the main wheat plant tiller. As it is a slow release fertiliser, it continued to release N late into the season. This was a compromise between optimal N fertilisation for good growth, and optimal conditions to observe any effect of *Bdellovibrio* treatment on wheat growth. Dr Vanessa McMillan and I also carefully monitored the wheat plants for signs of N (and other nutrient) deficiency throughout the season.

5.1.5.2 *Nitrogen cycling*

The natural soil bacterial/archaeal species also play important roles in N-cycling in the soil and thus impact wheat nutrition, as shown in Figure 25. N-fixing species, including some from the *Azotobacter* and *Rhizobium* genera, convert N_2 gas from the atmosphere to ammonium (NH_4^+) in the soil, thus increasing the amount of plant-available N [213]. In the soil, NH_4^+ is converted to NH_3 (ammonia), a reversible reaction driven by the pH of the water levels in the soil; Nitrifying species, such as Nitrosomonas, oxidise the NH_3 to NO_2^- (nitrite), and others, such as *Nitrobacter* and *Nitrospira*, oxidise NO_2^- to NO_3^- (nitrate) [214]. Although plants absorb NO_3^-, it is also liable to leaching from the soil in water, and so nitrification can potentially result in the loss of some plant-available N from the soil. Under farmed field conditions, NH_4^+ can also be lost from soil by erosion, through its association with negatively-charged clay particles. Finally, Denitrifying genera, such as *Paracoccus denitrificans* and *Pseudomonas stutzerii*, reduce Nitrite (NO_2^-) present in the soil to gaseous forms of nitrogen (including N_2) in the atmosphere, reducing the amount of plant-available N [214].

5.1.5.3 *Phosphorus*

Phosphorus (P) is an important component of phospholipids, which are present in the wheat grain [215], and DNA, but it also plays a central role in respiration: P-containing Adenosine-Tri-Phosphate (ATP) and Nicotinamide Adenine Dinucleotide (NAD, NADH) molecules are involved in glycolysis and the citric acid cycle, respectively, converting sugars into energy that fuels vital crop plant processes [216]. The plant-available form of P in the soil is orthophosphate, $H_2PO_4^{2-}$, but this can be converted into other forms relatively easily, resulting in lower available P in soil; in slightly alkaline soils such as Delafield, $H_2PO_4^{2-}$ reacts primarily with calcium ions (Ca^{2+}), reducing

the solubility and availability of P [217]. I considered the levels of P in my pot test soil in both the methods (Section 2.6.3 and 2.6.11) where it was added as fertiliser to the pot soil, and monitored the subsequent level of plant-available P during the experiment, as shown in the results (Section 5.4.4)

As shown in Figure 25, P is immobilised from plant-available HPO_4^{2-} by bacterial species naturally present in the soil, for use in bacterial phospholipids, DNA and respiration (in competition with plants for the same use), rendering it unavailable for wheat plant uptake. Conversely, many bacterial species including several *Pseudomonas* and *Bacillus* isolates can solubilise P contained within organic matter (e.g. dead bean plant tissue) in the soil, thus increasing the plant-available pool of P [218]. These processes may be affected by *Bdellovibrio* predation of Gram-negative P-metabolising species, and, if the P levels in the soil are found to be sub-optimal, may in turn affect wheat growth.

5.1.5.4 ***Potassium***

Amongst other functions, potassium ions (K+) bind to and alters the conformation of key enzymes involved in plant growth, such as those involved in making ATP [219]; K is a limiting factor in the rate of these enzyme reactions, and therefore plant growth. K+ also regulates stomatal opening and thus the influx of CO_2 and efflux of O_2 during photosynthetic respiration, and so is a limiting factor in plant growth: sufficient K levels are required to avoid slow growth and development of wheat. As shown in Figure 25, K is present in soil in mineral forms such as feldspar and mica; natural erosion occurs slowly, contributing minimal amounts of K to the wheat plant-available pool of K^+ ions, and as with P, some rhizobacterial species including members of the *Pseudomonas* and *Bacillus* genera can also solubilise mineral K from these natural mineral forms [220]. K levels in the soil were therefore considered in my methods in Section 2.6.11 and results in Section 5.4.4.

5.1.5.5 *Bdellovibrio treatment of wheat soil may affect macronutrient levels by preying upon Gram-negative wheat rhizosphere species.*

As N, P and K levels are all affected by the metabolic activity of bacterial species present in the soil, *Bdellovibrio* predation of Gram-negative wheat rhizosphere bacteria may in turn affect the levels of these macronutrients in the soil and impact upon wheat plant growth. This may be through the reduction of species that increase macronutrient levels in the soil, thus reducing good plant growth and yield; or through the reduction of those that compete with available NPK-increasing species, thus allowing them to flourish, and increasing plant growth and yield. Therefore, in the metagenomics analysis that I conducted of bacterial/archaeal 16s rDNA sequences isolated from the pot soil before and after *Bdellovibrio* treatment, I paid particular attention to any changes in the population of species that are known to affect macronutrient levels.

5.1.6 **What is known about Delafield soil structure?**

As my experiments involved adding a liquid inoculum of *Bdellovibrio* to soil, it is important to review issues of soil structure that may affect its void volume and therefore its capacity to retain liquids.

5.1.6.1 *Delafield is a typical agricultural silty clay loam type soil*

The soil initially used in the pots this study was collected from the top layer (to 50 cm depth) of the Rothamsted site Delafield in Hertfordshire [195]. Soils are classified in terms of their texture, described as the ratio of silt, clay and sand particles comprising them. This classification system is summarised in the textural soil triangle, shown in Appendix 3. Delafield is classified as a silty clay loam soil, primarily composed of silt (60-70%) with smaller percentages of clay and sand. The Land Information System (www.landis.org.uk) uses named classifications that include other components of the soil, e.g. the stone content; in this system, Delafield is classified as a Batcombe-Carstens type soil, which is slightly stony [221].

Unlike sandier soils, Silty clay loam soil such as Delafield retains more nutrients and water and are generally more fertile, due to an extensive pore

network between soil aggregates [68]; however, soils with a greater clay are harder to cultivate than silty clay loams as they are very sticky when wet and form a very hard cast when dry, containing fewer and smaller pores, which makes wheat root growth through the soil more difficult [68]. Other factors, particularly compaction by heavy agricultural machinery, are known to negatively affect wheat growth, with more compact soils being more difficult for roots to establish and grow through, potentially affecting growth and yield [222]. The Delafield soil used in this study was sieved to remove large stones and mixed thoroughly before putting in the pots (methods Section 2.6.3), to provide a uniform soil texture in each pot and avoid any negative effects of compaction in the field on wheat plant growth.

5.1.6.2 *Delafield soil is a habitat for diverse bacterial/archaeal species*

The stable, porous soil structure of Delafield also influences the diverse bacterial/archaeal rhizosphere species, including those that promote wheat growth, in the wheat soil. This population may also be affected by the addition of the Gram-negative bacterial predator *Bdellovibrio bacteriovorus*, which is the main focus of the metagenomic analysis I conducted in this study. Bacteria/archaea mostly reside in thin layers of moisture coating the inside of pores within the soil, which also contain dissolved nutrients, and air spaces in the pores provide a means of gaseous exchange (e.g. for aerobic species) [223]. Pore structure and the moisture-retaining capacity of the soil is therefore an important influence on soil microbial community structure, stability and load. The bacterial/archaeal community in turn affects the structure and stability of pores and aggregates, for example due to the production of exopolysaccharides by bacteria in biofilms, sticking soil particles together and promoting stable aggregate formation [224]. *Bdellovibrio* treatment of the wheat soil in this study may alter the structure of the microbial community by preying upon Gram-negative bacterial species, which could affect this relationship between bacterial/archaeal species and soil structure, and thus the growth of the wheat plant; this was part of my initial hypothesis for this project, as stated in Section 5.3.

Delafield soil is therefore an ideal, typical agricultural soil type in which wheat is commonly grown, comprising the best textural qualities of both sandy or

clay soils [68] and is predicted to harbour a diverse bacterial/archaeal community; it was thus chosen for my pot soil mesocosm experiment so that conditions were favourable for good wheat growth. This Delafield mesocosm experiment was developed for my project and was not previously used at Rothamsted, but it has since been adopted for other experiments after the first year of my pot tests.

5.1.7 **The soil bacterial and archaeal community**

In my study, I used a metagenomic analysis to assess the changes in bacterial and archaeal community structure in wheat rhizosphere soil throughout wheat plant development, both before and after different treatments with *Bdellovibrio* and *G. graminis* var. *tritici*.

5.1.7.1 ***DNA Extraction and Metagenomic analysis by Next-Generation Sequencing***

Metagenomic analysis involves sequencing short nucleotide sequences, with a high level of inter-species diversity but that are present in all species, from the total bacterial/archaeal DNA extracted from the soil; this is most commonly ribosomal DNA, such as the 16s ribosomal gene sequence chosen in my study. This technique has many advantages over more traditional culture-based techniques for assessing the microbial community in these environments: only 0.1-1% of bacterial species present in soil can be cultured in the laboratory [225], and so that approach is unsuitable for assessing the composition of environmental microbial communities. The lack of culturability in the laboratory is thought to be due to a high species specificity for pH, temperature, oxygen levels, or particular nutrients, that are not provided in most cases by the available media used for their culture; intra and inter-specific bacterial cell-cell signalling may also be disrupted, as the production of chemical signals that would normally promote (or inhibit) bacterial survival in the soil may be different in culture; and some bacteria may be overlooked in analysis due to slow growth or low natural numbers in their environment [226]. Despite the advantages, there are several factors

that affect the success of metagenomic analysis, which are summarised in Figure 26.

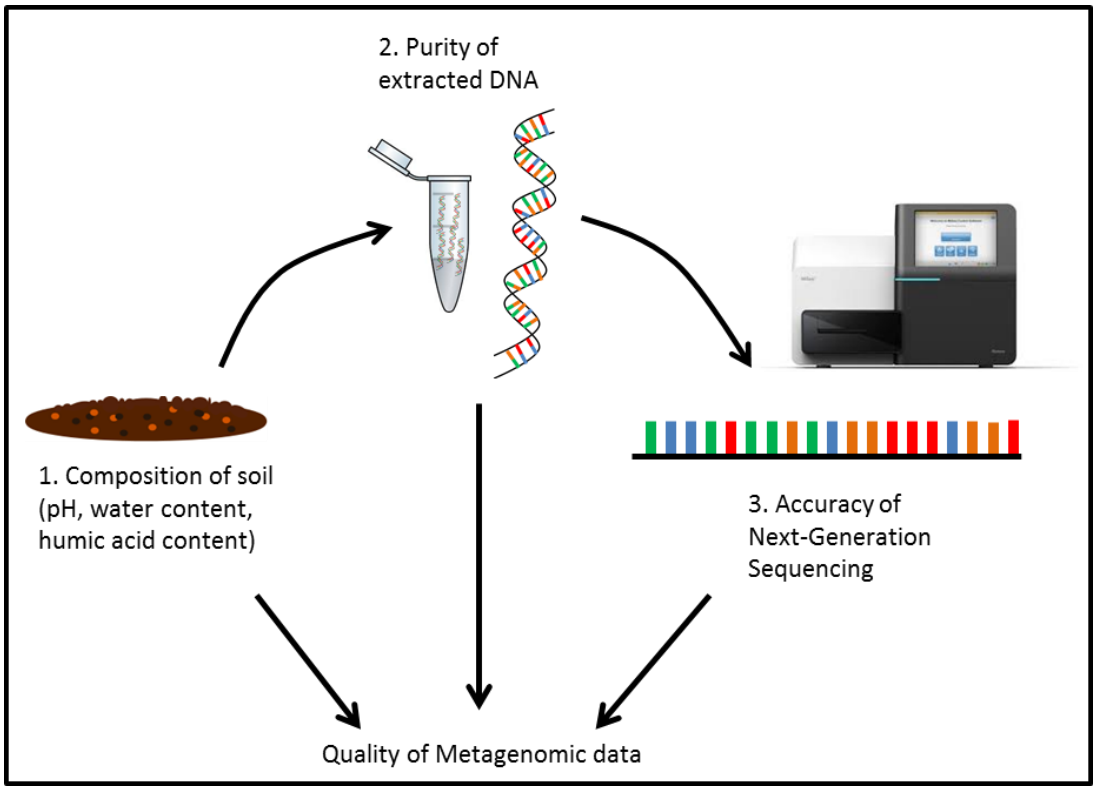


Figure 26. The factors affecting the quality of sequencing data in metagenomic analyses of the soil bacterial/archaeal community.

1. Soil structure and composition; e.g. the presence of clay or humic acid, and the pH and water level can affect the level and purity of DNA extracted from soils [227, 228]. Variation between samples was minimal in my study, as the soil in pots was all from the Delafield site in Rothamsted and was therefore similar in composition, and and humic acid levels in the Rothamsted soils are not known to be high (personal communication from Dr Ian Clark, RRes). The soil samples had all been prepared for extraction in the same way, by sieving through a 2 mm mesh sieve and storing at -20ºC; all DNA extractions were carried out within 5 days, to minimise any effect of the environment, e.g. temperature, in the laboratory. However, the addition of *Bdellovibrio* and take-all to some of the pots, and differences in moisture levels due to rainfall at the different time points that the soil samples had been collected, may have altered the water and pH levels in the soil; the pH of soil samples was measured after the DNA extractions were carried out in

case there was any unexplained variation between samples in terms of DNA amount or quality, or in the composition of the microbial community after 16s metagenomic sequencing. Meteorological data for the Rothamsted site, where the pots were kept, was also available for comparison in case of any unexplained variation.

2. Purity of extracted DNA. There are different protocols and kits available to carry out the DNA extraction, and some are more effective than others at extracting the maximum amount of DNA from the sample; the kit that I used was the MoBio Powersoil DNA kit, which was recommended by Dr Ian Clark (RRes) who routinely performs soil DNA extractions for 16s ribosomal metagenomic analysis. This kit has also been used successfully in several recent publications that explore the bacterial species present in wheat rhizosphere soil, and some studies used similar downstream 16s metagenomic analysis of the samples to those that I carried out [229-231].

The MoBio kit was recently included in studies comparing the effectiveness of different approaches (both kit-based and non-kit based) for extracting DNA from marine sediments, activated sludges (sewage treated with bacteria), and soils, in which the samples differed in composition e.g. texture, pH, and humic acid/organic matter content [227, 228, 232]. In the soil DNA extraction study, the MoBio kit was compared with an established, non-kit based extraction protocol to extract DNA from 14 different soils: the study measured the amount of DNA extracted, the success of PCR amplifying 16s rDNA genes from the extracted samples, and finally the bacterial species diversity using Terminal Restriction Fragment Polymorphism (T-RFLP), in which the 16s rDNA PCR product was digested and the number of different between-species fragment sizes were measured. Compared with an alternative non-kit based technique developed by the authors, the amount of DNA recovered and the success of PCR using the MoBio kit and non-kit based techniques was similar, though when a further DNA purification step was added to the non-kit based method, the success of the PCR increased (79% success in the MoBio kit compared with 95% in the non-kit method) and the diversity of bacteria recovered (particularly the actinomycete class) also increased. However, the protocol for the non-kit method is more time consuming than

the MoBio kit; I had a limited amount of time in which to complete the DNA extractions, and so the kit was the most appropriate option in this case.

The quality of extracted DNA, assessed by measuring the 260 nm and 280 nm absorbance ratio on a spectrophotometer, is important for producing high-quality metagenomic sequencing reads from extracted DNA samples. An acceptable quality 260/280 nm ratio is 1.8 or higher, while lower values indicate the presence of a contaminant. In a study that compared DNA extraction from activated sludge using different kits, the MoBio Powersoil kit consistently produced DNA samples with a 260/280 nm ratio greater than or equal to 1.8, which was better than 5 of the 7 kits in total that were tested; only one kit had a consistently higher ratio of ~2.5. All of my DNA samples were checked by spectrophotometer, and only 4 of 100 samples were repeated due to a 260/280 nm ratio lower than 1.8, to ensure that my samples were consistently high quality.

Recently, it has been noted that manufacturers of DNA extraction kits do not guarantee that the reagents included in their kit are sterile, which could affect the outcome of metagenomic analyses, particularly in samples containing low bacterial cell numbers: Salter and colleagues noted that contaminants were detected in DNA samples extracted using a kit from samples containing 1 x 10^3 cells of a single, pure strain of *Salmonella* bacteria, though the contaminant signal was not detected at higher numbers; this study was carried out as a contaminant was originally found to be the cause of a significant difference in bacterial communities in the throat swabs of infants compared with toddlers, due to the use of two different batches of the same kit [233, 234]. The study included a MoBio kit that was similar to (though not the same as) the Powersoil kit that I used. To minimise any potential contaminant effect in my study, I ensured that the DNA from all 100 soil samples was extracted using MoBio Powersoil kits ordered at the same time, from the same batch. As I was particularly interested in detecting an accurate *Bdellovibrio* count in the metagenomic analysis of my samples, I carried out the extractions in a laboratory at Rothamsted Research, where no work with *Bdellovibrio* had ever been carried out, to avoid any contamination with *Bdellovibrio*.

3. The accuracy of Next-Generation Sequencing (NGS) sequencing.

NGS technology allows an extremely large number of sequencing reactions to be carried out in parallel by either creating 'micro-reactors' that each accommodate one PCR from one template or, as in this study, by hybridising templates to a solid surface before sequencing. Those methods are therefore appropriate for large-scale sequencing projects such as metagenomic sequencing. The NGS platform that Argonne National Laboratory in the USA used to carry out my 16s rDNA metagenomic analysis was the Illumina MiSeq, which is one of three NGS platforms that are currently being used in large-scale sequencing studies such as this one.

In 2012, Loman and co-workers [235] tested the throughput (base pairs sequenced per sequencing run), error rate (the percentage of incorrectly called base pairs), and read length (the base pair length produced by individual sequencing reactions) of the Illumina MiSeq, the Roche 454 GS Junior, and the Ion Torrent PGM, by sequencing an isolate of *E. coli* for which the genome sequence is already known. They found that the MiSeq had both the highest throughput per run (1.6 GB per sequencing run compared with the next highest, 0.3 GB from the Ion Torrent PGM) and the lowest error rate (0.1%, compared with the next lowest, the Roche 454 Junior at 0.38%). The average length of sequencing reads produced by the MiSeq was 150 bp, compared with the 120 bp per read produced by the Ion Torrent PGM, and 500 bp by the Roche 454 GS junior. The MiSeq was therefore an ideal platform for my study; as I was sequencing a short part of the bacterial/archaeal 16s rDNA (variable region V4, 253 bp long, composed of two overlapping 151 bp sequences, one starting at each end) the shorter read length would not have a large impact on the resulting data set, but as the V4 sequence is so short, high accuracy is important to distinguish between closely related species. One run of the MiSeq platform takes 24 hours, and our sequencing could be performed in 2 runs; this short amount of time for data generation also made it appropriate for my sequencing because of the limited amount of time I had in which to carry out the analysis.

5.1.7.2 *Bacterial inoculant technology to increase wheat yield: an introduction*

The crop soil microbiome has important influences on the maintenance of agricultural soil 'health' (i.e. the intrinsic structural and nutritional qualities of soil that are conducive to crop plant growth), reflected in the recent emergence of several government programmes and initiatives to promote research in this area, such as the Soil Renaissance (http://soilrenaissance.org/) and the National Soil Health Initiative in the USA (http://www.nrcs.usda.gov/wps/portal/nrcs/main/national/soils/health/). In this study, I tested the effect of adding a liquid inoculant of laboratory-cultured *Bdellovibrio* to wheat rhizosphere soil as a liquid inoculant on:

- the rhizosphere microbiome;
- wheat infection with the take-all fungus *G. graminis* (which is known to be suppressed by bacterial PGPR species in the soil);
- and the growth and grain yield of wheat plants.

The addition of laboratory-cultured bacterial species to enhance crop growth and yield, called 'bacterial inoculant technology', has been the subject of recent reviews [236-238]. Recent studies have identified some bacterial species that improve wheat growth and/or wheat grain yield or nutritional quality (i.e. protein and nutrient content) when used experimentally as an inoculant, discussed in Sections 5.1.7.3 and 0. In those studies, relatively few inoculant species have been investigated so far; furthermore, these studies are often preliminary and do not extensively explore the effect of the bacterial inoculants on the growth and yield of different wheat cultivars at full maturity, in different soil types and growing seasons (where climate and weather conditions can be significantly different). The most in-depth studies of bacterial inoculants in wheat, which are discussed below, have mostly focussed on the nitrogen-fixing, free-living *Azospirillum brasiliense,* and the fungal pathogen-reducing bacterium *Pseudomonas fluorescens*, and a small number of other species.

5.1.7.3 *Bacterial inoculant species that increase wheat nutrient uptake and usage efficiency*

Piccinin and coworkers found that inoculating wheat seeds directly with the N_2-fixing, free-living bacterial species *Azospirillum brasiliense,* originally isolated from plant tissue, in either a liquid or peat carrier at 10^8 cells g^{-1} (peat) or ml^{-1} (liquid) prior to sowing increased wheat grain yield in the order of 20% at full (100 kg/Ha), half (50 kg/Ha) and 0 nitrogen fertiliser addition [239]. Similarly, Maeder and coworkers applied a combination of *Pseudomonas* spp. *jessennii* and *synxantha* to wheat grains, at 10^5-10^6 bacterial CFU per seed in a charcoal carrier, before planting, which increased wheat yield by 37% and 30% in the presence and absence of 70 kg/Ha nitrogen fertilisation; the protein and nutrient (most notably phosphorus and potassium as, along with nitrogen, these are three key nutrients required for good plant growth) concentrations in the grain were also significantly increased [240]. Taken together, these studies indicate that inoculation with bacterial species can improve nutrient uptake and usage, and therefore grain yield, in wheat.

5.1.7.4 *Bacterial inoculant species increase crop growth more effectively in their rhizosphere of origin*

Importantly, the *Pseudomonas* spp. in the latter study were originally isolated from the wheat rhizosphere, and were also tested in rice and black gram cereal crops; the yield did not increase to the same extent (5% and 10% with no added nitrogen, and 12% and 21% with 70 kg/Ha nitrogen, respectively) [240]. This indicates that PGPR bacterial species isolated from the rhizosphere of a specific crop may be more effective at promoting growth and yield when inoculated onto their crop of origin, rather than others. The fact that this bacterial species is asymbiotic, and therefore doesn't interact specifically with the roots of the wheat plant, suggests that this rhizosphere specificity effect may be due to these *Pseudomonas* spp. being able to occupy a niche in the wheat rhizosphere that is unavailable in that of other crop plants, possibly due to a difference in the microbiome community composition.

5.1.7.5 *Bacterial inoculant species that produce phytohormones that result in increased plant growth*

Apart from increasing nutrient uptake in wheat, another key mechanism by which PGPRs directly increase plant growth is through the production of phytohormones. These are chemicals that regulate plant growth processes, and fall into 5 different classes that perform different functions. These are:

- Auxin, including Indole-3-Acetic Acid (IAA), which promotes plant cell enlargement and division, root elongation, and increased nutrient and water uptake, amongst other functions;

- Gibberellins (including Giberellic Acid, GA_3), which promotes plant stem elongation,leaf and root growth, flowering, and senescence;

- Ethylene, which promotes fruit ripening;

- Cytokinins, which promote cell division and therefore tissue growth; and

- Abscisic acid (ABA), which is involved in stress responses e.g. drought tolerance.

To assess phytohormone production in two PGPR *Azospirillum brasiliense* strains, Perrig and coworkers (2007) [241] used gas-chromatography mass-spectrometry to determine the levels of IAA, Gibberellic Acid (GA_3) and ABA, Gas chromatography flame-ionisation detection to measure Ethylene production, and High-Performance Liquid Chromatography (HPLC) to measure the levels of Zeatin (a cytokinin) in the supernatants of the two strains cultured in exponential growth phase in liquid medium. They found that IAA, GA_3 and Zeatin were found in the supernatants of both strains, Cd and AZ39, but to a lesser extent in AZ39 than Cd; however, AZ39 also produced ABA, which was not detected in the Cd supernatant. Overall, the level of IAA produced by the both strains was higher than the levels of the other phytohormones (2.9 and 10.8 µg/ml in AZ39 and Cd, respectively, compared with the next highest levels of Zeatin, at 0.75 and 2.37 µg/ml in AZ39 and Cd).

This study demonstrates that phytohormone production by PGPRs can vary considerably, even between two strains of the same species, and so the contribution of phytohormone production to plant growth promotion may vary depending on the bacterial inoculant species. Additionally, the production of IAA at relatively high levels compared to other phytohormones is consistent with the findings of Piccinin and coworkers described in Section 5.1.7.3**;** IAA promotes nutrient uptake by the plant, which was demonstrated in their study. *Azospirillum* is also a nitrogen-fixing bacterium that reduces N_2 from the atmosphere into NH_{4+}, which also contributes to plant growth promotion; However, N-fixation only occurs in this species under microaerophilic, N-limited conditions, and is tightly regulated as it is a highly energy-demanding process [242]. Therefore, N-fixation is not considered to be the primary plant growth promoting mechanism of *Azospirillum* [243], which also fits with the study by Piccin and coworkers which demonstrated that it can significantly promote the growth of plants under well-nitrogen fertilised conditions.

Phytohormone production has also been detected in other wheat bacterial inoculant species, with a particular focus on IAA: Iqbal and Hasnain [244] found that three isolates of *Pseudomonas* from soil produced IAA (detected by HPLC), and their inoculation onto wheat seeds significantly increased the shoot length, root length, fresh and dry weights, and seed germination rate of wheat plants. Similarly, Narula and coworkers [245] found that *Azotobacter chroococcum*, *Pantoea agglomerans* and three unidentified soil isolates also produced IAA, and *A. chroococcum* also produced GA_3 and Kinetin (a cytokinin); wheat grains treated with these IAA producing species, or IAA alone, resulted in an increased number of root hairs in wheat seedlings; this could potentially result in increased nutrient uptake and usage by the plant.

Together, these studies demonstrate that phytohormones play an important role in plant growth promotion in wheat bacterial inoculant species, which may act in combination with other mechanisms of plant growth promotion, which includes increasing levels of available nutrients such as nitrogen.

5.1.7.6 *Bacterial inoculant species that reduce fungal diseases of wheat*

Another, more indirect means by which PGPR bacterial species have been shown to improve wheat yield is through antagonising agriculturally important, yield-reducing fungal pathogens of wheat. For example, Dal Bello, Monaco & Simon, showed that *Stenotrophomonas maltophilia* bacteria inhibit wheat infection with the growth-reducing seedling blight pathogen *Fusarium graminearum* when inoculated on to wheat seeds planted in soil containing the fungal inoculum, thus significantly increasing wheat plant growth (height and dry weight) compared with plants that had not received the *S. maltophilia* grain treatment [246].

Weller & Cook (1983) found that the *Pseudomonas fluorescens* strain 2-79 inhibits the pathogenicity of the yield-reducing take-all fungal pathogen *Gaeumannomyces graminis* var *tritici* when added to the soil in field studies. This observed suppressive effect of *P. fluorescens* has been mostly attributed to their production of the antibiotic 2,4-diacetylphloroglucinol (DAPG): Thomashow & Weller (1988) generated non-DAPG-producing *P. fluorescens* isolates using transposon mutagenesis, the mutant did not inhibit *G. graminis* var. *tritici* in vitro, and reduced take-all infection in wheat seedlings to a lesser extent than did the original non-mutant strain [247]. Later, Raaijmakers, Bonsall and Weller (1999) used High Pressure Liquid Chromatography/Mass Spectrometry to demonstrate the presence of DAPG in the rhizosphere of wheat in TAD soils, which was not present in virgin soils (containing no *G. graminis* var. *tritici*), and also that the amount of DAPG produced by *P. fluorescens* is proportional to the extent of their colonisation of wheat roots grown from wheat grains on to which they were inoculated, providing further biochemical evidence that DAPG is a major factor in the suppressiveness of TAD soils [248].

However, this is not the only means by which *P. fluorescens* suppress take-all infection in wheat. They have also been shown to alter the expression of pathogenicity-associated genes by *G. graminis* var, *tritici,* which encode enzymes involved in degrading the plant tissue (*Lac1, Lac2, Exo*) and signalling with plant tissue (*Gmk1*), important steps in the fungal infection process [19]; they also produce very high-affinity, iron-scavenging

siderophore molecules, and thus control *G. graminis* var. *tritici* by starving it for iron, in situations where iron is limited [200].

These studies that show some Gram-negative PGPR decrease fungal infection are particularly important for informing my study, as *Bdellovibrio* is able to prey on some species of *Pseudomonas* (as seen in **Chapter 3**), including *Pseudomonas fluorescens*. This led me to hypothesise that in our original study, the addition of *Bdellovibrio* to the wheat rhizosphere soil in combination with the *G. graminis* take-all fungus would exacerbate take-all infection in the wheat roots, which would therefore lead to decreased wheat plant growth and yield compared to *G. graminis* inoculated plants where *Bdellovibrio* was not added.

5.1.7.7 *Sources of problems associated with bacterial inoculant technology*

Often in bacterial inoculant studies, a much greater number of bacterial isolates are assessed for their PGPR activity in the rhizosphere than those that have a significant, positive effect on wheat growth and yield: some appear to be ineffective when used in a natural soil rhizosphere context. These candidate species are selected for PGPR characteristics based on the results of *in vitro* screening and preliminary *in vivo* tests, before their effects are fully tested *in vivo* in a natural wheat rhizosphere soil context. For example, Dal Bello and coworkers tested 52 species of bacteria isolated from the wheat rhizosphere for inhibitory activity against *F. graminearum* using a mycelial inhibition assay on Potato Dextrose Agar plates; 51 isolates significantly inhibited mycelial growth in this assay. The isolates were then inoculated on to wheat seeds, which were grown in sterilised and non-sterile field soils; 25 isolates significantly reduced symptoms of the fungal infection in sterile soil, but only one (*S. maltophilia*) also increased wheat plant growth (dry weight and height) while also decreasing infection with *F. graminearum* in the non-sterile soil [246].

5.1.7.8 *Considerations regarding bacterial inoculants*

There are several factors that together may result in a lack of inoculant effectiveness in a natural, agricultural soil context following positive *in vitro* PGPR screening studies. Firstly, the inoculant species is being

experimentally introduced into a pre-existing rhizosphere community; niches, both physical and nutritional, are already mostly occupied by the natural rhizosphere inhabitants, and so competition for nutrients (e.g. organic compounds produced by roots) and physical space (e.g. root and soil surfaces on which to form a stable bacterial biofilm) results in some inoculant cell death. Predation by protozoa is also important factor that reduces bacterial inoculant populations in field soil. Furthermore, the abiotic soil conditions (e.g. pH, moisture and temperature) may not be suited to the physiological characteristics of the inoculant species, and therefore exert stresses that significantly decrease their survival [249].

5.1.7.9 *Considerations regarding Bdellovibrio inoculants*

However, in my study, where the predatory species *Bdellovibrio bacteriovorus* was used as an inoculant in the wheat rhizosphere, I predicted that their survival in the soil would be increased compared with that of other, free-living bacterial inoculant species. *Bdellovibrio* spends a significant proportion of its life cycle in the periplasm of Gram-negative prey species, and are therefore partially sheltered from protozoan predation and abiotic stresses. Furthermore, as they use prey cell resources to grow and reproduce while in the periplasm, competition for nutrients is largely negated; *Bdellovibrio* competition for prey species with the natural predatory bacteria in the soil, which are typically present in low numbers, is the only source of competition for resources that would still apply.

5.1.7.10 *Levels of diverse, non-strictly-PGPR bacterial/archaeal species may affect wheat growth*

Microbial (and therefore bacterial/archaeal) diversity is positively associated with soil resilience, the ability of a soil to remain in a healthy state despite environmental change and abiotic stresses such as drought [250]. This indicates that many species that have not been assigned a PGPR status may contribute to important functions in the soil along with other non-strictly-PGPR species, and promote good plant growth as a community, but they may have no significant effect as single species. Although this is a separate phenomenon to single-species PGPR, the importance of species diversity is supported by microbial inoculant studies where adding mixed-species

microbial inoculants produces a better result than any of the species on their own. Additionally, some studies have shown a synergistic effect of bacterial species with Arbuscular Mycorrhizal Fungi in promoting the growth and yield of crop plants, including wheat [240].

One reason for the importance of microbial diversity is that one ecological function in soil may be performed by many species, which is known as functional redundancy. For example, there are many nitrifying bacterial species, that oxidise ammonia (NH_4^+, as described in Section 5.1.5.2), which belong to the genera *Nitrosomonas, Nitrosococcus, Nitrobacter*, and *Nitrococcus*; therefore, if the population of one species is disrupted, the nitrification process in the soil continues and single-species losses are buffered by the presence of other nitrifying species. Recently, nitrifying archaeal species have been identified in the soil: one study by Schauss and coworkers [251] demonstrated that archaeal nitrifiers were present in greater numbers in two different agricultural soils compared with their bacterial nitrifier counterparts (in a ratio of 7:1 and 37:1 archaea to bacteria), but that the rate of nitrification was slower in the archaeal than the bacterial group. The study also showed that when the antibiotic sulfadiazine (SDZ) was added to the soil at 10 mg/kg, growth of the bacterial nitrifiers was reduced, but growth of their archaeal counterparts was not affected to the same degree. The authors suggest that archaeal nitrifiers, belonging to the genera *Nitrososphaera, Nitrosopumilus, Nitrosotalea* and *Nitrosocaldus* [252], therefore have an important function in maintaining nitrification in soils, at a slower rate but with greater population numbers, where the nitrifying bacterial population are specifically disrupted (e.g. by a greater susceptibility to an antibiotic, as shown in that study).

Nitrification is therefore a good example of functional redundancy and the importance of diverse bacterial and archaeal species in crop plant soil to maintain an important nutrient cycling function. Such redundancy is also observed in other functions that affect nutrient availability to crop plants, such as phosphorus solubilisation (described in Section 5.1.5.3, and carried out by many different species of *Pseudomonas* and *Bacillus* [253]. The consideration of other, non-strictly-PGPR species such as those mentioned

in this Section is particularly important in the context of this study, where *B. bacteriovorus* added to the soil may prey upon and kill a wide range of Gram-negative species in the soil; therefore I predicted that the effect of *Bdellovibrio*, positive or negative, would be complex, affecting the levels of many different species to a small extent rather than having a large effect on the level of just one single species. I have considered this in my metagenomic analysis (in Section 5.4.3.8) by looking at the effect on population sizes of many species grouped by known ecological functions that they perform in soil that may promote or reduce wheat plant growth.

5.2 Specific Research Aims

The initial aims for the first wheat growing season were:

- To design and implement a wheat pot mesocosm experiment in a natural, outdoor setting, using two wheat varieties (Hereward and Cadenza), to test the effect of adding *B. bacteriovorus* HD100 to the wheat soil on the level of wheat plant infection with the yield-reducing take-all fungal pathogen *G. graminis* var. *tritici*, measured using a soil core bioassay;
- To assess the effect, if any, of *B. bacteriovorus* HD100 treatment (vs. a buffer control), in combination with a laboratory-cultured *G. graminis* var. *tritici* inoculation or a water control, on the Hereward wheat soil microbial community, using Next-Generation Sequencing (NGS) of bacterial/archaeal DNA extracted from the pot soil before, during, and after treatment with *B. bacteriovorus,* and at wheat plant harvest;
- To take harvest-time growth and yield measurements of Hereward and Cadenza wheat plants treated with *B. bacteriovorus* vs. a buffer control and inoculated with laboratory-cultured *G. graminis* var. *tritici* vs. a water control, using Analysis of Variance (ANOVA) to assess the effect, if any, of *B. bacteriovorus* HD100 on wheat plant growth and yield.

As the first experiment yielded some unexpected results, further aims (in the subsequent wheat growing season) were:

- To determine whether the effects of *B. bacteriovorus* HD100 treatment on growth and yield that were observed in the first wheat crop were sustained in a second, commercial variety (Conqueror) wheat crop, oversown into the original pots previously inoculated with *B. bacteriovorus* vs. a buffer control in combination with a *G. graminis* var *tritici* inoculation or a water control, using ANOVA to assess any significant differences in measurements of Conqueror wheat plant growth and yield;
- To design and implement a separate wheat pot mesocosm experiment, testing the effects of live vs. heat-killed *B. bacteriovorus* HD100 on measurements of Hereward wheat plant growth and yield, in fresh pots containing two different soils;
- To measure the levels of nitrogen, phosphorus, and potassium in the in *B. bacteriovorus*-treated and untreated soil, to determine whether the observed effects of *B. bacteriovorus* inoculation on wheat growth and yield resulted from altered soil nutrient content.

5.3 Hypothesis

My initial hypotheses in the first wheat growing season were that:

- *B. bacteriovorus* HD100 inoculation into the soil around wheat seedlings will reduce wheat plant growth and yield, by preying upon and killing Gram-negative wheat PGPRs such as *P. fluorescens*, known to protect wheat plants against the yield-reducing take-all fungal pathogen *G. graminis* var. *tritici* (and which I showed to be susceptible to predation in Chapter 3);
- *G. graminis* var. *tritici* infection levels will therefore be higher in *B. bacteriovorus* HD100-treated wheat plants compared with the buffer control;
- The extent of the wheat growth-reducing effect may be different in Cadenza, a low Take-All Building (TAB) variety, and Hereward, a high TAB variety, due to their natural, varietal differences in susceptibility to *G. graminis* var. *tritici*;

- The added *B. bacteriovorus* HD100 population in the soil will decrease after inoculation as Gram-negative prey numbers are rapidly depleted, limiting *B. bacteriovorus* growth and reproduction inside prey cells.

However, as the experiments yielded some unexpected results, further hypotheses for tests in the second wheat growing season were that:

- The wheat growth and yield effects observed in the first pot mesocosm experiment will not be sustained in a second wheat crop grown in the same, *B. bacteriovorus*-inoculated pots, due to a reduction in *B. bacteriovorus* HD100 back to natural levels resulting from rapid prey depletion and cold, over-winter temperatures;
- A short-term boost in Hereward wheat growth may be observed with the addition of heat-killed *B. bacteriovorus* HD100 due to the release of plant-available nutrients from the dead cells; however, a greater increase in yield will be observed with the addition of live *B. bacteriovorus* cells compared with heat-killed, due to a biological rather than a nutritional effect;
- The effect of *B. bacteriovorus* HD100 inoculation on Hereward wheat plant growth and yield may vary between the two different soil types, which may contain different ratios of prey and non-prey species, affecting the survival of the added *B. bacteriovorus*.

5.4 Results

5.4.1 calcium HEPES buffer does not affect wheat plant health or growth.

B.bacteriovorus are routinely cultured on Gram-negative (stationary phase) bacteria suspended in calcium HEPES (2 mM $CaCl_2$ 25 mM HEPES pH 7.6) buffer; 10 L predatory *B. bacteriovorus* HD100 was required for each inoculation, and the time taken to prepare the inoculant for transport to Rothamsted Research would need to be minimised in order to inoculate pots with optimally active, predatory *Bdellovibrio* cultures. To achieve this, *B. bacteriovorus* were to be inoculated into wheat pots, still suspended in the

buffer that they had been cultured in. This was to avoid the shearing off of predatorily required pili by centrifugation to suspend in alternative liquids.

Therefore, to ensure that the calcium HEPES buffer itself would not have any confounding effects on wheat plant health (necrosis or chlorosis due to toxicity or prevention of adequate nutrient uptake by the wheat plant) or growth (stunting) during **Pot Experiments 1 and 2**, I first added 150 ml calcium HEPES buffer or a water control to Hereward wheat plants, a variety to be used in the pot tests, at an early growth stage (Feeke's scale 2, Zadok's scale 21, Figure 24) as a pilot buffer control experiment. The pots were photographed 3, 10, 28 and 51 days after the buffer or water addition and assessed for any symptoms of poor health or growth of wheat plants between the buffer treatment and the control, shown in Figure 27.

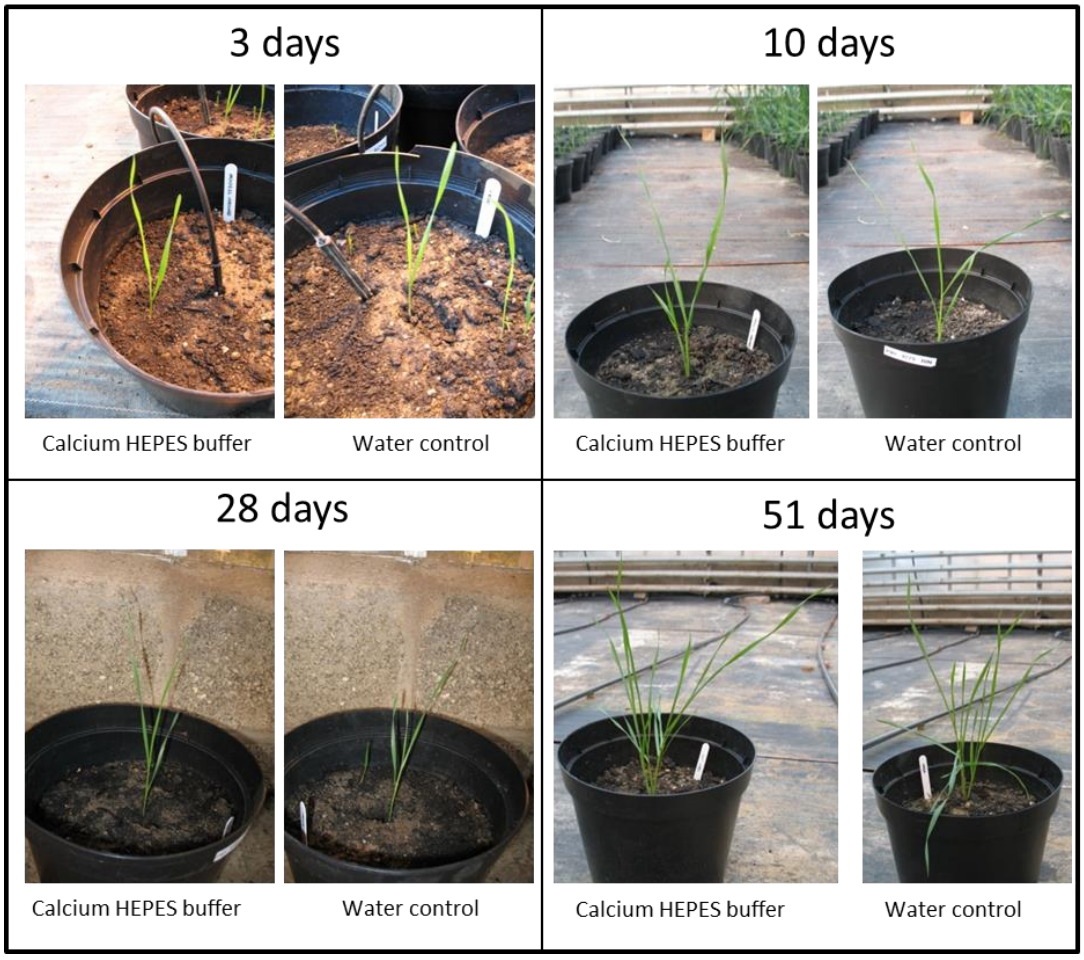


Figure 27 . Calcium HEPES buffer (2 mM $CaCl_2$ 25 mM HEPES pH 7.6) had no adverse effects on Hereward wheat plant growth or health. A typical representative plant from the buffer treated and water control groups are shown after 3, 10,28, and 51 days, with no signs of reduced growth or poor health under either treatment at any of these stages.

No signs of necrosis, chlorosis (grey/brown lesions or yellowing leaves) or growth-stunting were observed in either the calcium HEPES treated or control groups (Figure 27); this indicates that calcium HEPES buffer, in which *Bdellovibrio* is routinely cultured, does not affect wheat health or growth and would therefore be appropriate to use as a convenient carrier for the *Bdellovibrio* inoculant.

5.4.2 Experimental application of *B. bacteriovorus* HD100 to a wheat pot mesocosm

Pot experiment 1a was initially designed to test the following hypotheses:

- *Bdellovibrio* preys upon and kills PGPR *Pseudomonas* species in the soil that are known to protect wheat plants against wheat growth- and yield-reducing take-all fungal disease, caused by *G. graminis* var *tritici;*
- Thus the addition of large numbers of laboratory-cultured, predatory *B. bacteriovorus* HD100 to the soil around wheat plants increases wheat take-all infection levels and reduces the growth and grain yield of wheat grown in that soil.

5.4.2.1 *Standard statistical analysis methods of plant growth and yield measurements*

The measurements of wheat plants that were taken in **Pot experiment 1a** were the height and ear length of the main shoot, the length and number of additional tillers and ears, flag leaf length and senescence, single grain weight, the number of grains and total grain yield per plant, and take-all infection level. In **Pot experiment 1b and 2,** the flag leaf length and senescence measurements were excluded. This is because the flag leaf length data in **Pot experiment 1a** showed a similar pattern to the main shoot height after the data were analysed, so the two measurements were equivalent indicators of overall plant growth. Flag leaf senescence was excluded because flag leaves were all fully senesced at harvest, due to warmer temperatures earlier in the year in 2014 compared with 2013 (see **Appendix CD: Rothamsted weather reports 2012-2014**), so there was no difference between treatment groups.

Two different methods were used to assess take-all levels in the pots in **Pot experiment 1a**, both used by the wheat research group including Rodger White and Dr Vanessa McMillan at RRes, in previous publications [65, 80]. These were:

- Take-All Index (TAI) measurement [80]: **estimating** the take-all infection level of each root system of the mature plants from the pots, an indicator of the infection level of wheat growing in the soil when the *Bdellovibrio* and take-all inoculations were made;

- The soil core bioassay [65]: **calculating** the percentage of take-all infected roots (by counting all take-all infected and healthy roots) of new Hereward seedlings grown for 5 weeks in samples of the soil from the pots **after** the mature plants had been removed, an indicator of the potential of the take-all in the soil to infect wheat plants in the next growing season (as take-all infection is known to build up in the soil in successive seasons [65]).

The reason why these two methods were used was that *Bdellovibrio* may have affected take-all infection of the wheat plants grown in the pots in the season in which they were inoculated into the soil (mature plant TAI), but not in successive seasons (soil core bioassay), or vice versa. By using both methods, I could assess both the short-term and potential long-term effects of *Bdellovibrio* on wheat infection with take-all.

The extent and significance of differences in wheat growth and yield, and take-all infection levels, between treatments in these pot experiments were measured with help from Rodger White (a statistician at RRes) using Analysis of Variance (ANOVA) and Residual Maximum Likelihood (REML). All measurements in **Pot experiment 1a**, and all measurements apart from the length of side tillers and overall length of ears in **Pot Experiments 1b and 2,** were analysed using ANOVA, as the data were balanced (the number of measurement observations taken for each treatment group were similar).

REML was only used in the analysis of additional tiller and ear length in **Pot experiments 1b and 2**, where the number of individual measurements in each treatment group differed due to the small and highly variable number of side tillers that grew on plants between treatment groups, and thus the data were unbalanced.

5.4.2.2 *Treatment of non-significant results and sparse datasets in these results*

Under advice from Rodger White, the number of additional tillers and ears in **Pot Experiments 1b and 2** were not analysed, due to the very low number of count observations in each treatment group.

In the ANOVA and REML analyses, the effects of the different variables (*Bdellovibrio* treatment, take-all inoculation and wheat variety in **Pot Experiment 1a and 1b,** *Bdellovibrio* treatment and soil type in **Pot Experiment 2**) on the wheat plant measurements were analysed individually, but were also analysed in all possible combinations with each other. For example, in **Pot Experiment 1a and 1b,** any differences in wheat measurements between *Bdellovibrio* treated and buffer control treated plants were analysed for all plants regardless of variety, but were also assessed for Hereward and Cadenza variety plants separately. All ANOVA and REML results tables produced by the GenStat statistical analysis software package are included in the Appendix CD (in the folders: 2013 harvest raw data and ANOVA files, 2014 harvest raw data and ANOVA files); however, all but one assessment of differences between groups that took into account two or more treatments did not reach the significance threshold (where ANOVA/REML p-value of significance < 0.05), and so I have not included them in detail in the results that follow. The single significant difference between groups that took into account both *Bdellovibrio* treatment and variety in **Pot Experiment 1a** is included in Section 5.4.2.8.

5.4.2.3 *A Gram-positive contaminant was present in Bdellovibrio inoculum in Pot Experiment 2*

In **Pot Experiment 2**, a contaminant was discovered in the first inoculum that was prepared; this was present at a high level in one 1-Litre culture out of 10, while the other cultures had lower levels of contaminant present. It had caused no apparent effect on *Bdellovibrio* cell viability as *Bdellovibrio* motility and cell morphology was normal in that single culture and the mixed inoculum (Figure 28). The inoculum that was used contained 2.1×10^7 contaminant cells/400 ml, compared to 1.1×10^{11} *Bdellovibrio* cells, a contaminant to *Bdellovibrio* ratio of 1:5238 (0.02% cells were contaminants).

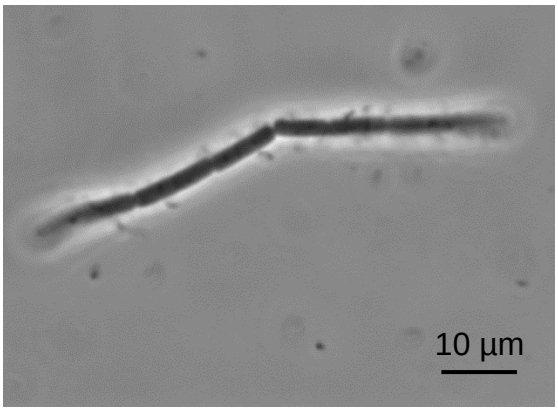


Figure 28 . The Gram-positive contaminant found in the first inoculation of Pot Experiment 2.

The 16s rDNA of the contaminant was sequenced and compared with 16s rDNA sequences of other species using the BLAST online search tool. The contaminant sequence was most similar to the 16s rDNA sequence of *Bacillus thuringiensis* (GenBank Accession No. KP006647.1), a Gram-positive, naturally soil-dwelling bacterium that produces insecticidal endotoxins.

It was crucial to add the *Bdellovibrio* inoculum to the wheat plants at a particular developmental stage (Feeke's scale 3-4/Zadok's scale 22-23, Figure 24), before the plants began to grow quickly in height after the winter, and to match the inoculation timings of **Pot Experiment 1a** in year 1; due to the culturing time of approximately 2 weeks for the *Bdellovibrio* inoculum, it wasn't feasible to culture more of this in time, so the difficult decision to use the cultures despite the presence of the contaminant was made, though the litre culture that had a higher contaminant level in it was discarded and not used in the inoculations. However, the metagenomic data showed that members of the *Bacillus* genus were present naturally in the soil at 0.38% of the total bacterial/archaeal population, at a count of 8.5×10^4 per g soil (As shown in the Appendix CD: metagenomic analysis/raw data.xlsx/all taxa- % and #: cells G-J436). It is therefore unlikely that the contaminant, present at just 0.02% of cells in the inoculum, would have affected the soil microbial community to any great extent in **Pot Experiment 2**.

5.4.2.4 *Take-all levels in soil*

The TAI of the mature Hereward and Cadenza wheat plants between treatments was significantly higher in plants where take-all had been added to the soil (86.9 in Hereward, 80.9 in Cadenza) compared with the water control (52.5 in Hereward, 38.7 in Cadenza) as shown in Table 10, though this water control level was unexpectedly high. The mean TAI was also higher in Hereward variety wheat (69.7) compared with Cadenza (59.8, ANOVA p = 0.008), which is consistent with previous findings that Hereward is more susceptible to infection with take-all than Cadenza [65]. However, the size of the difference in TAI between plants where take-all had been added compared with the water control was not significantly different between varieties (34.4 in Hereward, 42.2 in Cadenza as shown in Table 10; ANOVA p = 0.818 for the combined effect of variety x take-all treatment, as shown on Appendix CD, folder: 2013 Harvest raw data and ANOVA files, file name: Per plant grain yield and mature plant root take-all level). There were also no significant effects of *Bdellovibrio* treatment on the TAI: the difference was small with no significant difference in magnitude between the two varieties (3 in Hereward, 4.6 in Cadenza as shown in Table 10; ANOVA p = 0.818 for the combined effect of variety x *Bdellovibrio* treatment, as shown on Appendix CD, folder: 2013 Harvest raw data and ANOVA files, file name: Per plant grain yield and mature plant root take-all level). This suggests that *Bdellovibrio* had little effect on wheat plant infection with take-all.

Table 10 . The Take-All Index (TAI) of mature wheat plants of different varieties (Hereward or Cadenza), and under different *Bdellovibrio* treatments (inoculated *Bdellovibrio* or buffer control) and take-all treatments (added take-all or water control). TAI is expressed as a score out of 100 under each separate experimental condition : The effects of *Bdellovibrio* and take-all addition are shown separately for Hereward and Cadenza varieties. SED = Standard Error of the Difference between treatment conditions, $F_{1,28}$ = ANOVA F-statistic where number of Degrees of Freedom (DoF) = 1 and Denominator DoF = 28; $F_{1,28}$ significance = the p-value of significance associated with the $F_{1,28}$ statistic: differences were scored as significant where $F_{1,28}$ significance ≤ 0.05, highlighted in yellow.

	Variable	Condition	TAI	SED	$F_{1,28}$	$F_{1,28}$ significance
	Variety	Hereward	69.7	3.470	8.130	0.008
		Cadenza	59.8			
Hereward	Bdellovibrio	Added Bdellovibrio	71.2	3.470	0.050	0.757
		Buffer control	68.2			
	Take-all	Added take-all	86.9	3.470	1.250	<0.001
		water control	52.5			
Cadenza	Bdellovibrio	Added Bdellovibrio	62.1	3.470	0.050	0.695
		Buffer control	57.5			
	Take-all	Added take-all	80.9	3.470	1.250	<0.001
		water control	38.7			

The level of *G. graminis* var *tritici* inoculum in the soil was also measured using a soil core bioassay: as explained in Section 5.4.2.1, this provides a measure of the soil infectivity, i.e. the potential take-all infection level in the next crop of wheat to grow in the soil due to the presence of residual take-all inoculum), shown in Table 11 The percentage of roots infected with take-all was higher where take-all had been experimentally added to pots (back-transformed mean percentage of roots infected with take-all = 10.61%) compared to where it had not been added (5.49%), but this difference did not reach the significance threshold of ≤ 0.05 (ANOVA p = 0.111). As for the assessment of TAI, there was no significant difference between the infectivity of the soil treated with *Bdellovibrio* (7.819%) compared with the buffer control (7.569, ANOVA p < 0.936), so *Bdellovibrio* also did not affect the level of take-all inoculum left in the soil after the plants were removed.

Table 11 . **Take-all infection levels under different *Bdellovibrio* treatments (inoculated *Bdellovibrio* or buffer control), varieties (Hereward or Cadenza) and take-all treatments (added take-all or water control).** Infection levels are expressed as logit-transformed % roots infected with take-all (and back-transformed means) under each separate experimental condition (i.e. all plants under the "added *Bdellovibrio*" condition includes both take-all inoculated and non-take-all inoculated, and Hereward and Cadenza variety plants). SED = Standard Error of the Difference between treatment conditions, $F_{1,28}$ = ANOVA F-statistic where numerator Degrees of Freedom (DoF) = 1 and Denominator DoF = 28; $F_{1,28}$ significance = the p-value of significance associated with the $F_{1,28}$ statistic: differences were scored as significant where $F_{1,27}$ significance ≤ 0.05, and no differences were found to be significant.

Variable	Condition	Logit % roots infected with take-all (back-transformed means)	SED	$F_{1,28}$	$F_{1,28}$ significance
Bdellovibrio	Added Bdellovibrio	-1.2 (7.819)	0.205	0.010	0.936
	Buffer control	-1.216 (7.569)			
Variety	Hereward	-1.212 (7.631)	0.205	0.000	0.968
	Cadenza	-1.204 (7.756)			
Take-all	Added take-all	-1.04 (10.614)	0.205	2.700	0.111
	water control	-1.377 (5.488)			

Additionally, the TAI of the mature wheat plants shown in Table 10 and the infectivity of the pot soil after harvesting shown in Table 11 was similar where *Bdellovibrio* was added compared with the buffer control group (TAI = 66.7 vs 62.8 in mature wheat plants, % roots infected with take-all in the bioassay = 7.8 vs 7.6, respectively), though both measures of take-all level were slightly and non-significantly increased where *Bdellovibrio* were added.

5.4.2.5 *Take-all fungal inoculation did not significantly reduce wheat plant growth and yield compared with a water control.*

Natural take-all infection in commercially grown wheat results in visibly reduced wheat plant growth and smaller ears, along with the reduced grain yield at harvest; however, when I examined the mature plants there were no visible differences between those inoculated with take-all compared with those that received the water control (Figure 29), in ear length (a and b) or

whole plant size (c and d), and the plants in both groups looked equally healthy. This indicates that the difference in take-all level between take-all inoculated and water control plants, as shown in Table 10, was not sufficiently great as to result in any phenotypic differences.

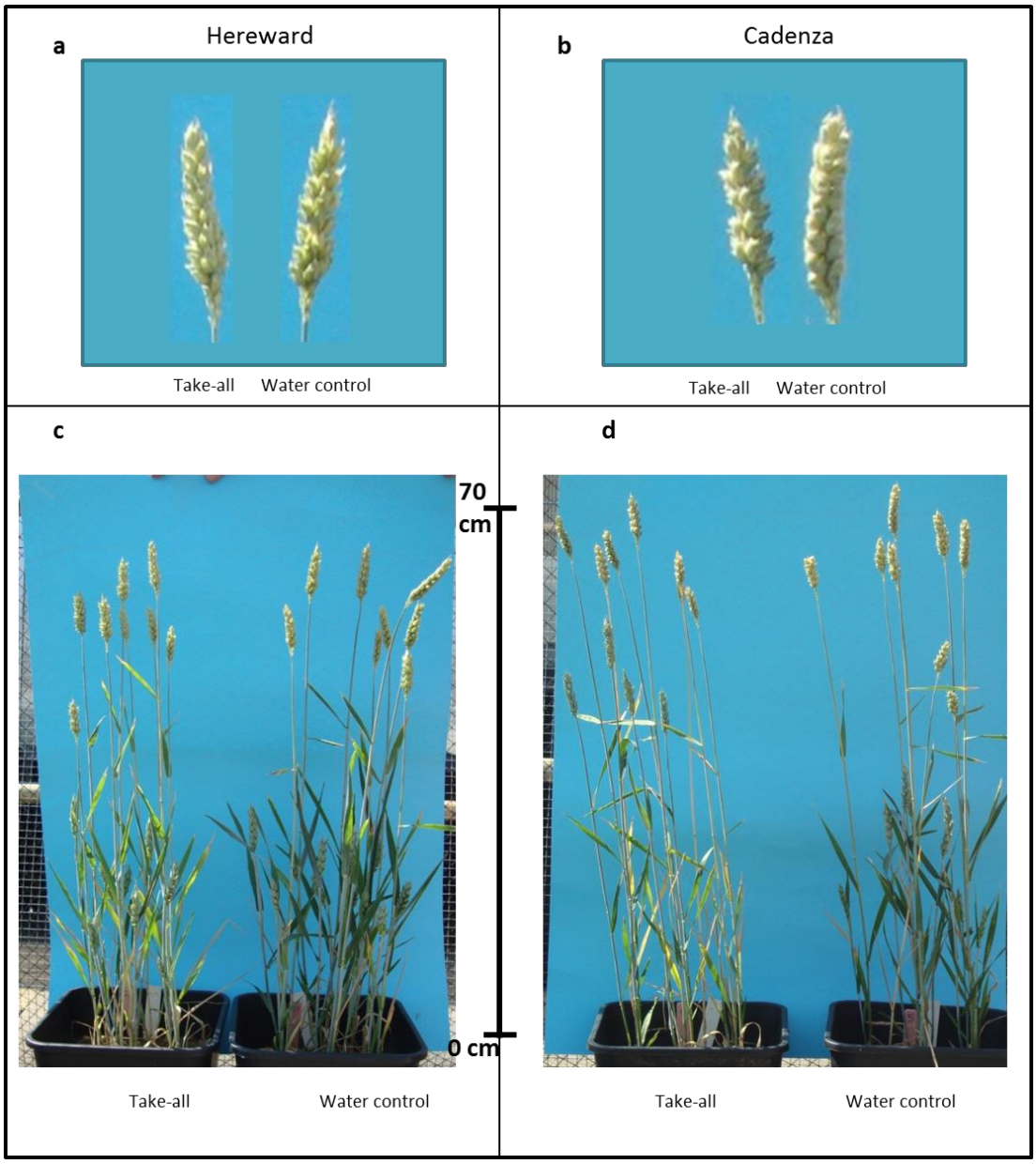


Figure 29 . Ear morphology and whole plant morphology between take-all inoculated and water control treated plants of both Hereward and Cadenza varieties. Differences in ear and plant morphology are not visible.

Consistent with this lack of a visible difference between plants in take-all added and water control treated plants, there were also no significant differences in any of the measurements of plant growth and yield between

these groups, as shown in Table 12. However, most measures of growth (height, flag leaf length, ear length and number, and tiller length) and yield (grain number, single grain weight and grain yield per plant) were slightly reduced with take-all addition, while the flag leaf senescence (an indicator of development/maturity) was increased with take-all addition. Although not significant separately, these trends are symptomatically typical of take-all infection, which is consistent with the increased take-all infection level in plants where take-all was added compared with the control group (Table 10). However, this lack of any significant difference in take-all symptoms precluded analysis of the effects of *Bdellovibrio* inoculation on the symptoms of take-all infection in wheat.

Table 12 . Key indicators of wheat plant growth and yield in all wheat pots inoculated with take-all compared with all water control pots showing differences in wheat plant growth and yield (mean values include *Bdellovibrio*-treated and non-*Bdellovibrio* treated, Hereward and Cadenza variety treatments) tested by Analysis of Variance (ANOVA). SED = Standard Error of Difference between the two *Bdellovibrio* treatments; $F_{1,28}$ = ANOVA F-statistic where numerator Degrees of Freedom (DoF) = 1 and Denominator DoF = 28; F probability (p) = ANOVA p-value of significance; there were no significant differences.

Measurement	*Unit/scale of measurement*	*Water mean*	*Take-all mean*	*SED*	$F_{1,28}$	*F probability (p)*
Height	cm	67.8	67.2	0.810	0.41	0.529
Flag leaf length	mm	93.9	92.7	1.848	0.43	0.517
Flag leaf senescence	score 1-10 **(see appendix)**	2.84	3.24	0.355	0.27	0.274
Primary ear length	cm	5.95	5.86	0.1163	0.51	0.481
Overall ear length	cm	5.59	5.59	0.910	0.00	0.977
Additional ear number	count	1.31	1.11	0.110	3.16	0.087
Tiller length	cm	47.6	50.9	2.820	1.35	0.256
Tiller number	count	1.29	1.12	0.107	2.65	0.114
Grain number per plant	count	30.2	27.8	1.911	1.64	0.211
Single grain weight	g	0.05	0.05	0.001	1.00	0.327
Grain yield per plant	g	1.47	1.32	0.085	3.01	0.094

5.4.2.6 *Bdellovibrio addition increased wheat plant growth and yield in both Hereward and Cadenza variety wheat.*

Although no significant effect of *Bdellovibrio* inoculation on take-all infection was found, there were still many significant differences in wheat plant growth and grain yield between the *Bdellovibrio*-treated and the buffer control group, which were easily observable in the whole plants before they were measured, shown in Figure 30. Furthermore, all growth and yield indicators that I measured were found through ANOVA testing to be significantly different where *Bdellovibrio* was added to pot soil compared with buffer control additions, regardless of take-all inoculation status or variety, which are displayed in Table 13**.**

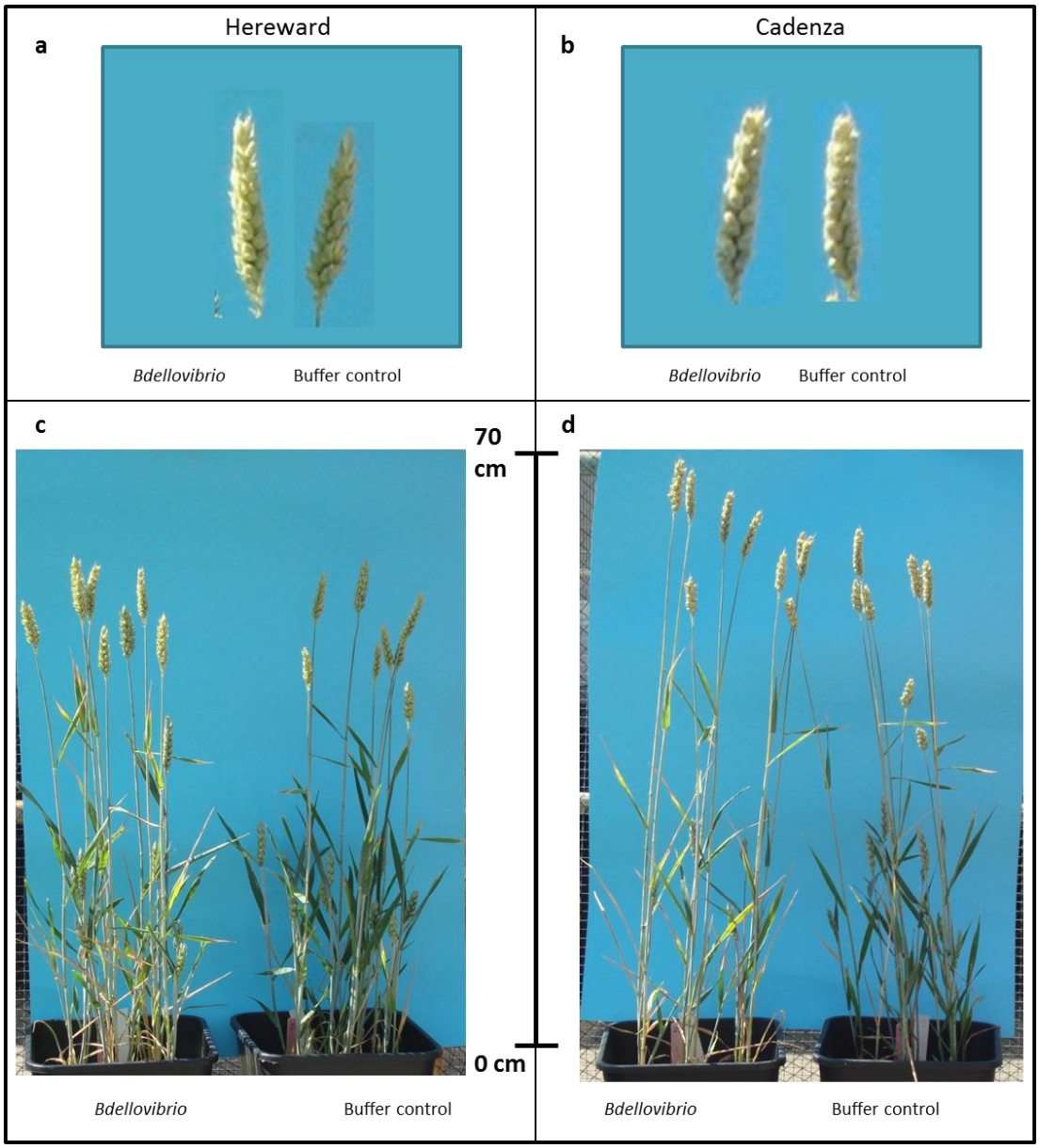


Figure 30 . Ear morphology and whole plant morphology between *Bdellovibrio* treated and buffer control wheat plants of both Hereward and Cadenza varieties showing differences in ear and plant morphology.

Table 13 . Key indicators of wheat plant growth and yield in all wheat pots treated with *Bdellovibrio* compared with all buffer control pots (mean values include take-all inoculated and non-take-all inoculated, Hereward and Cadenza variety treatments) tested by Analysis of Variance (ANOVA). SED = Standard Error of Difference between the two *Bdellovibrio* treatments; $F_{1,28}$ = ANOVA F-statistic where numerator Degrees of Freedom (DoF) = 1 and Denominator DoF = 28; F probability (p) = ANOVA p-value of significance. Significant differences (F-probability p < 0.05) are highlighted in yellow.

Measurement	Unit/scale of measurement	Buffer mean	Bdellovibrio mean	SED	$F_{1,28}$	F probability (p)
Height	cm	64.8	70.2	0.810	45.15	<.001
Flag leaf length	mm	83.8	103	1.850	104.71	<.001
Flag leaf senescence	score 1-10 **(see appendix)**	2.5	3.6	0.360	8.52	0.007
Primary ear length	cm	5.53	6.28	0.116	41.38	<.001
Overall ear length	cm	5.3	5.8	0.091	27.89	<.001
Additional ear number	count	1.37	1.05	0.110	8.51	0.007
Tiller length	cm	53.8	44.7	2.820	10.27	0.003
Tiller number	count	1.4	1.1	0.107	7.16	0.012
Grain number per plant	count	25.7	32.3	1.911	11.71	0.002
Single grain weight	g	0.05	0.05	0.001	25.00	<.001
Grain yield per plant	g	1.18	1.6	0.085	24.12	<.001

The height and flag leaf length of all the wheat plants combined were significantly increased (by 8.33% and 22.67%, respectively) where *Bdellovibrio* was added compared to the buffer control treatment, indicating that the overall growth of the plants increased with *Bdellovibrio* addition (shown in Figure 30 c and d). The flag leaf senescence at the time of harvest was also significantly increased, by 11%, with *Bdellovibrio* addition; flag leaf senescence is an indicator of overall senescence of the plant (and therefore wheat plant development and ripening of the grain), which in turn indicates

that *Bdellovibrio*-treated plants developed and ripened earlier than non-*Bdellovibrio* treated plants.

The length and number of side tillers produced by the wheat were reduced in wheat plants grown in *Bdellovibrio*-treated pots, by -16.91% and -21.43% respectively (also visible in Figure 30 c and d).

Furthermore, the mean length of all ears (including both primary and additional ears, present on the main and side tillers of the wheat plants) was significantly increased by 9.43% where *Bdellovibrio* was added to the pots; this difference was greater, at 13.55%, when only the primary ears were included in the analysis (this difference was visible in the plants, as shown in Figure 30 a and b).

This was confirmed by the increases in per-plant grain number (25.68%) and single-grain weight (6.38%) in wheat plants grown in *Bdellovibrio*-treated soil, compared with the buffer control plants; when combined, these increases in grain weight and number produced an increase in total grain yield per plant of 35.59%.

5.4.2.7 *The increased growth and yield in Bdellovibrio-treated plants occurred to the same extent in both Hereward and Cadenza wheat plants.*

There were several differences in growth between Hereward and Cadenza plants, detailed in Table 14, that were expected due to the differences in genotype and phenotype that resulted in their selection for this study. Hereward is a dwarf variety of wheat: its genome contains a mutation in a gene coding for a component of the gibberellin plant growth-promoting pathway, and consequently had a significantly shorter main tiller (63.40 cm vs 71.60 cm) and flag leaf (90.50 vs 96.10 mm) than Cadenza [189, 193]. Additionally, the significantly longer Hereward ear length (5.80 cm vs 5.30 cm overall, and 6.22 vs 5.59 when only primary ears are considered), greater additional ear number (1.39 vs. 1.03) and tiller number per plant (1.4 vs 1.00) is expected, given that the Hereward genome contains a translocation from Rye that is known generally to increase yield; however, no significant difference in per plant grain yield was observed between the two varieties.

Table 14 . Key indicators of wheat plant growth in all Hereward cultivar wheat pots compared with all Cadenza cultivar control pots showing differences between varieties (including take-all inoculated and non-take-all inoculated, *Bdellovibrio*-treated and non-*Bdellovibrio*-treated pots) tested by Analysis of Variance (ANOVA). SED = Standard Error of Difference between the two *Bdellovibrio* treatments; $F_{1,28}$ = ANOVA F-statistic where numerator Degrees of Freedom (DoF) = 1 and Denominator DoF = 28; F probability (p) = ANOVA p-value of significance. Significant differences (F-probability p < 0.05) are highlighted in yellow.

Measurement	*Unit/scale of measurement*	*Hereward mean*	*Cadenza mean*	*SED*	*$F_{1,28}$*	*F probability (p)*
Height	cm	63.40	71.60	0.810	100.52	<.001
Flag leaf length	mm	90.50	96.10	1.850	104.71	0.005
Flag leaf senescence	score 1-10 **(see appendix)**	3.28	2.80	0.355	1.79	0.192
Primary ear length	cm	6.22	5.59	0.116	29.29	<.001
Overall ear length	cm	5.80	5.30	0.091	25.87	<.001
Additional ear number	count	1.39	1.03	0.110	11.27	0.002
Tiller length	cm	46.80	51.70	2.820	2.96	0.097
Tiller number	count	1.40	1.00	0.107	13.01	0.001
Grain number per plant	count	31.89	26.09	1.911	9.22	0.005
Single grain weight	g	0.04	0.05	0.001	162.91	<.001
Grain yield per plant	g	1.40	1.38	0.085	0.09	0.767

5.4.2.8 *Additional tiller length decreased to a greater extent in Cadenza than in Hereward variety wheat with Bdellovibrio treatment.*

The potential interactive effects between *Bdellovibrio* inoculation, take-all inoculation and variety were all assessed by ANOVA, and all data are included on the Appendix CD (in the folders: 2013 harvest raw data and ANOVA files, 2014 harvest raw data and ANOVA files). However, only one significant interactive effect was found, on the length of additional tillers, which was affected differently between the two cultivars where pots were treated with *Bdellovibrio* compared with the buffer control (shown in Table

15): the tiller length was reduced to a lesser extent in Hereward (-5.81%) than in Cadenza (-25.80%).

Table 15 . A significant difference in the tiller length-reducing effect of *Bdellovibrio* on wheat plant growth in cv. Hereward compared to cv. Cadenza (including take-all inoculated and non-take-all inoculated plants) tested by Analysis of Variance (ANOVA). SED = Standard Error of Difference between the two *Bdellovibrio* treatments; $F_{1,28}$ = ANOVA F-statistic where numerator Degrees of Freedom (DoF) = 1 and Denominator DoF = 28; F probability (p) = ANOVA p-value of significance.

Measurement	Unit/scale of measurement	Hereward + buffer mean	Hereward +Bdellovibrio mean	Cadenza + buffer mean	Cadenza +Bdellovibrio mean	SED	$F_{1,28}$	F probability (p)
Tiller length	cm	48.20	45.40	59.30	44.00	3.99	4.89	0.036

5.4.2.9 *Pot Experiment 1b: wheat growth and yield increasing effects resulting from Bdellovibrio inoculation in soil only applied to crops growing in the soil at the time of application.*

Pot Experiment 1b was designed to investigate the longevity of the wheat growth and yield-enhancing effects of *Bdellovibrio* soil treatment, i.e. whether these effects would also be observed to any extent in a new crop of wheat planted into the pot soil treated in **Pot Experiment 1a**. I planted Conqueror cultivar wheat, a high-yield variety of wheat currently used in commercial wheat farming, in the pots from **Pot Experiment 1a** *in situ,* after all wheat (including roots) from **From Pot Experiment 1a** had been removed from the pots.

Only two measurements were significantly different at full growth in July 2014 where *Bdellovibrio* was inoculated in April 2013, compared with the buffer control (shown in Table 16: the ears were 17.46% shorter at full growth where *Bdellovibrio* had been added to the soil (5.7 cm vs 6.906 cm in the buffer control, ANOVA p = 0.003), and the single grain weight was 7.59%

greater where *Bdellovibrio* had been added (0.04006 g compared with 0.03702 g in the buffer control, ANOVA $p < 0.029$). All other measurements were not significantly different, and apart from grain number and grain yield plant, the values were extremely similar between *Bdellovibrio* treated and buffer control plants. No differences could be observed between *Bdellovibrio* and buffer control plants by eye as they were being measured, shown in the whole plant pictures in Figure 31.

Table 16 . Key indicators of wheat plant growth and yield in all wheat pots treated with *Bdellovibrio* compared with all buffer control pots (mean values include take-all inoculated and non-take-all inoculated, Hereward and Cadenza variety treatments) tested by Analysis of Variance (ANOVA) where data were balanced between treatment groups, and Residual Maximum Likelihood (REML) where data were unbalanced (shaded in grey). SED = Standard Error of Difference between the two *Bdellovibrio* treatments; $F_{1,28}$ = ANOVA F-statistic where numerator Degrees of Freedom (DoF) = 1 and Denominator DoF = 28; $Wald_{N,D}$ = REML Wald statistic where Numerator (N) and Denominator (D) DoF are given in the table for each measurement; F-probability (p) = ANOVA/REML p-value of significance. Significant differences (F-probability $p < 0.05$) are highlighted in yellow.

Measurement	Unit/scale of measurement	Buffer mean	Bdellovibrio mean	SED	$F_{1,28}$	$Wald_{N,D}$	F probability (p)
Height	cm	53.73	52.96	1.942	0.16	n/a	0.695
Primary ear length	cm	7.26	7.15	0.335	0.12	n/a	0.737
Overall ear length	cm	6.906	5.7	0.4784	n/a	$11.75_{1,19.4}$	0.003
Tiller length	cm	29.47	29.81	2.903	n/a	$0.17_{1,34.5}$	0.686
Grain number per plant	count	43.3	31.5	9.09	1.67	n/a	0.208
Single grain weight	g	0.03702	0.04006	0.00132	5.29	n/a	0.029
Grain yield per plant	g	1.62	1.25	0.364	1.01	n/a	0.323

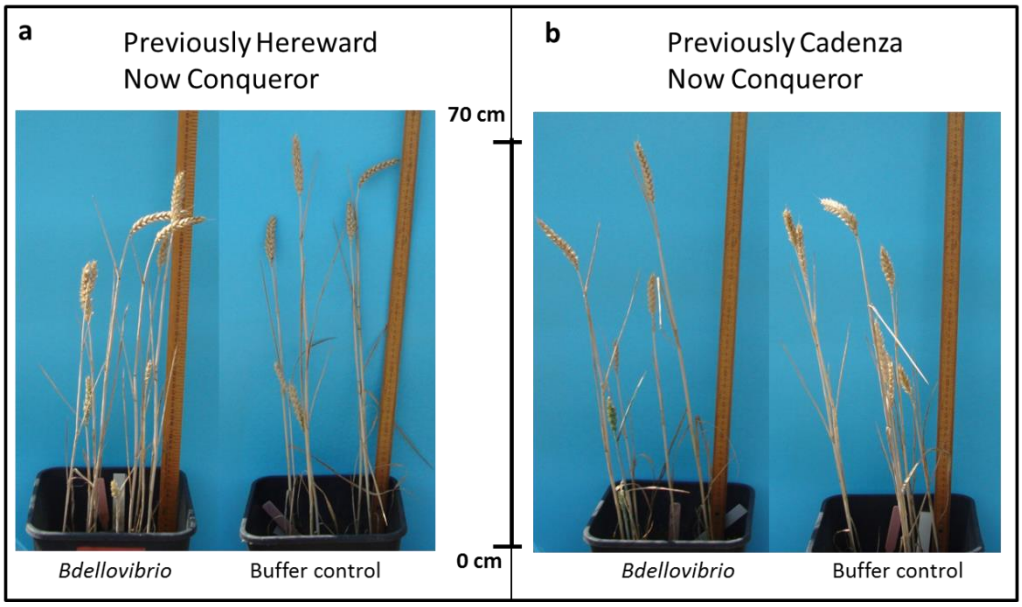


Figure 31 . Whole plant morphology of Conqueror wheat plants oversown in the pots from Pot experiment 1a, in which (a) Hereward or (b) Cadenza variety wheat was grown, and which were previously treated with *Bdellovibrio* or a buffer control. No differences in plant morphology between treatments were apparent.

The increase in grain number per plant in **Pot Experiment 1a**, which made a large contribution to the significant difference in the per-plant grain yield, was not observed in **Pot Experiment 1b**, where there were a greater number of grains per plant in the buffer control compared with pots to which *Bdellovibrio* had previously been added (43.3 compared with 31.5, respectively), though the difference was non-significant due to a high variation in grain number in the plants in both *Bdellovibrio* treated and buffer control groups (SED = 9.09).

Similarly, the per-plant grain yield was higher in buffer control groups than where *Bdellovibrio* had been previously added (1.62 g compared with 1.25 g), though the difference was also non-significant due to high variation between all plants (SED = 0.364). All other measurements, though non-significant, were slightly greater in the buffer control group than the *Bdellovibrio* treated group. In contrast to **Pot Experiment 1a**, however, the TAI of wheat plants in soil that had been treated with *Bdellovibrio* (81.1) was significantly higher than that of plants that had received the buffer control (69.9, ANOVA p = 0.048) (Table 17).

Table 17 . The Take-All Index (TAI) of mature Conqueror wheat plants grown in the soil from Pot Experiment 1a that had previously received different *Bdellovibrio* treatments (inoculated *Bdellovibrio* or buffer control), wheat varieties (Hereward or Cadenza) and take-all treatments (added take-all or water control). TAI is expressed as a score out of 100 under each separate experimental condition (i.e. all plants under the "added *Bdellovibrio*" condition includes plants grown in all pots previously treated with Take-all and the water control, and previously contained both Hereward and Cadenza variety wheat). SED = Standard Error of the Difference between treatment conditions, $F_{1,27}$ = ANOVA F-statistic where number of Degrees of Freedom (DoF) = 1 and Denominator DoF = 27; $F_{1,27}$ significance = the p-value of significance associated with the $F_{1,27}$ statistic: differences were scored as significant where $F_{1,27}$ significance ≤ 0.05, highlighted in yellow.

Variable	Condition	TAI	SED	$F_{1,27}$	$F_{1,27}$ significance
Bdellovibrio	Added Bdellovibrio	81.1	5.410	4.300	0.048
	Buffer control	69.9			
Variety	Hereward	74.4	5.410	0.150	0.699
	Cadenza	76.6			
Take-all	Added take-all	65.5	5.410	13.760	<0.001
	water control	85.5			

The only significantly different measurement where take-all had been added compared with the water control (displayed in Table 18) was in single grain weight, which was greater in the take-all added group (0.03821 g) compared with the water control (0.03999 g). The height and primary ear length were also increased in plants where take-all had been added compared with the water control, and the overall ear length, tiller length, grain number and yield per plant were slightly reduced. As shown in Table 17, the TAI of plants in soil inoculated with take-all (65.5) was significantly lower than that of plants in soil where the water control was added (85.5, ANOVA $p < 0.001$), which was the opposite of what was originally hypothesised; however this may explain the slightly higher grain number, height and primary ear length in take-all treated pots than water control.

Table 18 . Key indicators of wheat plant growth and yield in all wheat pots inoculated with take-all compared with all buffer control pots (mean values include *Bdellovibrio*-treated and non-*Bdellovibrio* treated, Hereward and Cadenza variety treatments) tested by Analysis of Variance (ANOVA) where data were balanced between treatment groups, and Residual Maximum Likelihood (REML) where data were unbalanced (shaded in grey). SED = Standard Error of Difference between the two *Bdellovibrio* treatments; $F_{1,28}$ = ANOVA F-statistic where numerator Degrees of Freedom (DoF) = 1 and Denominator DoF = 28; $Wald_{N,D}$ = REML Wald statistic where Numerator (N) and Denominator (D) DoF are given in the table for each measurement; F-probability (p) = ANOVA/REML p-value of significance. Significant differences (F-probability $p < 0.05$) are highlighted in yellow.

Measurement	*Unit/scale of measurement*	*Water mean*	*Take-all mean*	*SED*	$F_{1,28}$	$Wald_{N,D}$	*F probability (p)*
Height	cm	51.39	55.3	1.942	4.07		0.054
Primary ear length	cm	7.14	7.27	0.335	0.14		0.711
Overall ear length	cm	6.739	5.868	0.4785	n/a	$2.14_{1,19.4}$	0.16
Tiller length	cm	32.15	27.13	2.904	n/a	$2.37_{1,29.8}$	0.134
Grain number per plant	count	38.2	36.6	9.09	0.03	n/a	0.854
Single grain weight	g	0.03821	0.03999	0.00132	4.86	n/a	0.036
Grain yield per plant	g	1.44	1.43	0.364	0	n/a	0.968

There were no significant differences in any of the measurements between the Conqueror plants grown in the soil in which the cultivar Hereward had previously been grown, compared with the soil in which Cadenza had previously been grown, shown in Table 19. The variety of plant that had previously been grown in the soil also had no significant effect on take-all infection of the Conqueror oversow plants despite Hereward being a high-TAB and cadenza a low-TAB variety, theoretically building take-all levels in the soil to a greater and lesser extent, respectively, resulting in greater or lesser infection in subsequent crops. Here, the mean TAI values in the

second Conqueror crop were highly similar in soil previously planted with Hereward (74.4) and with Cadenza (76.6).

Table 19 . Key indicators of wheat plant growth and yield comparing all wheat pots in which Hereward was previously grown with all pots in which Cadenza was previously grown (mean values include *Bdellovibrio*-treated and non-*Bdellovibrio* treated, take-all inoculated and non-take-all inoculated treatments) tested by Analysis of Variance (ANOVA) where data were balanced between treatment groups, and Residual Maximum Likelihood (REML) where data were unbalanced (shaded in grey). SED = Standard Error of Difference between the two *Bdellovibrio* treatments; $F_{1,28}$ = ANOVA F-statistic where numerator Degrees of Freedom (DoF) = 1 and Denominator DoF = 28; $Wald_{N,D}$ = REML Wald statistic where Numerator (N) and Denominator (D) DoF are given in the table for each measurement; F-probability (p) = ANOVA/REML p-value of significance. Significant differences (F-probability $p < 0.05$) are highlighted in yellow.

Measurement	*Unit/scale of measurement*	*Previously Hereward mean*	*Previously Cadenza mean*	*SED*	*$F_{1,28}$*	*$Wald_{N,D}$*	*F probability (p)*
Height	cm	52.32	54.37	1.942	1.11	n/a	0.301
Primary ear length	cm	7.11	7.3	0.335	0.32	n/a	0.578
Overall ear length	cm	6.584	6.022	0.4766	n/a	$0.53_{1,18.2}$	0.475
Tiller length	cm	32.24	27.04	2.901	n/a	$2.42_{1,26.1}$	0.132
Grain number per plant	count	41.3	33.5	9.09	0.73	n/a	0.399
Single grain weight	g	0.03821	0.03886	0.00132	0.24	n/a	0.628
Grain yield per plant	g	1.58	1.3	0.364	0.59	n/a	0.449

5.4.2.10 *Pot Experiment 2: Bdellovibrio treatment of wheat soil had a biological effect on wheat grain yield in two different soil types, and across two growing seasons.*

Pot Experiment 2 was designed to assess whether the increased wheat plant growth and grain yield in pot soil inoculated with *Bdellovibrio* was attributable to *Bdellovibrio* cell death and release of nutrients, and their uptake by the wheat. This study was carried out in two different field soils

(Great Harpenden-1, A Batcombe-type silt loam/silty clay loam, and Delafield, a Batcombe-Carstens silty clay loam type soil with sandy inclusions, the same as was used in **Pot Experiment 1a**) to assess whether the wheat growth and yield effects are affected by soil type differences, such as texture and porosity. The climactic conditions (rainfall and temperature) were also very different between the growing season in 2013-2014, when this experiment was conducted, and 2012-2013, when **Pot Experiment 1a** was conducted: In 2013-2014, the rainfall level in the days immediately following *B. bacteriovorus* HD100 inoculation was higher than in 2012-2013; winter and spring temperatures were warmer in year 2013-2014 than 2012-2013, while July-August temperatures (when wheat plants usually reach maturity) were cooler (see Appendix CD: Rothamsted weather reports 2012-2014.xlsx). Thus, I could also assess the impact of climate and weather on the effect of *Bdellovibrio* addition.

As in **Pot Experiment 1a** and shown in Table 20, the per plant grain yield was increased in wheat plants where live *Bdellovibrio* was added to the soil (1.63 g), compared with the buffer control (0.90 g, ANOVA $p = 0.004$), an increase of 80.82%; this also increased in wheat plants where heat-killed *Bdellovibrio* was added compared with the buffer control (1.39 g compared with 0.90 g, $p = 0.004$), but to a lesser extent than where live *Bdellovibrio* was added: an increase of 53.89% compared with the buffer control. Live *Bdellovibrio* inoculation therefore accounts for a 26.93% increase in wheat yield. However, despite this larger significant increase with live *Bdellovibrio* treatment than heat-killed treatment relative to the buffer control described above, pairwise comparisons of the measurements between the three treatments indicated that the yield was not significantly different between plants treated with live *Bdellovibrio* compared with the heat-killed buffer control (Table 20, Student's t-test $p = 0.133$).

Table 20 . Differences in key indicators of wheat plant growth in all pots treated with live *Bdellovibrio* compared with a calcium HEPES buffer control and a heat-killed *Bdellovibrio* control, tested by Analysis of Variance (ANOVA- height, primary ear length, grain number per plant, single grain weight and grain yield per plant) or Restricted Maximum Likelihood Analysis (REML- Overall ear length, tiller length) where data were unbalanced due to high variation in the number of data points between treatment groups. P-values for Student's t-tests of difference between buffer and live *Bdellovibrio* treated, buffer and heat-killed *Bdellovibrio,* and live vs. heat-killed *Bdellovibrio* are also shown for each measurement. SED = Standard Error of Difference between the two *Bdellovibrio* treatments; $F_{2,20}$= ANOVA F-statistic where numerator Degrees of Freedom (DoF) = 2 and Denominator DoF = 20; Wald $_{N,D}$ = REML Wald statistic where N = numberator DoF and D = Denominator DoF; probability (p) = ANOVA/REML p-value of significance. Significant differences (probability $p < 0.05$) are highlighted in yellow.

Measurement	*Mean (buffer)*	*Mean (live Bdellovibrio)*	*Mean (heat-killed Bdellovibrio)*	*SED*	$F_{2,20}$	*Wald* $_{N,D}$	*probability (p)*	*buffer vs. live Bdellovibrio p*	*buffer vs. heat-killed Bdellovibrio p*	*live vs. heat-killed Bdellovibrio p*
Height	58.51	61.87	63.53	1.527	5.61	n/a	0.012	0.015	<.001	0.250
Primary ear length	5.922	6.912	6.813	0.2062	13.97	n/a	<.001	<.001	<.001	0.743
Overall ear length	5.949	6.3045	5.9415	0.1127	n/a	$2.91_{2,22.2}$	0.255	0.053	0.891	0.045
Tiller length	32.62	32.19	33.71	2.784	n/a	$0.61_{2,18.5}$	0.742	0.756	0.562	0.398
Grain number per plant	24.6	40.5	33.2	6.1	3.38	n/a	0.054	0.018	0.210	0.172
Single grain weight	0.0385	0.0413	0.0431	0.00271	1.48	n/a	0.251	0.302	0.129	0.590
Grain yield per plant	0.902	1.631	1.387	0.1922	7.46	n/a	0.004	0.001	0.021	0.133

The increases in per-plant grain number and single grain weight with live *Bdellovibrio* treatment compared to both the buffer control and the heat-killed *Bdellovibrio* control were not significant according to the ANOVA test when considered separately; however the grain number almost reached the ANOVA significance threshold, p = 0.054, and was significantly higher in plants treated with live *Bdellovibrio* compared with buffer control: 40.5 vs. 24.6 grains per plant, Student's t-test p = 0.018). The difference in grain yield between the live and heat-killed *Bdellovibrio* treatments must be attributable to an increase in grain number per plant (40.5 vs 33.2, respectively, compared with 24.6 in the buffer control) rather than an increase in the weight of single grains (0.0413 vs 0.0431 in the live and heat-killed *Bdellovibrio* treatments respectively, compared with 0.0385 in the buffer control), as was the case in **Pot Experiment 1a** (Table 6), demonstrating that live *Bdellovibrio* inoculation had a consistent effect on grain production between the two growing seasons. These results in **Pot Experiment 2** are also consistent with the increase in primary ear length in wheat plants treated with live *Bdellovibrio* (6.91 cm vs. 5.92 cm in the buffer control, ANOVA p <0.001) compared with those treated with heat-killed *Bdellovibrio* (6.81 cm), and could therefore accommodate a greater number of grains. However, despite the greater significant increase relative to the buffer control with live compared with heat-killed *Bdellovibrio* treatment, the difference in primary ear length between plants treated with live vs. heat-killed *Bdellovibrio* was not significant when considered separately (Table 20, Student's t-test p = 0.743), as was the case for the difference in grain yield (described above).

Conversely to grain number, yield and primary ear length, the height of the wheat was greater when the pot soil was inoculated with heat-killed *Bdellovibrio* (63.53 cm) compared with live *Bdellovibrio* (61.87 cm), which were both taller than the buffer control (58.51 cm, ANOVA p = 0.012). This was observable in the whole plants (shown in Figure 32 a, b and c), where plants treated with live *Bdellovibrio* are taller than the buffer control-treated plants, but the heat-killed *Bdellovibrio* control plants are taller still and bend slightly under their own weight. Again, despite this increase in height relative to the buffer control with heat-killed compared with live *Bdellovibrio*

treatment, the difference in height between plants treated with live compared with heat-killed *Bdellovibrio* was not significant when considered separately (Table 20, Student's t-test p = 0.250).

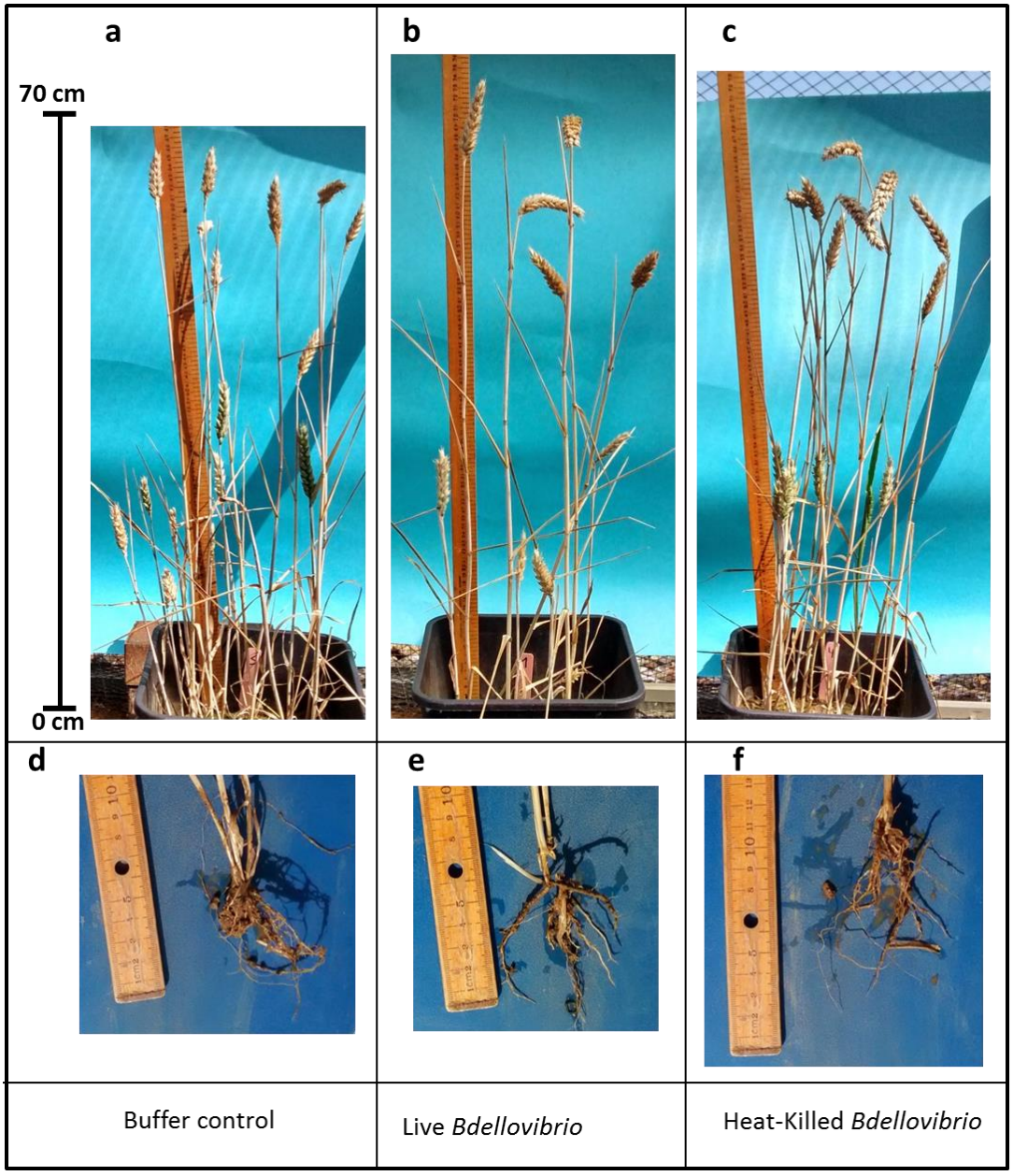


Figure 32 . Whole plant and root morphology of Hereward wheat plants in Pot Experiment 2 that received treatments with a buffer control (a + d), live *Bdellovibrio* (b + e), or heat-killed *Bdellovibrio* control (c + f).

As I measured the plants in each of the pots in **Pot Experiment 2**, I took a representative plant from each pot and examined the root system to look for any differences, such as root number, length or lateral branching, which might suggest differences in nutrition or response to plant hormones between

the treatment groups. However, all root morphologies appeared to be similar in all pots (examples shown in Figure 32 d, e and f); in addition, it is difficult to excavate the root systems without breaking off the smaller roots that presumably extend to the bottom of the pot. For these reasons, I did not examine root morphology any further.

Although no take-all was added to the soil, I measured the TAI of the mature Hereward wheat plants to check whether *Bdellovibrio* had an effect on the natural levels of take-all in the soil; there was no significant difference in TAI between live *Bdellovibrio* treatment, the heat-killed control and the buffer control, though the live *Bdellovibrio* treatment resulted in a slight, non-significant increase in TAI (52.1 compared with 47.5 and 47.4 in heat-killed *Bdellovibrio* and buffer controls, respectively, Table 21).

Table 21 . The Take-All Index (TAI) of mature wheat plants under different *Bdellovibrio* treatments (Live or heat-killed *Bdellovibrio* or buffer control) and in different soils (Great Harpenden-1 or Delafield). TAI is expressed as a score out of 100 under each separate experimental condition (i.e. all plants under the "*Bdellovibrio*" condition includes both Great-Harpenden-1 and Delafield soils). SED = Standard Error of the Difference between treatment conditions, $F_{N,D}$ = ANOVA F-statistic where N = numerator of Degrees of Freedom (DoF) and D = Denominator DoF ; $F_{N,D}$ significance = the p-value of significance associated with the $F_{N,D}$ statistic: differences were scored as significant where $F_{N,D}$ significance < 0.05, highlighted in yellow.

Variable	Condition	TAI	SED	$F_{N,D}$	$F_{N,D}$ significance
Bdellovibrio	Live Bdellovibrio	52.1	4.630	$0.68_{2,20}$	0.516
	Heat killed Bdellovibrio	47.5			
	Buffer control	47.4			
Soil	Great Harpenden-1	68.2	3.780	$102.62_{1,20}$	<0.001
	Delafield	29.9			

5.4.2.11 *Bdellovibrio treatment increased wheat grain yield in two different agricultural soils and in two growing seasons with different climate and weather conditions.*

There were some significant differences in wheat measurements between the two soil types, shown in Table 22; wheat height was significantly greater in Great Harpenden-1 soil (64.02 cm) compared with Delafield soil (58.59 cm,

ANOVA p < 0.001), as was primary ear length (6.88 cm compared with 6.22 cm, ANOVA p < 0.001) and single grain weight (0.044 g compared with 0.038 g, ANOVA p = 0.023), though no there was no significant difference in total per-plant wheat grain yield.

Table 22 . Differences in key indicators of wheat plant growth in all pots with Great Harpenden-1 soil compared with Delafield soil, tested by Analysis of Variance (ANOVA- height, primary ear length, grain number per plant, single grain weight and grain yield per plant) or Restricted Maximum Likelihood Analysis (REML- Overall ear length, tiller length) where data were unbalanced due to high variation in the number of data points between treatment groups. SED = Standard Error of Difference between the two *Bdellovibrio* treatments; $F_{1,20}$= ANOVA F-statistic where numerator Degrees of Freedom (DoF) = 1 and Denominator DoF = 20; $Wald_{N,D}$ = REML Wald statistic where N = numberator DoF and D = Denominator DoF; probability (p) = ANOVA/REML p-value of significance. Significant differences (F-probability p < 0.05) are highlighted in yellow.

Measurement	*Mean (Great Harpenden-1)*	*Mean (Delafield)*	*SED*	$F_{1,20}$	$Wald_{N,D}$	*probability (p)*
Height	64.02	58.59	1.246	18.99	n/a	<.001
Primary ear length	6.876	6.221	0.1683	15.14	n/a	<.001
Overall ear length	6.026	6.103	0.1127	n/a	$0.28_{1,23}$	0.601
Tiller length	32.39	33.29	2.781	n/a	$0.29_{1,19.1}$	0.595
Grain number per plant	28.4	37.1	4.98	3.07	n/a	0.095
Single grain weight	0.0437	0.0383	0.00222	6.09	n/a	0.023
Grain yield per plant	1.257	1.356	0.1569	0.4	n/a	0.534

THe TAI as shown in Table 21 was significantly higher in Great Harpenden-1 soil (68.2) than in Delafield (29.9, ANOVA p<0.001); Great Harpenden-1 soil is therefore more conducive to *G. graminis* var *tritici* survival and infection of wheat plants than Delafield soil. However, despite these differences between soil types, there were no interactions between the soil type and *Bdellovibrio*

treatment that significantly affected wheat growth or yield; and therefore the yield-increasing effect of inoculating live *Bdellovibrio* was found to be consistent between both soils.

The number of additional ears and tillers in **Pot Experiment 2** was highly variable between plants, pots, and treatment groups, and therefore the number and length data for ears and tillers could not be analysed using ANOVA. Instead, Restricted Maximum Likelihood analysis (REML) was used to compare overall ear and additional tiller length, taking into account the highly variable number of data points measured in each treatment group. No significant differences were found in overall ear or additional tiller length between *Bdellovibrio*-inoculated and control plants as shown in Table 20, nor between the soil types, shown in Table 22, due to the highly variable number of ears and tillers that grew on individual plants between treatment groups. This variation precluded the analysis of additional ear and tiller numbers, as advised by Rodger White (RRes), because in general there were so few of them in **Pot Experiment 2** compared with **Pot Experiment 1a**.

5.4.3 **Metagenomic analysis**

A metagenomic analysis of bacterial taxa in the wheat rhizosphere soil was carried out to assess the natural and added *Bdellovibrio bacteriovorus* levels in the soil and their impact on the rhizosphere microbial community, in order to address the questions as to whether:

- *B. bacteriovorus* preys upon and kills free-living bacteria in the soil that perform wheat growth-limiting functions, e.g. Denitrifying species;
- *B. bacteriovorus* preys upon and kills some Gram-negative species in the soil, providing a vacant niche for other, plant-growth promoting bacteria e.g. Nitrifiers that convert nitrogen into plant-utilisable nitrate ions;
- *B. bacteriovorus* itself carries out a process, not related to predation, that results in increased plant growth in the soil e.g. increased nutrient availability or improved soil texture; or, in part,

- Some of the *B. bacteriovorus*, added to the soil in extremely high numbers, had died and the nutrients contained within their cells had been released into the soil for use by the plant.

The laboratory cultured *B. bacteriovorus* HD100 cells in the inoculum were originally isolated from a soil environment [254]; we therefore hypothesised that *Bdellovibrio* would be able to locate suitable prey species and successfully prey upon them in order to survive in the soil.

5.4.3.1 *Analyses used and statistical treatment of the data*

In **Pot Experiment 1a,** *B. bacteriovorus* treatment had a positive effect on wheat growth and yield, rather than the negative effect that I had initially hypothesised. In addition, take-all inoculation had little impact on the growth of the wheat in pots. For these reasons, I focussed on analysing any statistically significant changes in the population of bacterial/archaeal taxa between samples taken before *Bdellovibrio* or buffer was added (on day 176), and 48 hours after the first *Bdellovibrio* or buffer addition was made (on day 184). This meant that I could include samples in groups that were to receive a take-all addition on day 187 in these analyses, as the take-all inoculation had not yet been made. This doubled the number of samples in each group (10 instead of 5), increasing statistical power.

The metagenomic analyses were carried out using soil samples from **Pot Experiment 1a**, comparing live *B. bacteriovorus* with buffer control treated wheat pot soil. However, this experiment was not initially designed to test the effect of *B. bacteriovorus* on the growth of wheat, and did not include any control for the large amounts of additional nutrients potentially added to the soil in the event of *B. bacteriovorus* cell death in the live treatment (discussed in section 5.4.4), which likely resulted in differences in the bacterial/archaeal community in the soil. **Pot Experiment 2** included a heat-killed *B. bacteriovorus* control treatment, which would have been a more appropriate comparison for the live *B. bacteriovorus* treatment; however, the metagenomic analysis took several months to complete, and **Pot Experiment 2** was carried out in the final year of my Ph.D., thus I could not

have completed a metagenomic analysis from this second experiment in time.

Due to financial limitations, I carried out the DNA sequencing using soil samples from Hereward wheat pots only: Hereward is used as a commercial variety of wheat today while Cadenza is not, and so I reasoned that these results would be more applicable to current farming practices.

I carried out the pairwise comparisons between the two sampling points (Section 2.6.3) for each treatment group separately, before comparing the significance of these differences between *Bdellovibrio*-treated and buffer control groups. Samples taken from the same set of pots at different time points were paired, and so any changes in their metagenomic profile were attributable to the treatment they received (*B. bacteriovorus* or buffer control), or alternatively to natural changes that occur over time due to e.g. wheat root development or weather, but not due to pre-existing differences in their metagenomic profiles. Subsequently, I compared the changes that I identified between the *Bdellovibrio*-treated and the buffer control pots, to identify and exclude any changes that occurred over time due to other, natural factors that were thus similar in both *B. bacteriovorus*-treated and buffer control pots, as they were both kept under the same environmental conditions (outdoors in a netted tunnel with natural weather exposure). This allowed me to identify population changes in taxa that were potentially associated with *B. bacteriovorus* treatment rather than natural factors or between-pot variation.

5.4.3.2 *Metagenomic sequencing calculations*

As I explained in Section 2.6.12, total bacterial/archaeal DNA was extracted from 250 mg of each sample taken from each pot in which Hereward was grown. I then measured the amount of DNA (ng/µl) in each extracted sample, and adjusted the amount (µl) of liquid sample, so that each contained 20 ng DNA, and sent these adjusted samples to Argonne National Laboratory (USA) for metagenomic sequencing (this process is also described in more detail in Section 2.6.12). In the metagenomic sequencing read data I received back for each sample, one read corresponded to the detection of one single bacterium/archaeon in the sample; I could therefore calculate how

many bacteria/archaea were detected in the DNA sample extracted from 250 mg soil using the known amount of liquid DNA extraction that was sent for sequencing, and multiply that value by 4 to find the number of bacteria/archaea in 1 g soil (250 mg x 4 = 1 g). The QIIME metagenomic analysis output gave the percentage of each bacterial/archaeal taxon in each sample (see Section 5.4.3.3), and so the number of each taxon per g of soil could be calculated by multiplying these percentage values by the number of total bacteria/archaea present, for each sample.

However, the metagenomic analysis likely gave a conservative estimate of the total number of bacteria/archaea present in each sample, due to losses incurred in the DNA extraction and metagenomic sequencing processes, as outlined in Section 5.1.7.1. To assess this in more detail, I calculated the number of *Bdellovibrio* that survived in soil by enumeration after addition under the same conditions as in Pot Experiment 1a and 2, up to 36 days after addition (Figure 33; this was carried out in a separate enumeration experiment, conducted in light of the metagenomic data).

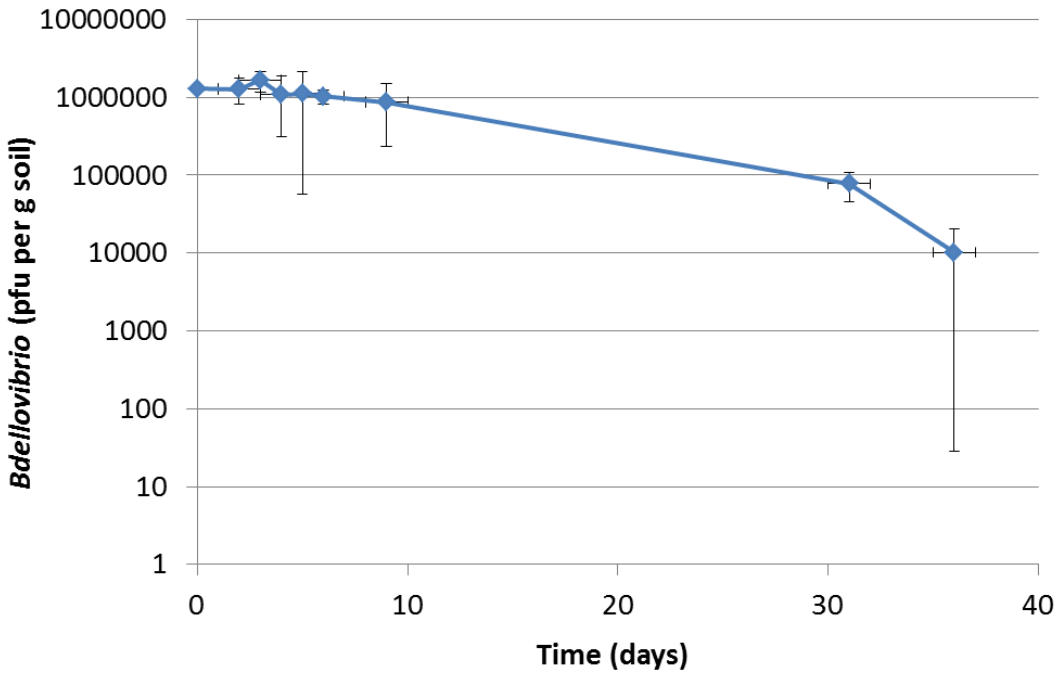


Figure 33 . Number of *B. bacteriovorus* recovered per g soil from an 8 kg pot inoculated with 1 x 10^{11} *B. bacteriovorus* HD100 (1.25 x 10^{7} cells per g soil), as in Pot Experiment 1a. *B. bacteriovorus* PFU were enumerated on a *P. putida* prey lawn from soil samples taken from 30 min to 36 days after addition. Error bars show 1 standard deviation.

It was therefore possible to calculate how many more *Bdellovibrio* were added to the soil than were recovered in pot soil samples after the first *B. bacteriovorus* addition was made in **Pot Experiment 1a**. Given that:

1. **1 x 10^{11}** *B. bacteriovorus* were added in total to the enumeration experiment pot containing 8 kg (8000 g) soil;
2. 48 hours after 1 x 10^{11} *B. bacteriovorus* had been added, the number of *B. bacteriovorus* cells recovered from the enumeration experiment pot was **1.2 x 10^{6}** per g soil;
3. In Pot Experiment 1a, **8.7 x 10^{10}** cells were added in to the pot in the first addition; and
4. *Bdellovibrio* comprised an average **3.95%** of the sequences in each sample corresponding to **8.3 x 10^{5}** cells per g soil;

I calculated that:

1. 1 x 400 ml addition in the enumeration experiment pot would have contained 1 x 10^{11} / 2 = **5 x 10^{10}** *B. bacteriovorus* cells;

2. 48 hours after 5 x 10^{10} *B. bacteriovorus* were added (at 6.25 x 10^{6} cells per g soil), the number of *B. bacteriovorus* recovered from the enumeration experiment pot would have been 1.2 x 10^{6}/ 2 = **6 x 10^{5}** cells per g soil if only 1 x 400 ml addition had been made, as in Pot Experiment 1a after the first addition;

3. 6.0 x 10^{5} / 6.25 x 10^{6} = **0.096** *B. bacteriovorus* survived in the enumeration experiment pot soil after 48 hours;

4. 8.7 x 10^{10} / 8000 = **1.09 x 10^{7}** *B. bacteriovorus* cells were added per g soil in Pot Experiment 1a;

5. (0.096 x 1.09 x 10^{7})/(8.3 x 10^{5}) = **1.26** times more *B. bacteriovorus* were likely added than were detected in the samples in Pot Experiment 1a.

The enumeration and calculation of *B. bacteriovorus* cell numbers present in the Pot Experiment 1a soil was based on inoculation of *B. bacteriovorus* HD100 into pots in which no wheat was planted (as the assay was conducted in light of the metagenomic analysis at the end of my Ph.D.), and therefore makes the assumption that the presence of wheat roots in the pots did not affect the survival or enumeration of *B. bacteriovorus* from the pots. It also makes the assumption that the *Bdellovibrio* were distributed evenly throughout the pot; although *Bdellovibrio* were enumerated from both central and outer edge soil samples of the enumeration experiment pot to account for this, it may still have affected the enumeration results, for example if the inoculum sank to the lower portion of the pot (as soil samples were taken to 10 cm depth). However, this calculated discrepancy should be borne in mind when considering the number of each taxon detected per g soil that are quoted in the results that follow.

5.4.3.3 *QIIME analysis*

The first outputs that were produced from the QIIME metagenomic analysis programme were bar chart plots of the percentage of each phylum, class, order, family or genus, relative to the whole bacterial population in each 20 ng DNA sample sent for metagenomic sequencing, with each sample taken from one pot. The number of sequence reads produced from each sample ranged from 5.33×10^4 to 2.81×10^5. Before any *Bdellovibrio* or buffer additions were made, the bar chart plots looked like that shown in Figure 34a.

It was easiest to distinguish between different members of the soil bacterial/archaeal community at the class level; so I first compared the levels of each class between the pre-treatment pot samples taken on Day 176, shown in Figure 34a, and samples taken 48 hours after *Bdellovibrio* or buffer control addition taken on day 184, shown in Figure 34b.

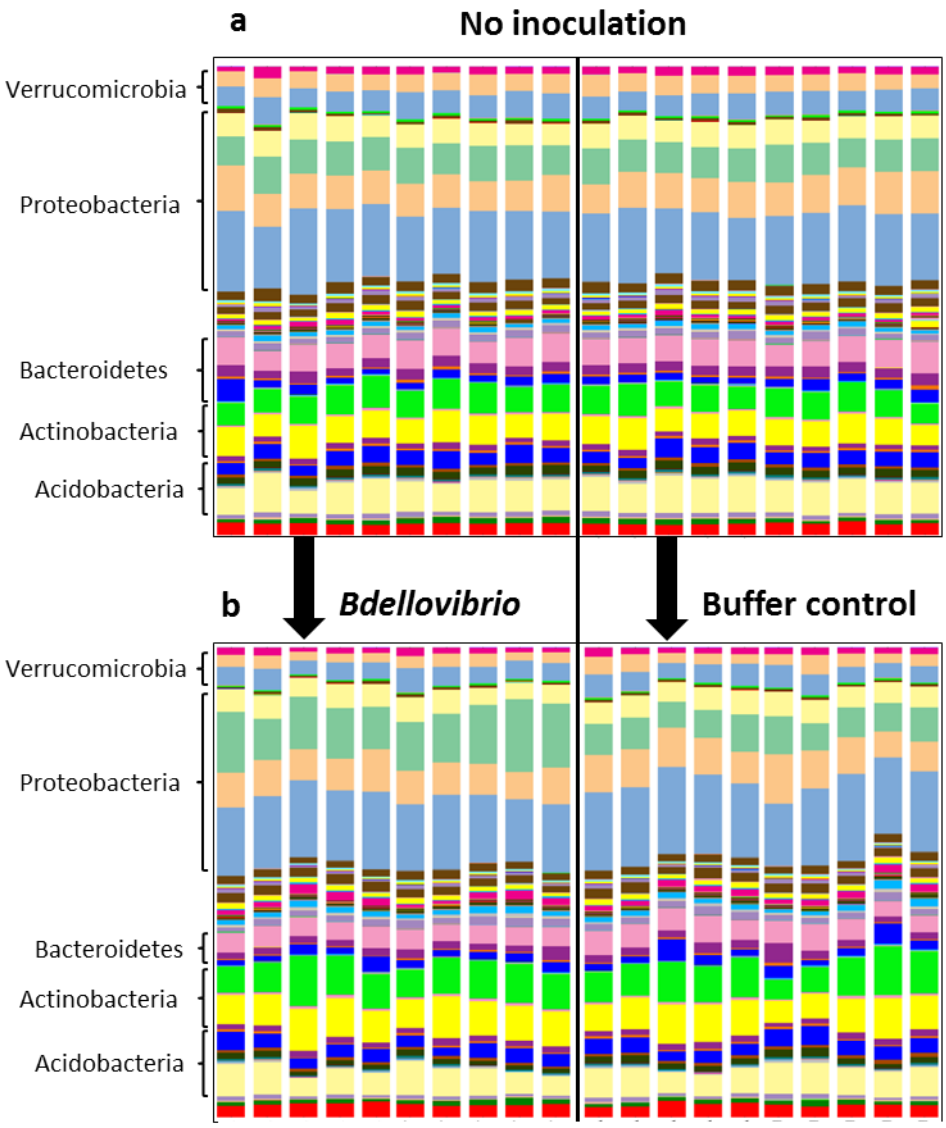


Figure 34 . QIIME output histogram summary showing the level of different bacterial/archaeal classes (represented by different colours) in soil samples taken (a) before and (b) after treatment with *Bdellovibrio* or a buffer control. Each sample of 20 mg DNA sent for sequencing, which yielded 5.33 x 10^4 - 2.81 x 10^5 sequence reads, is represented by one bar in the chart, and samples are grouped as shown according to treatment with *B. bacteriovorus* HD100 or buffer/water control that they received after the first inoculation had been completed on day 176. Key phyla groupings are shown on the left side.

There were some changes in the percentage of classes in samples from *Bdellovibrio*-treated pots that were different to those in buffer control pots that were visible between these graphs, in the range of 0.2-0.4%, such as in the *Bacteroidetes* and *Proteobacterial* classes; this gave me a first indication that the levels of *Bdellovibrio* (which is a species of *Deltaproteobacteria*, the *Proteobacterial* class shown in green in Figure 34) were increased in samples to which it was added, indicating their survival, and that the levels of other classes had been reduced in *Bdellovibrio*-added samples. Although these graphs were a good first idea of the changes in bacterial/archaeal community composition in the soil where *Bdellovibrio* had been added, this was at a general level, so I then began to analyse the specific levels of bacterial/archaeal genera in the soil between these time points.

In these analyses, I considered both the percentage of each genus relative to the total bacterial/archaeal population in each sample, and also the number of 16s rDNA reads (and therefore number of cells) associated with each genus in each sample, expressed as reads per g of soil (calculated from the known amount of soil the sequenced DNA was extracted from). This is because the number of reads per 20 ng sample as produced by the sequencing runs, and also per g soil calculated as above, varied considerably between time points (as shown in Figure 35 and Figure 36); the normalised percentage data was therefore more useful for comparative purposes. This reduced the level of variation between samples, although the percentage data was still relatively variable (I calculated the variation co-efficients for these data, as shown on the Appendix CD: 2013 metagenomic analysis/raw data.xlsx). Some research argues that percentage data is more robust and reproducible than numerical data [255], however as *Bdellovibrio* itself comprised a percentage of the samples it was added to, and thus alters the percentage representation of other species, I took the numerical data into account as well.

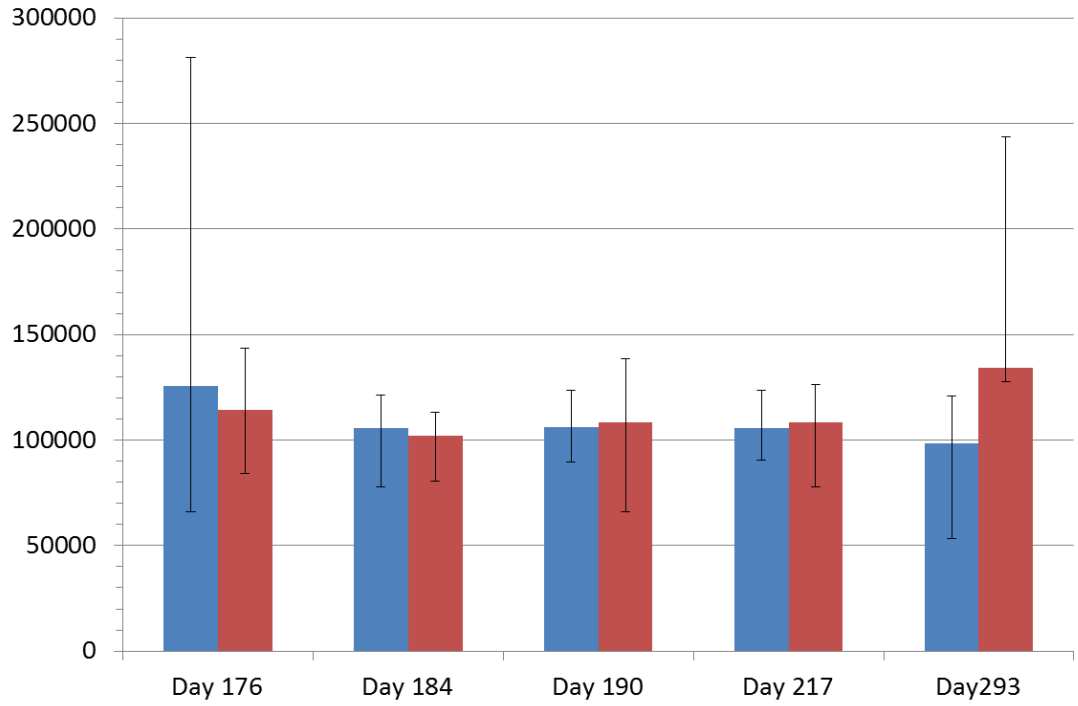


Figure 35 . Average number of reads in the 20 ng DNA sent for sequencing (X-axis) from each pot soil sample treated with *B. bacteriovorus* HD100 (shown in blue) compared with the buffer control group (red) collected over the 5 different sampling points (Y-axis). Error bars indicate the minimum and maximum number of sequence reads per g soil under each treatment at each sampling point.

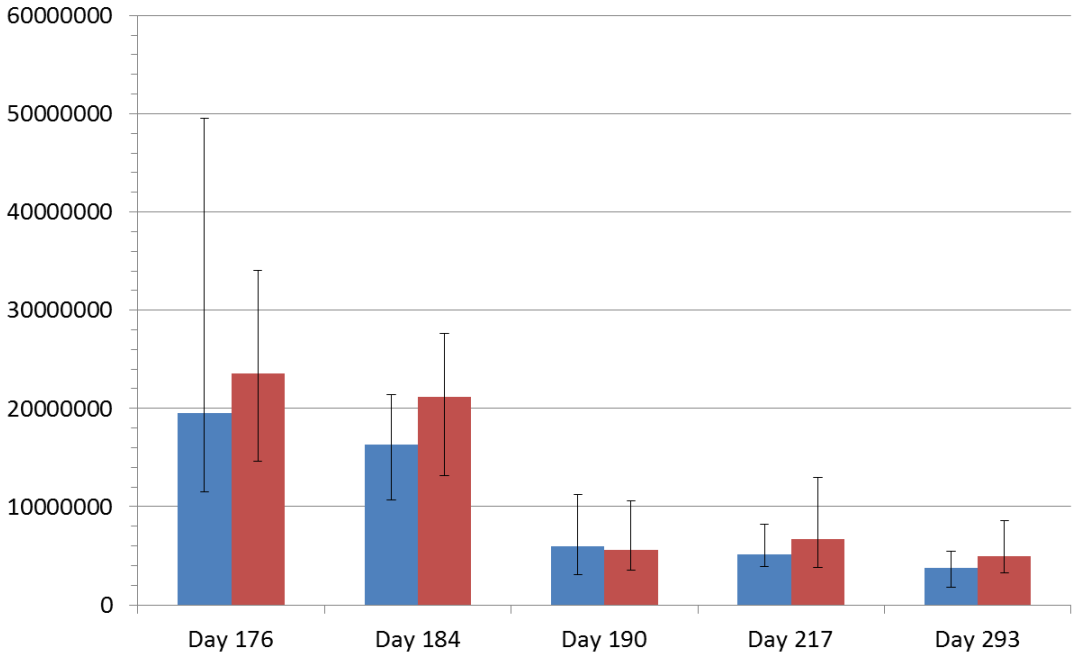


Figure 36 . Average number of reads per g soil, calculated using the number of reads produced per 20 ng sample sent for sequencing (shown in Figure 35) and the known amount of soil the sequenced DNA was extracted from (X-axis) from each pot soil sample treated with *B. bacteriovorus* HD100 (shown in blue) compared with the buffer control group (red) collected over the 5 different sampling points (Y-axis). Error bars indicate the minimum and maximum number of sequence reads per g soil under each treatment at each sampling point.

5.4.3.4 *Paired t-test with Benjamini-Hochberg correction*

In my statistical tests, I used a paired t-test of differences comparing samples before and after *Bdellovibrio* treatment or buffer control, for the percentage of all individual genera present in the samples. As there were several hundred different genera listed, the likelihood of Type I Errors was increased (where differences are incorrectly found to be significant). To account for this, I applied a Benjamini-Hochberg (BH) correction for multiple comparisons, suggested by Rodger White (statistician at RRes).

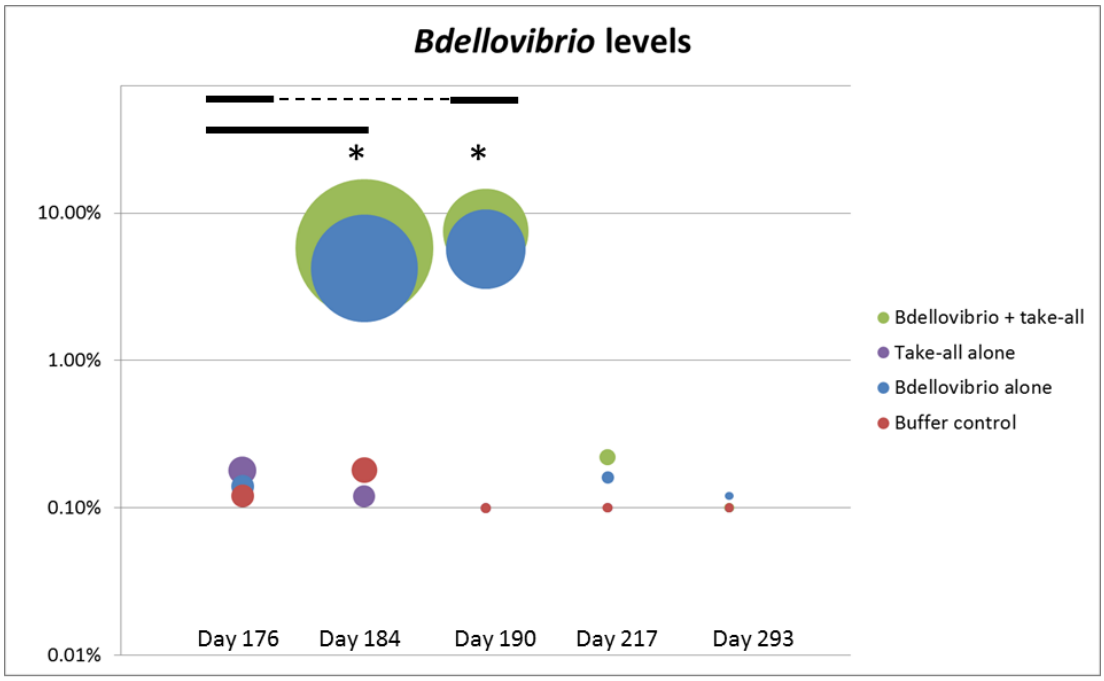
In the BH method, the data are ranked in order of decreasing t-test p-values, and each p-value is compared to a list of Critical Significance Values (CSV), which are calculated for each comparison based on the rank of its p-value in the list. If p < CSV, the difference in the group between pre- and post-*Bdellovibrio* treatment or buffer control samples is significant. The BH CSV corresponding to the smallest p-value in the list is equal to the Bonferroni

critical value (calculated as the threshold of significance, in this case 0.05, divided by the total number of comparisons), but the BH CSVs increase sequentially with increasing p-values. Thus, the test is less stringent than Bonferroni correction for multiple comparisons, the most simple and conservative correction for multiple comparisons, which can have an increased rate of Type II errors (where differences are incorrectly found to be non-significant) when the data set is large.

Due to the relatively high variation in the percentage and absolute number of each genus present across samples in the same treatment group at the same time of sampling, I have not displayed the variation on the graphs that follow in this Section (e.g. as error bars showing standard deviation around the mean, or by plotting individual samples). Instead, I show the mean values for each treatment group, unless otherwise specified. This is because general patterns in the data are difficult to see when the variation is included. However, I clearly indicate where a statistical test of significance has been carried out, so as to differentiate between significant and insignificant changes in the specific genera under different conditions.

5.4.3.5 ***Laboratory-grown predatory Bdellovibrio survives in the wheat rhizosphere soil up to 27 days after addition***

Firstly, I compared the percentage and number of *Bdellovibrio* present in the samples, to address the question of whether the *Bdellovibrio* we added to the soil survived after addition, and if so, how long it survived for. This data is displayed in Figure 37.



	% *Bdellovibrio* present in soil samples (Mean value from 5 pot replicates)			
Days after wheat sowing	*Bdellovibrio* + take-all	Take-all alone	*Bdellovibrio* alone	Buffer control
176 (pre-*Bdellovibrio* addition)	0.14%	0.18%	0.14%	0.12%
184 (post-*Bdellovibrio* addition 1)	5.80%	0.12%	4.22%	0.18%
190 (Post-*Bdellovibrio* addition 2	7.48%	0.10%	5.70%	0.10%
217 (27 days post-*Bdellovibrio* addition)	0.22%	0.10%	0.16%	0.10%
293 (wheat plant harvest)	0.10%	0.10%	0.12%	0.10%
	Number of *Bdellovibrio* present in 1 g soil (Mean value from 5 pot replicates)			
Days after wheat sowing	*Bdellovibrio* + take-all	Take-all alone	*Bdellovibrio* alone	Buffer control
176 (pre-*Bdellovibrio* addition)	25364	42652	29383	28186
184 (post-*Bdellovibrio* addition 1)	1031172	27125	625662	35703
190 (Post-*Bdellovibrio* addition 2	401133	5888	347253	5894
217 (27 days post-*Bdellovibrio* addition)	13894	4822	8822	5429
293 (wheat plant harvest)	5436	4373	3739	4533

Figure 37 . Graph showing the mean percentage (X-axis) and number (size of each bubble) of *Bdellovibrio* bacteria detected in samples in the different treatment groups at each sampling point (Y-axis). Percentage and numerical values are given in the tables below the graph. * = significant difference between pre- and post- *Bdellovibrio* treatment where difference is non-significant in the buffer control group (student's paired t-test with Benjamini-Hochberg correction for multiple comparisons).

Environmental members of the *Bdellovibrio* genus were present in the soil in all groups before inoculation on day 176, at a low level of 0.12-0.18% (2.54 x 10^4-4.27 x 10^4 *Bdellovibrio* per g soil), and these low levels persisted throughout wheat growth in the buffer control groups. This indicates that predatory *Bdellovibrio* species are able to survive naturally in Delafield soil, and thus suitable prey species are also likely to be present naturally. On day 184, 48 hours after the first *B. bacteriovorus* HD100 treatment was given, the level of total *Bdellovibrio* increased to 4.22-5.80% (6.26 x 10^5-1.03 x 10^6), a

statistically significant increase. Conversely, the levels in the buffer control, 0.12-0.18% (2.71×10^4-3.57×10^4), did not change significantly between day 176 and 184. The *Bdellovibrio* levels increased further to 5.70-7.48% (3.47×10^5-4.01×10^5) after the second *Bdellovibrio* addition had been made on day 190, indicating that the second dose added to *Bdellovibrio* that had survived from the first dose made 6 days prior. In the laboratory, the predatory cycle typically takes 4 hours when grown on *E. coli* or *P. putida* prey cells, and *Bdellovibrio* is unable to survive well after 48 hours in the absence of prey (Hobley et al. previously found that there was a 50% loss of viability of predatory cells after just 14-24 hours in the presence of very low prey cell numbers [33]). Additionally, 48 hours after inoculation, the proportion of *B. bacteriovorus* cells enumerated from an test pot inoculated separately to Pot Experiment 1a and 2 in April 2015 was 9.6% of the number of cells originally added addition (See section 5.4.3.2 and Figure 33), and the proportion of *B. bacteriovorus* in the pot remained at this level for up to 10 days; while this indicates some survival of the *B. bacteriovorus* cells due to successful predation in the soil, it also indicates a significant loss of *B. bacteriovorus* cells.The mean reduction in Gram-negative bacteria in samples to which *Bdellovibrio* was added compared with the buffer control was 1.35×10^6 after the first *Bdellovibrio* addition (excluding *Bdellovibrio* itself); Gram-positive and archaeal numbers increased in both the *Bdellovibrio*-treated (from 2.99×10^6 -3.12×10^6) and buffer control group (from 3.47×10^6 -3.93×10^6), so *Bdellovibrio* addition did not reduce the levels of Gram-positive bacteria and archaea, providing further evidence of some successful predation and killing of Gram-negative species by *B. bacteriovorus*.

27 days after the two *Bdellovibrio* inoculations had been made, at day 217, *Bdellovibrio* was present at 0.16-0.22% (8.82×10^3-1.39×10^3) in *Bdellovibrio*-treated soil (take-all inoculated and non-take-all inoculated), a higher level than the 0.10% (4822-5429) in the two corresponding buffer the *Bdellovibrio*-treated groups; *Bdellovibrio* had therefore reached a peak before declining again, though some still survived. This dynamic is supported by the comparable decrease in the number of *B. bacteriovorus* cells recovered from the soil in a pot by enumeration on a *P. putida* prey lawn (Figure 33); after 31

days, the number of *B. bacteriovorus* enumerated from the soil had decreased from 1.2 x 10^6 cells per g soil to 7.75 x 10^5 cells per g soil; after 36 days this had decreased more sharply to 1.02 x 10^5 cells per g soil. The dynamics of added *Bdellovibrio* in the soil, rising to a peak and declining slowly towards baseline levels again, strongly indicates that *Bdellovibrio* inoculated in the soil was able to prey upon other bacteria naturally present there. However, once the supply of Gram-negative prey bacteria had been reduced, the added *Bdellovibrio* population declined again, reaching levels comparable to the natural soil *Bdellovibrio* population in the buffer control groups at wheat plant harvest.

In order to further test for the longevity of the *B. bacteriovorus* HD100 treatment in the rhizosphere soil in the pots, samples of soil from all 40 pots in **Pot Experiment 1a** were enriched in liquid cultures with *P. putida* prey cells, on which the *Bdellovibrio* added to the soil were originally grown, before filtering and transferring these enrichments to overlay plates containing a lawn of *P. putida* prey. Enrichments from pots where *Bdellovibrio* had been added produced plaques of clearing on these overlay plates, indicating *Bdellovibrio* predation and killing of the prey cells in the lawn (examples shown in Figure 38 a, b and c), which did not appear on plates where enrichments were from pot soil receiving the calcium HEPES buffer control (shown in Figure 38 d). Additionally, when the original liquid enrichments were examined by light microscopy, small, highly motile *Bdellovibrio*-like bacterial cells were also observed in pots where *Bdellovibrio* had been added, but <u>not</u> in any of the control pots.

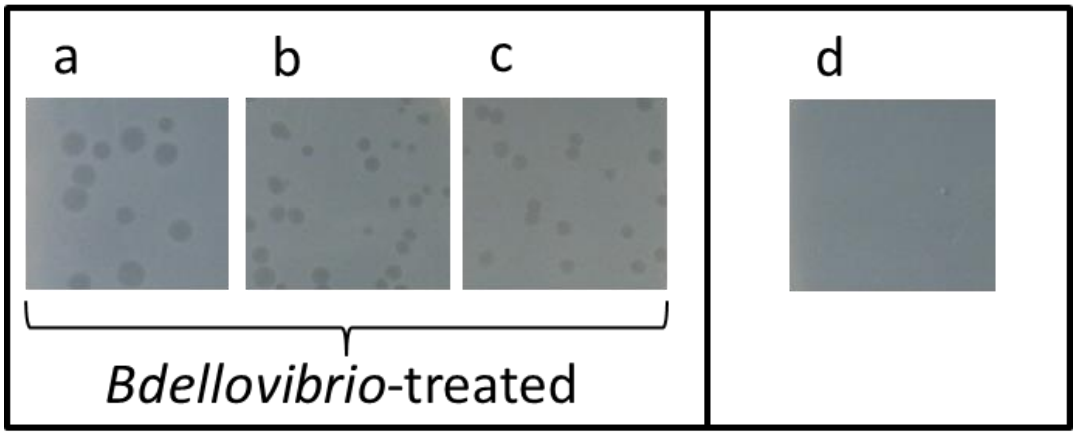


Figure 38 . 'Plaques' on overlay plates of *P. putida* lawns by *Bdellovibrio* from prey-enriched, *Bdellovibrio*-treated pot soil samples (a, b and c) and absence of plaques in the same from buffer control treated samples (d).

These plaque-producing, predatory, *Bdellovibrio*-like cells were identified by 16s rDNA sequencing as *B. bacteriovorus* HD100 using the BLAST online search tool, which gave 99% identity (1467/1469 bases) to the16s rDNA sequence in the *B. bacteriovorus* HD100 genome (GenBank accession no.: BX842648.2). While this level of identity could potentially be a different strain of *Bdellovibrio bacteriovorus,* it provides some further evidence for *Bdellovibrio* survival and persistence in soil, preying upon the species naturally present in the wheat rhizosphere.

5.4.3.6 *Analyses of bacterial diversity in Bdellovibrio treated and buffer control samples using Principal Co-ordinates Analysis (PCoA)*

In order to determine the taxa that contributed the most to variation between all of my samples, and to determine which (if any) taxa showed changes associated with *Bdellovibrio* addition, I conducted a weighted Principal Co-ordinates Analysis (PCoA) using the QIIME analysis software. This analysis uses all the differences in the numbers of each taxon between all samples to produce a set of three components (Principal Coordinates, PCs) that explain as much of the variation in metagenomic composition between samples as possible, with PC1 explaining the most variation, PC2 explaining the next most, and PC3 the third most variation. These PCs are used to produce a 3-

dimensional graph to visualise the relationships between the samples, shown for my data in Figure 39.

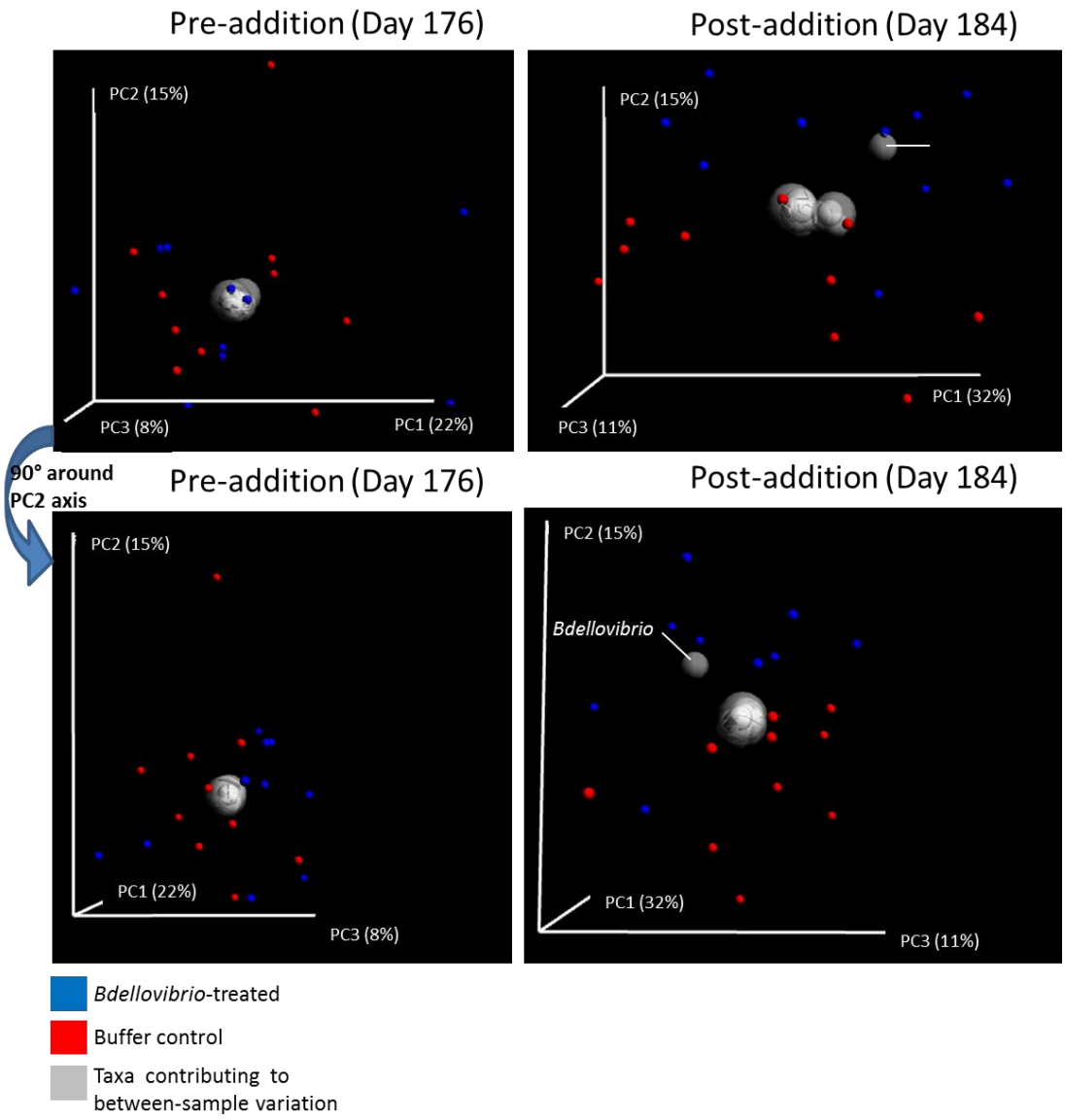


Figure 39 . Principal Coordinate Analysis (PCoA) of inter-sample variation, showing *Bdellovibrio* treated (blue) and buffer control (red) samples, before (left hand graphs) and 48 hours after (right-hand graphs) *Bdellovibrio* or buffer control treatments. The lower graphs are the same as the upper graphs, rotated 90º around the PC2 (vertical) axis to show variation along the PC3 axis. The 10 taxa contributing the most between-sample variation between metagenomic samples are shown as grey spheres: sphere size indicates size of contribution.

Taxa with the greatest contribution to between-sample variation before *Bdellovibrio* or buffer control treatment
k__Bacteria;p__Acidobacteria;c__Acidobacteria-6;o__iii1-15;f__;g__
k__Bacteria;p__Bacteroidetes;c__[Saprospirae];o__[Saprospirales];f__Chitinophagaceae;g__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Rhodoplanes
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Myxococcales;f__;g__
k__Bacteria;p__Actinobacteria;c__Thermoleophilia;o__Gaiellales;f__Gaiellaceae;g__
k__Bacteria;p__Verrucomicrobia;c__[Spartobacteria];o__[Chthoniobacterales];f__[Chthoniobacteraceae];g__DA101
Unassigned;Other;Other;Other;Other;Other
k__Bacteria;p__Verrucomicrobia;c__[Pedosphaerae];o__[Pedosphaerales];f__;g__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Sinobacteraceae;g__
k__Bacteria;p__Acidobacteria;c__[Chloracidobacteria];o__RB41;f__;g__

Taxa with the greatest contribution to between-sample variation after *Bdellovibrio* or buffer control treatment
k__Bacteria;p__Acidobacteria;c__Acidobacteria-6;o__iii1-15;f__;g__
k__Bacteria;p__Actinobacteria;c__Thermoleophilia;o__Gaiellales;f__Gaiellaceae;g__
k__Bacteria;p__Bacteroidetes;c__[Saprospirae];o__[Saprospirales];f__Chitinophagaceae;g__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Rhodoplanes
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Myxococcales;f__;g__
Unassigned;Other;Other;Other;Other;Other
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Bdellovibrionales;f__Bdellovibrionaceae;g__Bdellovibrio
k__Bacteria;p__Actinobacteria;c__Thermoleophilia;o__Solirubrobacterales;f__;g__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Kaistobacter
k__Bacteria;p__Verrucomicrobia;c__[Pedosphaerae];o__[Pedosphaerales];f__;g__

Figure 40 . List of the 10 taxa contributing most between-sample variation for the pre- and post-*Bdellovibrio* and buffer treatment samples shown as grey spheres on the PCoA plots in Figure 39 (taxa appearing in both pre-and post-treatment lists are highlighted in yellow).

The variation explained by each PC is shown on the graph in brackets next to each PC axis. The *Bdellovibrio*-treated and buffer control samples shown on the graph in blue and red, respectively, show no pattern of clustering on the graph together along any of the axes (shown in the left-hand graphs). However, 48 hours after *Bdellovibrio* addition or buffer treatment (shown in the right hand graphs), the *Bdellovibrio* and buffer control treated samples are divided clearly along the PC2 axis which explains the second highest amount of variation (15%).

The 10 taxa contributing the most amount of variation are shown as grey spheres on the graphs in Figure 39, and are listed by name in the tables in Figure 40. As the spheres were so close together on the graphs, and the only way to identify which sphere belonged to which taxon was to label them on the PCoA plots, it was impossible to determine which was which as the labels overlapped. Before any *Bdellovibrio* addition (blue) or buffer control treatments (red) as shown in the left-hand plots in Figure 39, these taxa are tightly clustered together in the centre of the sample distribution on the graph,

and are not distributed along any of the PC axes in particular, indicating that they are not associated with any of the samples more than any of the others. However, after *Bdellovibrio* addition on day 176 (right-hand graphs), the added *Bdellovibrio* is identified (and indicated on the right hand graphs) as a main taxon contributing to the variation between samples, and is higher up on the PC2 axis, more closely associated with *Bdellovibrio*-treated samples (shown in blue) than buffer control (red).

However, none of the other taxa appear to vary along the PC2 axis, and instead are located on the graph at the *Bdellovibrio*-buffer control sample division plane. This indicates that variation in these taxa is not primarily associated with *Bdellovibrio* or buffer control treatment. Instead, these taxa appear to vary slightly more along the PC1 axis; to determine the reason, I labelled each individual *Bdellovibrio*-treated or buffer control sample according to its Pot number, P, identifying the position it held in the randomised plot in the netted tunnel we grew the wheat in; the resultant labelled graph is shown in Figure 41**.**

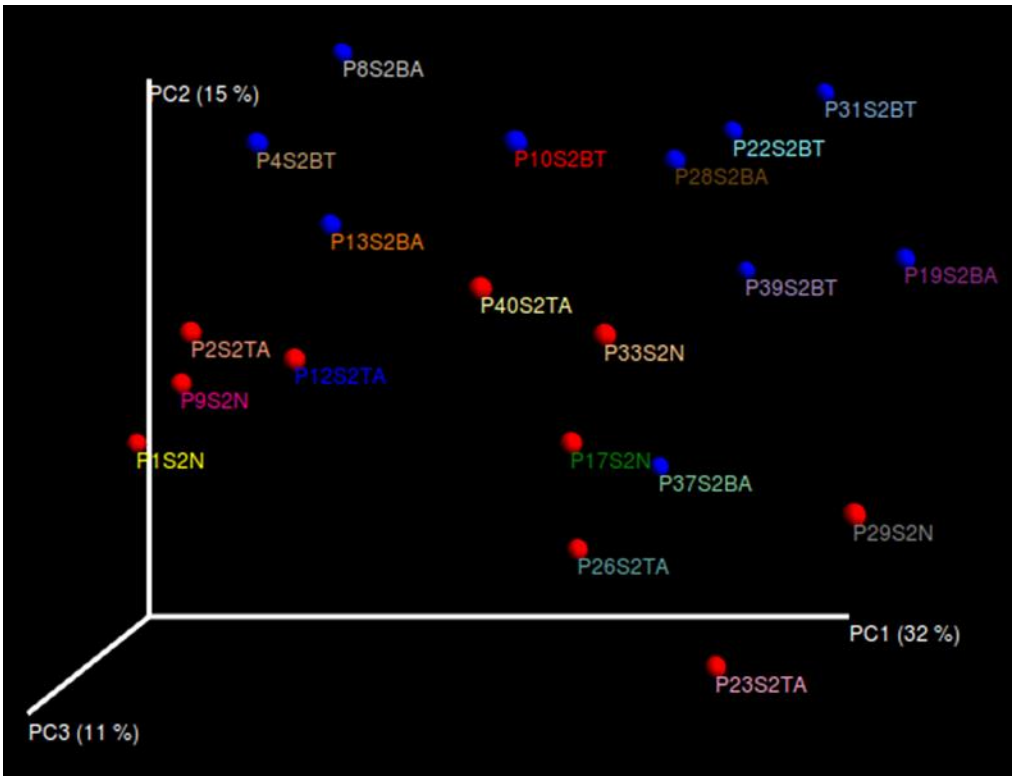


Figure 41 . PCoA plot of all samples 48 hours after *Bdellovibrio* (blue) or buffer control treatment (red), labelled with identifiers containing the pot number (P).

The samples that are further along the PC1 axis are all from higher-numbered pots, which were positioned further north-west in the netted tunnel than the lower numbered pots, which are closer to the origin of the PC1 axis on the graph, and therefore further south-east in the tunnel. Thus there appears to be a positional effect on the microbial community in the pots, likely due to differences in environmental factors, such as sun or wind exposure. This also indicates that the position of the pots accounted for the most variation between samples, as this separation occurs along the PC1 axis (explaining the majority, 32%, of variation); in comparison, *Bdellovibrio* treatment varies along the PC2 axis which explains less variation than PC1 (15%). *Bdellovibrio* treatment therefore appears to be only a secondary contributing factor for variation in the bacterial/archaeal community in the wheat pot rhizospheres, after pot position.

Some taxa explained the most variation in both pre- and post- *Bdellovibrio* or buffer control addition samples, which are highlighted in the tables in Figure 40**.** The majority (7/10) were associated with between-sample variation both

before and after *Bdellovibrio* or buffer treatment, which indicates that *Bdellovibrio* addition didn't alter between-sample variation to any great extent. However, since I observed a drop in Gram-negative bacterial numbers but not Gram-positive or archaea, there may have been many, small changes in taxa rather than singular, large changes, which would not appear in PCoA. This led me to investigate the changes in all individual genera between pre- and post- *Bdellovibrio*-treated samples compared with the buffer control in Section 5.4.3.7, which would help me to find any small, yet significant, changes associated with *Bdellovibrio* addition.

5.4.3.7 *Significant, specific changes in bacteria/archaea in Bdellovibrio-treated versus buffer control samples.*

The QIIME analysis yielded percentage and count data for taxa classified at the lowest rank possible (to the order, family, genus or species level). In the automated assignment of phylogeny carried out in QIIME, as described in Section 2.6.19, could not always resolve sequence differences to the genus or species level, and consequently some of the sequences were classified only by their family or order level.

To determine which specific taxa were significantly affected by *Bdellovibrio* treatment, I conducted a Student's t-test of significance (with Benjamini-Hochberg correction for multiple comparisons) between pre- and post-treatment samples for the percentage amounts of each of the taxa at their lowest level of classification, in each of the two treatment groups separately. I then identified which taxa had increased or decreased significantly, and to a greater extent, in *Bdellovibrio*-treated samples compared with buffer control (the rationale for this approach is explained in Section 5.4.2.1). This was an unbiased approach to analysis, as all taxa were assessed, and none were specifically targeted for analysis because of a wheat-growth promoting process that they are known to carry out (e.g. nitrification or plant hormone production).

This analysis identified 59 taxa for which the percentage amount in the samples were significantly reduced or increased between pre- and post-treatment samples, as shown on the Appendix CD: metagenomic

analysis/raw data.xlsx/Pre vs Post-add 1. Many of these differences, though significant, were small, such as in the genera *Arthrobacter* and *Nostocoidea* and the family *Peptostreptococcaceae* (0.01% increase/decrease corresponding to approximately 10 out of the 1-1.5 x 10^5 16s rDNA sequences that the sequencing process yielded for each 20 ng DNA sample, as shown in Figure 35**,** and corresponding to approximately 2 x 10^3 cells per g soil). As this was such a small difference in the number of sequences (and therefore cells) given that *B. bacteriovorus* was added at 1.4 x 10^{11} per pot and detected at 6.26 x 10^5 - 1.03 x 10^6 cells per g soil (Section 5.4.3.5), it is highly unlikely that such a difference would have any significant effect on plant growth, and so I did not focus on these in this section of the analysis; however, the possibility of an additive effect of small differences in many taxa is considered in Section 5.4.3.8.

The percentages of 25 of these taxa were also significantly different between pre- and post- treatment samples in the buffer control; in some, the differences observed were similar to those observed in *Bdellovibrio*-treated samples, e.g. the genus *Sporosarcina* was increased by 0.01%, (corresponding to approximately 10 cells) in both *Bdellovibrio*-treated and buffer control samples; similarly, the genus *Bacillus* was increased by 0.49% (490 cells) in *Bdellovibrio*-treated samples and by 0.47% (470 cells) in buffer control samples, a difference of just 0.02% (20 cells). However in other taxa, significant differences in percentage between pre- and post- treatment samples had different magnitudes in *Bdellovibrio*-treated and buffer control samples: for example, the genus *Balneimonas* was increased by 0.10% (100 cells) in *Bdellovibrio*-treated samples, compared with an increased 0.05% (50 cells) in buffer control samples; the genus called ‘DA101’ from the family Chthoniobacteriaceae was decreased by 0.98% (980 cells) in *Bdellovibrio*-treated samples, but only by 0.69% (690 cells) in buffer control samples.

Taking these observations into account, I chose 9 taxa for a more detailed analysis, for which larger differences were observed between pre- and post-*Bdellovibrio* treatment samples, but where the difference either had a different magnitude or was not significantly different, or both, in buffer control samples. 3 of these taxa of interest were Gram-positive, and their numbers

increased to a greater extent with *Bdellovibrio* addition, while the remaining 6 were Gram-negative, and their numbers decreased to a greater extent with *Bdellovibrio* addition. This pattern was true for most Gram-positive vs. Gram-negative taxa, as shown on the Appendix CD: metagenomic analysis/raw data.xlsx/Pre vs Post-add 1 (B-H)**.**

To assess the impact of *Bdellovibrio* addition on these taxa over the whole season, I plotted the mean percentage and number of cells per g soil for each taxon in *Bdellovibrio*-treated (blue) and buffer control samples (red) over all 5 sampling points that I took throughout wheat growth, shown in Figure 42 to Figure 44. The accompanying tables, Table 23 to Table 25, show the percentage population changes observed between pre- and post-treatment in close relatives of my 9 chosen taxa, which were largely non-significant, small, and similar between *Bdellovibrio* and buffer control groups; if not, the changes were similar to those observed in the 9 taxa I originally selected, confirming these 9 as suitable candidates for further analysis.

The graphs are grouped firstly by known Gram status (positive or negative), and secondly by the shape of the graph (and therefore the pattern of change in the taxon over time). One of the most apparent differences in the graphs is that the plots of percentage data are very different to those of the count data: the percentage levels fluctuate up and down, but the count data falls over time in all cases. This reflects the drop in the total number of sequence reads in the metagenomic data in later-collected samples, as shown in Figure 35, and may have been due to a number of reasons:

1. There was a natural drop in bacterial numbers in the rhizosphere soil in transition from spring to summer over the main wheat growth period in the pots;

2. Bacteria move from the rhizosphere soil to the wheat root surface over the wheat growth period, attaching themselves to roots, and thus fewer cells remained in my soil samples;

3. It is an artefact of the length of sample storage time (all samples were stored at -20ºC after they were taken, but were all extracted within the same

week, so each set was frozen for a different length of time) or soil quality changes affecting the DNA extraction process, discussed in Section 5.1.7.1.

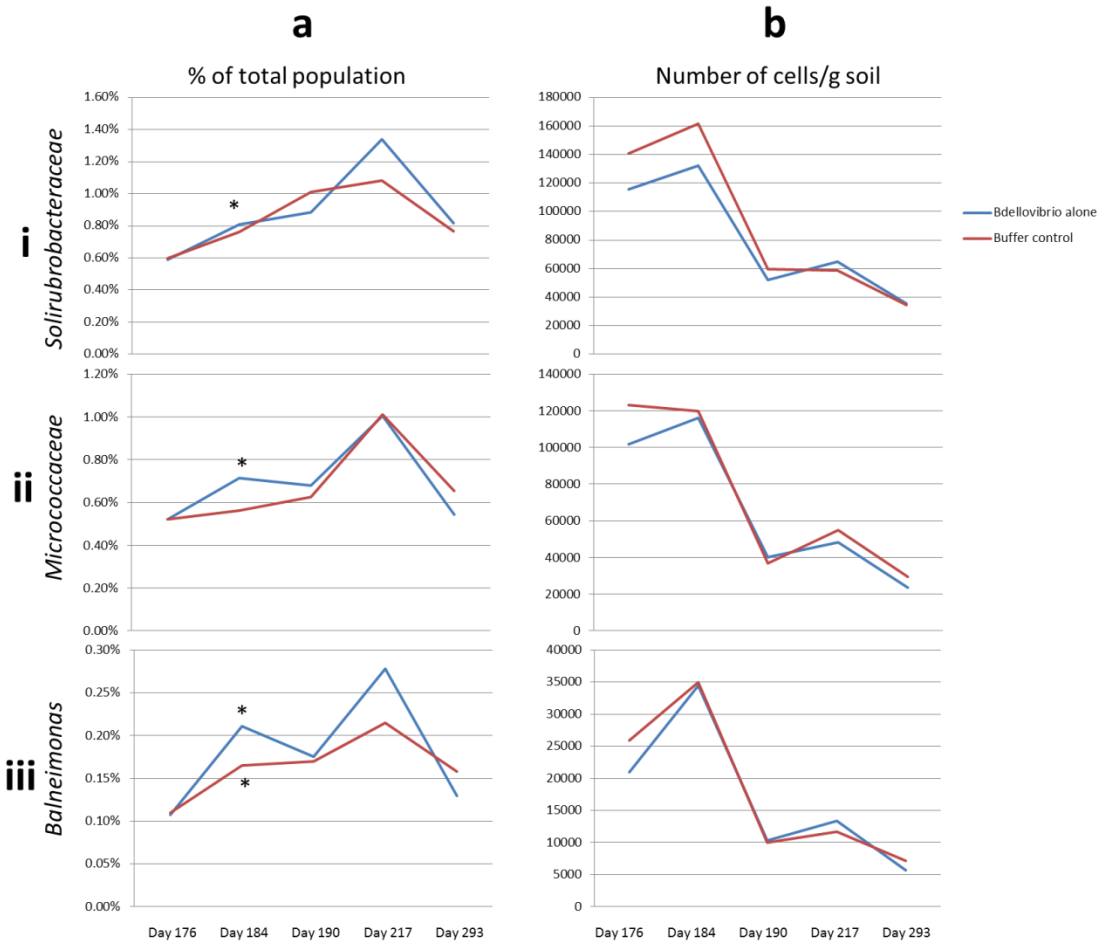


Figure 42 . Three <u>Gram-positive</u> taxa (i, ii, and iii) whose percentage of the total population (a) and number of cells/g of soil (b) were <u>increased</u> to a greater extent in *Bdellovibrio* treated (blue) than buffer control (red) samples. Significant changes between Day 176 (pre- *Bdellovibrio* or buffer control treatment) and Day 184 (48 hours post-treatment) are shown as an asterisk (*) next to the relevant line on the graph.

Table 23 . Excerpt from the analysis of % change in population between samples taken on day 176 (pre-treatment) and day 184 (48 hours after the first treatment) with corresponding Benjamini-Hochberg corrected t-test significance values (BH sig, significant = * non-significant = N/S) in *Bdellovibrio*-treated and buffer control samples. Taxa shown in Figure 42 are highlighted in yellow, in the context of their closest phylogenetic relatives.

	Bdellovibrio		Buffer control	
	% change	BH Sig	% change	BH Sig
;c__Thermoleophilia;o__Solirubrobacterales;f__Patulibacteraceae;g__	0.09%	*	0.11%	*
;c__Thermoleophilia;o__Solirubrobacterales;f__Solirubrobacteraceae;g__	0.22%	*	0.16%	N/S
;c__Thermoleophilia;o__Solirubrobacterales;f__Solirubrobacteraceae;g__Solirubrobacter	0.00%	N/S	0.00%	N/S
;c__Thermoleophilia;o__Solirubrobacterales;f__Solirubrobacteraceae;Other	0.01%	N/S	0.01%	*
;c__Thermoleophilia;o__Solirubrobacterales;Other;Other	0.02%	*	0.01%	N/S
;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;Other	0.00%	N/S	0.01%	N/S
;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae;g__	0.19%	*	0.04%	N/S
;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae;g__Arthrobacter	0.01%	*	0.01%	N/S
;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae;g__Microbispora	0.00%	N/S	0.00%	N/S
;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae;g__Rothia	0.00%	N/S	0.00%	N/S
;c__Actinobacteria;o__Actinomycetales;f__Micromonosporaceae;g__	0.05%	N/S	0.02%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae;g__Afipia	0.00%	N/S	0.00%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae;g__Balneimonas	0.10%	*	0.05%	*
a;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae;g__Bosea	-0.03%	N/S	0.00%	N/S

The mean percentage of the three Gram-positive taxa I identified, shown in Figure 42, increased in *Bdellovibrio*-treated samples between pre-treatment (Day 176) and 48 hours after the first *Bdellovibrio* treatment (Day 184), to a greater extent than in the buffer control samples. This may be due to predatory killing of Gram-negative species by *Bdellovibrio,* reducing their occupation of niches that may subsequently have been occupied by these Gram-positive species.

After the initial rise, there was a drop in the percentage of *Micrococcaceae* and *Balneimonas* from 48 h after the first *Bdellovibrio* addition (Day 184) until 48 h after the second *Bdellovibrio* addition (Day 190), but a rise in the corresponding buffer controls; conversely, there was a rise in *Solirubrobacteraceae* over the same period in *Bdellovibrio*-treated samples, but this was smaller than the rise that occurred in the buffer control. That the first *Bdellovibrio* addition was associated with a rise in percentage of these Gram-positive taxa, but that a drop in *Balneimonas* and *Micrococcaceae* occurred after the second addition was initially surprising; however, dispersal or death of these bacteria could have occurred due to competition with other Gram-positive bacteria or archaea inhabiting the same niche that were also increased by *Bdellovibrio* addition, thus limiting their population growth. This

could also have been due to the blooming of a *Solirubrobacteraceae* species-specific phage in the soil.

Between 48 hours and 29 days after the second *Bdellovibrio* addition (Day 190-217), the percentage levels of all three genera in the total population peaked before declining again from 29 days after the second addition until wheat harvest (Day 293); in *Balneimonas* and *Solirubrobacteraceae,* the peak 29 days after *Bdellovibrio* addition was greater than in the buffer control samples, but in the *Micrococcaceae* the peak was similar. In *Balneimonas* and *Solirubrobacteraceae,* this exaggerated peak compared with the buffer control may have been due to a disruption of normal bacterial growth patterns in the soil relative to the levels of other bacterial species and possibly phage in the soil, thus making the cycle of rises and falls that naturally occur in the balance of species more pronounced; the overall shape of the buffer control curves in all three Gram-positive species in Figure 42 undulates to a lesser extent over time than the curves for *Bdellovibrio*-treated samples, which supports this explanation. However, it is important to note that the data were very variable (as explained in Section 5.4.3.3), which may have affected some of these differences between *Bdellovibrio*-treated and buffer control samples.

In the case of the 6 Gram-negative taxa that I identified, shown in Figure 43 and Figure 44, the percentage that each taxon represented relative to the whole bacterial/archaeal population decreased significantly in *Bdellovibrio*-treated samples between pre-treatment (Day 176) and 48 hours after the first *Bdellovibrio* treatment (Day 184); but as with the Gram-positive taxa, this change occurred to a greater extent than in the buffer control. The overall pattern of change in percentage of each taxon across the 5 sampling points throughout wheat growth largely followed two different shapes. In Figure 43, the curves produced by joining the percentage that each taxon represented at the different time points is shaped like a reclining man, with a drop in percentage followed by a rise followed by another drop or a plateau, while in Figure 44, the curves are hammock-shaped, with a drop in percentage early in the season followed by a rise later on.

As with the Gram-positive bacteria, these patterns in bacterial numbers appeared to be exaggerated in the *Bdellovibrio*-treated samples compared with the buffer control; in Figure 43a, the initial significant decrease in percentage of the 3 taxa from pre-*Bdellovibrio* addition to 48 hours after addition was greater than in the buffer control, but, for *Chthoniobacteraceae* and *Rhizobiales* there was less of a decrease from 48 hours after the first *Bdellovibrio* addition to 48 hours after the second addition in *Bdellovibrio*-treated samples than in the buffer control. Crucially, this pattern was also observable in the corresponding cell number/g soil graphs shown on the right hand side of Figure 43b. The pattern observed here may be a result of the relative abundance of prey in the pot soil before the first *Bdellovibrio* addition compared with the second; as prey cell numbers were reduced, likely by predation, prey encounter by the second dose of *Bdellovibrio* cells would also be reduced, and the rate of predation would have decreased.

This pattern is slightly different for the *Chitinophagaceae*; there was an increase in their percentage relative to the whole bacterial/archaeal population from 48 hours after the first *Bdellovibrio* treatment to 48 hours after the second treatment, but the number of cells per g soil decreased at a rate similar to that observed between pre-addition and 48 hours after the first *Bdellovibrio* addition; conversely, the cell number per g soil in the corresponding buffer control decreased to a greater extent from 48 hours after the first treatment to 48 hours after the second treatment, compared with *Bdellovibrio*-treated samples. Despite these slight differences in population patterns between the 3 taxa shown in Figure 43, the net result is the same: a more rapid initial decline in percentage and cell number per g soil where *Bdellovibrio* was added, which subsequently slowed, resulting in percentage and cell number reaching similar values between *Bdellovibrio*-added and buffer control samples by 48 hours after the second treatment.

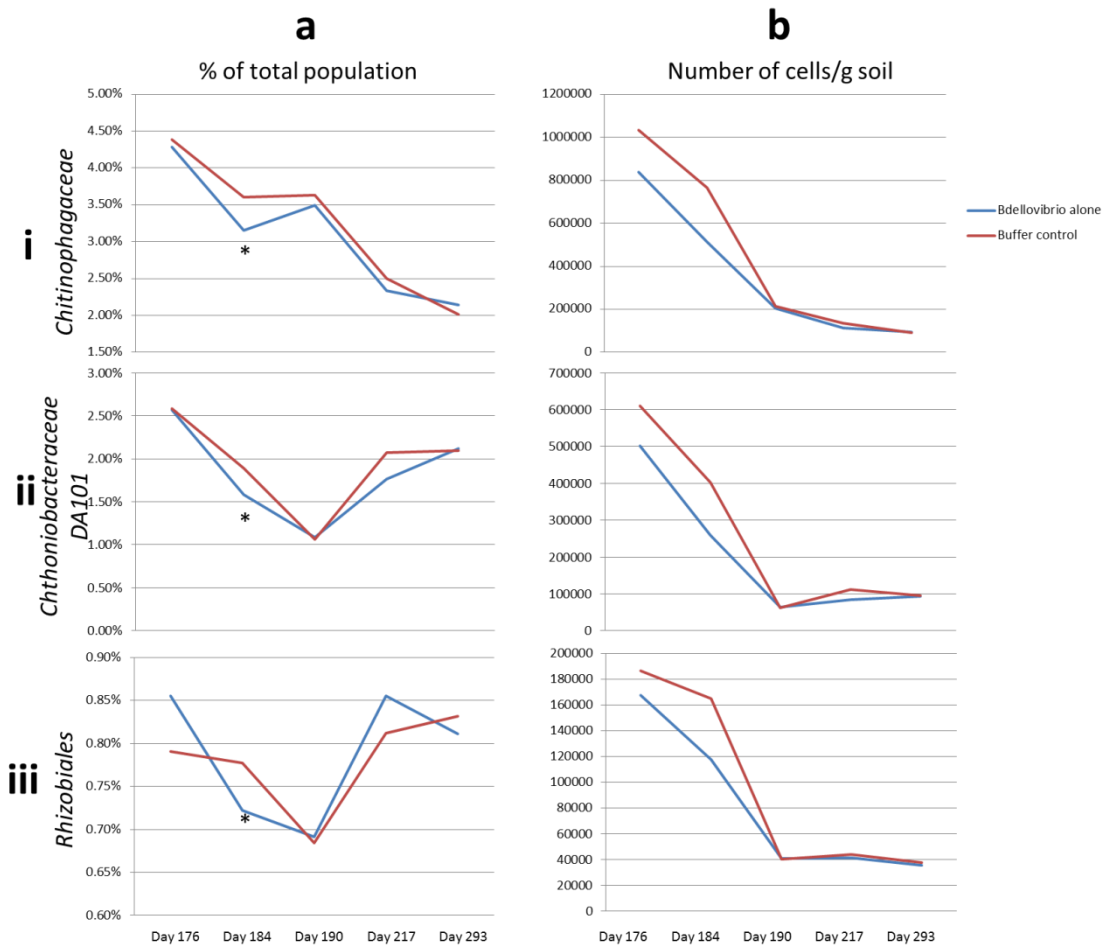


Figure 43 . Three Gram-negative taxa (i, ii, and iii) whose percentage of the total population (a) and number of cells/g of soil (b) were decreased to a greater extent in *Bdellovibrio* treated (blue) than buffer control (red) samples. The lines made by joining the percentage values over the 5 sampling points are shaped like a reclining man, with a fall-rise-fall pattern. Significant changes between Day 176 (pre- *Bdellovibrio* or buffer control treatment) and Day 184 (48 hours post-treatment) are shown as an asterisk (*) next to the relevant line on the graph.

Table 24 . Excerpt from the analysis of % change in population between samples taken on day 176 (pre-treatment) and day 184 (48 hours after the first treatment) with corresponding Benjamini-Hochberg corrected t-test significance values (BH sig, significant = * non-significant = N/S) in *Bdellovibrio*-treated and buffer control samples. Taxa shown in Figure 43 are highlighted in yellow, in the context of their closest phylogenetic relatives.

	Bdellovibrio		Buffer control	
	% change	BH Sig	% change	BH Sig
;c__[Saprospirae];o__[Saprospirales];f__;g__	-0.03%	N/S	-0.03%	N/S
;c__[Saprospirae];o__[Saprospirales];f__Chitinophagaceae;g__	-1.13%	*	-0.78%	N/S
;c__[Saprospirae];o__[Saprospirales];f__Chitinophagaceae;g__Chitinophaga	0.00%	N/S	-0.02%	N/S
;c__[Saprospirae];o__[Saprospirales];f__Chitinophagaceae;g__Flavisolibacter	-0.03%	N/S	-0.03%	N/S
;c__[Saprospirae];o__[Saprospirales];f__Chitinophagaceae;g__Niabella	0.00%	N/S	0.00%	N/S
;c__[Saprospirae];o__[Saprospirales];f__Chitinophagaceae;g__Niastella	0.00%	N/S	0.00%	N/S
;c__[Saprospirae];o__[Saprospirales];f__Chitinophagaceae;g__Sediminibacterium	-0.02%	N/S	-0.02%	N/S
;c__[Saprospirae];o__[Saprospirales];f__Chitinophagaceae;g__Segetibacter	0.00%	N/S	0.02%	N/S
;c__[Saprospirae];o__[Saprospirales];f__Chitinophagaceae;g__Terrimonas	0.00%	N/S	0.00%	N/S
;c__[Saprospirae];o__[Saprospirales];f__Saprospiraceae;g__	-0.18%	*	-0.16%	N/S
;c__At12OctB3;o__;f__;g__	0.01%	*	0.01%	N/S
ɔia;c__[Pedosphaerae];o__[Pedosphaerales];Other;Other	-0.03%	N/S	-0.01%	N/S
ɔia;c__[Spartobacteria];o__[Chthoniobacterales];f__[Chthoniobacteraceae];g__	-0.16%	*	-0.10%	N/S
ɔia;c__[Spartobacteria];o__[Chthoniobacterales];f__[Chthoniobacteraceae];g__Candidatus Xiphinematobacter	-0.17%	*	-0.15%	*
ɔia;c__[Spartobacteria];o__[Chthoniobacterales];f__[Chthoniobacteraceae];g__Chthoniobacter	-0.06%	*	-0.03%	N/S
ɔia;c__[Spartobacteria];o__[Chthoniobacterales];f__[Chthoniobacteraceae];g__DA101	-0.98%	*	-0.69%	N/S
ɔia;c__[Spartobacteria];o__[Chthoniobacterales];f__[Chthoniobacteraceae];g__Ellin506	-0.01%	N/S	-0.01%	N/S
ɔia;c__[Spartobacteria];o__[Chthoniobacterales];f__[Chthoniobacteraceae];g__OR-59	-0.02%	N/S	-0.01%	N/S
ɔia;c__[Spartobacteria];o__[Chthoniobacterales];f__01D2Z36;g__	0.00%	N/S	0.00%	N/S
a;c__Alphaproteobacteria;o__Kiloniellales;f__;g__	0.00%	N/S	0.00%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__;g__	-0.13%	*	-0.01%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Aurantimonadaceae;g__	0.00%	N/S	0.00%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Aurantimonadaceae;Other	0.00%	N/S	0.00%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Bartonellaceae;g__	0.00%	N/S	0.00%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Beijerinckiaceae;g__	0.01%	N/S	0.02%	*
a;c__Alphaproteobacteria;o__Rhizobiales;f__Beijerinckiaceae;g__Beijerinckia	0.00%	N/S	0.00%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Beijerinckiaceae;g__Chelatococcus	0.00%	N/S	0.00%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae;g__	-0.27%	N/S	-0.03%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae;g__Afipia	0.00%	N/S	0.00%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae;g__Balneimonas	0.10%	*	0.05%	*
a;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae;g__Bosea	-0.03%	N/S	0.00%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae;g__Bradyrhizobium	-0.01%	N/S	0.00%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae;g__Nitrobacter	0.00%	N/S	0.00%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae;Other	-0.01%	N/S	0.01%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Brucellaceae;g__Ochrobactrum	0.00%	N/S	0.00%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Brucellaceae;Other	0.00%	N/S	0.00%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__	-0.30%	*	-0.09%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Devosia	-0.01%	N/S	0.04%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Hyphomicrobium	-0.02%	N/S	0.01%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Pedomicrobium	-0.01%	N/S	-0.02%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Rhodobium	0.00%	N/S	0.00%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Rhodoplanes	-0.41%	N/S	-0.12%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;Other	-0.03%	*	-0.02%	*
a;c__Alphaproteobacteria;o__Rhizobiales;f__Methylocystaceae;g__	-0.01%	N/S	0.01%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Methylocystaceae;g__Methylopila	0.00%	N/S	0.00%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Methylocystaceae;g__Methylosinus	0.00%	N/S	0.00%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Phyllobacteriaceae;g__	0.03%	N/S	0.04%	*
a;c__Alphaproteobacteria;o__Rhizobiales;f__Phyllobacteriaceae;g__Mesorhizobium	0.01%	N/S	0.01%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Phyllobacteriaceae;g__Phyllobacterium	0.02%	N/S	0.01%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Phyllobacteriaceae;Other	0.01%	N/S	0.01%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__	0.00%	N/S	0.02%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__Agrobacterium	0.03%	N/S	-0.01%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__Kaistia	0.00%	N/S	0.01%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__Rhizobium	0.01%	N/S	0.01%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;Other	0.04%	N/S	0.03%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Rhodobiaceae;g__Afifella	-0.01%	N/S	0.00%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Xanthobacteraceae;g__	0.01%	N/S	0.00%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Xanthobacteraceae;g__Blastochloris	0.00%	N/S	0.01%	*
a;c__Alphaproteobacteria;o__Rhizobiales;f__Xanthobacteraceae;g__Labrys	0.00%	N/S	0.00%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;f__Xanthobacteraceae;Other	0.00%	*	0.00%	N/S
a;c__Alphaproteobacteria;o__Rhizobiales;Other;Other	-0.01%	N/S	0.00%	N/S
a;c__Alphaproteobacteria;o__Rhodobacterales;f__Hyphomonadaceae;g__	-0.03%	N/S	-0.03%	N/S

By 48 hours after the second *Bdellovibrio* or buffer control additions on Day 190, the percentage and number of each taxon shown in Figure 43 was similar between the two treatments, and the pattern for the two treatment groups from this time point onwards was also relatively similar, indicating that the effect that *Bdellovibrio* treatment had was limited to a short period of time after it was added. The *Rhizobiales* were a potential exception, as their percentage increased to a greater extent from 48 hours to 29 days after the second *Bdellovibrio* addition (at day 217) compared with the buffer control; however, the number of cells/g soil remained relatively similar, so, if this difference is not due to between-sample variation, it only indicates that the *Rhizobiales* population was at a higher level relative to other species in the soil in *Bdellovibrio*-treated samples compared with buffer control samples, and may have survived or recovered more rapidly after *Bdellovibrio* treatment of the soil.

As in Figure 43, Figure 44 shows that after the initial significant reduction in percentage of these three Gram-negative taxa, there was a smaller reduction between 48 hours after the first *Bdellovibrio* addition (Day 184) and 48 hours after the second *Bdellovibrio* addition (Day 190) in all three taxa, particularly *Solibacter* and *Pirellulaceae,* compared with the buffer control in which the decreases in percentage of all three taxa are more consistent from pre-buffer-addition to 48 hours after the second buffer addition. A larger drop in cell number per g soil is observed between pre-treatment and 48 hours after the first treatment in *Bdellovibrio*-treated samples when compared with the buffer control, but larger decreases in cell number per g soil were observed in the buffer control relative to the *Bdellovibrio*-treated samples from 48 hours after the first treatment to 48 hours after the second treatment, so the cell number per g soil were at similar levels between the two treatments for all 3 taxa. These data therefore show that *Bdellovibrio* treatment results in a larger initial drop in percentage and cell number than the buffer control in these soil mesocosms, but that this more rapid decrease is not sustained when more *Bdellovibrio* were added in the second inoculation.

All three Gram-negative taxa in Figure 44 were present at a lower percentage and cell number in *Bdellovibrio*-treated samples compared with buffer control

29 days after the second *Bdellovibrio* or buffer control treatment (Day 217), suggesting that *Bdellovibrio* treatment has a small longer-term effect on population stability or growth in these taxa. However, at wheat harvest, the mean percentage and number of *Pedosphaerae* and *Pirellulaceae* were similar in *Bdellovibrio*-treated and control samples, so this longer-term effect was still limited. In contrast, the percentage and number of *Solibacter* remained lower in *Bdellovibrio*-treated compared with buffer control samples at wheat harvest; this indicates that the population stability and growth may have been reduced by *Bdellovibrio* addition to a more long-lasting extent in *Solibacter* than in other taxa that were reduced by *Bdellovibrio* predation. It may therefore be that when the *Solibacter* population was initially reduced, another species may have inhabited the niche it left behind, constraining *Solibacter* population recovery once the *Bdellovibrio* population had been reduced.

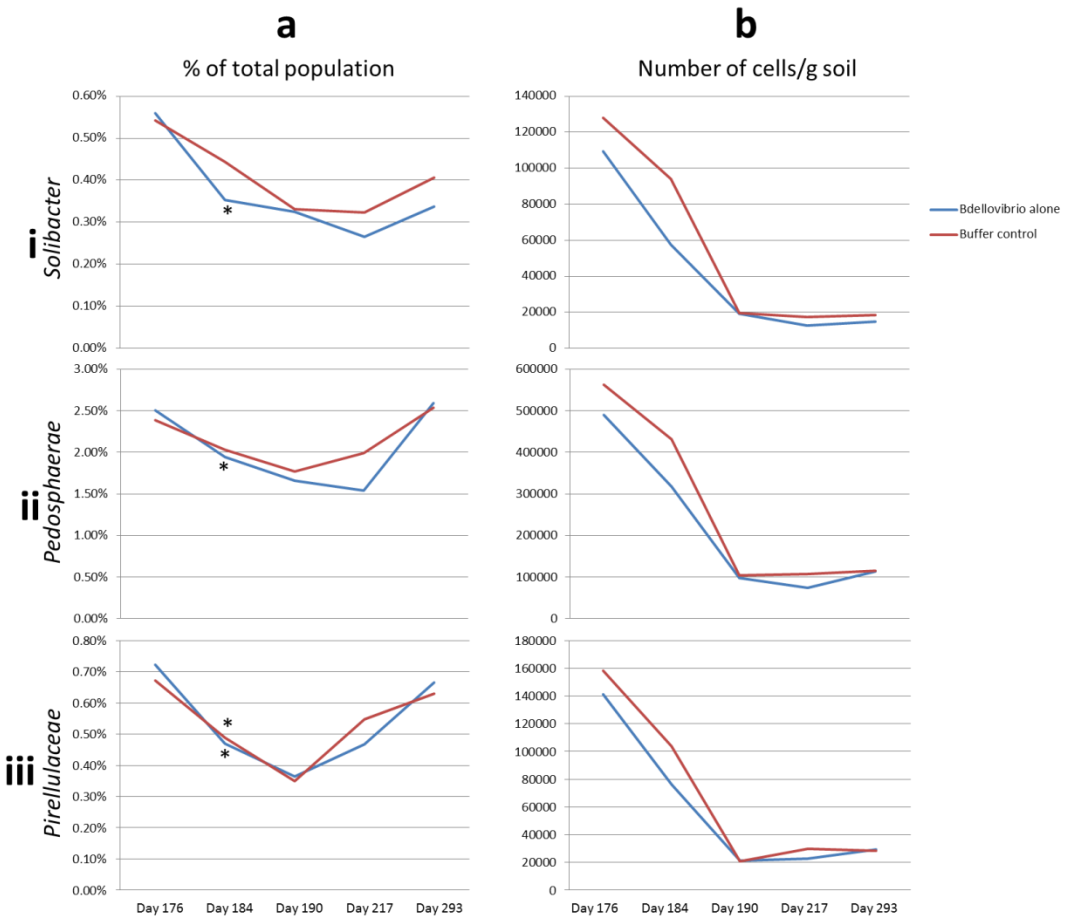


Figure 44 . Three Gram-negative taxa (i, ii, and iii) whose percentage of the total population (a) and number of cells/g of soil (b) were decreased to a greater extent in *Bdellovibrio* treated (blue) than buffer control (red) samples. The lines made by joining the percentage values over the 5 sampling points are U or hammock-shaped, with a fall followed by a rise. Significant changes between Day 176 (pre- *Bdellovibrio* or buffer control treatment) and Day 184 (48 hours post-treatment) are shown as an asterisk (*) next to the relevant line on the graph.

Table 25 . Excerpt from the analysis of % change in population between samples taken on day 176 (pre-treatment) and day 184 (48 hours after the first treatment) with corresponding Benjamini-Hochberg corrected t-test significance values ('BH sig', significant = * non-significant = N/S) in *Bdellovibrio*-treated and buffer control samples. Taxa shown in Figure 44 are highlighted in yellow, in the context of their closest phylogenetic relatives.

	Bdellovibrio		Buffer control	
	% change	BH Sig	% change	BH Sig
__Solibacteres;o__Solibacterales;f__Solibacteraceae;g__	0.00%	N/S	0.00%	N/S
__Solibacteres;o__Solibacterales;f__Solibacteraceae;g__Candidatus Solibacter	-0.21%	*	-0.10%	N/S
__Solibacteres;o__Solibacterales;f__Solibacteraceae;Other	0.00%	N/S	0.00%	N/S
a;c__[Methylacidiphilae];o__S-BQ2-57;f__;g__	-0.01%	N/S	-0.01%	N/S
a;c__[Pedosphaerae];o__[Pedosphaerales];f__;g__	-0.56%	*	-0.35%	N/S
a;c__[Pedosphaerae];o__[Pedosphaerales];f__[Pedosphaeraceae];g__	0.00%	N/S	0.00%	N/S
a;c__[Pedosphaerae];o__[Pedosphaerales];f__[Pedosphaeraceae];g__Pedosphaera	0.00%	N/S	0.01%	N/S
a;c__[Pedosphaerae];o__[Pedosphaerales];f__auto67_4W;g__	-0.16%	N/S	-0.19%	N/S
a;c__[Pedosphaerae];o__[Pedosphaerales];f__Ellin515;g__	-0.14%	*	-0.03%	N/S
a;c__[Pedosphaerae];o__[Pedosphaerales];f__Ellin517;g__	-0.14%	N/S	-0.07%	N/S
a;c__[Pedosphaerae];o__[Pedosphaerales];f__R4-41B;g__	-0.01%	N/S	-0.02%	N/S
a;c__[Pedosphaerae];o__[Pedosphaerales];Other;Other	-0.03%	N/S	-0.01%	N/S
a;c__[Spartobacteria];o__[Chthoniobacterales];f__[Chthoniobacteraceae];g__	-0.16%	N/S	-0.10%	N/S
;c__Planctomycetia;o__Gemmatales;f__Isosphaeraceae;Other	0.00%	N/S	0.00%	N/S
;c__Planctomycetia;o__Pirellulales;f__Pirellulaceae;g__	-0.25%	*	-0.18%	*
;c__Planctomycetia;o__Pirellulales;f__Pirellulaceae;g__A17	-0.03%	*	-0.03%	*
;c__Planctomycetia;o__Pirellulales;f__Pirellulaceae;g__Pirellula	-0.08%	*	-0.06%	*
;c__Planctomycetia;o__Pirellulales;f__Pirellulaceae;Other	0.00%	N/S	0.00%	N/S
;c__Planctomycetia;o__Planctomycetales;f__Planctomycetaceae;g__Planctomyces	-0.07%	*	-0.06%	*

5.4.3.8 *Trends in genus-level changes in Aerobic, Anaerobic, nitrogen-fixing, Nitrifying, and PGPR species in the soil*

In Section 5.4.3.7, I looked at significant changes in genera after *Bdellovibrio* treatment without first targeting specific genera for functions that may improve wheat growth, which provided an unbiased initial analysis. However, next, I used Brock Biology of Microorganisms, and MicrobeWiki (https://microbewiki.kenyon.edu/index.php/MicrobeWiki) and my previous literature review in Section 5.1.7 to search for soil bacteria/archaea that could be grouped as strictly Aerobic or Anaerobic, nitrogen-fixing, Nitrifying or Denitrifying, and wheat plant growth-promoting (PGPR). Once I had found these groups of bacteria/archaea, I searched for them in the metagenomic data from my soil samples, including genera that had not changed significantly with *Bdellovibrio* treatment between pre-addition and 48 hours after the first addition (and therefore weren't included in the analysis in Section 5.4.3.7). Instead, my criteria for their inclusion in this analysis were:

- That the taxon belonged to one of the functional groups mentioned above; and
- The taxon was present in **both *Bdellovibrio* and buffer control** sample; and
- The taxon was detected in my sequence reads at **at least one time point** (before treatment on day 176 or 48 hours after the first treatment on day 184- some were reduced to 0 sequence reads at 48 hours after the first treatment).

I then plotted these grouped genera together in bubble charts, to show the difference in levels between pre- and post- treatment samples in both *Bdellovibrio*-treated and buffer control groups (plotted on the x-axis), in both percentage level (y-axis) and number/g soil (size of each bubble) taken together, because these values were not correlated perfectly with one another, as I showed in Section 5.4.3.7. I do not differentiate between genera on the graph by name, because the purpose of this analysis was instead to look at trends in groups of bacterial/archaeal genera and avoid taking individual patterns into account.However, the list and corresponding percentages and numbers/g soil of the genera I included in each group are given on the Appendix CD: metagenomic analysis/raw data.xlsx/Aerobic vs. Anaerobic, N-ifiers + N-fixers, PGPR.

It was sometimes difficult to see the differences in genera present at a very low level in the soil (<1 x 10^3 cells/g soil, equivalent to approximately 10 reads in my samples of 1 x 10^5 reads) when plotted alongside those that were present at a higher level (>1 x 10^4 cells/g soil, corresponding to 100); therefore, alongside the plots of untransformed data, which showed the differences in high-level genera very well, I have included graphs on which the percentage data has been transformed by taking $\log_{10}$ of each value. Thus, genera present at a low level were more spread out along the y-axis in these graphs, and differences were more easily observed between *Bdellovibrio*-treated and control samples, before and after treatment. I also tried taking $\log_{10}$ of the number of cells/g soil, but this transformation was not particularly helpful when looking at differences, as all the circle sizes were large. Nevertheless, I have included these graphs for completion.

It is important to note that the high levels of some genera are due to the fact that **the QIIME phylogenetic assignments that were performed frequently do not reach the genus-level, and sometimes only reach order or family level, and thus may include several genera.** However, I made sure to only include order or family-level classified bacteria/archaea when I was certain that all genera contained within that classification could be included in the groups that I had made. Conversely, the QIIME phylogenetic assignments rarely reached the species-level in my metagenomic data set; this precluded the analysis of denitrifying bacteria, as they are phylogenetically spread out between different genera and are therefore only identified as denitrifiers by their species classification. Thus I was unable to find any denitrifiers in my data set, though they may have been present in the soil but counted along with other species in the same genus.

The purpose of this part of my analysis was thus to determine whether any patterns of response to *Bdellovibrio* addition were unique to any particular functional group of genera that may explain the differences in wheat growth that I observed in **Pot Experiment 1a**. The first group of bacteria/archaea I looked at were nitrifiers and nitrogen-fixers, shown in Figure 45; this is because N levels in the pot soil were relatively low, as shown in my chemical analyses, and adequate N is vital for good wheat plant growth, as I explained in Section 5.1.5.1**.** The improved growth of *Bdellovibrio*-treated wheat that I observed may have resulted from positive changes in the level of bacteria/archaea in these groups that increase N levels in soil.

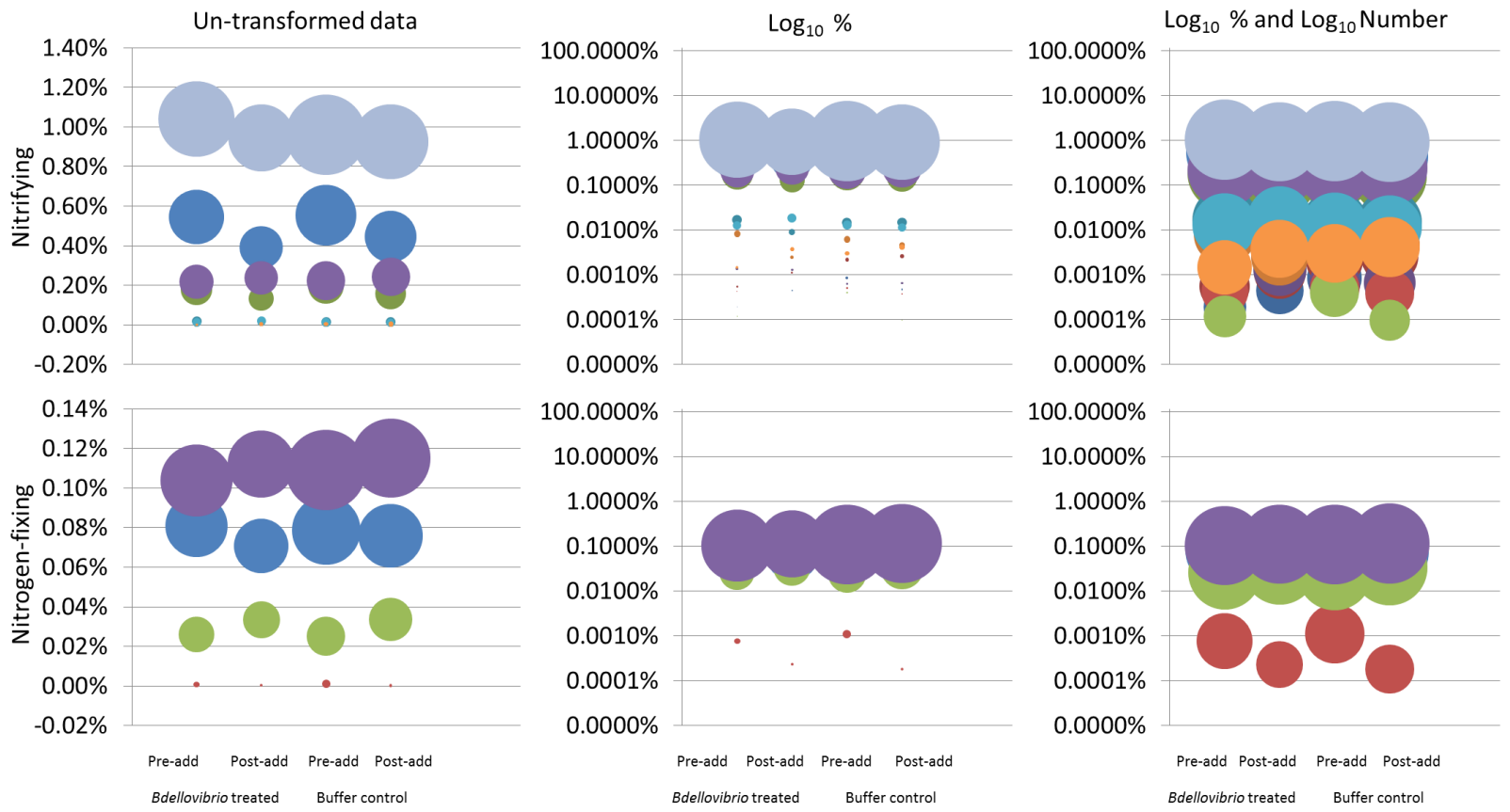


Figure 45 . The percentage (%, y-axis) of total reads and number/g soil (displayed as bubble size) of Nitrifying (i) and nitrogen-fixing (ii) genera in the soil from Pot Experiment 1a, showing levels in *Bdellovibrio* and buffer control treated samples, both before (pre-add) and 48 hours after the first addition (post-add). Data are shown un-transformed (a), with Log_{10}-transformed percentage (b), and with Log_{10}-transformed % and number/g of soil (c) for ease of analysis.

The percentage and number of Nitrifying genera (top-left and top-middle graphs) were reduced slightly in the buffer control from pre-addition to 48 hours after the first addition was made. However, these levels were reduced to a greater extent where *Bdellovibrio* was added, for genera that were present at a high level in the samples (shown most clearly in Figure 45a (i)) and at a low level (Figure 45b (i)). The percentage and number of some low-level genera were increased after *Bdellovibrio* addition, but they were present at such a low level (0.0001%-0.001% and <100 cells per g soil) that these changes would have an extremely small impact on N levels in the soil compared with the large relative decreases in percentage and number of high-level genera.

Similarly, the level of N-fixing genera decreased to a greater extent (or increased to a lesser extent) after *Bdellovibrio* addition compared with the buffer control, shown in the bottom-left and middle-left graphs, particularly in genera present at a high starting level (there was one taxon present at a low level, for which the change with *Bdellovibrio* addition was relatively similar to that with the buffer control, if a slightly smaller decrease). It should be noted that these genera are classified as legume-symbiotic N-fixers that are not known to be associated with wheat (no free-living N-fixers could be found in my data set due to classifications not reaching the required taxonomic level to distinguish from non-N-fixing genera), but I included them anyway in case their pattern helped explain the increased wheat growth that I observed in **Pot Experiment 1a**.

The next groups of bacteria/archaea I looked at were those I describe as aerobic and anaerobic bacteria, shown in Figure 46 and named in Figure 47**.** An anaerobic or aerobic environment in the soil depends largely on the moisture content in the soil; waterlogged soils are anaerobic environments, while very dry soils are largely aerobic. Moisture content is affected by the structure of the soil, as I explained in Section 5.1.6: soils that have a greater network of interconnected pores and variable sizes of soil particles, which are generally considered ‘good’ agricultural soils conducive to wheat growth, retain water well and thus spend more time as anaerobic environments. Cohesive, clayey soils with a reduced network of pores do not retain water

well and dry out easily in sunny weather to form a hard, dry cast, thus spending more time as aerobic environments. The level of aerobic vs. anaerobic genera in the soils treated with *Bdellovibrio* compared with a buffer control may therefore give some indication of the soil moisture content and structure, and whether this may have been different between treatments.

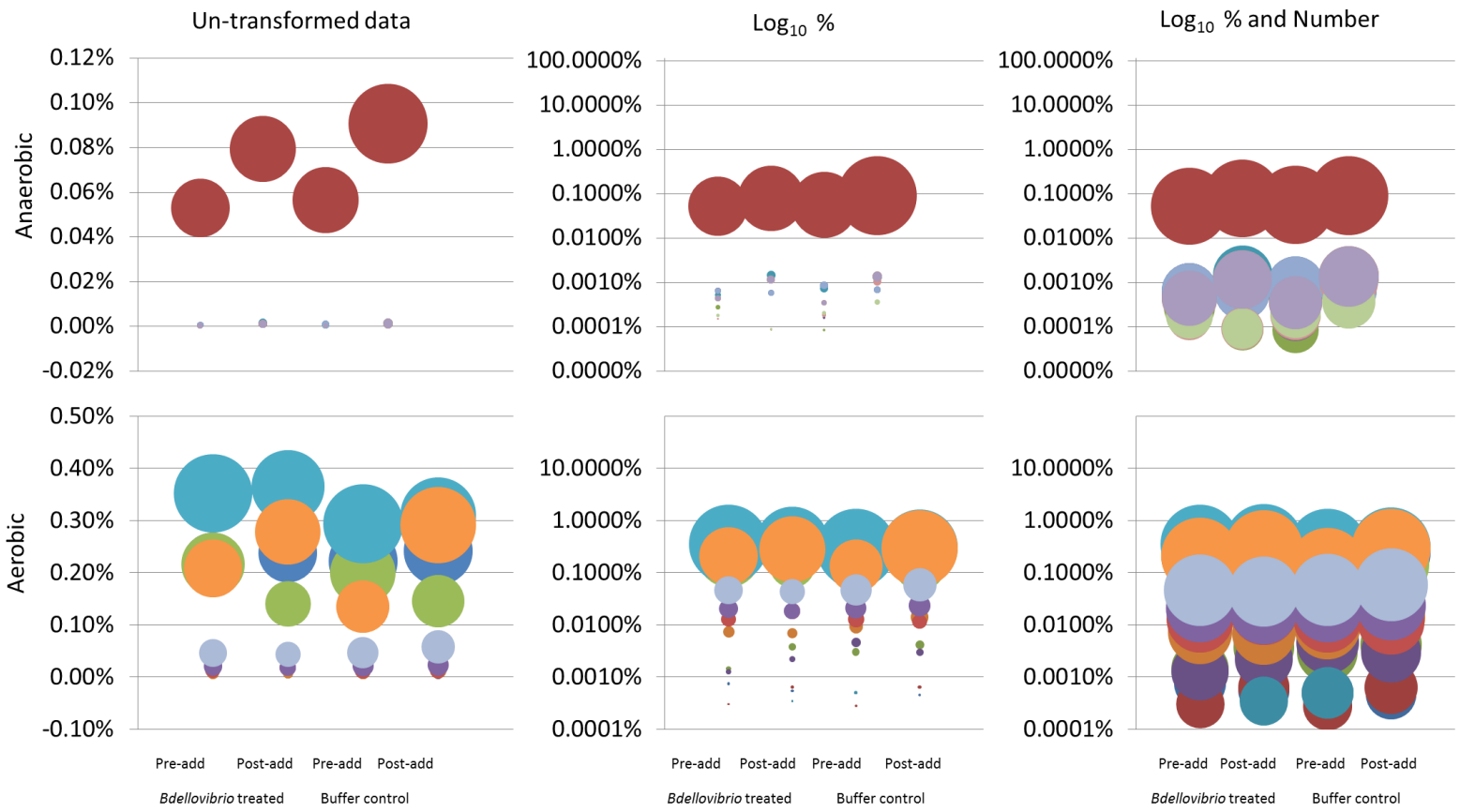


Figure 46 . The percentage of total reads (%, y-axis) and number/g soil (displayed as bubble size) of anaerobic (i) and aerobic (ii) genera in the soil from Pot Experiment 1a, showing levels in *Bdellovibrio* and buffer control treated samples, both before (pre-add) and 48 hours after the first addition (post-add). Data are shown un-transformed (a), with Log_{10}-transformed percentage (b), and with Log_{10}-transformed % and number/g of soil (c) for ease of analysis.

Anaerobic taxa
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfovibrionales;f__Desulfovibrionaceae;g__Desulfovibrio
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus
k__Archaea;p__Euryarchaeota;c__Methanomicrobia;o__Methanosarcinales;f__Methanosarcinaceae;g__Methanosarcina
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides
k__Archaea;p__Euryarchaeota;c__Methanobacteria;o__Methanobacteriales;f__Methanobacteriaceae;g__Methanobrevibacter
k__Archaea;p__Euryarchaeota;c__Methanobacteria;o__Methanobacteriales;f__Methanobacteriaceae;g__Methanosphaera
k__Archaea;p__Euryarchaeota;c__Methanomicrobia;o__Methanosarcinales;f__Methanosarcinaceae;g__Methanosarcina

Aerobic taxa
k__Bacteria;p__Spirochaetes;c__[Leptospirae];o__[Leptospirales];f__Leptospiraceae;g__Turneriella
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__Methylobacterium
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae;g__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Nitrosomonadales;f__Nitrosomonadaceae;g__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Nitrosomonadales;f__Nitrosomonadaceae;g__Nitrosovibrio
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Nitrosomonadales;f__Nitrosomonadaceae;Other
k__Bacteria;p__Planctomycetes;c__Planctomycetia;o__Pirellulales;f__Pirellulaceae;g__Pirellula
k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae;o__Verrucomicrobiales;f__Verrucomicrobiaceae;g__Prosthecobacter
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Sphingomonas
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;g__Staphylococcus
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Hydrogenophilales;f__Hydrogenophilaceae;g__Thiobacillus
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Nocardiaceae;g__Rhodococcus

Figure 47 . List of anaerobic and aerobic taxa detected in my metagenomic analysis, categorised using Brock Biology of Microorganisms and MicrobeWiki (https://microbewiki.kenyon.edu/index.php/MicrobeWiki).

The anaerobic genera were, overall, reduced to a greater extent (or increased to a lesser extent) where *Bdellovibrio* was added compared with the buffer control, from pre-addition to 48 hours after the first *Bdellovibrio* addition (top-left and top-middle graphs). In the anaerobic genera with lower pre-treatment levels in the soil, there is a dichotomy between those that were reduced with *Bdellovibrio* addition and those that were increased, presumably due to their occupation of a niche left behind by genera whose levels were decreased due to *Bdellovibrio* predation; the post-addition levels are bunched into a higher group and a lower group with *Bdellovibrio* treatment, while in the buffer control group, post-addition levels fall in between these two extremes. This indicates that where *Bdellovibrio* addition affected the levels of anaerobic bacteria/archaea, some were increased and some decreased, and so the net effect on the number of anaerobes in the soil was relatively small overall.

This same effect is observed in the aerobic genera, shown in the bottom graphs of Figure 46. Some were reduced with *Bdellovibrio* addition while some were increased, from pre-addition to 48 hours after the first addition, in

the *Bdellovibrio*-treated group relative to the buffer control. This difference is clearly observable in genera that had a higher pre-treatment level (bottom-left graph) and in those that had a lower pre-treatment level (bottom-middle graphs); however, as with the nitrifying, N-fixing, and anaerobic genera, the overall effect is neither an increase nor a decrease in aerobic bacteria relative to the buffer control between pre- and post-addition samples, just a difference in their distribution between the high and low levels.

Finally, I looked at the levels of any PGPR genera that were present in the soil. However, I could only find one with certainty in my metagenomic data, again due to the fact that there is a species-level distinction between PGPRs and non-PGPRs from a given genus. The exception to this that I found in my data was *Azospirillum*, a PGPR that produces plant growth promoting hormones and that fixes N; the percentage and number/g soil of this genus are shown in Figure 48.

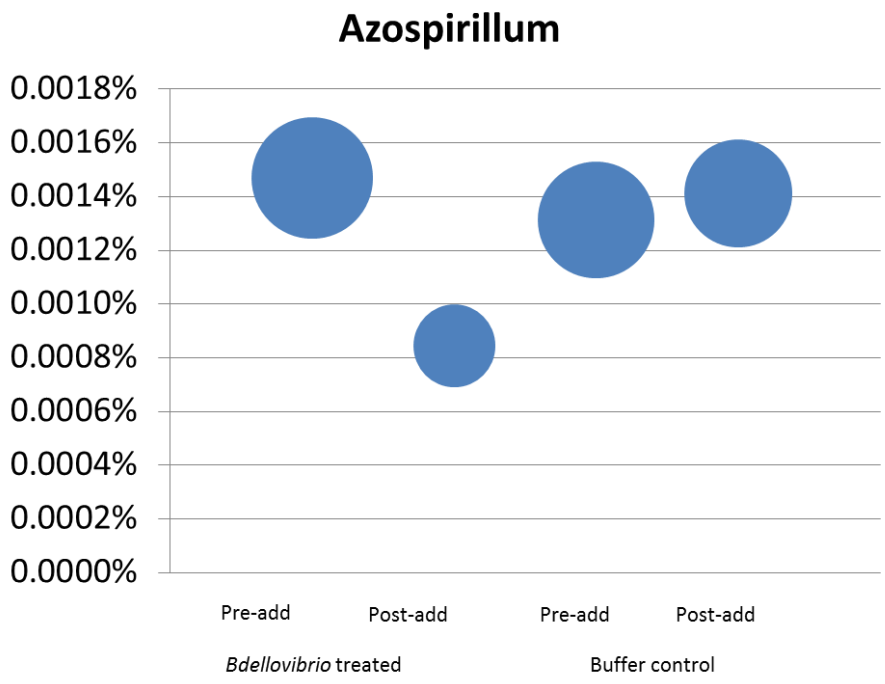


Figure 48 . The percentage (%, y-axis) and number/g soil (displayed as bubble size) of the Plant Growth-Promoting Rhizobacteria (PGPR) species *Azospirillum* in the soil from Pot Experiment 1a, showing levels in *Bdellovibrio* and buffer control treated samples, both before (pre-add) and 48 hours after the first addition (post-add).

The level of *Azospirillum* decreased where *Bdellovibrio* was added, from 0.0015 to 0.0008 (corresponding to a very small mean decrease from 1.5 to 0.8 sequences detected out of the 1 x 10^5 in total that were sequenced in each 20 ng DNA from each soil sample, as shown in Figure 35**)**, but increased only slightly from 0.0013 to 0.0014 (corresponding to an increase from 1.3 to 1.4 sequences out of the total 1 x 10^5) where the buffer control was added, from pre-addition to 48 hours after addition of these treatments. However, this change was not significant in either case, because the data were very variable and the percentage extremely low, and therefore this did not show up in the analysis of significant changes that I carried out in Section 5.4.3.7. Nevertheless, this effect is the opposite of what would be expected if *Bdellovibrio* addition affected wheat plant growth through altering the levels of PGPRs in the soil, as an increased level would result in increased wheat growth. However, it should be borne in mind that other non-identified PGPR species may have been increased by *Bdellovibrio* addition, but as their distinction from non-PGPR species required species-level classification, the phylogenetic assignments given in this study to the genus level meant that I could not include them in this analysis.

This analysis shows that none of these groups of bacteria/archaea appear to be affected by *Bdellovibrio* addition to the extent that they might cause or reflect changes in the community or soil characteristics to promote wheat growth. Given that the significant changes I found in specific genera in Section 5.4.3.7 were small, and therefore could have only contributed to increased wheat plant growth as part of a larger set of factors, *Bdellovibrio* itself may have PGPR characteristics distinct from predation that may more directly affect plant growth than I initially hypothesised. Dr Penny Hirsch (RRes) suggested that that *Bdellovibrio* may produce phytohormones, and that this may account for its positive effect on wheat growth, and so I investigated this possibility in Section 5.4.5.

5.4.4 Levels of nitrate (NO_3^-) and ammonium (NH_4^+) in the soil were slightly increased with *Bdellovibrio* inoculation.

Given that *Bdellovibrio* increases the growth and yield of wheat plants, but the metagenomic analysis did not identify any obvious increases or decreases in one (or a small number) of species whose activity is known to affect plant development, the levels of some key nutrients were measured in the soil collected from pots in which Cadenza plants were grown (Table 26). These measurements of nutrient content were not originally planned, but carried out in light of the metagenomic analysis, and so there was only sufficient soil collected from Cadenza pots in which to carry out these analyses.

Table 26 . Levels of key nutrients and present in soil, and pH levels, from Cadenza wheat pots treated with *Bdellovibrio*. All values are expressed only as milligrams per kilogram of soil (mg/kg). Separate levels of nitrate (NO3-N) and ammonium (NH4-N) are also shown. nitrogen levels were determined by potassium chloride extraction , and potassium levels by ammonium nitrate extraction [84]. Due to limited amounts of soil available for the extractions, soil from the two post-*Bdellovibrio* samples was pooled, as were soils from the two samples at 27 days post-*Bdellovibrio* inoculation and at wheat harvest.

Nutrient content of pot soil (ppm)						
	NO3-N	NH4-N	Total Nitrogen (N)	Potassium (K)	Phosphorus (P)	pH
Pre-*Bdellovibrio* addition (day 176)	0.51	2.21	2.71	206.87	44.51	7.5
Post-*Bdellovibrio* addition (day 184 + 190, pooled)	0.90	4.13	5.04	197.61	45.58	8
Post-*Bdellovibrio* addition (day 217 + 293, pooled)	5.07	3.35	8.42	196.79	46	8

The level of exchangeable K+ ions in the soil required for adequate wheat growth is 125 mg/kg [256], and the optimum level of P in the soil to support good wheat growth is 15-20 mg/kg of soil [257]. As shown in **Table 26**, the levels of Potassium (K) and Phosphorus (P) were higher than this in the soil before *Bdellovibrio* addition, and the difference between the pre- and post-*Bdellovibrio* added added soil samples was small; therefore it is unlikely that the increased growth of the wheat where *Bdellovibrio* was added is attributable to differences in K and P.

The total level of inorganic, wheat plant-available N in the soil increased from 2.71 mg/kg (equivalent to 10.84 kg/Ha NH_4NO_3) to 5.04 mg/kg (20.16 kg/Ha NH_4NO_3) after the addition of *Bdellvibrio* into the soil (Table 26; kg/Ha NH_4NO_3 = mg/kg plant-available N x 4 [210]). The amount of N that could have been added into the soil in each pot in 2 additions of *Bdellovibrio* was 52.74 mg/kg (210.96 kg/Ha NH_4NO_3), calculated from the Lowry protein assay. Given that about 10% of the *Bdellovibrio* cells could be detected after inoculation, this could correspond to a release of N equivalent to about 190 kg/Ha NH4NO3. This can alternatively be calculated by taking into account the N added to the *Bdellovibrio* inoculum used in these experiments in the YT medium (described in Section 2.6.6): 65 ml of YT medium was used to grow the *P. putida* cells that were added to each of the the 1 L cultures, which contained 5 g peptone (typically 15.2% N) and 8g tryptone (13.3% N). A total of 1.73 g N was therefore present in 1 L YT medium, and therefore 0.11 g N was contained in the 65 ml YT that was added to the 1 L *Bdellovibrio* cultures. 2 x 400 ml of this inoculum was added to each pot, which together contained (0.11 g N/1000 ml) x 800 ml = 0.088 g or 88 mg N. The %N in NH_4NO_3 fertiliser is approximately 34%, calculated using the molecular weights of N,H and O; thus the equivalent of 88 mg N x 0.34 = 258.82 kg/Ha NH_4NO_3 would have been added to each pot if all *Bdellovibrio* in the inoculum had died.

Using the estimate of N content from the Lowry assay, if all *Bdellovibrio* cells had died after inoculation, this would have manifested in the Post-*Bdellovibrio* pooled sample as an increase in N content of 39.55 mg/kg (158.2 kg/Ha), because the pooled sample contained 50% soil taken after only 1 *Bdellovibrio* inoculation (this soil therefore contained half of the final amount of *Bdellovibrio* that was added) and 50% soil taken after 2 *Bdellovibrio* inoculations (this soil contained all of the final amount of added *Bdellovibrio*); the pooled sample thus contained only 75% of the final amount of added *Bdellovibrio* ((50% of the pooled sample x 50% final *Bdellovibrio* amount)+(50% of the pooled sample x 100% final *Bdellovibrio* amount) = 75%) , and therefore contained only 75% of the N in the *Bdellovibrio* cells that could be potentially added to the soil through *Bdellovibrio* cell death.

That the N levels rose by 2.33 mg/kg (9.32 kg/Ha) from the pre-*Bdellovibrio* to this Post-*Bdellovibrio* sample may indicate that *Bdellovibrio* were able to survive in the soil, though the slight increase in N suggests that some died, as would be expected when adding bacteria in to the soil in these large numbers. However, it could have been that a large increase in N occurred in the soil due to *Bdellovibrio* cell death, but this N was taken up by the plants before the soil was sampled for analysis, and so they were not observable in my analyses of soil N content described here. The extent of a fertiliser effect such as this could have been tested by adding an equivalent amount of additional NH_4NO_3 fertiliser to an additional set of control plants and taking the same measurements of growth and yield that were taken for all other test and control plants; however, this control was not included in my pot tests as the effect of *B. bacteriovorus* on the growth of wheat was unexpected in **Pot Experiment 1a**, and I then had limited space and time in which to conduct **Pot Experiment 2**, in which buffer and heat-killed *Bdellovibrio* controls were chosen as a priority in consultation with my supervisors at Rothamsted Research.

In the pooled sample of soil sampled at 217 days (the day after N containing fertiliser was applied) and 293 days post-*Bdellovibrio* inoculation (77 days after fertiliser application), the level of available inorganic N in the soil had risen to 8.42 mg/kg (33.68 kg/Ha, Table 26). As these samples were pooled, the levels in soil at day 217 is likely to be lower than this value as N fertiliser had only just been added to soil, effectively diluting the soil from day 293, in which this value is likely to be higher. The increase in N at this stage was primarily in the form of NO_3^-: 5.07 mg/kg (20.28 kg/Ha) NO_3^- compared with 3.35 mg/kg (13.4 kg/Ha) NH_4^+, despite the slow-release Osmocote fertiliser containing 93.75 mg/kg (375 kg/Ha) NH_4^+, compared with 81.25 mg/kg (325 kg/Ha) NO_3^-. This could indicate increased Nitrification in the soil due to the activity of nitrifying bacterial/archaeal species.

5.4.5 The *Bdellovibrio* genome includes genes for putative plant hormone (Indole-3-Acetic Acid)-producing proteins.

Given that the metagenomic soil analysis showed that changes in the bacterial/archaeal community in the soil were relatively small where *Bdellovibrio* was added compared with the buffer control, I hypothesised that the large plant growth-promoting effects that we observed in the wheat in **Pot Experiments 1a and 2** could be due to a direct effect of *Bdellovibrio,* rather than an indirect effect of *Bdellovibrio* predation of other species in the rhizosphere. As the nutrient content of the soil did not considerably alter between soils before and after *Bdellovibrio* inoculation as shown in Section 5.4.4, and the increased nitrogen is likely due to some *Bdellovibrio* cell death, the wheat growth promoting effect may instead be due to plant hormone production by *Bdellovibrio.*

I consulted my Rothamsted supervisor Dr Penny Hirsch and she suggested that I test the hypothesis that *Bdellovibrio* might produce phytohormones that might promote wheat plant growth. To investigate whether *Bdellovibrio* produces any proteins involved in the synthesis or degradation of plant hormones, I conducted BLASTP (protein sequence) searches in the *Bdellovibrio bacteriovorus* HD100 genome to determine whether there were any genes that could theoretically produce proteins in *Bdellovibrio* which had significant homology to proteins from other species of rhizosphere bacteria known to be involved in plant hormone production pathways. The relevant bacterially produced phytohormone protein sequences were collected from two recent reviews of the molecular basis for interactions between rhizosphere bacteria and their host plants [258, 259]. All five major classes of phytohormone (auxin, ethylene, abscisic acid, cytokinin and gibberellin) were represented in the set of protein sequences that I included in these comparisons. The proteins with shared homology between the published phytohormone-producing species and *Bdellovibrio* are shown in Table 27, and also show the percentage identity, query coverage (the length of the homologous sequence shared between the *Bdellovibrio* and rhizosphere species proteins), and the expected (E) value (the number of homology matches with the same BLAST statistics expected due to chance).

Table 27 . Putative phytohormone proteins in *Bdellovibrio,* with homology to known phytohormone proteins in other rhizosphere bacterial species.

Phytohormone-associated protein	Bacterial species of origin	Annotated Function	Pathway	Homologous *Bdellovibrio* protein	Annotation in *Bdellovibrio*	% Identity to phytohormone protein	% BLAST Query coverage	BLAST E value
IaaH	*Agrobacterium tumefaciens*	Indole Acetimide Hydrolase	Auxin (Indole-3-Acetic Acid) Production	Bd0059	GatA Glutamyl-tRNA (Gln) amidotransferase A subunit	30	91	2×10^{-38}
IaaL	*Pseudomonas syringae*	Indole-3-Acetic Acid lysine synthetase	Auxin (Indole-3-Acetic Acid) Regulation	Bd1990	Auxin-regulated protein	23	57	0.010
IpdC	*Pseudomonas fluorescens*	Indolepyruvate decarboxylase	Auxin (Indole-3-Acetic Acid) Production	Bd2647	carboxylase	28	48	9×10^{-13}
Ipt	*Agrobacterium tumefaciens*	Isopentyl transferase	Cytokinin production	Bd1565	MiaA delta(2)-isopentenyl-pyrophosphate tRNA-adenosine transferase	33	19	9×10^{-5}
Fas5	*Rhodococcus opacus* PD630	Cytokinin oxidase	Cytokinin metabolism	Bd2890	oxidoreductase	24	43	3×10^{-8}
Efe	*Pseudomonas syringae*	Ethylene forming enzyme	Ethylene production	Bd0452	oxidoreductase	25	82	2×10^{-12}

A survey of relevant literature found that the BLAST E-value generally accepted as indicating a significant similarity is 1×10^{-5} [260]. According to this criterion, two of the protein sequences of interest listed in Table 27, the putative auxin production protein Bd1990 and the putative cytokinin production protein Bd1565, do not have significant homology with the relevant bacterially produced phytohormone sequences. Of the remaining 4 putative proteins in *Bdellovibrio* that were identified in these BLAST protein searches, two show homology to proteins involved in the production of Indole-3-Acetic Acid (IAA), an auxin with plant-growth promoting effects; one had homology to a protein involved in the breakdown of cytokinin, which promotes plant cell division and therefore tissue development; and the final protein sequence has homology to a protein involved in the production of ethylene, which promotes fruit ripening in some plants.

To determine whether the putative phytohormone proteins in Table 27 were expressed in prey-dependent *B. bacteriovorus*, I carried out RT-PCRs of total RNA extracted at different time points throughout the *B. bacteriovorus* HD100 predatory cycle and during Attack Phase (AP), as well as from 4 HI strains (HI2, 13, 22, and 26). This detected any transcription of the genes encoding these proteins, as a proxy for gene expression. I did not include Bd1565 in this analysis as it did not reach the significance threshold for similarity to the known cytokinin production protein; however, I did include Bd1990, because it is putatively involved in IAA production, as are the majority of the hypothetical proteins listed in Table 27, and it is also annotated in the *B. bacteriovorus* genome as an IAA-regulated protein. Even though Bd1990 does not show significant homology to the known IAA-associated protein, it may therefore be involved in a multi-protein, IAA production/detection system in *B. bacteriovorus* HD100. The results of the RT-PCRs are shown in Figure 49.

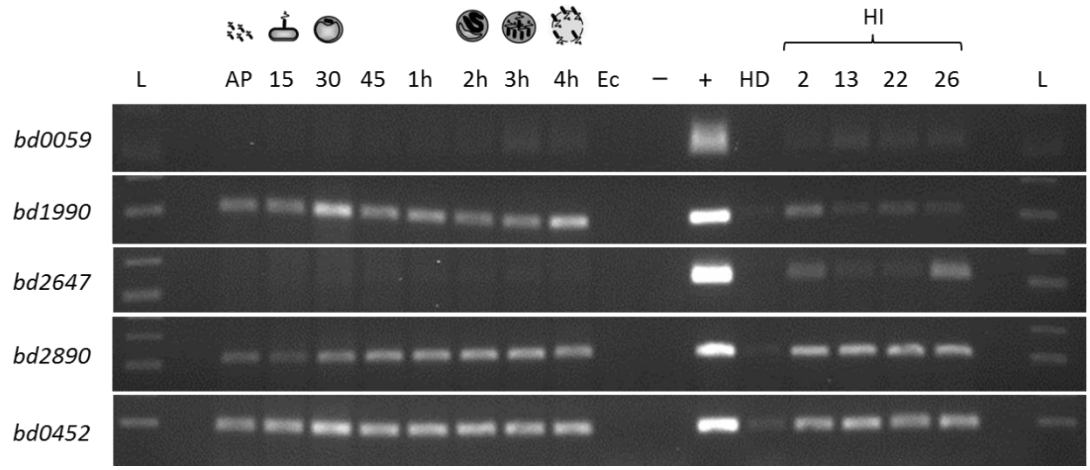


Figure 49 . RT-PCR showing transcription of putative phytohormone genes (as an indicator of protein expression) in *B. bacteriovorus* HD100, as listed in Table 27, across different time points in the predatory cycle (AP = attack phase, 15 = 15 min, 30 = 30 min, 45 = 45 min, 1h = 1 hour, 2h = 2 hours, 3h = 3 hours, 4h = 4 hours). Ec = *E. coli* total RNA control, - = no template (negative control), + = *B. bacteriovorus* HD100 genomic DNA (positive control). HD = Host Dependent *B. bacteriovorus* HD100 RNA, matched to the same concentration of RNA as the Host Independent (HI) strains 2, 13, 22 and 26, isolated as naturally-ocurring mutants of *B. bacteriovorus* HD100. L = 100 bp ladder.

The genes bd0059 and *bd2647* were minimally transcribed throughout the *B. bacteriovorus* HD100 predatory cycle, and transcribed at low levels in the HI strains. The other genes were transcribed throughout the predatory cycle and in AP, as well as in the HI strains; *bd1990* (a putative IAA lysine synthetase) showed slight peaks of transcription at 30 min and 4 hours, while *bd2890* (a putative cytokinin oxidase) and *bd0452* (a putative ethylene-forming enzyme) were constitutively transcribed throughout the predatory cycle (though *bd2890* was transcribed at slightly lower level until 30 min).

As most of the homologous proteins in Table 27 were hypothetically involved in IAA production, I used the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (Version 1, http://www.genome.jp/kegg/) to determine whether any other *Bdellovibrio* proteins were implicated in the IAA production pathway. The KEGG pathway for IAA production is contained within the Tryptophan metabolism map, as IAA is synthesised from Tryptophan via several different pathways as shown in Figure 50 (IAA is annotated and highlighted in yellow here as the synonym ‘indoleacetate’). Highlighted in green are two more *Bdellovibrio* proteins that are involved in IAA production

but that weren't identified in the BLAST searches; these were Bd2540, a monoamine oxidase, and Bd2266, an aldehyde dehydrogenase. These two proteins are involved in the last two steps of a three-step pathway by which Tryptophan is converted to IAA, shown in Figure 50.

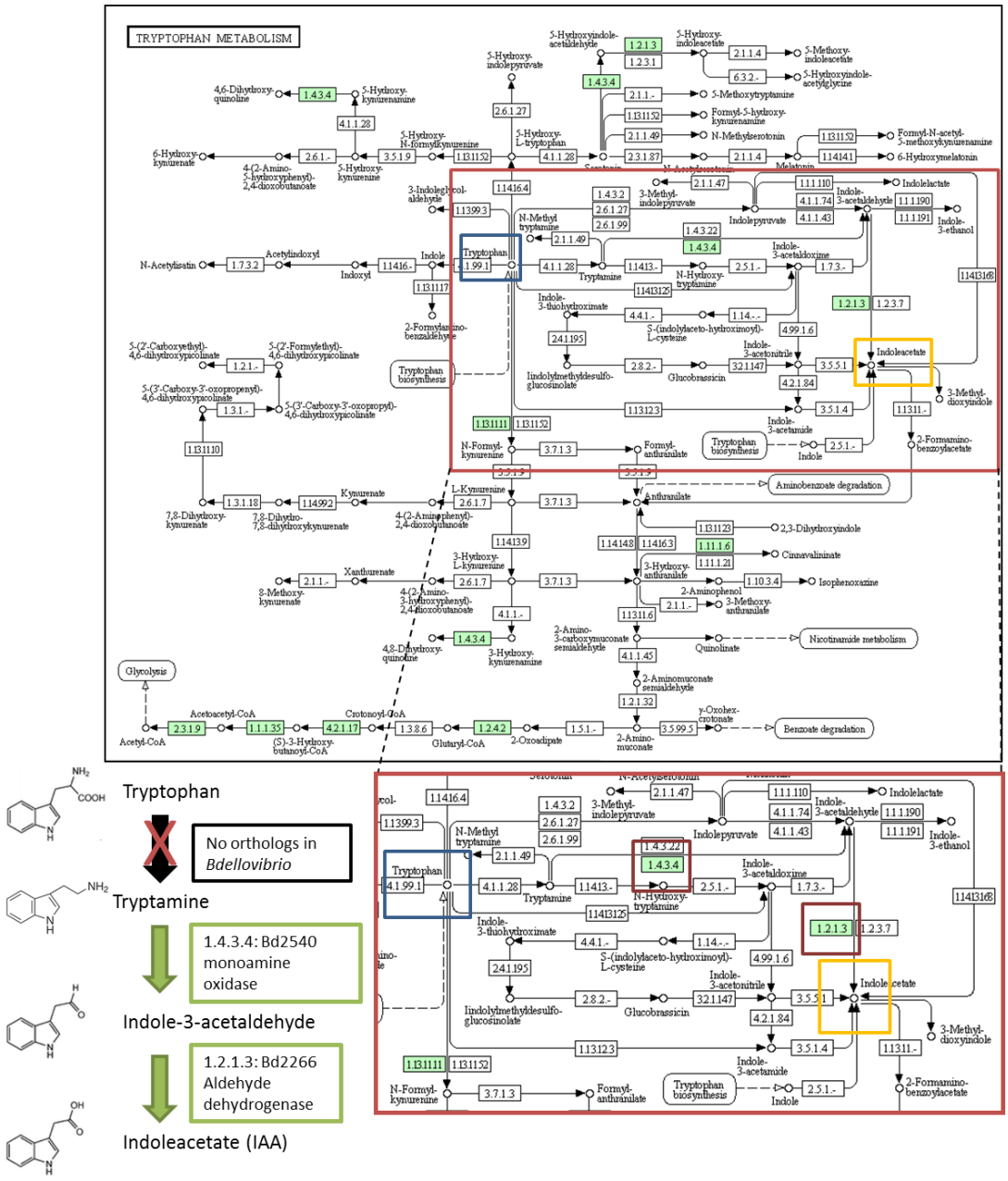


Figure 50 . The KEGG pathway showing production of Indole-3-Acetic Acid (IAA, highlighted in yellow) from tryptophan (highlighted in blue) via the tryptamine-containing pathway, with putative *Bdellovibrio* IAA production proteins shown highlighted in green.

Although there were no *Bdellovibrio* homologues that perform the first step that converts tryptophan to tryptamine, it is possible that *Bdellovibrio* could produce IAA if the tryptophan to tryptamine conversion step was carried out by other rhizosphere bacteria or the wheat plant itself, or if tryptamine, originally inside the bdelloplast, is liberated from prey digestion, thus promoting plant growth. The genes encoding Bd2540 and Bd2266 are transcribed during the *B. bacteriovorus* HD100 predatory cycle inside the bdelloplast, as shown in Figure 51; furthermore, a peak of *bd2540* transcription is observed at 2 h and a peak of *bd2266* transcription at 3 h, so their order of transcription peaks (and therefore expression) is the same as their order in the tryptamine-containing IAA production pathway shown in Figure 50. Taken together, this supports the hypothesis that *B. bacteriovorus* may produce IAA in the bdelloplast, if the tryptamine precursor is present.

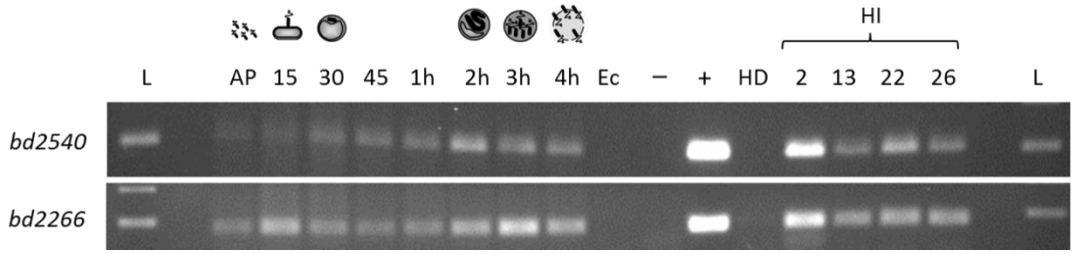


Figure 51 . RT-PCR showing transcription of putative phytohormone genes (as an indicator of protein expression) in *B. bacteriovorus* HD100, identified in the KEGG Tryptophan metabolism pathway (which includes IAA production), across different time points in the predatory cycle (AP = attack phase, 15 = 15 min, 30 = 30 min, 45 = 45 min, 1h = 1 hour, 2h = 2 hours, 3h = 3 hours, 4h = 4 hours). Ec = *E. coli* total RNA control, - = no template (negative control), + = *B. bacteriovorus* HD100 genomic DNA (positive control). HD = Host Dependent *B. bacteriovorus* HD100 RNA, matched to the same concentration of RNA as the Host Independent (HI) strains 2, 13, 22 and 26, isolated as naturally-ocurring mutants of *B. bacteriovorus* HD100. L = 100 bp ladder.

Similarly, the hypothetical proteins Bd0059 and Bd2647 show sequence homology (Table 27) to proteins that catalyse the last steps of two alternative pathways of IAA production from tryptophan, catalysing the conversion of indole-3-acetamide and indolepyruvate, respectively, to IAA (Figure 52); however, the transcription (and therefore likely protein expression) of *bd0059*

and *bd2647* was extremely low throughout the predatory cycle, and relatively low in *B. bacteriovorus* HI strains (Figure 49 and Figure 51, and so these proteins are unlikely to contribute to IAA production as much as Bd2540 and Bd2266. However, as with tryptamine, if these precursors were available to *Bdellovibrio* in the soil or liberated from bdelloplasts, some IAA production via these pathways may be possible.

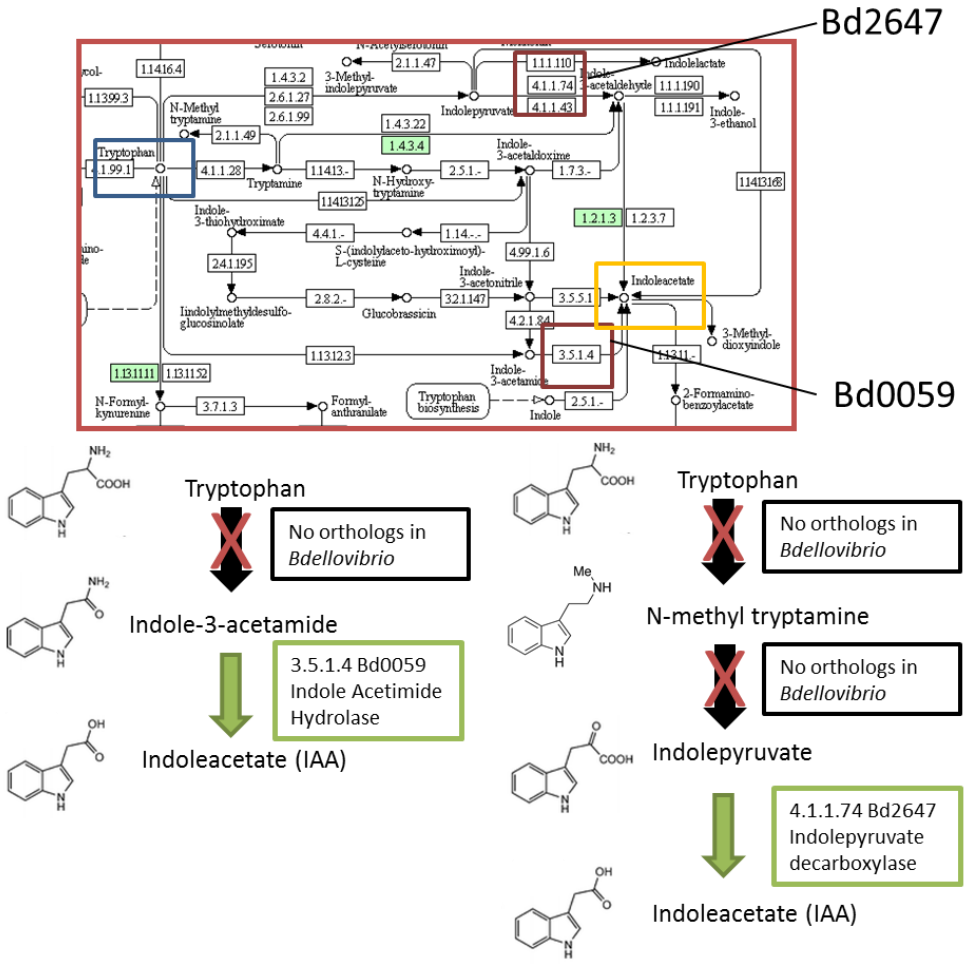


Figure 52 . An extracted section from the KEGG pathway showing production of Indole-3-Acetic Acid (IAA, highlighted in yellow) from tryptophan (highlighted in blue) via the indole-3-acetamide and indolepyruvate-containing pathways, with putative *Bdellovibrio* IAA production proteins shown highlighted in green.

The availability of IAA precursors to *Bdellovibrio* is unknown, but some studies [261, 262] have successfully detected tryptamine, indole-3-acetamide and indolepyruvate in extracted fractions of cell culture supernatants from PGPR *A. brasilense* and *Bradyrhizobium* species. This could be carried out using the supernatant of host-dependent *Bdellovibrio* cultures grown on soil-

dwelling bacteria, to test for tryptamine after the bdelloplasts have been lysed, and confirm or reject this tryptamine liberation hypothesis. Due to time constraints at the end of my Ph.D., however, I did not carry this out.

If *Bdellovibrio* were able to produce IAA in attack-phase cells when cultured in the laboratory, it is important to note that IAA is heat-labile, and therefore would not survive microwaving; therefore, the increased plant stem growth that I observed in the wheat plants treated with the heat-killed *Bdellovibrio* control in Section 5.4.2.10 relative to the live *Bdellovibrio* and the calcium HEPES control treatments is not due to IAA production, and is more likely due to increased nutrient availability to the plants from the killed *Bdellovibrio* cells after they were added.

The putative protein Bd1990 is involved in the regulation of IAA, however when I searched the KEGG pathway database there were no entries containing Bd1990. However, the protein with which it shares homology (shown in Table 27), IaaL, was present in the KEGG 'Indoleacetate:L-lysine ligase (ADP-forming)' chemical reaction entry, in which N(6)-[(Indol-3-yl)acetyl]-L-lysine is produced through an ATP-dependent reaction between Indole-3-acetate and L-lysine, shown in Figure 53. As Bd1990 is likely involved in downstream regulation of IAA, discussed in greater detail below, I consider Bd1990 to be in a separate plant hormone regulation category from the other putative proteins involved in plant hormone production.

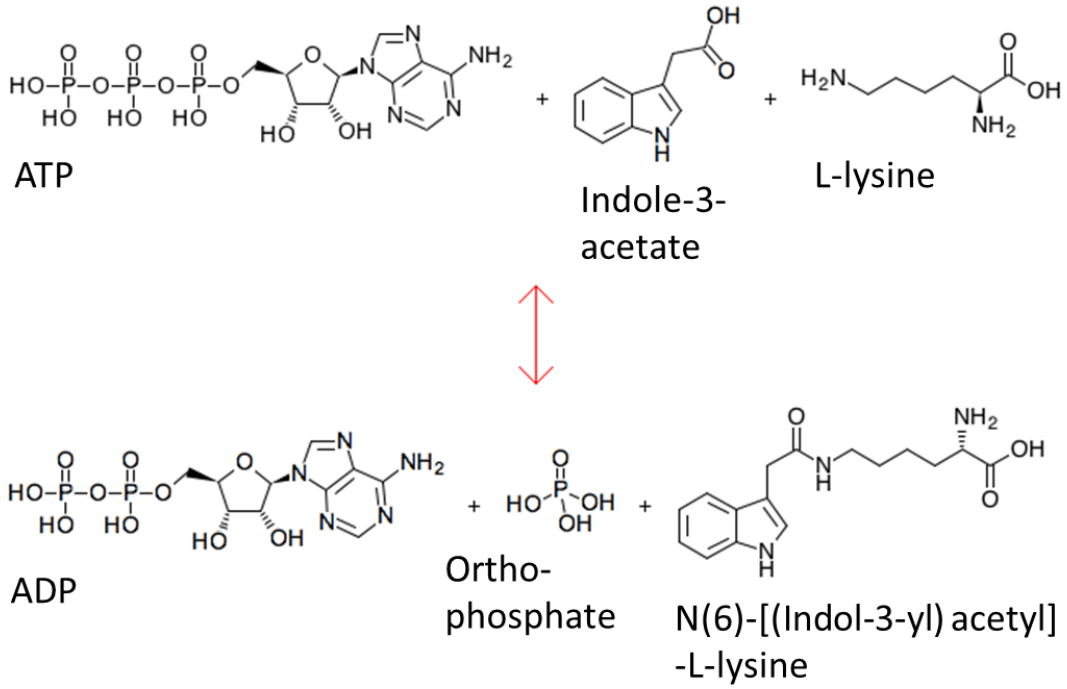


Figure 53 . The ATP-dependent 'Indoleacetate:L-lysine ligase (ADP-forming)' pathway, catalysed by IaaL Indole-3-acetic acid lysine synthetase, with which Bd1990 shares significant homology. Chemical reaction adapted from the original KEGG diagram (http://www.genome.jp/dbget-bin/www_bget?rn:R03095).

In addition to RT-PCR described above, I checked the transcription levels (as an indicator of expression levels) of the putative plant hormone encoding genes in *B. bacteriovorus* HD100 using RNA-seq data that had been generated previously by my research group. Briefly, a database containing this data displays graphically the level of RNA transcription across the whole HD100 genome of attack-phase cells (Figure 54, yellow), grown on *E. coli* prey cells in calcium HEPES buffer, as well as in 3 prey-independent HI strains in log-phase growth grown in PY medium (HI13, HI22, as well as a Δ*bd0108* HI strain [59]), shown in green, blue and red in Figure 54, respectively. All corresponding genes for the proteins in Table 27, apart from *bd1990,* showed no transcription in the RNA-seq data (non-expression of the gene in that condition), but these genes were all transcribed in the HI strains (expression of the gene). An example of the RNA-seq data chart showing this expression pattern is shown in Figure 54a; however, the RT-PCR data showed that there was some transcription (and therefore likely some gene expression) of *bd2890, bd0452, bd2540,* and *bd2266*, as shown in figures

Figure 49 and Figure 51, in attack-phase *B. bacteriovorus* HD100. However, *bd1990* was transcribed in HD100 as well as in all three HI strains to a similar extent, which is shown in Figure 54b, and is consistent with the RT-PCR data.

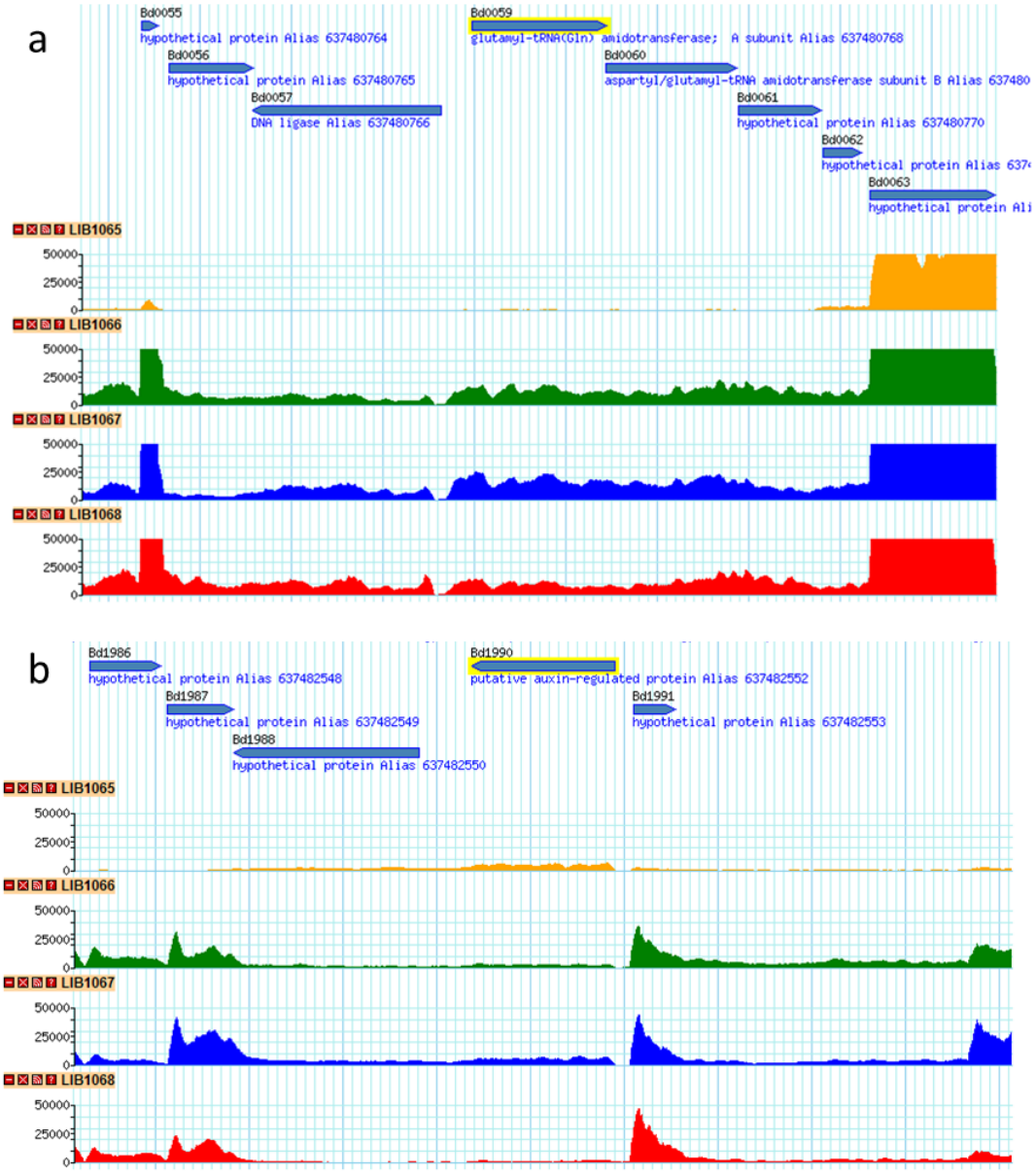


Figure 54 . RNA-seq transcription data showing the transcription levels of (a) *bd0059*, typical of most putative plant hormone production genes I found in the predatory *Bdellovibrio* genome, and (b) *bd1990*. The y-axis indicates the RNA-seq read coverage; 'LIB' Numbers are the library identifier numbers for attack-phase *B. bacteriovorus* HD100 genome (yellow) and three HI strains (green, blue and red, details in text).

5.5 Discussion

5.5.1 Live *B. bacteriovorus* increased wheat plant yield in a pot soil mesocosm

Bdellovibrio treatment of the pot soil mesocosms increased the yield of Hereward and Cadenza wheat by 35.59% in the first growing season (**Pot Experiment 1a,** Table 13), and of Hereward by 80.82% in the second growing season (**Pot Experiment 2)** compared with a buffer control (Table 13). The yield increase in the second growing season with live *B. bacteriovorus* treatment was 26.93% compared with a heat-killed *B. bacteriovorus* control treatment, which was used in this study to account for any nutritional effects of the added live *B. bacteriovorus* (discussed in Sections 5.5.2 and 0).

This yield increase with live *B. bacteriovorus* treatment was an unexpected result, as my original hypothesis was that *B. bacteriovorus* addition would increase take-all inoculum in the soil by preying upon the take-all reducing PGPR *P. fluorescens*, thus hypothetically decreasing yield. The yield increase was primarily due to an increase in grain number (and thus ear length to accommodate more grain) rather than grain weight (Table 13, Table 20). These results indicate that *B. bacteriovorus* treatment promoted an increase in the number of florets, from which individual grains are produced, during the flowering stage of wheat development as outlined in Section 5.1.4. It is therefore possible that the live *B. bacteriovorus* treatment could have had a biological effect on grain development and yield.

However, these pot experiments were not initially designed to account for the possibility of a large, unexpected positive effect of *B. bacteriovorus* on wheat growth and yield*;* because of this, there are some caveats associated with the experimental design of the pot tests that preclude any definitive conclusions about the possible biological effects of the added *B. bacteriovorus.* This is mainly due to low nitrogen levels in the soil, which was discovered after the pot tests were completed, and thus the significant possibility of a nutrient ‘boost’ effect in wheat grain yield due to increased

nitrogen in the soil resulting from the death of a proportion of the added *B. bacteriovorus,* discussed in section 5.5.2.

5.5.2 Low nitrogen levels in the soil in Pot Experiment 1a likely affected the increased wheat yield observed with live *B. bacteriovorus* addition

The level of the key wheat nutrients potassium and phosphorus in the pot soil were sufficient for good wheat growth (Table 26), and wheat plants showed no sign of nitrogen deficiency when the *Bdellovibrio* and take-all additions were made (Figure 4) or at full growth (Figure 29 and Figure 30). However despite the measures taken to ensure adequate N fertilisation of the wheat plants in the pot experiments described in Sections 2.6.3 and 2.6.11, the level of inorganic, plant-available N in the soil in **Pot Experiment 1a** was found to be unexpectedly low in chemical analyses of pot soil samples taken throughout wheat growth, which were conducted after the pot tests were complete (Table 26, Section 5.4.4).

Crop residues from the winter beans grown in the soil prior to its use as mesocosms in this experiment added organic N into the soil, which may have been converted at a steady rate by bacterial decomposers and nitrifiers, as shown in Section 5.1.5. Therefore, the slow metabolic breakdown of N from plant tissue may have contributed to a steady N supply to the wheat plants before the Osmocote® fertiliser was added during wheat growth. However the low N levels in the soil raised the distinct possibility of a nutrient 'boost' effect of adding *B. bacteriovorus* to the soil (i.e. through the death of some of the 2.3×10^{11} *B. bacteriovorus* cells in the pots and the subsequent release of nutrients into the soil, which the plants could take up and use to grow). The chemical analyses did not reveal any large increase in N after *B. bacteriovorus* addition; however, this may be because the soil samples were taken 48 hours after *B. bacteriovorus* addition, and so the wheat plants could have already taken up N from the *B. bacteriovorus* inoculum, which would mask a greater increase than that indicated by the chemical analysis.

If all *B. bacteriovorus* cells in the inoculum had died on addition to the pots, they would have released the equivalent of 210.96 kg/Ha NH_4NO_3 into the soil, a substantial amount compared to the 10.84 kg/Ha NH_4NO_3 present in the soil before any inoculations were made (Section 5.4.4). In a field trial study by Barraclough and coworkers, which included Hereward and Cadenza variety wheat grown in soil with similar starting levels of N to this study, an addition of 200 kg/Ha NH_4NO_3 to the soil increased grain yield by approximately 2 to 3 times compared with control plants to which no fertiliser was given [64].

Additionally, adding fertilizer at the tillering stage was recently shown to produce the greatest increases in wheat yield (discussed in Section 5.1.5); I initially chose to add the *B. bacteriovorus* at the end of the tillering stage before the main plant growth, as any effects of the treatment on the take-all infection levels in the plants, and therefore wheat plant growth, would be more clearly observable. Addition of the Osmocote Fertiliser was then postponed until the late main shoot growth stage to avoid disrupting the soil environment to which the *B. bacteriovorus* had been added, on the assumption that there was sufficient N already in the soil to support good wheat growth. Other studies, designed specifically to test the effects of proposed bacterial inoculants added to wheat soil, have previously incorporated different levels of N-containing fertiliser into their study design, as described in Section 5.1.7.3. This alternative design would have allowed us to account for any nutrient 'boost' effects on wheat growth and the soil microbial community, and conversely to determine the extent of the biological effects of *B. bacteriovorus* inoculation into the wheat soil discussed below.

The wheat yield in this study could thus have been increased due to increased N in the soil from the *B. bacteriovorus* cultures, the effects of which were not measurable in my study design. This should be taken into account when considering the other possible effects of *B. bacteriovorus* addition on the wheat growth measurements and the soil metagenomic analysis in the discussion that follows.

5.5.3 Live *B. bacteriovorus* soil treatment decreased the development of wheat side tillers and increased ripening rate of the main tiller

The height of the main wheat plant tiller was increased where live *B. bacteriovorus* were added, but the length and number of side-tillers produced by wheat plants were reduced (Table 13). This indicates a reduced nutritional resource investment by the wheat into producing side-tillers where live *B. bacteriovorus* was added; a recent study described a mutant wheat strain with a similar reduced tillering pattern (*tin* for tillering inhibition), shown to be due to altered expression of genes involved in metabolism and cell cycle progression, resulting in a diversion of sucrose away from the tillers [263]. In contrast, the length of side tillers increased with the addition of heat-killed *B. bacteriovorus* compared with the buffer control, which was the opposite effect to the live *B. bacteriovorus* treatment (Table 13); the height of the main wheat tiller was also increased to a greater extent with the addition of the heat-killed control compared with the live *B. bacteriovorus* treatment. This suggests a possible developmental effect of live *B. bacteriovorus* addition.

Side tillers can be missed by the combine harvester during conventional harvesting as they are generally shorter than the main plant tiller; live *Bdellovibrio* treatment could therefore increase the production efficiency of wheat plants in a commercial agricultural field context in terms of converting nutrient reserves into harvestable grain through conventional harvesting practices.

The wheat flag leaf senescence scores at harvest were also advanced in *B. bacteriovorus* treated pots (Table 13), indicating faster ripening; this could speed up the growing season if *B. bacteriovorus* were used as a commercial treatment, allowing busy farmers greater turnover time before the next crop, e.g. for ploughing fields. These specific changes in growth and development compared with both the buffer and heat-killed controls suggest that live *B. bacteriovorus* treatment could have had a biological effect on the wheat plants to increase yield, possibly via predation of other species in the soil, or production of plant hormones that promote growth and development, discussed in Sections 5.5.9 and 0.

The heat-killed *B. bacteriovorus* treatment was included alongside the buffer control in **Pot Experiment 2** to account for the possibility of a nutrient 'boost' effect of *B. bacteriovorus*, as outlined in section 5.5.2. The live *B. bacteriovorus* treatment increased wheat grain yield to a greater extent than did the heat-killed *B. bacteriovorus* treatment when compared with the buffer control (Table 13); however the heat-killed *B. bacteriovorus* control also increased wheat plant yield, even though it was to a lesser extent than the live *B. bacteriovorus* treatment. This may have been due to the fact that the heat-killing process would have produced Maillard reaction products [264] between amino acids and reducing sugars from the bacterial cells, which are readily available nutrients for plant cells. This may have resulted in a nutritional 'boost' effect on the wheat plants at a crucial time in development, particularly as the live and heat-killed *B. bacteriovorus* treatments were added just after tillering, the point at which fertilisation has previously been shown to be most effective at increasing wheat growth (See Section 5.1.5.1).

Alternatively, if the wheat yield increase observed with the live *B. bacteriovorus* treatment was due largely to cell death and the release of N as discussed in Section 5.5.2, the smaller yield increase observed with the heat-killed control could have been due to some effects of heat treatment resulting in the reduced availability of N. For example, heat treatment would have resulted in the denaturation of N-containing proteins in the inoculum, which is known to reduce N availability and thus the rate of N release to the plants [265]; additionally, the heat itself and reactive oxygen species released from bacterial cells in the heat-killing process would have increased oxidation of the amino groups of bacterial proteins to ammonia [266], which is volatile and could have subsequently been lost from the inoculum.

Bacterial cell wall Lipopolysaccharides (LPS) and flagellin proteins, which contain conserved Pathogen-Associated Molecular Patterns (PAMPs) that are recognised in plant cells through binding to PAMP Recognition Receptors (PRRs), stimulate the production of specific resistance gene (*R*-gene) products in plants, protecting them against infection with pathogenic bacteria [267, 268]. Thus, my Rothamsted supervisor Dr Penny Hirsch suggested that the LPS and flagellin in the heat-killed *B. bacteriovorus*/residual *P. putida*

prey treatment may have also had an immune priming effect on the wheat plants, protecting them against other, soil-dwelling pathogenic species that may have had a detrimental effect on general wheat plant growth and yield in the buffer control plants. This immune-priming effect could also have been augmented in the heat-killed control by the melting and cooling of *B. bacteriovorus* LPS during the heat-killing process, breaking down the LPS into small particles, which would then collide with plant cell receptors more frequently and thus elicit an immune response more readily.

B. bacteriovorus flagellins are not likely to elicit an immune response in plant cells in this way, as their flagella are sheathed, and therefore the wheat PRRs would not have been directly exposed to the flagellin contained within [269]. Some prey cell flagellins may have remained after *B. bacteriovorus* predation and clearing of the prey in the live *B. bacteriovorus* culture added to the wheat soil; however, this would not have been more than that present on the outside of soil bacteria normally, and the presence of residual flagella has not been quantitatively studied (Dr Carey Lambert, University of Nottingham: personal communication). Therefore, the immune priming effect is not likely to have occurred (to the same extent) in wheat plants treated with live *B. bacteriovorus*, and the growth and yield effects that I observed are likely due to other effects, e.g. predation of certain species in the soil (discussed in Section 5.5.9), plant hormone production (discussed in Section 5.5.10), and the nutrient 'boost' effect of added N from the *B. bacteriovorus* cells described in Section 5.5.2.

The *B. bacteriovorus* cells in the inoculum used in this study were cultured on *P. putida* prey; this is because *P. putida* is naturally present in wheat soil, and any residual *P. putida* added to the wheat soil would not have as large an effect on wheat growth as other potential prey species. In the *B. bacteriovorus* inoculum, residual *P. putida* were present in the inoculum and added at 9.6×10^5 per pot, 120 cells per g soil (Section 2.6.8). Although *P. putida* is a known PGPR species, that is able to colonise root surfaces and degrade some environmental pollutants with negative effects on plant growth [270], is unlikely that the residual *P. putida* would have had any significant

effect on wheat plant growth when added at these low levels, constituting only 0.0002% of the total bacterial cell number in the inoculum.

Despite the potential confounding effects of heat-killing the cultures described above, heat-killing was the most appropriate method of sterilisation available in this study; the alternative to heat-based methods for sterilising large quantities of bacterial cultures with minimal loss of the bacterial cell constituents would have been irradiation (e.g. gamma-irradiation, [271]); however with such large quantities of inoculum, this would have taken longer, with an additional transportation step to an irradiation facility. It was important in this study to prepare the heat-killed cultures quickly so that they could be transported to Rothamsted Research along with the live cultures, without compromising the viability of the live cells.

5.5.4 *B. bacteriovorus* treatment increased yield and affected growth similarly in Hereward and Cadenza variety wheat

There was only one growth measurement that was differently affected by *B. bacteriovorus* treatment in Hereward and Cadenza wheat varieties: a greater reduction in Cadenza variety wheat side tiller length with *Bdellovibrio* treatment was observed compared with Hereward (Table 15). This could be due to the fact that Hereward, unlike Cadenza, is a dwarf variety with a reduced response to the plant growth-promoting hormone Gibberellin (discussed in Section 5.1.2.2), suggesting that the effects of *B. bacteriovorus* treatment may be mediated through a plant growth promoting hormone pathway, as discussed in Section 5.5.10. In all other measurements, however, there was no significant difference in the effects of *Bdellovibrio* treatment between Hereward and Cadenza, shown in the ANOVA analyses in the Genstat files on the Appendix CD: 2013 harvest raw data and ANOVA files; the consistent effect of *Bdellovibrio* treatment between different cultivars would be a useful characteristic if *Bdellovibrio* were to be used as a growth and yield-promoting agent, given that many different cultivars are grown commercially [74].

5.5.5 *B. bacteriovorus* inoculation around wheat roots did not affect wheat infection with *G. graminis* var. *tritici* in the first wheat crop

I found that *Bdellovibrio* treatment did not significantly affect added or natural *G. graminis* var. *tritici* (take-all) infection levels in the wheat crop to which it was added (Table 10). Along with the yield increase described in Section 5.5.1, this was also contrary to the original hypothesis, that *Bdellovibrio* treatment would increase take-all disease by reducing the levels of *P. fluorescens,*which protects wheat plants against infection with take-all (Section 5.1.7.6). However, this result could be explained by my finding in the metagenomic analysis that no single Gram-negative taxon was specifically reduced by *Bdellovibrio* by a very large amount compared with other taxa (Section 5.4.3.7), and so any decrease in *P. fluorescens* was likely small (though the QIIME taxonomic assignment programme I used was not able to differentiate between *Pseudomonas* 16s rDNA sequences to the species level, as explained in Section 5.4.3.7).

Another unexpected result was that take-all infection levels in the control plants (which were given water instead of the *G. graminis* var. *tritici* inoculum, as this was suspended in water), though significantly lower than take-all added plants, were still relatively high in Pot Experiment 1a (Table 10): as this experiment was carried out in non-sterilised field soil that has previously been used to grow wheat (though winter beans were grown in it in the year immediately prior to use in **Pot Experiment 1a** to minimise the natural take-all inoculum level), it is possible that some take-all inoculum was present in the soil in addition to that which was added experimentally, which may have affected the results reported here.

5.5.6 The yield-promoting effects of the live *B. bacteriovorus* soil treatment were limited to the season in which it was added

When Conqueror variety wheat plants were oversown into *B. bacteriovorus*-treated pots saved from the previous wheat growing season, the single-grain weight was significantly increased but the total per-plant grain yield was non-significantly decreased (Table 16). This shows that the yield-promoting

effects of *Bdellovibrio* inoculation in soil are apparently restricted to the wheat plants grown in the same season in which the *Bdellovibrio* treatment was administered, supported by the metagenomic analysis, which showed that the number of added *Bdellovibrio* present in the soil reduced back to natural levels one month after their addition (Figure 37). Furthermore, in pots previously treated with *Bdellovibrio,* the overall ear length was significantly reduced, and there were also non-significant reductions in grain number and plant height (Table 16). This could suggest that the original hypothesis, that *Bdellovibrio* would reduce wheat plant growth and yield by increasing take-all levels as discussed in Section 5.5.7, could be true for successive growth seasons, as take-all inoculum builds in the soil; or it could be due to other long term effects on soil bacteria that were not studied here.

5.5.7 Previous live *Bdellovibrio* treatment of the soil in the first wheat crop increased take-all infection in a second, oversown wheat crop

The slight increase in take-all infection in the *Bdellovibrio*-treated compared with buffer control plants at maturity in **Pot Experiment 1a** (Table 10), the infectivity of the pot soil after harvesting in **Pot Experiment 1a** (Table 11), and the significant increase in take-all infection of the subsequent Conqueror wheat crop planted in the pots previously treated with *Bdellovibrio* compared with the buffer control (Table 17) provides some evidence that *Bdellovibrio* addition might increase take-all incidence, as I originally hypothesised. The lack of a significant difference in take-all infection between *Bdellovibrio*-treated and buffer control pots until the second growing season suggests that this potential effect of *Bdellovibrio* may only affect subsequent wheat crops, and may build up over the years, as explained in Section 5.1.3. This is supported by the moderate decreases in growth and yield parameters (grain number, total grain yield, and ear length) that I observed in the second wheat crop, as discussed in Section 5.5.6. This is an important possibility to consider, as *Bdellovibrio* treatment may thus have some longer-term detrimental effects on wheat plant health, growth and yield. However, the second wheat crops were grown in my final Ph.D. year, and so I could not

run a third season of experiments to test whether this effect continued in further wheat crops.

The take-all infection level of Conqueror plants, oversown in the soil saved from **Pot Experiment 1a**, was higher in water control pots than in take-all inoculated pots, which was at first counterintuitive. However, the results of the soil core bioassay at the end of **Pot Experiment 1a**, which measured the infectivity of the soil after mature plant harvest, showed that there was no significant difference in take-all levels in the soil between take-all added pots and water control pots. One possible explanation for this is that pouring the *G. graminis* var. *tritici* inoculum into the soil directly around the wheat plant, as was carried out in my study, may have facilitated take-all establishment on the wheat roots, thus out-competing the natural take-all fungal strain(s) present in the soil; harvesting the mature wheat plants before the soil core bioassay would thus have resulted in removal of the take-all fungus that had established on the mature plant roots, which would usually be ploughed back into the soil in fields after the wheat is harvested.

This may have lowered the take-all inoculum in the experimentally inoculated pots to a level comparable with water control plants, which would explain the lack of a significant difference in take-all levels in the pot soil in **Pot Experiment 1a**. As the natural take-all present in the pots may have been better adapted to survival over winter in the soil, and the natural take-all levels had not been interrupted by adding laboratory-cultured *G. graminis* var. *tritici* in the water control pots, take-all levels may have built up to a greater extent in the water control than in the take-all inoculated pots by the following year, thus resulting in higher take-all levels observed in the subsequent crop of oversown Conqueror wheat. This effect, along with the unexpected yield increases observed with *B. bacteriovorus* addition, was the reason I chose to exclude *G. graminis* var. *tritici* inoculation from **Pot Experiment 2**, and instead focussed on the questions as to whether the wheat yield increasing effects observed with *B. bacterivorous* addition would also be observed in different soil types, and in a different growing season.

5.5.8 *B. bacteriovorus* treatment increased wheat yield in different soil types and in different growing seasons

The grain yield promoting effect I observed with *B. bacteriovorus* treatment of the pot soil occurred regardless of soil type in **Pot Experiment 2** (Section 5.4.2.11) despite some wheat growth differences in Delafield compared with Great Harpenden-1 soil. These were that Hereward plants grown in Delafield soil were significantly reduced in height, primary ear length, and single grain weight compared with those grown in Great Harpenden-1 (Table 20); conversely, plant infection with take-all was significantly higher in Great-Harpenden-1 soil than in Delafield soil (Table 21). The properties of Great Harpenden-1 soil appear to thus be slightly more conducive to wheat growth, but also more conducive to *G. graminis* var. *tritici* build-up than Delafield soil, though the soil structural properties were not objectively measured because this was not the main focus of my study. However, if Great Harpenden-1 soil was less dense with more pore spaces, wheat roots could grow further down in the soil to give greater nutrient acquisition potential, but *G. graminis* var. *tritici* growth would be increased [272]. However, as soil type did not affect the yield increases observed with *B. bacteriovorus* treatment, I did not pursue any further analysis of this difference.

The growth-promoting effect of the live *B. bacteriovorus* HD100 was observed under natural, outdoor weather conditions, in two different seasons where the weather was different (see Section 5.4.2.10 and Appendix CD: Rothamsted weather reports 2012-2014.xlsx), as in real, agricultural field conditions. This suggests that, if its effect was biological rather than nutritional, *B. bacteriovorus* could be used commercially as a wheat yield-promoting agent.

5.5.9 *B. bacteriovorus* treatment reduced the levels of diverse Gram-negative taxa in the soil

To determine whether the yield increases and growth differences with *B. bacteriovorus* treatment were due to predation of general Gram-negative bacterial taxa in the soil, I conducted a metagenomic analysis of the pot soil

from Pot Experiment 1 before and after *B. bacteriovorus* addition, and throughout the growth of the wheat until harvest. As mentioned in Section 5.4.3.1, comparing live *B. bacteriovorus* addition with a buffer control rather than a heat-killed *B. bacteriovorus* control meant that the possible effects of a large N addition due to *B. bacteriovorus* cell death was not accounted for, and should be taken into account when considering the results of the metagenomic analysis described here. However, the added *B. bacteriovorus* survived in the soil in high numbers 48 hours after addition as shown in the metagenomic analysis (Figure 37), and up to 10 days as shown by enumeration after adding to the pot soil in a separate experiment (Figure 33). *B. bacteriovorus* treatment also decreased the overall number of Gram-negative bacterial taxa in the soil (by 1.35×10^6 relative to the buffer control), but not those of Gram-positive taxa or archaea, as shown in Section 5.4.3.5. This indicated that some predation was taking place, as *B. bacteriovorus* survival (only tested in buffer) in the presence of low prey cell numbers was previously shown to be reduced by 50% after just 14-24 hours [33].

Furthermore, **Live, predatory *B. bacteriovorus* HD100 were recovered from the soil 1 month after inoculation** in all *B. bacteriovorus*-treated but in none of the buffer control pots (Figure 38), indicating some longer-term survival in the pot soil, though their numbers as indicated in the metagenomic analysis and in separate enumerations from pot soil were almost reduced to natural, pre-treatment levels of *Bdellovibrio*, as shown in Figure 37 and Figure 33, respectively. Together, these data support one of my original hypotheses, that *B. bacteriovorus* would prey upon and kill Gram-negative species in the soil and reduce their numbers.

The proportion and number of some Gram-negative taxa in the bacterial soil community were significantly reduced with *Bdellovibrio* treatment, to a greater extent than the buffer control: these were *Chitinophagaceae, Chthoniobacteraceae* DA101, *Rhizobiales, Solibacter, Pedosphaerae,* and *Pirellulaceae* (Figure 42 and Figure 43). Conversely, some Gram-positive taxa were significantly increased: these were *Solirubrobacteraceae, Micrococcaceae,* and *Balneimonas* (Figure 41). This effect was largely short-term, lasting as long as *Bdellovibrio* survived in the soil, but with some

potential longer-term impact on the bacterial community composition in the soil, as is the case for *Solibacter* where cell numbers remained lower in *Bdellovibrio*-treated samples compared with buffer control at harvest time (Figure 43).

The Gram-negative *Pedosphaerae* spp., which were reduced with *B. bacteriovorus* treatment, are reported to be associated with late flowering in *Arabidopsis thaliana* [273]. Flowering time in wheat is controlled by the same pathway as in *A. thaliana,* involving the Vernalisation (Vrn) and Photoperiod (Ppd) genes, ensuring that winter wheat does not flower until the summertime, at full growth of the main wheat plant [274]. A reduction in *Pedosphaerae* may therefore have contributed to the early senescence and ripening that I observed, allowing time for greater numbers of floret and therefore grain production; this may provide a (partial) mechanism by which *B. bacteriovorus* increased wheat grain yield.

However, other bacterial species identified as changing in numbers in this analysis have known functions that were not as likely to affect wheat plant growth or yield, and thus do not help to explain the increases that were observed with *B. bacterivorous* treatment. The Gram-positive *Micrococcus luteus,* which is a member of the *Micrococcaceae* that were increased with *B. bacteriovorus* treatment, produces a trehalose tetraester biosurfactant that can degrade petroleum products including fuel [275], which are reported to have detrimental effects on wheat growth; however, the increase in *Micrococcaceae* possibly occupying Gram-negative niches emptied by predation would only have affected wheat growth if the Delafield soil had become unknowingly contaminated with fuel before the pot soil was collected, which is unlikely, as a detailed history of the soil was known to Prof Kim Hammond-Kosack and Vanessa McMillan (RRes) who chose this soil for my pot mesocosm tests.

Some Gram-positive *Balneimonas* (recently re-classified as *Microvirga*) species, which were increased with *B. bacteriovorus* treatment, are legume-associated N-fixers [276]; these species would not associate with the roots of wheat to affect N uptake, so any changes in population would have no effect

on wheat yield. The Gram-negative *Solibacter* reduces nitrate and Nitrite in the soil, but is not a denitrifier (and so does not convert these ions to N_2, so N would not be lost from the soil in this case) [277]. Thus, their reduction by *B. bacteriovorus* predation would likely not affect wheat growth either.

Some Gram-negative *Chitinophagaceae* species, which were decreased with *B. bacteriovorus* treatment, degrade chitin in the soil [278], which may include the chitin cell walls of fungal pathogens or commensals. Thus it is ambiguous whether the effect of *Chitinophagaceae* reduction in the soil, as in this study, would be beneficial or detrimental to wheat plant growth, but it may help to explain why the level of the fungal pathogen *G. graminis* var. *tritici* was slightly higher in *B. bacteriovorus*-treated pot soil than in the buffer control.

An analysis of the effects of *Bdellovibrio* treatment on the proportion and number of functional groups of bacteria showed that the levels of genera known to increase N levels (N-fixers and nitrifiers) in the soil were actually decreased, shown in Figure 45; therefore, it is unlikely that the mechanism of *Bdellovibrio* action to improve wheat growth is through the alteration of nitrogen-metabolising bacterial/archaeal populations, as their decreased number and proportion in the soil would theoretically manifest as decreased wheat growth. However, it is important to note that I could not analyse the levels of denitrifying genera in this way, due to a lack of species-level classification in the QIIME phylogeny assignment; increased wheat growth could still be explained by a decrease in denitrifiers, reducing N loss from the soil. Similarly, there may have been an increase in free-living N-fixers that could not be observed for the same reasons as for denitrifiers, and their levels may have increased, thus increasing N levels in the soil. Further analysis of denitrifier and free-living N-fixer levels, for example with qPCR using species-specific primers on the original DNA extracted from the soil, could shed light on this; however, due to time limitations, such an analysis was beyond the scope of my study.

I also analysed the levels of collated aerobic/anaerobic bacterial/archaeal taxa in the same way, which I hypothesised may be indicators that reflect soil

composition: increased anaerobic species with *B. bacteriovorus* treatment, for example, could indicate increased moisture retention in the soil, reducing soil aerobicity and help to explain the yield increases that were observed through increased or more consistent wheat hydration. However, the overall level of both aerobic and anaerobic bacterial/archaeal species remained unchanged by *B. bacteriovorus* treatment, shown in Figure 46, which suggests that the increased wheat growth and yield I observed was not due to its alteration of soil composition in this way.

Only one known wheat PGPR genus, the Gram-negative *Azospirillum,* could be identified in the metagenomic analysis: its natural, pre-*B. bacteriovorus* levels in soil were generally very low, but decreased (non-significantly) during *B. bacteriovorus* treatment, shown in Figure 47. This suggests that the yield-promoting effects of *B. bacteriovorus* were not a result of an indirect, promoting effect on the known wheat PGPR population in soil; however, some wheat PGPRs may not have been identified as the metagenomic analysis could not resolve the identity of some taxa to the species level.

5.5.10 ***B. bacteriovorus*** **expresses putative wheat phytohormone production genes, which may promote wheat growth and yield**

Given that the effects of *B. bacteriovorus* predation in the wheat pot soil mesocosm on individual Gram-negative taxa were relatively small, as discussed in Section 5.5.9, I investigated whether *B. bacteriovorus* may have a more direct effect on wheat growth by producing phytohormones. Through RT-PCR expression studies, I found that that *B. bacteriovorus* HD100 expressed several proteins, in attack-phase and during the predatory cycle, with significant homology to known bacterial phytohormone-producing proteins (Figure 49, Figure 51, and Table 27). One of these was a putative protein involved in the breakdown of cytokinin, a plant growth-promoting hormone, and one homologous to Ethylene forming enzyme (Efe), involved in the production of ethylene, which promotes grain ripening in wheat [279], however a protein sequence alignment of the homologous *P. putida* and *B. bacteriovorus* sequences showed that some histidine residues that are key for the function of this enzyme were not conserved in *B. bacteriovorus*

(Appendix 4) . Four putative proteins (Bd0059, Bd2647, Bd2540 and Bd2266), however, were encoded in the *B. bacteriovorus* HD100 genome that are involved in sequential steps in the production of Indole Acetic Acid, IAA, by other bacteria. IAA production is well documented in other rhizosphere bacterial species, including *Azospirillum*, a known PGPR used as an inoculant to increase crop growth and yield [243, 258, 259, 280].

Two of these proteins that are reported in other bacteria to catalyse the final steps in two alternative IAA production pathways (Figure 52), Bd0059 and Bd2647, were minimally expressed in the *B. bacteriovorus* predatory cycle (Figure 49). In her Ph.D. thesis, Sarah Basford (University of Nottingham) showed that the gene *bd0059,* which is annotated as glutamyl-tRNA amidotransferase subunit A (*gatA*) in *B. bacteriovorus* HD100, is co-transcribed with its neighbouring genes, *gatB (bd0060) and gatC (bd0058),* suggesting that their annotated co-related functions in the HD100 genome with respect to tRNAs are correct. A protein sequence alignment between Bd2647 and the homologous *P. fluorescens* IpdC protein sequence showed that few key residues were conserved in *B. bacteriovorus*. These two proteins (Bd0059 and Bd2647) are therefore likely not part of an IAA production mechanism in *B. bacteriovorus* HD100 (Appendix 5).

However, the two remaining proteins, which I found listed on the KEGG tryptophan metabolism map (containing IAA production pathways), Bd2540 and Bd2266, putatively catalyse the last two steps in the three-step ‘tryptamine-containing’ IAA pathway. They were expressed throughout the predatory cycle (Figure 49), with sequential peaks of expression in the same order as their position in the pathway, at 2 and 3 hours respectively (in the mid to late bdelloplast stage, when the daughter cells separate). It is possible that the first step in this pathway (the conversion of tryptamine to tryptophan) is carried out by other Gram-negative prey species in wheat soil and could therefore be present in the prey bdelloplast, where *Bdellovibrio* could produce IAA and promote wheat plant growth.

However, it is important to note that there were no published crystal structures of any homologous IAA-related proteins in bacterial or plant

species (I found these genes using KEGG annotations rather than through BLAST searches against known protein sequences), so I could not determine whether any key functional residues were conserved in *B. bacteriovorus* HD100. Additionally, some species of rhizosphere bacteria are known to produce IAA in the soil (Section 5.1.7.5), and IAA is also an endogenous compound in wheat plant roots [281, 282] and therefore may be released from plant matter when it decomposes. To my knowledge there are no published studies that have investigated the levels of tryptamine precursor in soil or in the prey bdelloplast. Further assays in which tryptamine/IAA levels are measured (such as in studies by Tien and coworkers and Sekine and coworkers [261, 262] throughout the predatory cycle of *Bdellovibrio* growing on prey species isolated from soil would verify whether *Bdellovibrio* could produce IAA. The functionality of the individual genes could also be confirmed using a gene complementation assay: putative IAA-production genes in the *B. bacteriovorus* genome could be cloned into individual plasmids, which could then be transformed separately into a known IAA-producing bacterial species (such as those shown in Table 27) in which the homologous gene is mutated, to determine whether IAA production can be restored in the complemented mutant. However, due to time limits at the end of my Ph.D. I was unable to carry this out.

Another putative phytohormone protein, Bd1990 has homology to IaaL in *P. syringae*, which is an IAA-Lysine synthetase, including a TSG-rich region that is key to the function of IaaL as an Adenosine monophosphate (AMP)-dependent synthase enzyme (Appendix 6). This protein is known to **inactivate IAA** through conjugating it with Lysine [283], as shown in Figure 53; the annotation of the Bd1990 protein in BLAST shows that it contains an IAA-amido synthetase domain and a GH3 auxin (IAA)-responsive multi-domain, providing support for the hypothesis that the IAA-Lysine synthetase activity is conserved in the Bd1990 protein in *Bdellovibrio* (Figure 55). However, given that *Bdellovibrio* addition resulted in wheat plant growth promotion, I was initially surprised that this IAA-inactivating protein might expressed by *Bdellovibrio* in the soil. However, It is important to recall that as the attack-phase *B. bacteriovorus* HD100 used in the RNA-seq and RT-PCR

studies were grown on *E. coli* cells in calcium HEPES buffer, and not in soil (from which *B. bacteriovorus* HD100 was originally isolated), the level of expression indicated by these methods shown in Figure 54 may not be representative of the transcriptome of this isolate when in a natural rhizosphere soil environment such as the pots in my study.

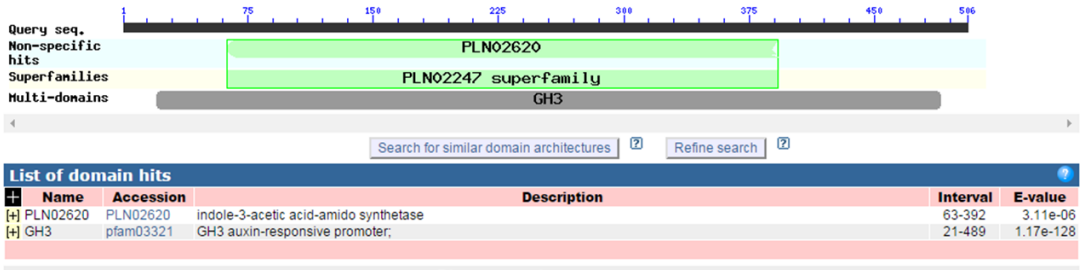


Figure 55 . A BLAST annotation of Bd1990 identifies an Indole-3-Acetic Acid-amido synthetase domain, and a GH3 auxin-responsive promoter multi-domain.

Additionally, inactivation of IAA by conjugation with Lysine may not be the primary function of this protein in *Bdellovibrio;* as Bd1990 is expressed in attack-phase cells, in which few prey cells remain, it may act instead as an IAA sensor, detecting IAA produced by other rhizosphere bacteria or by the plant root which is colonised by these bacteria. This may allow *Bdellovibrio* to use IAA as a chemotactic signal to move towards areas rich in potential prey cells (e.g. the root surface); a role for IAA as a bacterial signal has previously been suggested, and IAA sensing by *Azospirillum* acts as a feedback mechanism to promote its own synthesis [282]. If this is the case, then *B. bacteriovorus* itself may establish close to the root, where it might prevent other, potentially pathogenic bacteria from accessing and infecting the plant; this is another mechanism by which *B. bacteriovorus* could have improved wheat plant growth and yield. This hypothesis could be tested in additional pot mesocosm inoculation tests, in which a metagenomic analysis is carried out to assess the composition of the bacterial community associated with the wheat root before and after *B. bacteriovorus* addition, compared with soil samples taken from around the roots. It could also be tested *in vitro* using IAA as a test attractant for *B. bacteriovorus* cells, using similar methods to those in assays previously carried out by Lambert and coworkers [284].

Alternatively, as IAA is known to function as a signalling mechanism in other bacterial species, and in some cases has been shown to promote bacterial survival e.g. by enhancing colonisation of plant surfaces as in *Erwinia herbicola* and *Pseudomonas savastanoi* [280], disruption of IAA signalling by IAA-Lys conjugation may be beneficial for *Bdellovibrio* in preventing potential prey species from establishing a stable biofilm population, in which cells might be harder to prey upon due to the protective polysaccharides and proteins produced in this context, though previous studies have shown that predation still occurs at a slower rate when prey are in a biofilm [94].

IAA-amino acid conjugations are reversible by hydrolysis of the amide bond between the protein and amino acid by enzymes within plant cells, well-documented in several plant species including wheat [285]; before this takes place, the conjugated form may protect the IAA from enzymatic degradation and allow its transportation and storage [282]. Thus, *Bdellovibrio* Bd1990 may convert IAA produced by itself, as previously explained, or by other rhizosphere species to a form that is utilisable by plant cells that can convert it back into IAA, but affords IAA some protection before it reaches the plant, e.g. from other bacterial species such as *Pseudomonas* and *Bradyrhizobium* in the rhizosphere that can catabolise IAA [282].

Taken together, these phytohormone protein expression studies suggest that *B. bacteriovorus* HD100 could potentially produce the plant growth-promoting hormone IAA, if starting materials were available inside bacterial prey. Previous studies have shown that IAA is involved in floret development in wheat [286], with higher IAA levels associated with a greater number of florets [287]; as each floret develops into a grain, and grain number was the main contributing factor to wheat yield increase in this study, IAA production is another potential mechanism by which *Bdellovibrio* may have increased wheat yield.

5.6 Chapter conclusions

B. bacteriovorus HD100 increased the growth and yield (primarily due to increased grain number) of two different wheat varieties in pot mesocosm

tests, in two different growing seasons and two different agricultural field soils. I identified four possible mechanisms for this yield-increasing effect in my experiments, which may have acted alone or in combination with one another:

1. *B. bacteriovorus* HD100 preyed upon and killed *Pedosphaerae* in the soil, which are associated with late flowering in *Arabidopsis*; thus *B. bacteriovorus* treatment resulted in early flowering of the wheat, allowing time for more florets (and therefore more grains) to develop;

2. *B. bacteriovorus* HD100 died in large numbers in the soil, which would have added large amounts of nitrogen from the dead bacterial cells, thus stimulating the growth of the wheat in low-N pot soil;

3. *B. bacteriovorus* HD100 itself produced Indole-3-Acetic Acid (IAA) in the soil, thus promoting wheat growth, floret development, and an increased grain yield;

4. *B. bacteriovorus* HD100 established on the wheat roots, possibly in response to IAA signalling by the plant or root-associated bacterial species, preventing other (wheat pathogenic) bacterial species from infecting the wheat, thereby increasing wheat growth and yield.

Therefore, *B. bacteriovorus* HD100 could potentially be used as a treatment to promote the growth and yield of wheat. However, as the pot tests were not initially designed to account for the unexpected growth-promoting effects of *B. bacteriovorus*, and therefore did not adequately control for the possibility of the nutrient ‘boost’ effect described in point 2 of these conclusions, further experiments are required to determine the extent of the biological effects proposed here, and thus the potential of *B. bacteriovorus* as a yield-promoting agent under these circumstances.

6 Overall Discussion

The *Bdellovibrio bacteriovorus* type strain (HD100) was originally isolated from soil, but few studies have investigated its predatory activity against soil-dwelling, Gram-negative pathogen and Plant Growth-Promoting Rhizobacterial (PGPR) species that are known affect the growth, yield, and shelf-life of agriculturally important crops [23, 35]. In this project, I assessed the potential of *B. bacteriovorus* as a 'food security guard' to protect crops against pre-and post-harvest, yield-reducing bacterial infections, and to increase crop yields. This was tested in three systems, building in their complexity throughout the project: firstly a simple, *in vitro* system containing prey species monocultures in buffer; then a semi-sterile, post-harvest *in vivo* system growing on the surface of *Agaricus bisporus* mushrooms; and finally in a complex microbial community in a growing *Triticum aestivum* wheat pot soil mesocosm.

The initial *in vitro* assays revealed that the predatory activity of *B. bacteriovorus* against a range of crop pathogens and PGPRs was varied. Four species were found to be highly susceptible to predation (*Pseudomonas avellanae* 48 (hazelnut canker), *P. syringae pv. phaseolicola*: halo blight in bean; *P. tolaasii* 2192^{T}: brown blotch in mushroom; and *P. agarici* 2289: drippy gill in mushroom), and three species appeared to be completely resistant to predation (*B. vietnamiensis* G4: a PGPR; *P. marginalis* 667: soft rot in various hosts; and *Pectobacterium atrosepticum* SCRI1143: blackleg in potato). The species in susceptible and resistant groups were phylogenetically diverse, and were isolated from a range of host crops and soil environments, indicating that resistance to *B. bacteriovorus* predation was not due to any single, conserved mechanism. Resistance may be achieved through either physically blocking predatory attachment of *B. bacteriovorus* to the cell, for example in the presence of an S-layer or other outer membrane structures, or through the production of bacterially toxic effectors, killing *Bdellovibrio* or otherwise disabling its predatory activity.

As some potentially bacterially toxic effectors produced by Gram-negative plant pathogenic species may only produced by the pathogen in its natural context, I then developed an assay to test *B. bacteriovorus* predation of *P. tolaasii* on the surface of a post-harvest, *Agaricus bisporus* mushroom host. *B. bacteriovorus* treatment reduced the of characteristic brown-blotch lesion symptoms resulting from *P. tolaasii*-infection of the mushroom caps, which was found to be due to predatory killing and reduction of the *P. tolaasii* population.

B. bacteriovorus also preyed upon and killed a probable pathogenic *Pseudomonas* species isolated from organic mushroom cap tissue *in vitro*, but predation was less efficient against probable commensal species that were isolated from *Bdellovibrio*-treated mushroom tissue, which appeared to occupy the niche left behind by the inoculated *P. tolaasii*. Therefore, in addition to reducing the pathogenic symptoms of *P. tolaasii* infection, which is the most problematic disease of cultivated mushrooms, the effects of *B. bacteriovorus* predation on the populations of other mushroom-associated bacterial species could combine fortuitously to maximise post-harvest mushroom yield and shelf-life, by reducing pathogen numbers but sparing potentially beneficial commensals.

Finally, *Bdellovibrio* increased wheat plant grain yield in an outdoor pot mesocosm, in natural field soil containing a complex microbial community. However, this was an unexpected result, as this study was initially designed to test the effect of *B. bacteriovorus* on wheat infection levels with the take-all fungus, *G. graminis* var *tritici.* The soil also contained unexpectedly low levels of nitrogen, which is important for good wheat growth, which was discovered after completing the experiments; the study was not initially designed to account for any effects of added nitrogen, i.e. from the death of *B. bacteriovorus* cells in the inoculum when added to the soil. Thus it is not possible to make any strong conclusions about any biological effects the added *B. bacteriovorus* could have had on wheat growth at this stage.

However, the growth and yield-promoting effect was coupled with an increased number of grain harvested from *Bdellovibrio*-treated wheat, and

earlier wheat ripening and senescence, which suggested that *Bdellovibrio* could have moderated wheat plant development, possibly by promoting earlier flowering, allowing time for more grain to develop. The metagenomic analysis showed that *Pedosphaerae* species in the soil, recently found to be associated with late flowering in *Arabidopsis*, were significantly reduced with *Bdellovibrio* addition, which is one biological mechanism by which *Bdellovibrio* could have increased wheat yield by promoting early flowering. *Bdellovibrio* also expresses genes involved in producing and responding to Indole-3-Acetic Acid during the predatory life cycle, which suggests two other possibilities by which *Bdellovibrio* could have possibly increased wheat yield: the first is a direct effect of IAA production by *Bdellovibrio,* which would promote wheat growth, flowering and grain development; however, *Bdellovibrio* only expresses proteins that are putatively involved in the last two steps of an IAA production pathway, and would require the presence of an IAA intermediate either in the prey bdellopast to successfully produce IAA. The second is that *Bdellovibrio* could respond to IAA produced by the plant roots or other bacterial species, possibly through a putative IAA-Lysine synthetase, known to be involved in bacterial signalling; this response could allow *Bdellovibrio* to detect and move towards areas of high prey density at the plant root surface, where it could prey upon wheat pathogenic Gram-negative bacterial species. Further studies incorporating a nitrogen fertilisation regime are required to determine whether any of these potential biological effects of *B. bacteriovorus* have a significant impact on wheat growth in well-fertilised soil.

The choice of host crop systems may have been serendipitous in testing *Bdellovibrio* as a ‘food security guard’: mushrooms were chosen as a first test host because they were easily acquired in large numbers and manipulated in the laboratory, and because *P. tolaasii* was susceptible to *Bdellovibrio* predation *in vitro*, and wheat was initially chosen as a host in which to test a different hypothesis, that *Bdellovibrio* would increase take-all infection of wheat. In the latter tests, the *Bdellovibrio* were added just before the main period of wheat plant growth and development; as *Bdellovibrio* may have had a developmental effect on wheat, these growth and yield-promoting effects

may not have been observed to the same extent if the *Bdellovibrio* had been added earlier or later. Future studies will determine the extent of any biological effects of *B. bacteriovorus* on wheat growth, and whether the choice of mushrooms as a first test host was truly fortuitous, or whether *B. bacteriovorus* could be used to the same effect in a range of other pre- or post- harvest crops.

The mechanisms behind the crop growth, yield, and shelf-life promoting effects of *Bdellovibrio*, particularly the extent of a nutrient 'boost' effect due to *B. bacteriovorus* cell death in the wheat soil inoculum, and the effectiveness of *B. bacteriovorus* in a commercial setting remain to be elucidated. However, my Ph.D. thesis demonstrated that the naturally soil-dwelling *B. bacteriovorus* HD100 can reduce pathogen spoilage in a post-harvest crop (mushrooms) and survive to some extent in wheat soil, where it could potentially promote growth and yield as a pre-harvest crop soil treatment (though this requires further tests controlling for any nutrient 'boost' effects); these are two complementary means of securing more food for the growing global human population. *Bdellovibrio* could therefore play a role the food supply chain in future as a naturally derived 'food security guard', particularly when used in post-harvest crops to prevent spoilage.

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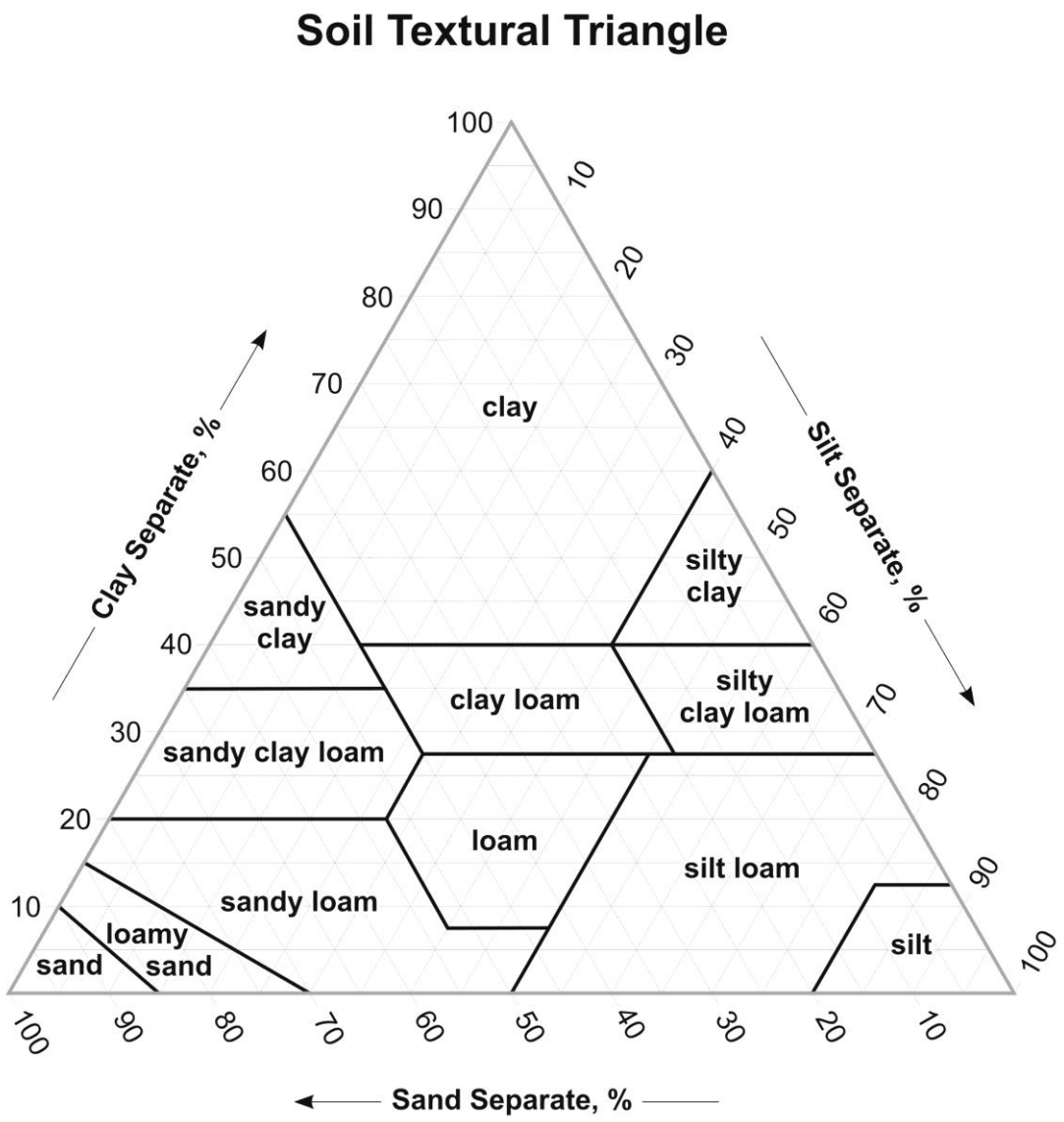
Appendix

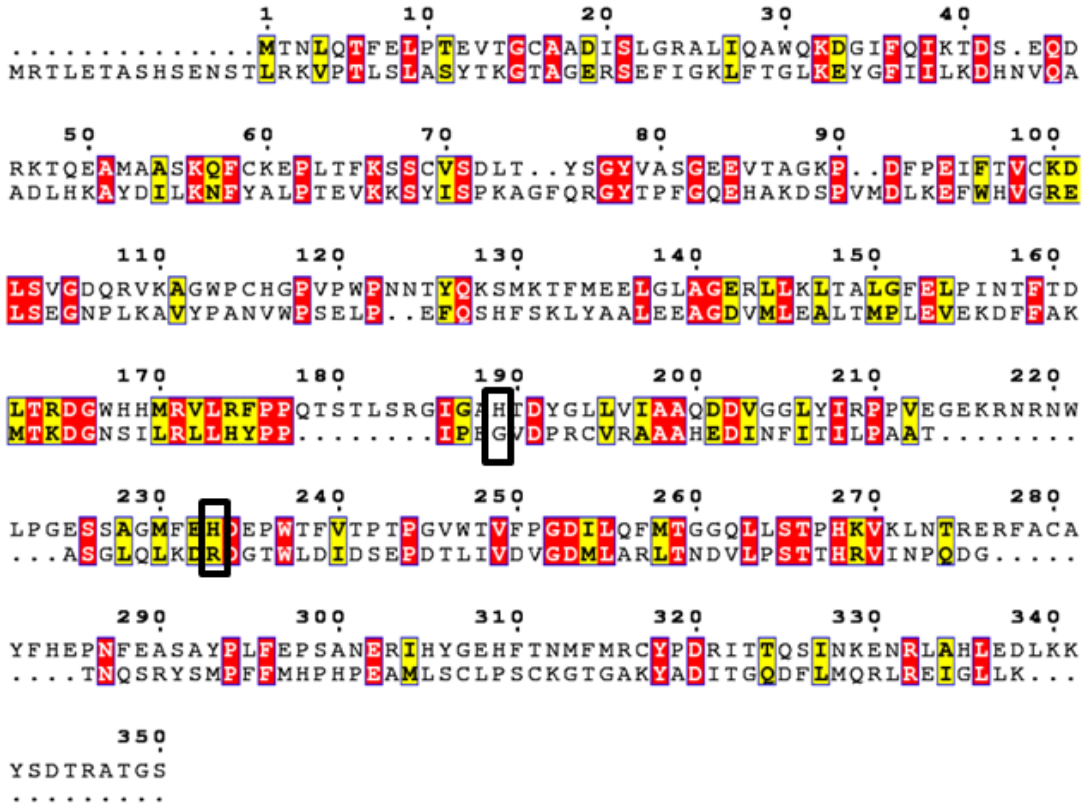
Step	Program used	Settings	Notes
Alignment	MUSCLE 3.7	• Find diagonals option disabled • Maximum number of iterations: 16 • No duration limitation • No more than 200 sequences	Several studies and especially the BAliBASE benchmark showed that MUSCLE achieved the highest ranking of any method at the time of publication.
Alignment refinement	Gblocks 0.91b	• Minimum number of sequences for a conserved position: half the number of sequences + 1 • Minimum number of sequences for a flank position: 85% of the number of sequences • Maximum number of contiguous nonconserved positions: 8 • Minimum length of a block: 10 • Allowed gap positions: none	This step is optional. Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise). Parameters are set to their default values in Gblocks. These are rather stringent; e.g. all positions with gaps are removed.
Phylogeny	PhyML 3.0	• Substitution model: HKY85 for DNA/RNA, WAG for proteins • aLRT test: SH-like • Number of substitution rate categories: 4 • Gamma parameter: estimated • Proportion of invariable sites: estimated • Transition/transversion ratio (DNA/RNA): 4	PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. HKY fits well in most cases as it modelizes the main features of DNA substitutions: transition/transversion and unequal base frequencies. LG has been shown to be the best amino-acid replacement matrix to date. Gamma distributed rates are mandatory in most (if not all) analyses, and using invariant sites generally improves (never degrade) the fit.
Tree rendering	TreeDyn 198.3	• Rectangular tree • Display branch support values (in red) • Font: Times, 10pt	TreeDyn offers many tree customization options compared to other tree rendering tools and especially for tree annotations. The starting output tree is rooted using mid-point rooting method (performed by Retree from PHYLIP package) but the user can reroot the tree using our dynamic tree editing interface.

Appendix 1 . Recommended (default) settings of the “one click” option of the Phylogeny.fr phylogenetic tree construction programme [62], as used in Sections 2.5.8 and 2.5.9.

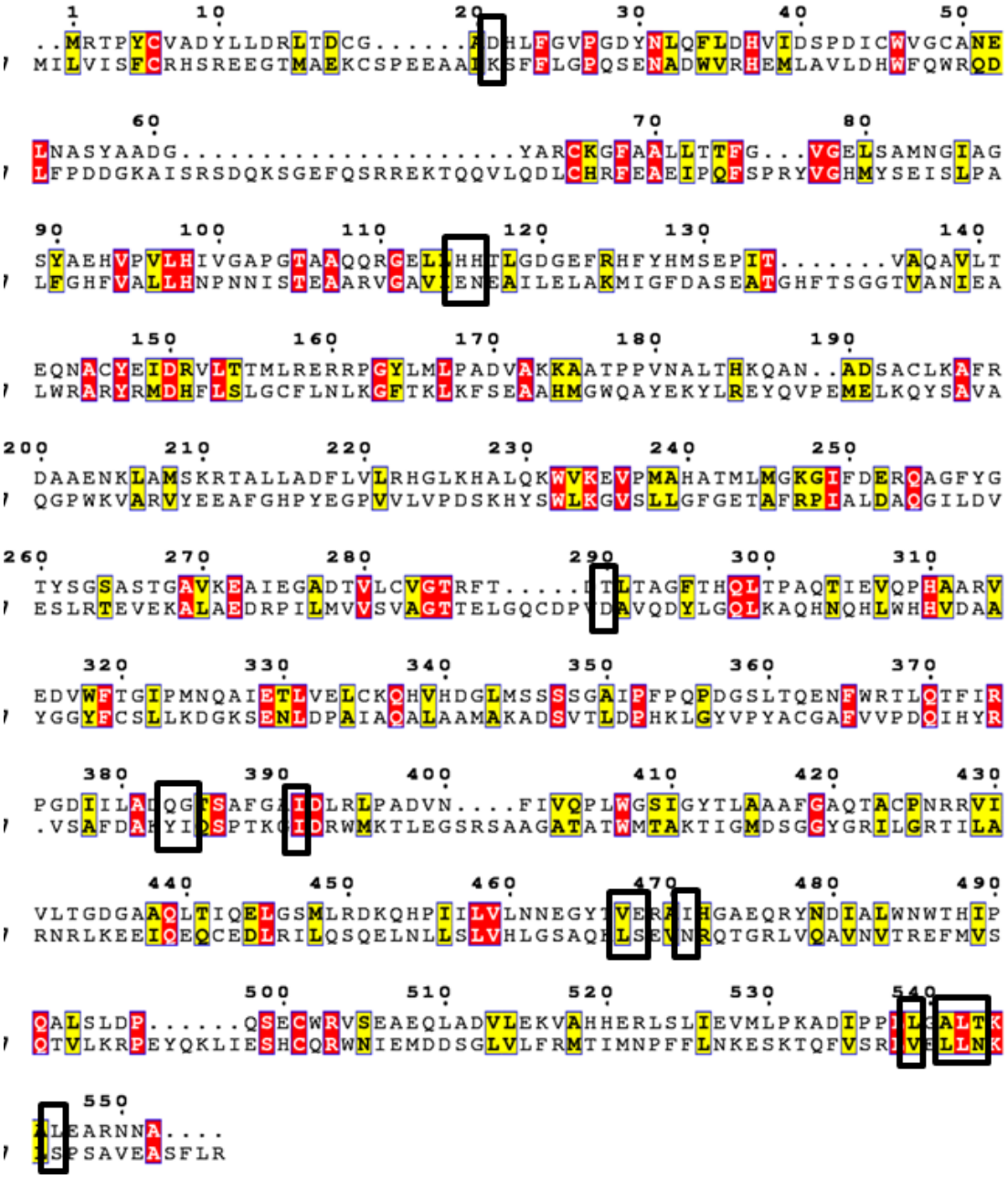
Appendix 2 . Visual scale of flag leaf senescence used as a reference to assess wheat plants in Section 2.6.14.



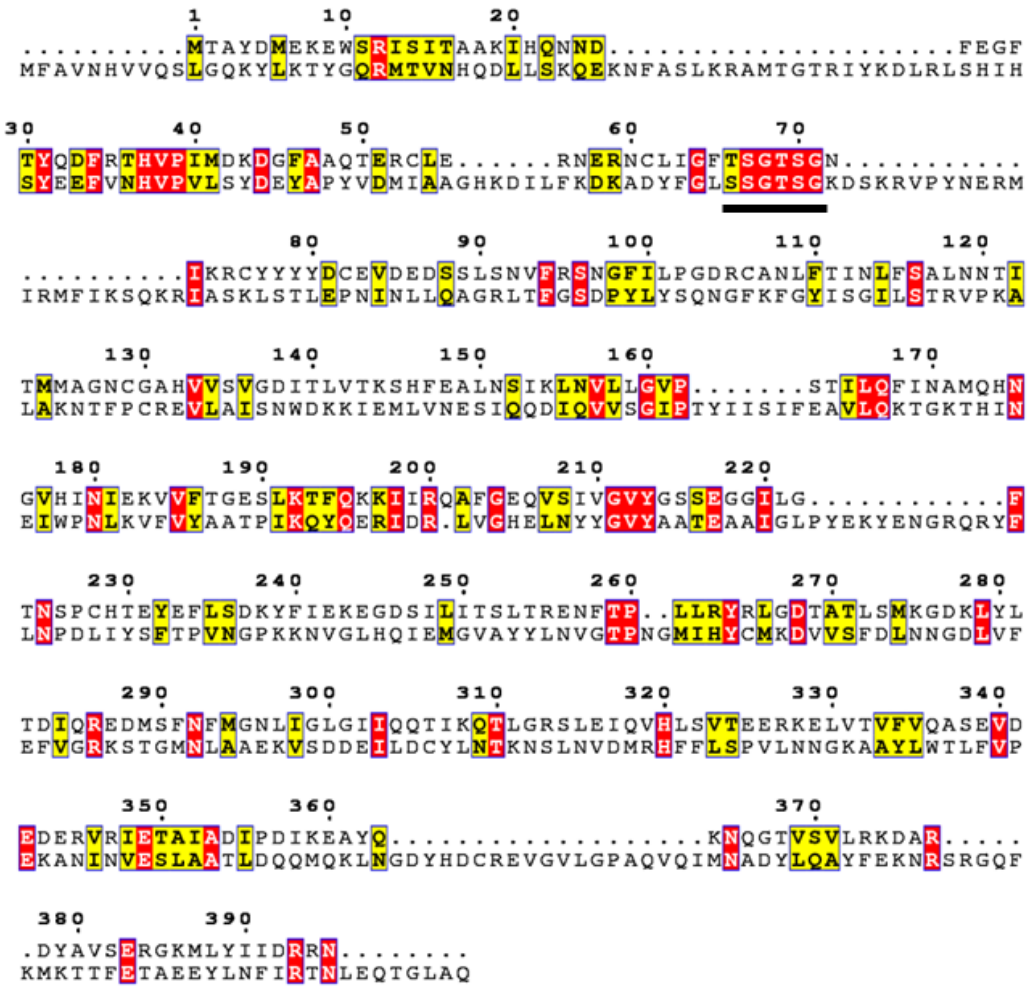
Appendix 3 . The "soil textural triangle" [68], showing the percentage clay, silt and sand composition in each soil classification group described in the centre of the triangle.



Appendix 4 . Protein sequence alignment between the *Pseudomonas syringae* Ethylene-forming enzyme Efe (top) and *B. bacteriovorus* Bd0452 (bottom); Histidine residues that are key for Efe function,highlighted in black boxes [288], are not conserved in Bd0452. The alignment was created using the ClustalW online tool (http://www.ch.embnet.org/software/ClustalW.html), and this figure was produced using ESPript (http://espript.ibcp.fr/ESPript/ESPript/).



Appendix 5 . Protein sequence alignment between the *Pseudomonas fluorescens* Indole-3-pyruvate decarboxylase IpdC (top) and *B. bacteriovorus* Bd2647 (bottom); residues that are key for IpdC function,highlighted in black boxes[289], are mostly non-conserved in Bd0452. The alignment was created using the ClustalW online tool (http://www.ch.embnet.org/software/ClustalW.html), and this figure was produced using ESPript (http://espript.ibcp.fr/ESPript/ESPript/).



Appendix 6 . Protein sequence alignment between the *Pseudomonas syringae* Indoleacetic acid Lysine synthetase IaaL (top) and *B. bacteriovorus* Bd1990 (bottom); a TSG-rich region key for function as an Adenosine Monophosphate (AMP)-dependent synthase [290],underlined, is conserved in Bd1990. The alignment was created using the ClustalW online tool (http://www.ch.embnet.org/software/ClustalW.html), and this figure was produced using ESPript (http://espript.ibcp.fr/ESPript/ESPript/).