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The Microflora of the Huhu Grub

A thesis
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of the requirements for the degree
of
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Abstract

New Zealand's endemic longhorned beetle larvae, the Huhu grub (*Prionoplus reticularis*) feeds on dead coniferous wood. No studies have been conducted on its gut microflora. Given that the Huhu grub feeds solely on lignocelluloses, it is likely that there are microorganisms present in its gut which are capable of degrading lignocelluloses to release energy rich sugars. This process of lignocelluloses release is the rate limiting step in the utilisation of woody material for bioprocesses such as bioethanol production. Microbial communities from wild grubs were compared with those raised on laboratory diets of either: lignocellulose, cellulose, or complex nutrients. Bacterial gut communities were surveyed using 454 pyrosequencing of the variable 5 and 6 regions (400nt reads) of bacterial 16S rRNA genes as well as clone library analysis of the full length gene (1500bp). Fungal gut communities were analysed using cloning and Sanger sequencing of amplified fungal intergenic spacer (ITS) regions. The wild type gut bacterial population was highly diverse, with no known cellulose or lignin degraders detected in any abundance, although a strain of *Burkholderia* thought to be capable of nitrogen fixation was detected. No methanogenic archaea or acetogenic bacteria were detected. Fungal ITS sequences had high similarity with those of known lignin, hemicellulose and cellulose degraders in the public databases, and an uncultured *Basidiomycete* made up 51% of the wild type community, while species of the *Penicillium* genus dominated the grubs reared on laboratory diets of lignocellulose. When grubs were reared on a diet of only cellulose the fungal community was dominated by a single species identified as *Candida shehatae*, a hemicellulose degrader known to associate with other longhorned beetle larvae. These fungi may be of interest for the biological conversion of lignocelluloses.

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I want to dedicate this thesis to my late grandparents Carl and Joy Duignan, who I believe would have been very proud of my achievements and who, for me, give new meaning to Isaac Newton’s famous words:

“If I have seen a little further it is by standing on the shoulders of Giants.”

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1 Introduction

Fossil fuels are a non-renewable resource and their combustion is contributing to the greenhouse gas effect, causing temperatures to rise around the globe.

Bioethanol made via the fermentation of starches from corn and sugar-cane has been proposed as a renewable substitute for fossil fuels. But doubts have arisen over their sustainability, as this use brings direct competition for these substrates with the animal and human food chain. Lignocelluloses are a better substrate for bioethanol production as they are found in waste materials such as wood chips, grass cuttings, organic domestic refuse and pulp and paper. However, there are currently no known microorganisms which are capable of efficiently degrading lignocelluloses to release the energy rich sugars at a rate applicable to bioethanol production. In nature lignocellulosic biomass is decomposed and there must be a great diversity of microorganisms with this capability. Huhu grubs (*Prionoplus reticularis*) feed on the lignocellulose component of dead trees and are endemic to New Zealand. A recent study on a termite and a wood boring beetle larva has suggested that the breakdown of lignocelluloses in the insect digestive tract is achieved by the microorganisms which inhabit the insect gut (Geib, *et al.*, 2008) and the same is presumed to be the case with Huhu grubs.

1.1 Biofuels

1.1.1 Climate change

Earth receives light and heat energy from the sun, some energy is absorbed by the planet, the rest is reflected off the surface. Some of the radiation which is reflected is absorbed by Earth's atmosphere, while the rest is radiated into space. The reflection and absorption of radiation by the Earth's atmosphere warms the planet's surface and is called the greenhouse gas effect. Gases such as carbon dioxide, methane, and water vapour increase the atmosphere's capacity to absorb heat radiated from the earth's surface (Shindell, *et al.*, 1998).

Global surface temperatures in the last 100 years have increased by $\geq 0.5^{\circ}\text{C}$ (Moberg, *et al.* 2005) and an additional 2°C warming is predicted for the next 50 years (Schnoor, 2005). It is widely accepted that global warming has at least in

part been due to anthropogenic climate forcing agents (Shindell, *et al.*, 1998, Schnoor, 2005, Houghton, 2007, Kharecha & Hansen, 2008). Carbon dioxide is a greenhouse gas and is the most significant source of anthropogenic global warming. According to Hansen and Sato (2004) carbon dioxide has accounted for 90% of the increased greenhouse gas climate forcing in recent years. Carbon dioxide is a product of fossil fuel combustion which is the world's primary source of transportation energy (Antoni, *et al.*, 2007). Fossils fuels are not only a non-renewable resource, but their use is contributing to the greenhouse gas affect, causing global temperatures to rise (Himmel, *et al.*, 2007). It is therefore pertinent that a renewable and environmentally responsible fuel source be found as soon as possible.

1.1.2 Biofuels from starch biomass

Fuels such as hydrogen and methane have been proposed as alternatives to fossil fuels. However, both hydrogen and methane use would require new storage and transportation infrastructure at a high cost (Buckley & Wall, 2006). Bioethanol is already being mass produced around the world, particularly from the fermentation of corn starch in the U.S.A and from the fermentation of sugar cane in Brazil. Bioethanol is regarded as a carbon neutral energy source because the plants which it is derived from have a net consumption of carbon dioxide during their lifetime which "cancels out" the carbon dioxide released when the ethanol derived from their fermentation is combusted. However, the carbon budget would take time to become neutral if densely forested land were cleared for biofuel crop production (Gibbs, *et al.*, 2008). As Koh and Ghazoul (2008) stated, the production of bioethanol from food crops such as corn and sugar cane is not sustainable because it competes with these resources from the food chain and occupies valuable land, ultimately driving up food prices. The most attractive solution to this problem is to utilise the energy rich cellulose and hemicelluloses contained in lignocellulosic biomass which can be sourced from waste materials such as wood chips, pulp and paper, agricultural residues, and forestry wastes and thinning (Wyman, 1999).

1.2 Lignocelluloses

1.2.1 Lignocellulose biomass

Lignocelluloses are one of the most abundant forms of biomass on the planet and are a key component in woody plants as well as in plant cell walls and waste materials (Lee, 1997). Lignocellulose biomass is typically composed of; cellulose (35-50%), hemicelluloses (20-35%), and lignin (12-20%) and is much more complex, and difficult to degrade than starch. The carbohydrate polymers are tightly bound to lignin mainly by hydrogen bonding but also by some covalent bonding, where lignin is interspersed with hemicelluloses forming a matrix which surrounds the cellulose microfibrils (Kirk & Farrell, 1987, Lee, 1997). Cellulose comprises β 1-4 linked D-glucose subunits in a two molecule repeating unit (cellobiose) which can be degraded by cellulase enzymes, acid hydrolysis or other aggressive physical and chemical treatments. Cellulose exists in both crystalline and amorphous forms, with the amorphous form being less abundant and more susceptible to enzymatic degradation (Beguin & Aubert, 1994). Hemicellulose is an amorphous chain of a variety of sugars such as: arabinose, glucose, galactose, mannose, and xylose. Rather than being a sugar based structure, lignins are heterogeneous substances based on a phenol propene backbone (Wyman, 1999). Lignin is biosynthetically derived from coniferyl, coumaryl, and sinapyl alcohols (Ragauskas, *et al.*, 2006).

1.2.2 Biomass recalcitrance

Lignocelluloses are highly recalcitrant to enzymatic degradation and have not yet been efficiently used for bioethanol production. This is firstly due to the presence of plant cuticles and the structural diversity of cell walls, as well as the arrangement of vascular bundles and the degree of lignification (Cosgrove, 2005). In order to utilise the cellulose component of lignocelluloses, they must first be separated from lignin, which binds cellulose sheets in an enzymatically inaccessible matrix (see figure 1-1). Aside from lignin removal being a barrier, cellulose itself is a relatively intractable polymer with strong inter glucose hydrogen bonding making it resistant to enzymatic degradation, as well as hydrophobic sheet formations imparting resistance to acid hydrolysis via the

presence of a dense water layer near the hydrated cellulose surface (Matthews, *et al.*, 2006).

1.2.3 Current pre-treatments

Lignin's presence in lignocelluloses provides a protective barrier which prevents plant cell degradation by bacteria and fungi. For the successful conversion of biomass to fuel, lignin must be removed or degraded before celluloses and hemicelluloses are hydrolysed to their monomeric sugar components which can then be fermented to ethanol (Kumar, *et al.*, 2009). Pre-treatments aim to expose the cellulose in the plant fibres, ideally with low cost, and little loss of substrate or production of toxic by-products. The goal of exposing cellulose fibres in lignocellulose pre-treatment is depicted in figure 1-1. Current pre-treatment of lignocellulosic biomass involves high pressure (~10atm) steam treatment or weak acid exposure at high temperatures (240 degrees Celsius) for up to two hours (Himmel, *et al.*, 2007).

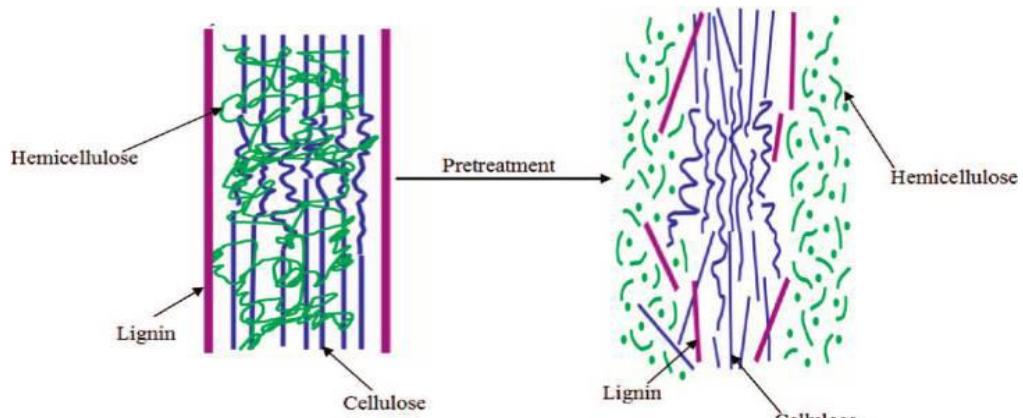


Figure 1-1. Cellulose exposure after lignocellulose pretreatment (Kumar, *et al.*, 2009)

Another pre-treatment is the ammonia fibre expansion (AFEX) method which leaves hemicelluloses in place but renders the rest of the cell wall much more susceptible to enzymatic degradation. These pre-treatments are very energy expensive and time consuming, they also result in the loss of 30 to 40% of fermentable substrate (Torget, *et al.*, 1991). They also result in by-products which are inhibitory to fermenting organisms and contribute to a high BOD in waste

water. Depending on the energy source used for the high pressure or temperature used in lignocellulose pre-treatment, net greenhouse gas emissions may actually be increased. An alternative way forward is to find organisms and/or enzymes which can efficiently degrade lignocelluloses.

1.2.4 Biological lignocellulose degradation

Much of the research done on “white rot” and “brown rot” wood degrading fungi (Kirk & Farrell, 1987, Dutton & Evans, 1996, Goodell, *et al.*, 1997, Hammel, *et al.*, 2002, Martinez, *et al.*, 2009) and most of what is known about lignin biodegradation is from pure culture studies of Basidiomycete fungi (Geib *et al.*, 2008). White rot fungi can degrade the three major plant cell wall components; lignin, cellulose and hemicelluloses simultaneously. It appears that lignin peroxidase (ligninase) is an important enzyme in this process as it randomly disassembles lignin via radical intermediates formed in the presence of oxygen (Kirk & Farrell 1987). In contrast, brown rot fungi are able to surmount the lignin barrier by removing cellulose and hemicellulose with only slight modifications to lignin polymers (Geib *et al.*, 2008, Martinez *et al.*, 2009). Despite the widely categorised nature of wood degrading fungi and their enzymes, it appears that the rate and efficiency of decay is not great, which limits their application to lignocellulose degradation for bioethanol production (Kumar, *et al.*, 2009).

In nature there are many animals that are xylophagous and thrive on lignocellulosic food sources. Beetle larvae, termites, cockroaches, wood wasp larva, limnoriidae marine isopods (shipworms), marine bivalves, beavers, and giant pandas all feed on lignocelluloses. While the degradation of cellulose in complex environments such as the insect gut has been well described (Martin, 1983) little is understood about lignocellulose degradation and the fate of lignin, although it is thought that a consortia of symbiotic bacteria and fungi are involved (Geib, *et al.*, 2008).

The termite has been the focus of much research into symbiotic lignocellulose digestion. In termites the digestion of lignocellulose is aided by the physical dispersion of wood particles in the gizzard which increases substrate surface area in the foregut, before alkali conditions further aid lignin depolymerisation in the

hindgut (Breznak, *et al.*, 1994). Some cellulases produced by the termite itself have also been reported, as have cellulases of gut protist origin (Tokuda, *et al.*, 2004). It is likely that the Huhu grub also provides conditions conducive to lignocellulose degradation, but is ultimately dependent on its microbiota. It is possible that factors other than a Huhu grub's microbiota may be at least in part responsible for lignocellulose degradation. As Breznak *et al.*, (1994) have stated; some termites have a symbiotic relationship with Basidiomycete fungi, which partially degrade wood before consumption by termites, which in turn provide faeces as a nutrient source for the fungi.

1.3 Wood boring insects

The genus *Prionoplus* has only one species, the Huhu grub (*Anamalia arthropoda insect coleopteran prioninae prionoplus reticularis* White) and is endemic to New Zealand. However, *Coleoptera* (beetles) are the group of insects with the largest number of known species at ~350 000 (Droogmans, 2002) and are found everywhere except marine and polar regions. Many of the Cermabycidae (longhorned beetle) family have a wood boring larval stage (like the Huhu grub) before metamorphosing into beetles. While the Coleoptera have been the subject of a vast amount of research on their morphology and taxonomy, there is still much to be discovered about the gut microflora of the wood boring larval stages of particular beetles, including the cerambycid family to which the Huhu grub belongs. However there have been some intriguing insights made concerning the microbial biodiversity of the intestinal tracts of these animals via the use of molecular ecological techniques.

1.3.1 Coleoptera gut flora

One study of the New Zealand grass grub, (*Costelytra zealandica*) used denaturing gradient gel electrophoresis (DGGE) to analyse microbial 16S rDNA extracted from the mid and hindguts of grubs fed on different diets as well as grubs from different geographic locations (Zhang & Jackson, 2008). It was found that the bands appearing from the midgut samples were less conserved than the bands from the hindgut samples. Grubs fed on carrot, or starved, had fewer bands present when compared to soil fed grubs, with the midgut again showing greater

variation than the hindgut. Grubs collected from different geographic locations did have band variation, with some conserved bands and the midgut having greater variation than the hindgut. Sequencing of DNA bands from DGGE analysis revealed that most were associated with clostridia.

Like DGGE, terminal restriction fragment length polymorphism (TRFLP) is a community profiling technique commonly applied to microbial 16S rDNA. This method was used extensively to profile the microbial gut community of *Pachnoda ephippiata* larvae (Egert, *et al.*, 2003), which have a similar lifecycle to the Huhu grub. It was found that the midgut community differed from that of the hindgut, and that these communities differed significantly from the grub's soil diet.

Sequencing of bacterial 16S genes demonstrated that members of the Actinobacteria dominated in the alkaline midgut while members of the cytophaga flavobacterium-bacteriodes (CFB) group were dominant in the hindgut. A Methanobacteriaceae related archaeal 16S sequence was also found in the hindgut which suggested that methanogenesis is a process associated with the hindgut. Physical conditions in the gut were also measured and it was found that they varied not only along the length of the gut (Egert, *et al.*, 2003), but also across the width of the gut (Egert, *et al.*, 2005). The midgut had higher alkalinity and oxygen concentration, which are thought to be important community determining factors (Egert, *et al.*, 2003).

Research has also been conducted on the gut flora of two cerambycids in tandem. Bacterial 16S genes were cloned and sequenced from DNA extracted from the guts of *Anoplophora glabripennis* and *Servaea vestita* (Schloss, *et al.*, 2006). In *A. glabripennis* one hundred 16S rDNA clones were analysed with sequences indicating the following proportions of community members: 48% Firmicutes, 45% Proteobacteria, 4% Actinobacteria, and 3% Bacteriodetes. However, it was duly noted that a greater number of sequenced clones would be likely to reveal higher diversity. From amplified DNA extracted from the gut flora of *S. vestita*, eighty clones containing bacterial 16S rDNA genes were screened. All eighty genes were from members of the γ -proteobacteria. This group of bacteria is a common commensal gut organism which aids the host via vitamin biosynthesis (Schloss, *et al.*, 2006).

These studies have demonstrated longhorned beetle larvae microbial communities which are species, diet, and physiologically specific. This suggests that it is valuable to investigate the gut communities of related beetle larvae, as they may harbour organisms of interest and biotechnological significance.

1.3.2 Termites

It has long been known that termites are able to consume wood, and many old decayed houses stand (perhaps not any more) as evidence to this fact. However, the mechanisms by which lignocellulose degradation is achieved in the termite gut have long been the subject of intense research.

Geib, *et al.*, (2008) used tetramethylammonium hydroxide (TMAH) thermo chemolysis to analyse lignin degradation in the Asian longhorned beetle (*Anoplophora glabripennis*) and the Pacific dampwood termite (*Zootermopsis angusticollis*). When frass lignin structure was compared with undigested wood, significant lignin side chain oxidation was observed in both systems. The oxidation/depolymerisation of lignin occurred at similar levels to those observed in white rot fungi, except at a higher rate (hours versus weeks). The total gut DNA of *A.glabripennis* (from grubs found at different locations, living in different tree species) was analysed for the presence of fungi using PCR clone libraries of amplified fungal ITS regions. The one OTU present was identified as an ascomycete fungus, *Fusarium solani/Nectria haematococca* species complex. This is a soft rot fungus and may be contributing to lignin degradation. This was supported by metatranscriptomic data in which active fungal and prokaryotic transcripts were observed.

(Warnecke, *et al.*, 2007) carried out a metagenomic and functional analysis of the hindgut microbiota of a *Nasutitermes* termite species (which does not contain cellulose-fermenting protozoa). Total DNA from pooled luminal hindgut contents was cloned and 71 mega-bases were sequenced. The cloned inserts were highly fragmented, with the largest being 15kb. This meant that linkage of phylogenetic marker genes to functional genes was limited. 1750 bacterial 16S rRNA genes were analysed with 12 phyla and 216 phylotypes being identified at the 99% threshold. *Fibrobacter* was the most commonly found phylum. Analysis of the

community metagenome revealed no evidence of enzymes linked to lignocellulose degradation. However, cellulase genes were common with over 100 gene modules being identified, although no clostridium like dockerin or cohesion modules were found. CO₂ reductive acetogenesis was identified as the major electron sink, with archaeal primers giving no amplification, which suggested the absence of methanogens. Wood is highly depleted in nitrogen, an element essential for life. Nitrogen fixation is therefore a vital process in the termite gut community and fittingly 12 near full length nitrogen fixation genes were found in the metagenomic analysis.

While the above examples highlight the insight that culture independent methods can achieve, traditional culture based methods have also been successfully employed in the quest for understanding of termite lignocellulose degradation. Harazono *et al.*, (2003) were able to isolate and characterise two strains of aromatic degrading bacteria from the gut of the termite *Coptotermes formosanus*. An aerobic mineral salt enrichment culture containing the lignin catabolic intermediates vanillin and veratraldehyde was used to isolate a *Burkholderia* species and a *Citrobacter* strain. The *Citrobacter* strain was identified as a new “species” via 16S rRNA gene sequence analysis, and interestingly neither strain was able to hydrolyse starch. Similarly, Ramin *et al.*, (2009) were able to isolate cellulolytic bacteria from the termite *Coptotermes curvignathus*. Isolates were cultured in a medium containing carboxymethyl-cellulose and cellulose. Five species were identified by 16S rRNA gene homology: *Bacillus cereus*, *Enterobacter clocae*, an *Acinetobacter sp*, *Enterobacter aerogenes*, and *Chryseobacterium kuangyangense*.

Although thorough investigations of termite microbial communities have hinted at the role these communities play in lignocellulose degradation, the exact mechanism, and organisms responsible remain elusive.

1.3.3 Huhu grubs

Previous research done on New Zealand’s native Huhu beetle larvae (*Prionoplus reticularis*) has focussed on morphology and lifecycle (Edwards, 1959, Edwards,

1959, Edwards, 1960, Morgan, 1960, Hosking, 1978) with no published work on the intestinal microbiota. Female Huhu beetles oviposit 250-300 eggs onto dead tree stumps and logs. Huhu beetle larvae (Huhu grubs) feed predominantly on the dead wood of gymnosperms and are not restricted to native species, as grubs are commonly found in exotic timbers such as *Pinus radiata*. The cessation of active sap secretion is thought to set the earliest limit to colonisation, with the wood not required to be in an already decayed condition (Edwards, 1959). Huhu grubs then tunnel through the wood, consuming it and egesting faecal frass behind them. Tunnel orientation is dependent on the state of decay of the wood with tunnels in fresh wood being longitudinal (with the grain) and tunnels in more decayed wood being increasingly random (Edwards, 1959). This continues until the grub reaches full size (up to 7cm long) after 2 to 3 years with a non feeding, overwintering final instar (Rogers, *et al.*, 2002). Huhu grubs have previously been raised in the laboratory on an artificial pine sawdust based diet and were shown to reach maturity after only 250 days, much faster than in their natural environment (Rogers, *et al.* 2002). Mature Huhu beetles can be observed flying nocturnally in the drier summer months from late spring to early autumn. They live for approximately two weeks and do not feed during this period, although it is thought that water uptake does occur (Edwards, 1960). Mating usually occurs between 10pm and 1am and is preceded by a 15 minute period where the male has mounted the female and inactivity is interrupted by the twitching of antennae by both parties. Coitus may then occur for up to 12 minutes, although it is thought that only 12-15 seconds are required for the female's eggs to become fertilised (Edwards, 1959).

There is no published literature on the gut flora of the Huhu grub, or the mechanism by which it is able to consume, and thrive on lignocellulose.

1.4 Methods of Microbial community analysis

1.4.1 Culture based methods

Growing microorganisms under laboratory conditions allows for physiology and function to be observed and assayed directly via the use of microscopy and varied physical parameters such as nutrient and oxygen content, salinity, pH,

temperature, etc. However, mimicking environmental conditions in order to culture organisms adapted to specific substrate and nutrient types and concentrations has proven challenging (Kaeberlein, *et al.*, 2002), because when high substrate concentrations are used, frequently only the fast growing bacterial ‘weed’ species are obtained. The molecular ecological revolution has hinted that the true diversity of microorganisms on earth is orders of magnitude greater than culture based studies had previously estimated. Amann, *et al.*, (1995) estimated that >99% of microorganisms in nature cannot be cultivated using standard methods.

1.4.2 Culture independent methods

With the development of the polymerise chain reaction (Mullis & Faloona, 1987), dideoxy terminator sequencing (Sanger, 1988), and the use of the small subunit 16S rRNA gene as a biomarker for microbial life (Woese, *et al.*, 1990), came a revolution in microbial ecology. These techniques allowed for the identification of microorganisms relative to each other via the differences in base pair sequences in variable regions of their 16S rRNA genes, which are essential for protein synthesis and therefore life (non viral). The variable sequence regions are thought to drift under neutral evolution (Kimura, 1969), as they do not function in the secondary structure of the rRNA molecule (Figure 1-2) which is coded for by the conserved regions, which are convenient for developing PCR primers. Differences in 16S rRNA gene sequences are therefore interpreted as evolutionary distances between strains and are used to make phylogenetic inferences.

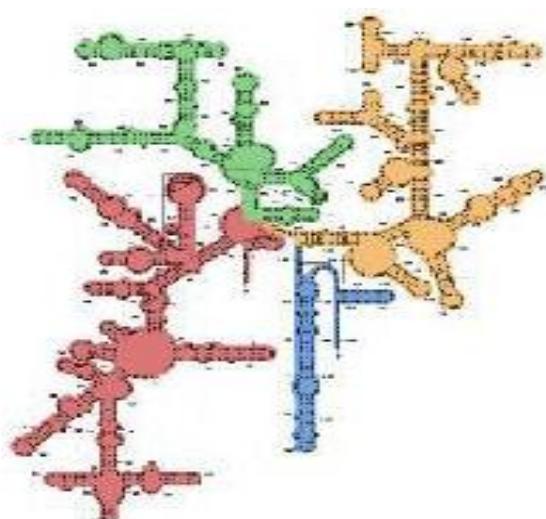


Figure 1-2. 16S rRNA molecule secondary structure (Garrett & Grisham, 2005).

Red corresponds to the 5' domain (nucleotides 27-556), green to the central domain (nucleotides 564-912), yellow (923-1391) and blue (1392-1541) correspond to the 3' domain.

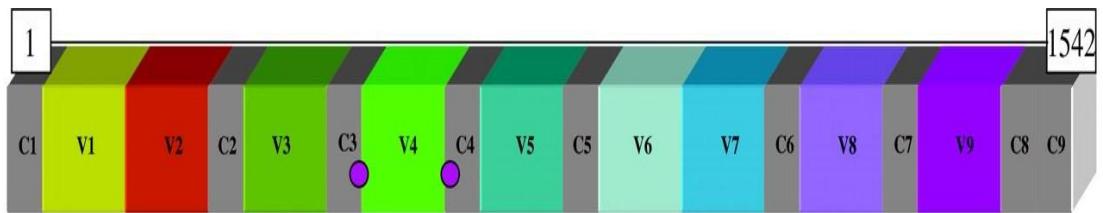


Figure 1-3. Conserved (C) and variable (V) regions of the bacterial 16S rRNA gene (Petrosino, *et al.*, 2009)

1.4.3 Community profiling

Community genetic profiling has become a valuable tool for identifying differences in microbial diversity and community structure which can be correlated to environmental conditions and factors. Terminal restriction fragment length polymorphism (TRFLP) profiling, developed by Liu, *et al.*, (1997) utilises PCR with one primer fluorescently labelled, so that when amplified biomarker genes are digested with sequence specific restriction enzymes, terminal fluorescently labelled fragments of different lengths are generated (ribotypes). Different ribotypes represent different bacterial strains and are separated along with internal size standards by capillary electrophoresis using an automated DNA sequencer. The fluorescent label is detected and signal intensity is related to fragment concentration. The resultant community profiles are then screened for noise before being analysed statistically in a software package such as PRIMER6 (Clarke & Gorley, 2006) which can carry out cluster and multidimensional scaling analysis. TRFLP is a powerful technique for detecting and explaining microbial community differences, but does not give any information regarding the types of organisms present. For this, sequencing of biomarker genes is required.

1.4.4 Clone library analysis

Sanger sequencing employs a polymerase chain reaction with a proportion of dideoxy nucleotide tri-phosphates (ddNTPs) which once incorporated into a replicating DNA strand prevent any further dNTP addition to that particular

molecule (Sanger, 1988). Amplicons of every possible length (for the primers used) are generated prior to being separated by capillary electrophoresis and detected using an automated DNA sequencer. If the PCR primers can bind to more than one DNA template then the sequencing will not work as different terminal ddNTPs will be detected in amplicons of the same length. It is for this reason that PCR amplified biomarker genes must be separated before sequencing. This can be achieved by inserting amplified genes into a plasmid vector and transforming the vector into an *E.coli* host. Invitrogen's TOPO® TA Cloning Kit for Sequencing uses a linear topoisomerase enzyme bound to a plasmid coding for antibiotic resistance and with a single terminal 3' T overhang to insert a PCR product with a terminal A overhang (produced by a longer final PCR extension at 72°C). The vectors can then be transformed into individual *E.coli* hosts via heat shock or electroporation before cells are selectively grown on antibiotic containing Luria Bertani plates. Plasmid DNA can be directly sequenced or PCR amplified for restriction digest analyses using primer sites which flank the insert.

1.4.5 454 pyrosequencing

Nyren P (1987) and Ronaghi *et al.* (1996) were the first to couple DNA polymerase base incorporation, ATP sulfurylase ATP production and subsequent light and inorganic phosphate (PPi) release with the firefly luciferase enzyme in order to sequence DNA *in vitro*. The fact that sequencing reactions are carried out simultaneously on single DNA molecules attached to beads in separate wells means that there is no need for expensive and time consuming cloning. In metagenomic or shotgun 454 sequencing, cell community DNA is simply fragmented and attached to beads prior to sequencing. However, for the targeting of a particular DNA sequence such as the 16S rRNA gene, PCR and sample label attachment are still necessary prior to sequencing. These principles have been developed into a high throughput sequencing technology capable of producing reads of over 400 bases at a fraction of the cost of Sanger sequencing (Rothberg & Leamon, 2008).

The main error which occurs in pyrosequencing involves the accurate determination of homopolymer lengths, as the intensity of fluorescence does not accurately resemble the quantity of a single base incorporated consecutively in a

single wash. Similar flowgrams can result in different sequences, for example when thymine (T) is washed over a plate an observed light intensity of 4.65 would denote a homopolymer of TTTTT whereas the true sequence could actually be TTTT. Quince and Lanzen *et al.*, (2009) have developed an algorithm called “Pyronoise” which clusters flowgrams using a distance measure that models noise. Flowgram probability distributions were calculated by observing homopolymer signal intensities from pyrosequencing of the 5th variable region of 23 clones of known sequence. The sequences differed by $\geq 7\%$ in order to aid unambiguous flowgram-sequence assignment. The probability that a flowgram was generated by a sequence was defined as a distance by using the flowgrams and their distribution of intensities. This process was compared with the ribosomal database project and other pyrosequencing pipelines on 90 16S rRNA genes, obtained from a eutrophic lake, which were sequenced by the Sanger method. Pyronoise was the best at preventing an over-estimation of the number of OTUs.

1.4.6 DNA extraction and PCR biases

No scientific method is without fault, and culture independent microbial ecological methods are no exception. There are particular biases associated with DNA extraction and downstream analyses which are reviewed by Von Wintzingerode, *et al.*, (1997). The most obvious argument against the use of such techniques is that just because a particular organism’s DNA is present in an environment it does not prove that it is active in a process or even alive, although the use of RNA probes in FISH (fluorescent in situ hybridisation) can be used to resolve this issue. DNA extraction bias is another important issue, as in a community of microorganisms some cells will be less or more resistant to whichever cell lysis method is employed along with DNA being present in variable states of degradation. This has been shown to occur to the extent where analysis of the same sample with different DNA extraction methods yields significantly different results in regard to community structure and composition (Leff, *et al.*, 1995).

PCR biases are also considerable and should never be disregarded. Universal primers which anneal to highly conserved gene regions are often used to amplify a biomarker gene (such as bacterial 16S rDNA) for an entire domain of life. Given

the small amount that is known about microbial diversity from sources other than biomarker gene amplification and sequencing there is always the possibility that a large proportion of novel organisms are excluded from analysis. Even within the realm of known organisms, primers will bind to particular sequences with different affinities and subsequent extension will occur with variable levels of bias for different template molecules, leading to potentially skewed OTU abundances (Suzuki & Giovannoni, 1996). Another bias associated with DNA extraction and PCR is the co-extraction of DNA polymerase inhibitors from an environmental sample along with DNA (Tebbe & Vahjen, 1993). This can result in a severe under estimation of diversity as the less abundant DNA molecules may not undergo amplification, while the molecules present in a greater concentration do.

Biases also extend into analysis procedures, particularly in regard to screening for chimeric PCR artifacts. PCR chimeras form due to in vitro recombination of homologous DNA and lead to amplified DNA comprising of sequences from two different genes. Most algorithms for removing chimeric DNA sequences from a dataset compare 5' and 3' sequences of the same molecule to a reference database separately and decide whether or not the sequence is chimeric based on the whether the two ends appear to be from different organisms or not. This method is therefore dependent on the reference database being fully representative of microbial diversity in nature.

Despite the limitations of current methodologies, well designed experiments can be employed to ask even the most difficult scientific questions.

1.5 Aims of this thesis

- Capture Huhu grubs from the wild and raise them in the laboratory on controlled diets.
- Catalogue gut microbial community composition in wild type grubs.
- Determine whether grub diet affects microbial community composition.
- Deduce which microbes are associated with lignocellulose, hemicellulose, and cellulose degradation.
- Identify any microbes with the potential for important community function such as nitrogen fixing, methanogenesis, acetogenesis, acetic acid synthesis etc.
- Begin cultivation efforts targeted towards organisms of interest identified in culture independent analysis.

2 Materials and Methods

2.1 *Huhu larvae rearing*

2.1.1 Grub capture

Dead trees or logs which had been down for at least three years were ideal locations to search for Huhu larvae, as this time period would allow for larger larvae to be present. Holes in the end of a log wide enough for an adult larvae to fit in were good indicators of Huhu infestation, as was a decayed state of the wood.

Logs which complied with these parameters had sections cut off using a medium sized chainsaw (16 inch bar). If larvae were not immediately obvious then the section was broken apart along the grain using a small axe. If larvae were found they were transferred to a plastic container along with some wood or sawdust from nearby. Forceps were often required to extract the larvae from their tunnels in the wood. Grubs were given individual containers to prevent biting inflicted injuries during their transition from log to lab.

If no Huhu larvae were found from the initial off cut, then the chainsaw was used to segment the log further until either larvae were found or it was determined that they were not present in a particular log.

The Huhu larvae used for all of the molecular analysis were found at two locations. Sixteen were taken from a pine log on a farm outside Hamilton at 291 Karakariki Rd. The pine log had been down for approximately five years and was about one metre in diameter. These larvae were raised on nutrient rich diets in the laboratory for two months. The three wild type larvae, and the eleven larvae raised on pine block and cellulose diets were sourced from Jubilee Park off Boundary Rd in Claudelands, Hamilton. They were found, using only an axe, in a recently felled, half metre in diameter segment of Kahikatea tree which had been decaying whilst upright for some time. Figures 2-1 and 2-2 show a Huhu larva tunnelling in the wild, and then being measured in the lab prior to being introduced to a laboratory diet.



Figure 2-1. Huhu larvae tunnelling in pine wood.



Figure 2-2. Larval measurement prior to transfer to artificial diet

Initially, grubs were raised on nutrient rich artificial pine, cellulose, and starch diets in the laboratory in order to make microbial community comparisons based on diet differences. These laboaratory media were based on the description in Rogers, *et al.*, (2002). In subsequent work, when the limitations of this approach were appreciated, a second cohort of grubs was grown on either a pine block or compressed cellulose, only with no other amendments.

**Table 2-1. Ingredients for 2.5kg of pine sawdust based medium for Huhu grubs
(Rogers, *et al.*, 2002)**

Ingredient	Amount
Wheat germ	60.8g
Wesson's salt mix	20.3g
Agar (Difco granulated)	81.3g
Cellulose powder (sigma cell 50µm particle size)	182.5g
Pinus radiata sawdust (Untreated)	121.8g
Glucose (D) anhydrous	30.5g
Yeast (Edmond's baking grade)	60.8g
Casein (Lactic)	60.8g
Sucrose	50.7g
Distilled water	1745ml
Mould inhibitor	36.5ml
7 vitamin solution	40.5ml

Table 2-2. Wesson's salt mix

Ingredient	Amount
Magnesium sulphate 7H ₂ O	45g
Calcium carbonate	105g
Aluminium potassium sulfate 12H ₂ O	0.045g
Cupric sulfate	0.195g
Ferric phosphate	7.35g
Manganese sulfate	0.1g
Potassium chloride	60g
Potassium phosphate monobasic	155g
Potassium iodide	0.025g

Table 2-3. Mould inhibitor

Ingredient	Amount
p-hydroxybenzoate	4.49g
Sorbic acid	5.88g
Ethanol (drum grade)	50ml

The p-hydroxybenzoate and the sorbic acid were dissolved in the ethanol using a magnetic stirrer then filter sterilised through a 0.2µm membrane into a sterile Schott bottle.

Table 2-4. Vitamin solution

Ingredient	Amount
Cyanocobalamine B12	50mg
p-Aminobenzoic acid	40mg
D(+) Biotin	10mg
Nicotinic Acid	100mg
Calcium pantothenate	50mg
Pyridine hydrochloride	150mg
Thiamine hydrochloride	100mg
Ascorbic acid	105mg
Distilled water	500ml

The above components were dissolved together in the water before being filter sterilised through a 0.2µm membrane into sterile 25ml universal tubes. Tubes were kept in tinfoil at 4°C.

The cellulose medium was made using the same ingredients outlined in table 2-1 except with TORK facial tissue paper used as cellulose in place of pine sawdust. The tissue paper was weighed dry before being saturated with distilled water and autoclaved at 121°C for 20 minutes. It was then shredded before being added to the other ingredients in Table 2-1.

In the starch diet both pine sawdust and cellulose powder (from the original pine based medium) were replaced with potato starch. The amount of agar was twice that of the other media in order to provide comparable solidity to the mix. Larvae would not gain weight on the original starch diet with 3.25% agar (same concentration as pine diet) as it was too soft to accommodate effective larval tunnelling and ecdysis (cuticle moulting between instars).

These media were made by mixing all of the ingredients apart from the mould inhibitor and vitamin solution together in a clean 5L glass conical flask and autoclaving at 121°C for 20 minutes. When the mixture had cooled to approximately 80 °C the vitamin solution and mould inhibitor where added in a laminar flow fume hood using sterile pipette tips. The liquid medium was mixed thoroughly by swirling before being poured hot into clean 200ml clear plastic screw cap containers to about the half way mark. Prior to the medium setting hard, 1.5ml eppendorf tubes were pushed into the surface of the medium in each container. These tubes were removed once the medium had set hard in order to provide a groove in which Huhu larvae were placed to encourage tunnelling and diet consumption.

Three Wild Type grubs from Boundary Rd were dissected and had gut DNA extracted immediately following capture for use as wild type representatives. The larvae which were raised on the laboratory prepared media shown in (figure 2-3) were weighed and transferred to fresh containers of media monthly, or as required, for two months. The larvae were then dissected and had DNA extracted from their gut contents as well as their most recent frass, collected on the day of dissection.

Six additional grubs from kahikatea logs on Boundary Road were captured for laboratory rearing on two different diets. Three grubs were fed into segments of untreated pine timber which had holes drilled in their ends and had been autoclaved in water (figure 2-4.) The other three grubs were fed into test tubes containing wet autoclaved tissue paper (figure 2-5.) which contained cellulose but no lignin. DNA was extracted from each grub's frass and gut contents once after one month of feeding.

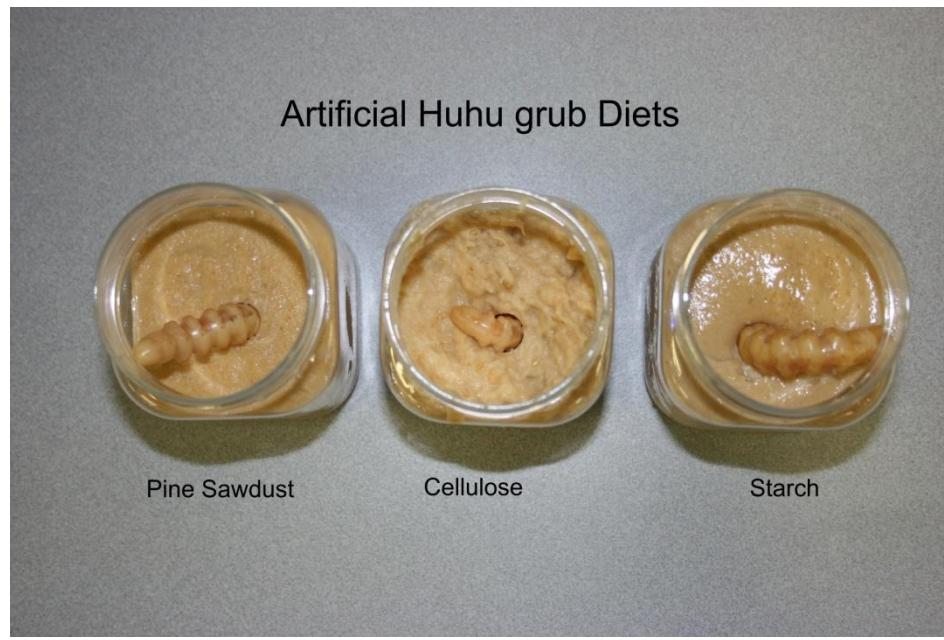


Figure 2-3. Artificial Huhu grub media, developed from the protocol of Rogers et al., (2002).

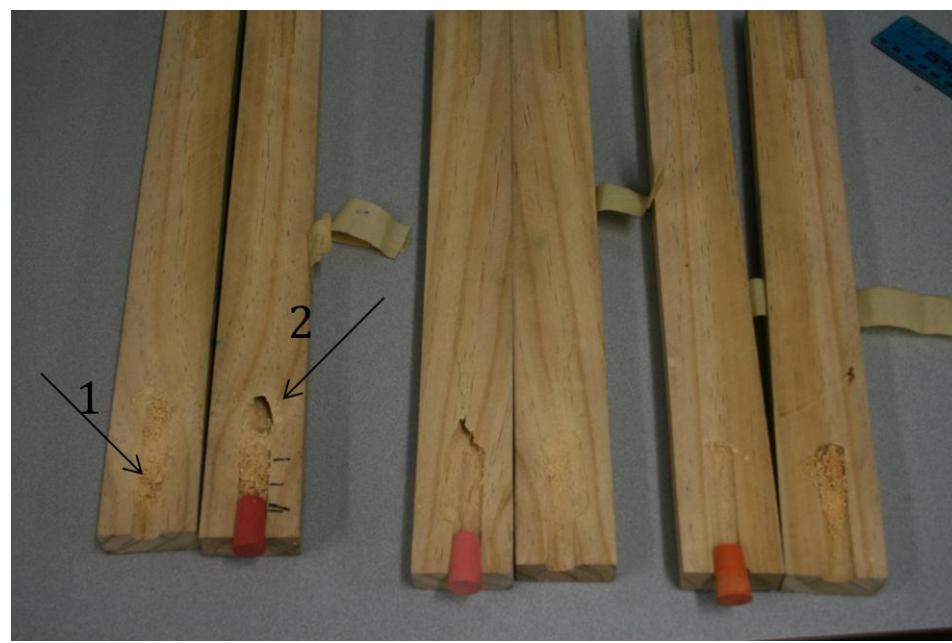


Figure 2-4. Untreated pine timber grub diet.

The two halves of each block are normally placed together to create a dark enclosed tunnel. Frass can be seen in the tunnels (1), and the extensions of the tunnels created by the Huhu grubs are also visible (2).



Figure 2-5. Grub diet of wet autoclaved cellulose tissue paper.

2.2 DNA extraction

2.2.1 Grub dissection

Table 2-5. Phosphate buffered saline (1x)

Ingredient	Amount
Sodium chloride	4g
Potassium chloride	0.1g
Sodium pyrophosphate (Na_2HPO_4)	0.72g
Potassium dihydrogen phosphate (KH_2PO_4)	0.12g
Distilled water	500ml

The above ingredients were dissolved in 400ml of water, the pH was adjusted to 7.4 then the solution was made up to 500ml with distilled water before being autoclaved at 121°C for 20 minutes.

All dissections were carried out in a laminar flow fume hood. All dissecting instruments used were dipped in 70% ethanol and flamed using a Bunsen burner before and after each grub was dissected. The wax dissecting tray was sprayed with an excess amount of 70% ethanol and wiped down before and after each use. Fresh areas of parafilm were sprayed with 70% ethanol before use and discarded afterwards.

The larva to be dissected was placed on the wax tray and immersed in PBS. With its dorsal surface facing upwards the larva had two pins inserted each side of its head and tail respectively. One pin was used to destroy the larva's brain. Using fine tipped forceps, flesh from about three quarters of the way down the body was pulled up so that scissors could be used to slice open the cuticle. The scissors were then used to cut along the middle of the larva's dorsal surface in a straight line, exposing the intestine and fatty tissue. Two pins were used to spread open the cuticle on each side and further expose the gut. Scissors were used to separate the head from the body while leaving it attached to the intact intestine. Connective tissue and malpighian tubules were cut away from the intestine along its entire length with care being taken not to cause a puncture. The intestine was separated from the anus and lifted out by the head onto a piece of parafilm. Figure 2-6 (Edwards, 1960) shows the gut layout of the Huhu larvae and figure 2-7 shows the actual gut of a grub mid way through a dissection.

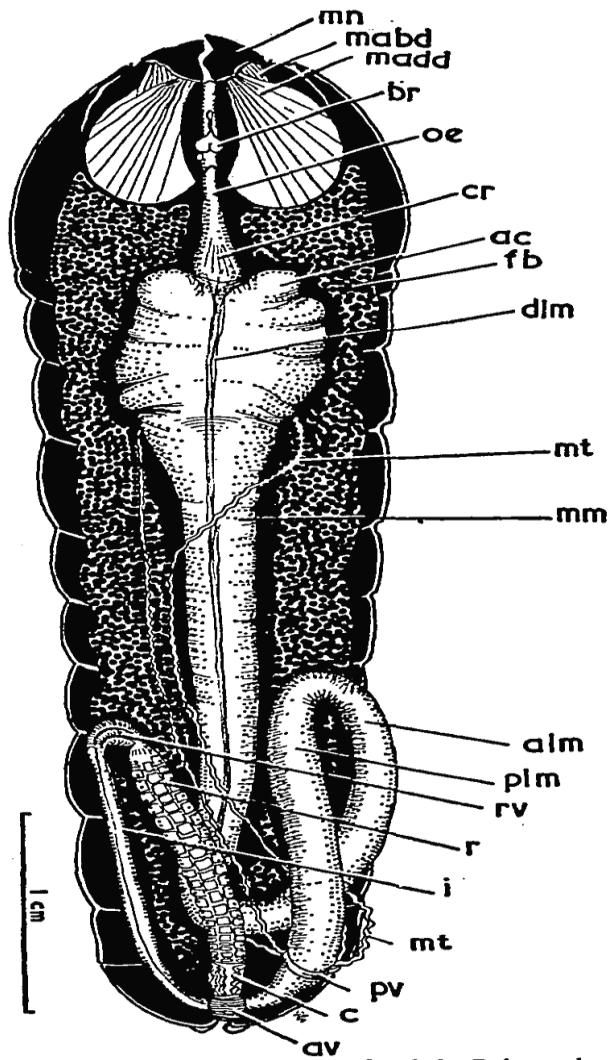


Figure 2-6. Gut of larval *Prionoplus reticularis*.

ac, anterior crypt; alm, ascending limb of midgut; av, anal valve; br, brain; c, cryptonephridial tubules; cr, crop; dlm, dorsal longitudinal muscle; fb, fat body; i, ileum; mabd, mandibular abductor muscle; madd, mandibular adductor; mm, mid mesenteron; mn, mandible; mt, malpighian tubule; oe, oesophagus; plm, posterior limb mesenteron; pv, pyloric valve; r, rectum; rv, rectal valve. (Edwards, J S. Observations on the ecology and behaviour of the Huhu Beetle, *Prionoplus reticularis* White. (Col. Ceramb.) 1960)



Figure 2-7. Huhu larva dissection.

2.2.2 DNA extraction

The dissected intestine was cut at about halfway and held by the head using forceps above an open 1.5ml eppendorf tube to collect any liquid from the intestinal lumen. The head was severed and any liquid surrounding the gut segment was pipetted into the eppendorf tube. The upper half of the intestine was levaged (liquid forced through the lumen for collection) twice with 200µl of PBS, and the lower half once with 200µl PBS. A 200µl pipette was used to force the liquid through the intestinal lumen and into the 1.5ml eppendorf tube.

The 1.5ml eppendorf tube was vortexed before being centrifuged at 16,100g for 5 minutes at 4°C. The supernatant was discarded and the pellet resuspended by vortexing in 600µl of PBS. This was centrifuged as above with the pellet being resuspended in 400µl PBS. The entire contents of the eppendorf tube (400-500µl) were pipetted into the 1st tube in the MoBio Powersoil DNA extraction kit (catalog # 12888-100). DNA extraction was carried out according to the user's manual. The DNA extraction kit excluded humic acids and other PCR inhibitors.

Larvae were dissected and had DNA extracted from their gut contents.

Prior to dissection, an amount of frass of approx 0.2-0.4g was collected and used to extract frass DNA again using the MoBio Powersoil kit. An extraction blank was also taken by following the user's manual with no input of frass into the first tube in the kit.

All DNA samples were analysed for quantity and quality using a NanoDrop ND-1000 (NanoDrop technologies, Montchanin. DE).

2.3 16S rRNA gene and fungal ITS amplification and community profiling

2.3.1 TRFLP

The fungal ITS (18S-23S intergenic spacer) TRFLP PCR reactions were carried out in triplicate 25 μ L volumes. Each tube contained 3 μ L of 2 to 10 ng/ μ L template DNA, 1.5 μ L of 25mM MgCl₂, 0.75 μ L of each primer at 10 μ M (Invitrogen Ltd, New Zealand), ITS1F hexachlorofluorescein (HEX) labelled (5'CTTGGTCATTAGAGGAAGTAA) and ITS4R (5'TCCTCCGCTTATTGATATGC), 2.5 μ L of 10x PCR buffer (Invitrogen Ltd, New Zealand), 2.5 μ L of 2mM dNTPs, 2.5 μ L of 0.2mg/mL bovine serum albumin (BSA), 0.2 μ L of 5U/ μ L Platinum Taq polymerase (Invitrogen Ltd, New Zealand), and autoclaved MilliQ water up to 25 μ L (Millipore, Billerica, MA, USA).

Thermal cycling conditions were; Initial denaturation at 94°C for 3 min, 30 cycles of 94°C for 20 sec, 55°C for 20 sec, 72°C for 1 min 15 sec, and a final extension of 5 min at 72°C.

All PCR reactions were run on an eppendorf master cycler (EPPENDORF AG) thermal cycler. A four microlitre aliquot from one of the triplicate reactions from each sample was visualised after electrophoresis on a 0.8% agarose gel in 1x TAE buffer (80 volts for 30 to 60 minutes) with a 0.2mg/L ethidium bromide wash. Bromophenol blue was used as a loading dye and Lambda/HindII fragments (Invitrogen Ltd, New Zealand) were used as a size standard ladder. See appendix C for TAE buffer composition.

Triplicate reactions were combined before being purified using a QuickClean PCR Purification Kit (Genscript Corporation, New Jersey, USA). The purified PCR products were analysed for quantity and quality using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA).

The fungal ITS PCR products were digested with the MSP1 enzyme (Invitrogen Ltd, New Zealand). Enzyme digestions were carried out in 10µL reactions with 1µL of MSP1 enzyme and 2µL of buffer and made up to 10ul with autoclaved MilliQ water for five hours at 37°C and twenty minutes at 80°C. The products of these enzymatic digestions were diluted 1:15 with autoclaved milliQ water.

Genotypes were resolved along with the internal size standard 1200Liz, using a MegaBACE 500 DNA Analysis System fitted with 40 cm capillary arrays (Amersham Biosciences) loaded with linear polyacrylamide Long Read Matrix (Amersham Biosciences).

2.3.2 Statistical analysis

The raw genotyping data was processed using the freeware programs Perl (Wall, Copyright (C) 1993-2005) and R (Team, 2009) with a peak height cut off of five fluorescence units. This process filtered out peaks which had peak areas less than three standard deviations greater than the ‘zero’ (calculated using background noise), and binned peaks within one base pair of each other. In all TRFLP analyses, fragment peaks which were shorter than the length of the fluorescent primer (eluted before it) were disregarded.

Fungal frass and gut TRFLP profiles were analysed both separately and together. Any sample peaks in common with peaks present in DNA extraction blank samples were disregarded as contaminants.

The PRIMER6 software package (Clarke & Gorley, 2006) was used for comparison of the resultant community profiles. Data was first transformed into presence/absence in order to account for the effects of PCR bias and the semi quantitative nature of genotyping. Relative similarities between TRFLP profiles

were measured using Bray-Curtis similarity index based non-metric multidimensional scaling (MDS) analysis with 100 random restarts and results plotted in two dimensions. Relative distances between samples (labelled by their diet) in the ordination plots denoted relative similarity between TRFLP profiles. Plots with a stress value less than 0.20 provide interpretable information (Clarke, 1993). The CLUSTER method was used to carry out agglomerative, hierarchical clustering of the Bray-Curtis similarities, along with similarity profile (SIMPROF) permutation tests which identify statistically significant clusters. These results were superimposed onto MDS plots at 20%, 40%, and 50% similarity levels. In order to check whether the major peaks were conserved between gut and frass samples, cluster and MDS analyses were carried out on untransformed data and peak height contribution to community similarities was considered using SIMPER analysis.

2.4 454 pyrosequencing

2.4.1 DNA sample preparation and sequencing

Individual DNA samples from each treatment group were quantified on a NanoDrop ND-1000 (NanoDrop technologies, Montchanin. DE) before being pooled together in equal amounts to provide between 200 and 300ng DNA suspended in sterile MilliQ water. This resulted in pooled samples each containing gut DNA from three grubs from each of the treatment groups: wild type (WT), pine (P), cellulose (C), starch (S), and pine frass (Pf) where the latter groups refer to the nutrient rich artificial diets. These pooled DNA samples were cooled to -80°C along with dry and normal ice and couriered to Taxon Biosciences, Inc. Tiburon, California.

50,000 reads for each sample were requested. 454 GS FLX Titanium PCR amplicon pyrosequencing of the V5/V6 region was carried out using 29ng of barcoded PCR product for each sample.

2.4.2 Data processing and analysis

Pyronoise (Quince, *et al.*, 2009) was used to group similar sequences according to their flow-grams before the data was checked for chimeras using the perseus

algorithm (Quince, unpublished). Non chimeric sequences had their sample labels removed and were parsed into a non redundant tab delimited FASTA file and submitted to the MAFFT server for alignment (Katoh, *et al.*, 2002). Subsequent analysis was performed using mothur (Schloss, *et al.*, 2009). A column formatted distance matrix was calculated and sequences were clustered into OTUs at the 97% level using the furthest neighbour method. OTU richness estimates (sobs, ace, chao) were calculated for each sample, and single sample rarefaction analysis was carried out in order to estimate the degree of community coverage attained by the sequencing. Shared sobs and chao indices were used to construct Venn diagrams showing OTUs common or distinct between samples. Dendograms displaying the dissimilarity (1-similarity) between samples were constructed using “Yue and Clayton theta” and “Jaccard” coefficient using richness” estimators. OTU abundance and community similarity heatmaps were also generated using mothur. Sequences representative of each OTU were extracted and submitted to the rdp classifier for taxonomic assignment. The output file was tab delimited before being imported in Microsoft excel and filtered together with OTU read abundance data. The resulting spreadsheet proved to be a powerful tool for assigning the dominant taxa in each sample and constructing graphical representations of community diversity and taxa distribution. The OTUs which contained greater than 1% of the total reads in the Wild Type sample were imported into Geneious and used to construct a Jukes and Cantor neighbour joining tree. Wild Type OTUs with greater than 0.1% of the total sample reads were imported into ARB (Ludwig, *et al.*, 2004) and used to construct a phylum level phylogenetic tree.

2.5 Cloning & sequencing

2.5.1 PCR

Each clone library consisted of 16S rDNA or fungal ITS regions amplified from the pooled gut or frass DNA from three grubs fed on the same diet. 7 μ L aliquots of DNA were combined from each individual grub's DNA within a group of three grubs fed on each diet (including wild type). Water was used in place of DNA as a negative control in both PCR protocols.

The pooled DNA was amplified in triplicate 50 μ L volumes. For 16S rDNA gene amplification each tube contained 7 μ L of template DNA at 2-10ng/ μ L, 3 μ L of 25mM MgCl₂, 1 μ M each of primer 27F (5'AGATTGATCCTGGCTCAG) and primer 1522R (5'AAGGAGGTGATCCARCCGCA), 5 μ L of 10x PCR buffer (Invitrogen Ltd, New Zealand), 5 μ L 2mM dNTPs, 8 μ L of 0.2mg/ml bovine serum albumin (BSA), 0.2 μ L of 5U/ μ L Platinum Taq polymerase (Invitrogen Ltd, New Zealand), and autoclaved milliQ up to 50 μ L. Thermal cycling conditions were; Initial denaturation at 94°C for 3 min, 31 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 2 min, and a final extension of 30 min at 72°C.

For fungal ITS PCR reactions each 50 μ L tube contained 3 μ L of 2 to 10 ng/ μ L template DNA, 1.5 μ L of 25mM MgCl₂, 0.75 μ L of each primer at 10 μ M (Invitrogen Ltd, New Zealand), ITS1F (5'CTTGGTCATTAGAGGAAGTAA) and ITS4R (5'TCCTCCGCTTATTGATATGC), 2.5 μ L of 10x PCR buffer (Invitrogen Ltd, New Zealand), 2.5 μ L of 2mM dNTPs, 2.5 μ L of 0.2mg/mL BSA, 0.2 μ L of 5U/ μ L Platinum Taq polymerase (Invitrogen Ltd, New Zealand), and autoclaved MilliQ water up to 50 μ L (Millipore, Billerica, MA, USA). Thermal cycling conditions were; Initial denaturation at 94°C for 3 min, 30 cycles of 94°C for 20 sec, 55°C for 20 sec, 72°C for 1 min 15 sec, and a final extension of 5 min at 72°C.

All PCR reactions were run on an eppendorf master cycler (EPPENDORF AG) thermal cycler. Agarose gel electrophoresis was used to visualise the amplified DNA at the expected length and assess its quality. Aliquots from one of the

triplicate reactions from each sample were visualised on agarose gel as described in section 2.3.1. 16S rDNA triplicates were combined and run on a single lane agarose gel using conditions as above. The band was visualised under UV light and cut out of the gel using a sterile scalpel blade. DNA was extracted from the gel using a PureLink Quick Gel Extraction Kit (Invitrogen Ltd, New Zealand) and stored at -20°C. Fungal ITS triplicate 50µL reactions were combined and column purified using a QuickClean PCR Purification Kit (Genscript Corporation, New Jersey, USA) according to the user's guide. Fungal ITS regions can vary in length and therefore were more suited to column purification than to multiple gel band excisions.

2.5.2 Transformation

Four microlitre aliquots of gel or column purified PCR product were cloned into the plasmid vector pCR® 4-TOPO® using a TOPO® TA Cloning Kit for Sequencing (Invitrogen Ltd, New Zealand) according to the user's manual guideline for electrocompetent *E.coli*, which uses a dilute salt solution in order to prevent arcing during electroporation. This solution was left at room temperature for half an hour in order to maximise the number of transformants. 2µL from each cloning reaction was transformed into One Shot® TOP10 electrocompetent cells using the protocol in the user's manual. A 0.2cm cuvette containing the 50µL cell/plasmid vector mix was electroporated for ~5 seconds at; 200 ohms, 25 farads, 2.5 volts. After shaking at 37°C with 250µL S.O.C medium for at least one hour to allow for antibiotic resistance expression, 20 and 50µL cell aliquots were spread onto selective LB agar plates containing 50µg/ml kanamycin and incubated at 37°C overnight.

2.5.3 Colony screening

Each colony found growing after overnight incubation was firstly transferred (up to a total of 120) to a numbered patch plate of LB kanamycin agar. Initially a simple boiling lysis method was used to extract plasmid DNA for subsequent PCR analysis. A large portion of each colony was picked with a sterile toothpick and placed in 50µL aliquots of sterile MilliQ water in a sterile 96 well plate. The plate was heated to 96°C for 10mins to lyse cells before being centrifuged at 1500g for

10mins to pellet cell debris, leaving the DNA in the supernatant. Plates were stored at -20°C.

The plasmid insert was amplified for restriction digest analysis in 25µL reaction volumes. Each well in a 96 well plate contained: 5µL of template DNA, 2.5µL of 25mM MgCl₂, 0.625µL of 10µM primer aliquots M13F (5'GTAAAACGACGGCCAG) and M13R (5'CAGGAAACAGCTATGAC), 2.5µL of 10x PCR buffer (Invitrogen Ltd, New Zealand), 2.5µL 2mM dNTPs, 2.5µL of 0.2mg/ml BSA, 0.2µL of 5U/µL Tfi polymerase (Invitrogen Ltd, New Zealand), and autoclaved milliQ water up to 25µL. Thermal cycling conditions were; Initial denaturation at 94°C for 2 min, 30 cycles of 94°C for 30 sec, 55°C for 20 sec, 72°C for 2 min, and a final extension of 7 min at 72°C.

11.5µL aliquots of amplified 16S rDNA samples were digested over night at 37°C using 5 units each of XbaI and HaeIII enzymes (in a double digest, single tube reaction), 2.5µL of React2 buffer (Invitrogen Ltd, New Zealand), and 5µL of sterile MilliQ water. Banding patterns were visualised on 1.2% agarose gels (1x TAE) run at 70 volts for 45 minutes. Gel patterns were discerned manually via visual inspection.

10µL aliquots of amplified fungal ITS DNA samples were digested over night at 37°C using 1 unit each of AluI (Roche diagnostics) and HaeIII (Invitrogen Ltd, New Zealand) enzymes in separate tubes, along with 2.5µL of their corresponding buffer, and 8µL of sterile MilliQ water. Banding patterns were visualised as above.

Colonies with distinct banding patterns were grown overnight at 37°C in 20mL of LB broth supplemented with Kanamycin (50µg/mL) before having DNA extracted from 1mL of culture using an alkaline lysis plasmid mini-prep method developed by the Preuss lab at the university of Chicago (Preuss, 2008). DNA samples were resuspended in sterile MilliQ water and checked for purity (260:280) using a NanoDrop ND-1000 (NanoDrop technologies, Montchanin. DE).

2.5.4 Sanger sequencing & analysis

Two representative samples from each banding pattern were sent to the University of Waikato DNA sequencing facility for bi-directional sequencing using M13F and/or M13R primers. DNA sequences were resolved using a MegaBACE 500 DNA Analysis System fitted with 40 cm capillary arrays (Amersham Biosciences) loaded with linear polyacrylamide Long Read Matrix (Amersham Biosciences). DNA templates were prepared using DYEnamicET dye terminator chemistry (Amersham Biosciences).

Sequence editing, alignment, and phylogenetic tree construction were carried out using the Geneious Pro software package (Drummond, *et al.*, 2009). Sequence electropherograms were examined for quality and the presence of vector sequences and trimmed accordingly. The greengenes bellorophon tool (DeSantis, *et al.*, 2006) was used to check for chimera formation in the bacterial 16S rRNA gene clone library, while fungal ITS1 and ITS2 Basic Local Alignment Search Tool (BLAST) (Altschul, *et al.*, 1998) hits were compared in order to identify fungal ITS chimeras. Sequence alignment was achieved using the Genious Pro aligner. Sequences with greater than 97% pairwise identity were considered the same OTU and a single representative was used in subsequent phylogenetic analyses. Sequences were compared with the genbank database using BLAST. Pairwise evolutionary distances were calculated from percent similarities using Jukes and Cantor (1969) correction, and phylogenetic trees were constructed by the Neighbour-joining method (Saitou & Nei, 1987). The support for each node was determined by assembling a consensus tree of 100 bootstrap replicates.

2.6 Archaeal detection

Using DNA extracted (as in section 2.2) from two different grubs (not those used for the rest of the study), archaeal 16S rRNA genes were targeted for PCR amplification using the following protocol. For 16S rDNA gene amplification each 25 μ L reaction contained 1 μ L of template DNA at 2-10ng/ μ L, 1.5 μ L of 25mM MgCl₂, 1.5 μ L of each primer (10 μ M) 347f (5'CCAGGCCCTACGGGGCGCA) and primer 1335R (5'GTGTGCAAGGAGCAGGGAC), 2.5 μ L of 10x PCR buffer (Invitrogen Ltd,

New Zealand), 2.5 μ L 2mM dNTPs, 0.75 μ L of 0.2mg/ml bovine serum albumin (BSA), 0.2 μ L of 5U/ μ L Platinum Taq polymerase (Invitrogen Ltd, New Zealand), and autoclaved milliQ up to 25 μ L. Thermal cycling conditions were; Initial denaturation at 94°C for 3min 30 seconds, 32 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 2 min, and a final extension of 6 min at 72°C. An environmental DNA sample previously shown to give archaeal 16S rRNA gene amplification was used as appositive control. PCR products were visualised using agarose gel electrophoresis as in section 2.3.1.

Methanogenic archaea were targeted for isolation using DSMZ liquid medium 734 (appendix B), described by Leadbetter and Breznak (1996). Dissected Huhu gut was homogenised in the anaerobic culture media and 0.1ml of this was used to inoculate two 150ml bottles containing 20ml of DSMZ 734 medium which were then pressurised to 1atm using 4:1 H₂/CO₂. Bottles were incubated at 25°C with daily pressure checks for 1 week. Samples were also extracted for viewing under the phase contrast microscope throughout the week.

2.7 Fungal culturing & identification

Fungi capable of lignocellulose degradation were targeted for isolation by using DSMZ mineral medium 457 (appendix B) agar plates supplemented with 0.26g/L pine sawdust as the only carbon source prior to autoclaving. Fresh frass from pine block fed Huhu grubs was mixed into autoclaved MilliQ water and 100 μ L was pipetted into agar pour plates of the above medium, and plates were incubated at 25°C for 1 week. Colonies found growing in the plates were extracted using sterile scalpel and tweezers and were used to inoculate R2A (appendix B) medium plates which were incubated at 25°C for 1 week. Any growth on the plates was used to spread plate onto fresh R2A medium in order to isolate single colonies; plates were incubated at 25°C for 1 week.

Large individual colonies were picked using a flamed loop and used to extract DNA using the following cetyltrimethylammonium bromide (CTAB) protocol. Colonies were mixed with 0.5ml CTAB and 2 μ L of β -mercaptoethanol in a sterile 1.5ml eppendorf tubes and centrifuged at 16000g for one minute. The tube was incubated at 100°C for twenty minutes. Tubes were allowed to cool before an

equal volume of chloroform/isoamyl alcohol (24:1) was added and tubes were mixed on a rocker/rotator for 20 minutes at 180rpm. Tubes were centrifuged at 16000g for 15 minutes and the aqueous phase was transferred to a new tube. A half volume of 5M NaCl and then an equal volume of isopropanol were added in order to precipitate DNA. Tubes were inverted several times and incubated at -80°C overnight. After thawing tubes were centrifuged at 16000g for 30mins, and the resulting supernatant was discarded. The pellet was washed with 200µL of 80% ethanol and centrifuged at 16000g for 30 seconds before the supernatant was discarded. The pellet was air dried at room temperature for 30mins and the pellet was resuspended in 30µL of autoclaved MilliQ water. DNA samples were stored at -20°C.

In order to identify the colonies, fungal ITS regions were PCR amplified for sequencing. The PCR and electrophoresis protocol outlined in section 2.5.1 were used and all PCR product bands were cut out of gels and purified as in section 2.5.1 prior to sequencing using the fungal ITS1f primer. An environmental DNA sample previously shown to give fungal ITS amplification was used as a positive control.

3 Results & Discussion

3.1 Larval growth

After ten days on the nutrient rich media, six of sixteen grubs collected from Karakariki had died, giving a mortality rate of ~ 38%. Of 11 grubs introduced to the pine block and cellulose diets, nine survived giving a mortality rate of ~18%, with only grubs introduced to the pine block dying.

Table 3-1 shows that grub weight gain varied significantly between individuals and figure 3-1 shows that when weight gain was averaged according to diet and standard deviations were considered, there were no significant differences according to diet.

Table 3-1. Grub Growth

Grub	Diet	Weight gain per month (g)	Mean weight gain per month (g) ±SD
1	Cellulose	0.02	
3	Cellulose	0.43	0.35 ±0.3
7	Cellulose	0.61	
4	Pine Block	0.15	
9	Pine Block	0.05	0.07 ±0.07
11	Pine Block	0.01	
V	Nutrient Pine	0.36	
W	Nutrient Pine	0.56	0.41 ±0.13
T	Nutrient Pine	0.31	
N	Nutrient Cellulose	0.32	
P	Nutrient Cellulose	0.1	0.43 ±0.4
Q	Nutrient Cellulose	0.87	
Z	Nutrient Starch	0.12	
R	Nutrient Starch	0.26	0.22 ±0.09
X	Nutrient Starch	0.29	

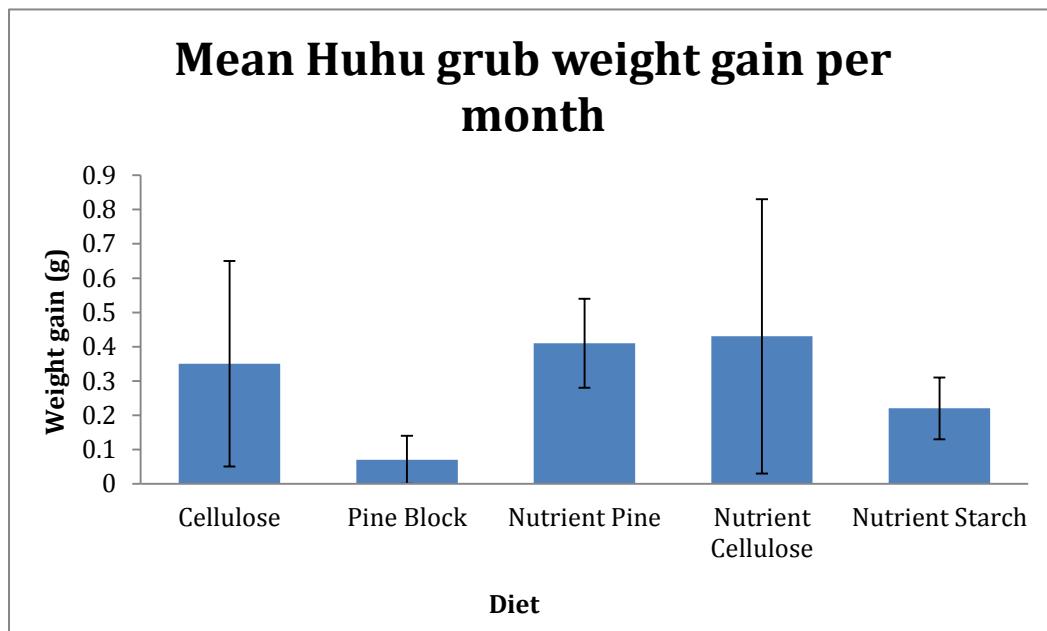


Figure 3-1. Mean Huhu grub weight gain per month.

3.2 TRFLP fingerprinting analysis

Gut fungal TRFLP fragment length data (Table 3-2.) did not indicate any diversity trend between the different diet groups, with great variation between individuals. However, larvae in the wild type group had their two strongest peaks (fluorescence) conserved between each individual. These two peaks were not found in grubs other than Wild Type.

Table 3-2. Larval gut Fungal ITS TRFLP restriction fragment data

Larvae	Group	Number of restriction fragments	Group mean (±SD)
81	Wild	4	
82	Wild	3	28
83	Wild	78	
V	Pine	11	
W	Pine	93	46
T	Pine	34	
N	Cellulose	0	
P	Cellulose	30	11
Q	Cellulose	2	
Z	Starch	24	
R	Starch	109	44
X	Starch	1	

Table 3-3 shows larval frass fungal TRFLP diversity. Fungal frass diversity varied greatly between individuals, with no discernable pattern between groups. Individual peak numbers ranged from 1 to 142 across all groups.

Table 3-3. Larval frass Fungal ITS TRFLP restriction fragment data

Larval frass	Group	Number of restriction fragments	Group mean ($\pm SD$)
Vf	Pine	43	
Wf	Pine	142	64 \pm 70
Tf	Pine	8	
Nf	Cellulose	43	
Pf	Cellulose	3	17 \pm 23
Qf	Cellulose	4	
Zf	Starch	86	
Rf	Starch	82	56 \pm 48
Xf	Starch	1	

Figures 3-2 and 3-3 indicate that fungal gut and frass communities are significantly different between individuals and cannot be deemed diet specific when grubs are fed nutrient rich diets. This is highlighted by the SIMPROF test function of the cluster analysis which is visualised as dotted red lines in figure 3-2. This indicates that individual fungal communities from larval guts and frass, and all four diets cannot be significantly differentiated from one another (samples: 81, Ng, x, 82, Q, Pf, Xf, Qf, Tf, Vf, T, W). Although diet specific fungal communities are not evident here with the nutrient rich diets, highly diet specific fungal communities were observed in the guts of grubs grown on pine block and cellulose tissue paper diets which contained only lignocellulose, or cellulose respectively.

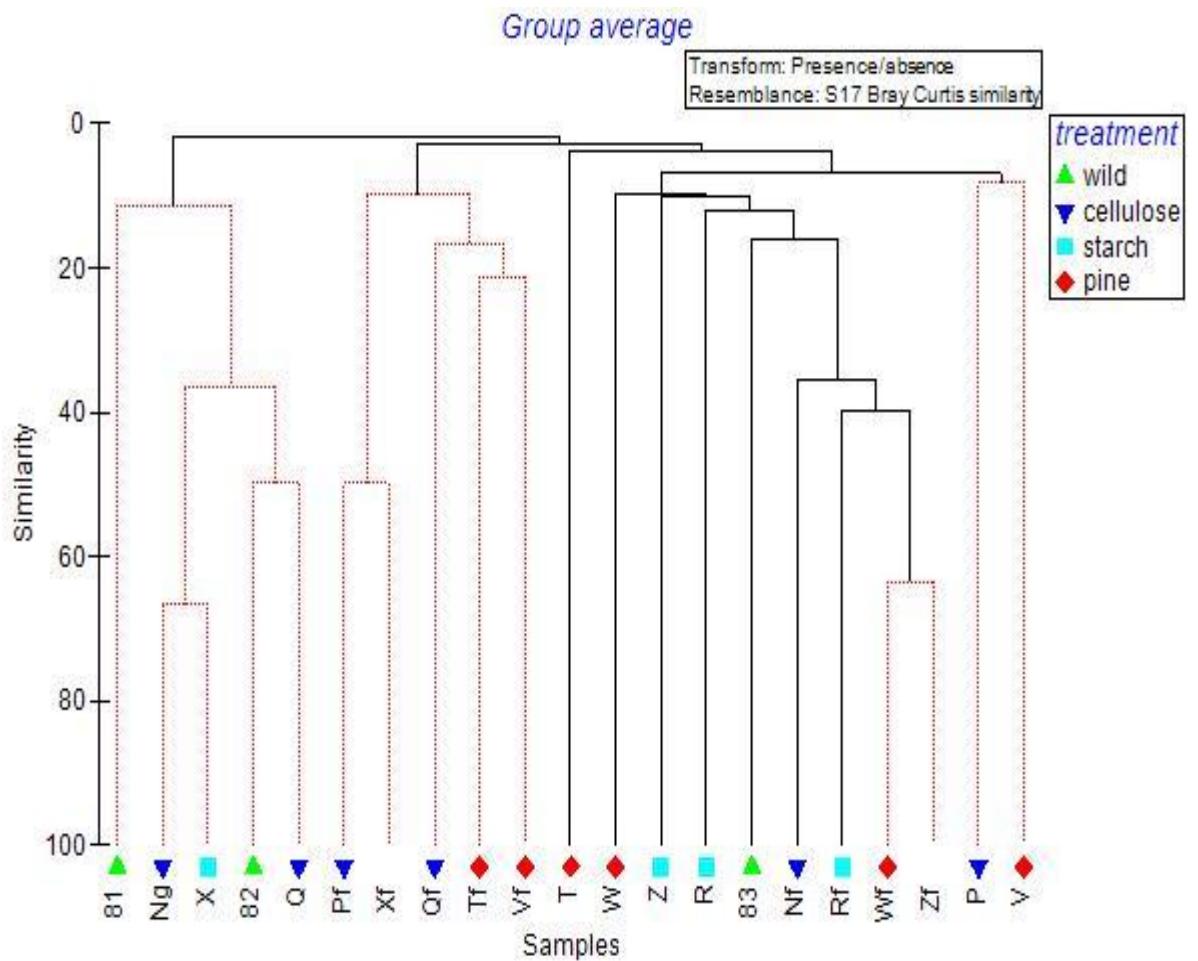


Figure 3-2. Cluster plot showing Bray-Curtis similarity between fungal ITS region TRFLP community profiles from grubs grown on different diets.

Samples connected by dotted red lines were generated using the SIMPROF test, indicating that they cannot be significantly differentiated.

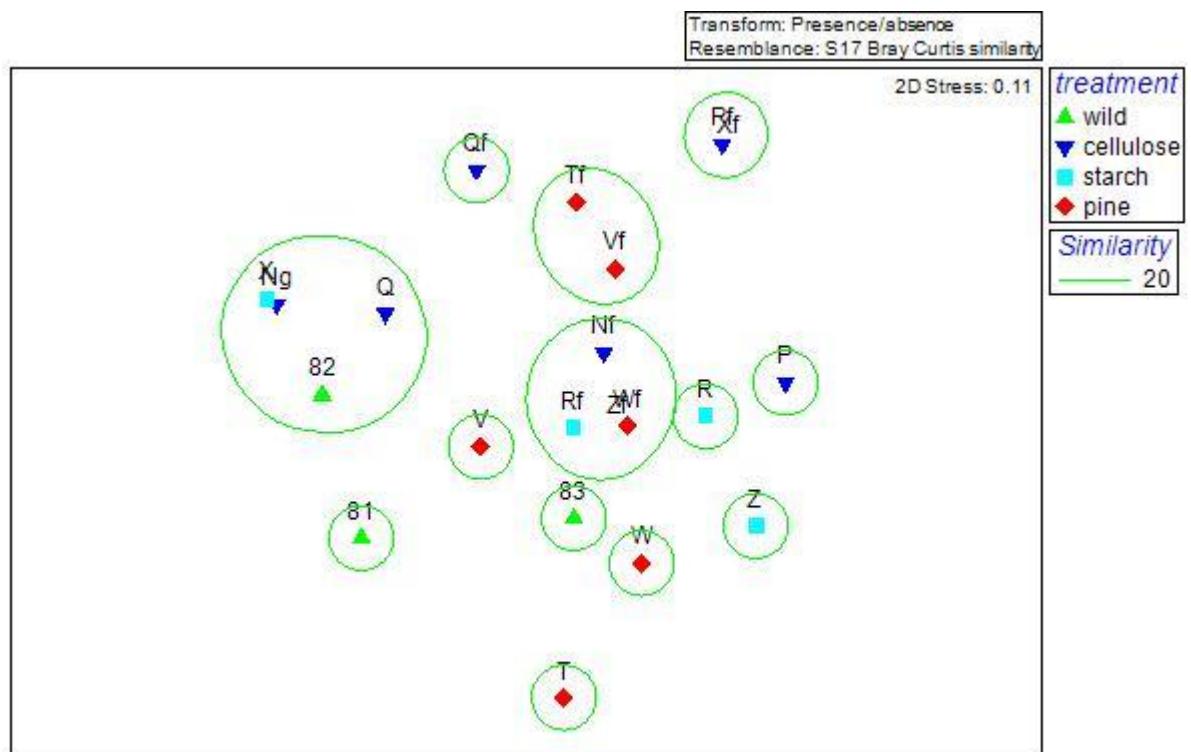


Figure 3-3. Presence/absence transformed Fungal TRFLP profile multidimensional scaling (MDS).

Overall presence/absence transformed two-dimensional, non-metric MDS ordination (stress = 0.11) based on Bray-Curtis similarities of TRFLP profiles of fungal communities. Points within solid green circles cluster at the 20% similarity level.

3.3 454 pyrosequencing

3.3.1 Alpha Diversity

Figure 3-4 shows the number of OTUs recovered as a function of sampling effort (in the form of number of sequence reads). In the case of the Wild Type Huhu grubs, the slope of the curve is initially steep as new OTUs are encountered, but levels off as new OTUs are recovered at a rate of four per one thousand reads. This demonstrates that the sampling effort (number of reads) given was sufficient to cover the majority of the biodiversity present. The rarefaction curves for the other samples reach asymptotes after a low number of reads, indicating that there is low biodiversity compared to the Wild Type, and that biodiversity was sampled sufficiently.

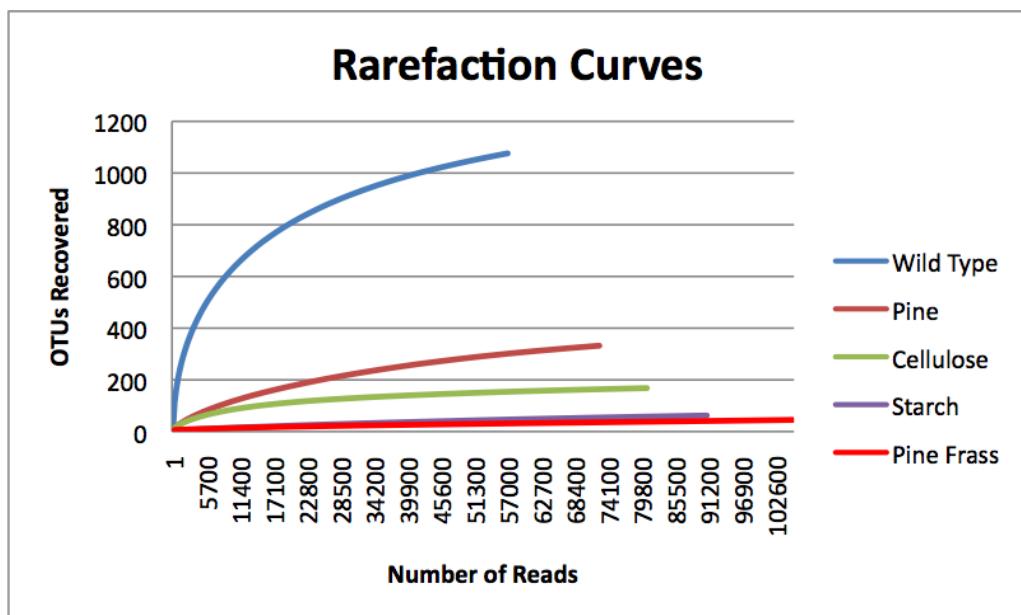


Figure 3-4. Rarefaction curve of bacterial species richness.

3.3.2 Beta Diversity

When figures 3-5, 3-6, and 3-7 are considered it is apparent that the relatively low diversity observed in those grubs grown on all three nutrient rich laboratory media is due to one extremely abundant OTU which is absent from the Wild Type grubs. This is shown in figure 3-10 where each vertical line in each row (one row for

each grub) represents a different OTU and has a potential colour spectrum ranging from white (absent) through to black/grey (low abundance) and bright orange (high abundance). At the left side of the figure there is a dark orange line (OTU) which is present in all of the samples apart from the Wild Type. The rest of the figure as a whole shows many vertical lines in the Wild Type row which correspond to absent OTUs in the other rows, indicating that the Wild Type has higher biodiversity. Closer inspection of the taxonomically assigned sequence data (not shown) shows that the OTU shared by all samples except the Wild Type is a strain of lactobacillus (*Weisella paramesenteroides*) which is represented in each sample by the following proportions of reads; Starch 99%, Cellulose 68%, Pine 81%, Pine Frass 50%, Wild Type 0%. Figures 3-8 and 3-9 show the community similarity between each sample. Figure 3-8 indicates that the Wild Type is distinct from the other samples, which group more closely together. The colour of the tiles in figure 3-9 similarly demonstrates that the Starch, Cellulose, Pine, and Pine Frass communities are very similar to one another but dissimilar to the Wild Type community. This is likely due to the laboratory diets (table 2-1, page 19) fed to the Huhu grubs promoting the proliferation of gut bacteria which can most competitively consume the easily metabolised sugars, which are common in all the nutrient rich laboratory media, rather than the recalcitrant lignocellulose or cellulose components of the different diets.

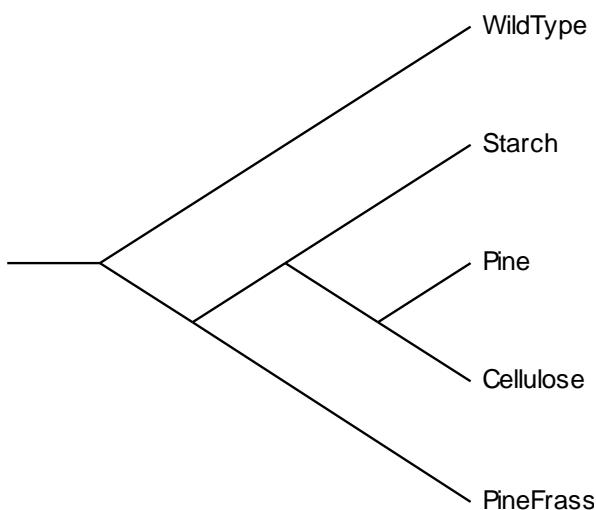


Figure 3-5. Dendrogram showing bacterial community dissimilarity.

Calculated using the “Jaccard coefficient using richness” estimator displaying the dissimilarity (1-similarity) between samples.

Heatmap showing community composition similarity at distance 0.03

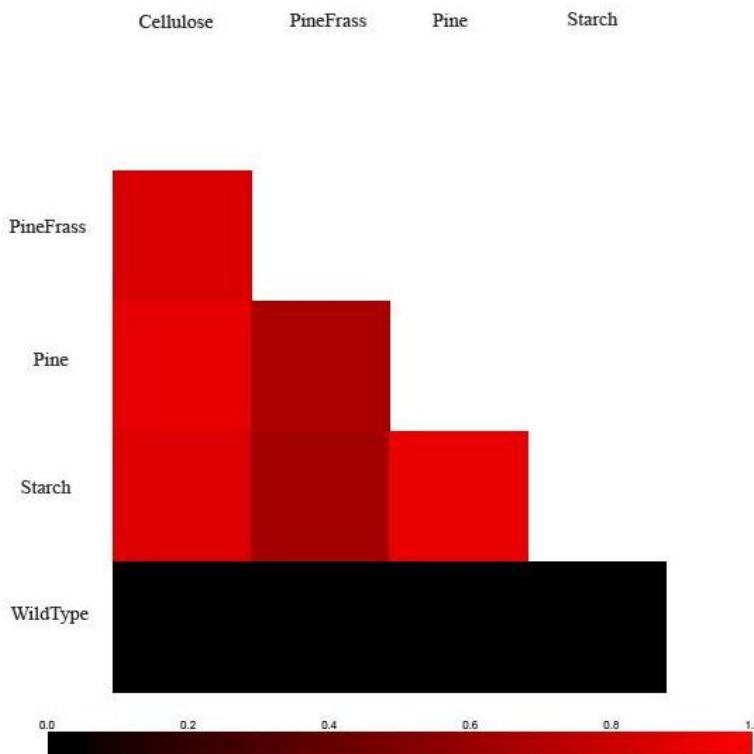


Figure 3-6. Heatmap showing community composition similarity at distance 0.03.

Community similarity is compared two samples at a time (each tile). The darker a tile the more dissimilar the two communities are, the brighter a tile the more similar they are.

Despite the prevalence of one particularly abundant OTU in the grubs raised on the laboratory media there is still an interesting trend related to the one ingredient which differs in each of these different media, i.e. Pine (lignocellulose), cellulose, or starch. The Venn diagrams in figures 3-8 and 3-9 show the number of OTUs shared between, and unique to each of the respective laboratory diets. The total number of OTUs increases with increasing diet complexity from Starch (62), Cellulose (168), Pine (332), to Wild Type (1076) (figure 3-10). Pine Frass had the lowest number of OTUs at 45 and only 8 of the total 1381 OTUs were common to all treatments. Figures 3-8 and 3-9 show that bacterial diversity is greatly decreased when Huhu grubs are raised on laboratory diets, while figure 3-7 shows that this difference is mainly attributable to the abundance of a single OTU (*Weisella sp.*) which is present only in the guts and frass of grubs fed on the laboratory media.

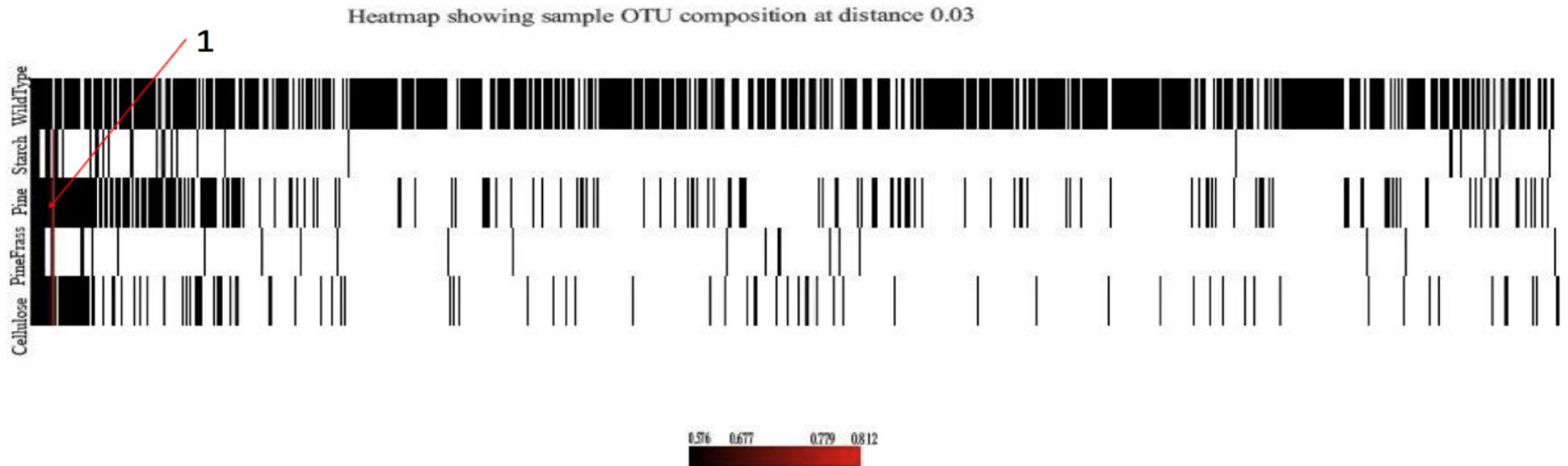
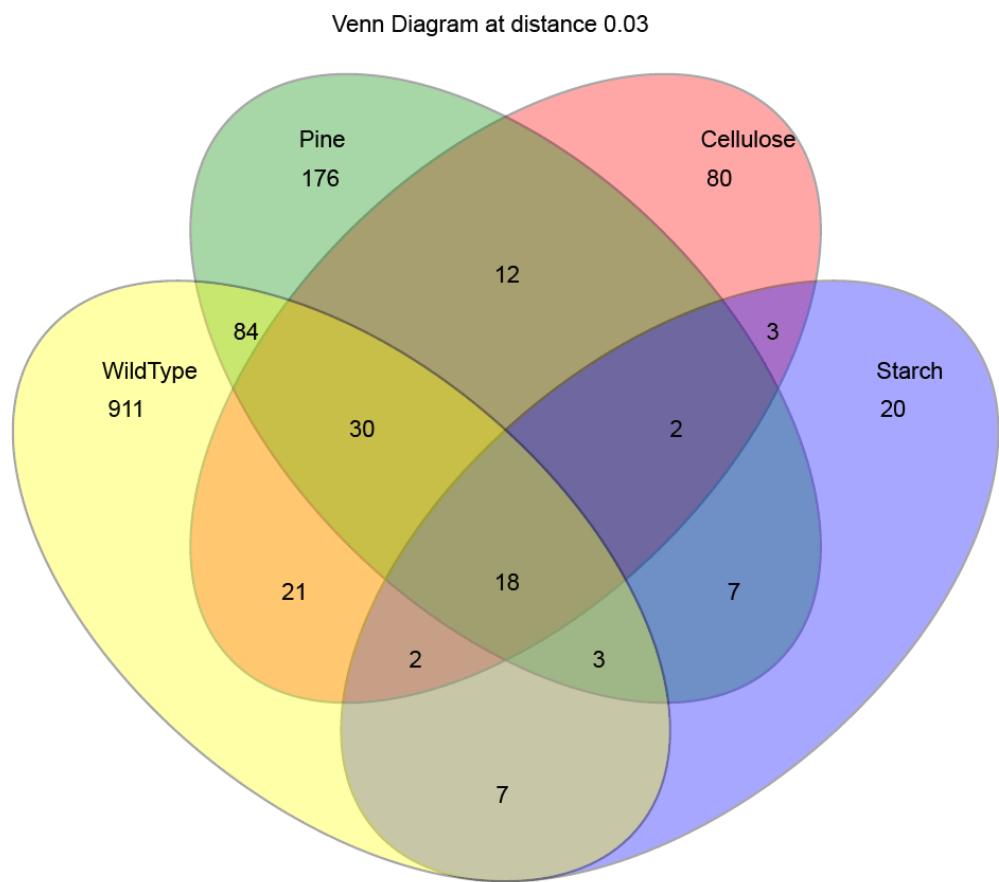


Figure 3-7. Heatmap of Wild Type, Pine, Cellulose, Starch, and Pine Frass gut 454 OTU abundances.

The redness of each band in the heatmap reflects the abundance of that particular OTU in a particular sample, darker bands indicate less abundant OTUs, and white indicates that a given OTU is not present in a particular sample. The red arrow labelled 1 indicates the presence of the only red band in the heatmap. The orange arrow (1) points to the OTU common to the nutrient rich media fed grubs which is absent in the Wild type grubs.



The number of species in group Cellulose is 168

The number of species in group Pine is 332

The number of species in group Starch is 62

The number of species in group WildType is 1076

The number of species shared between groups Cellulose and Pine is 62

The number of species shared between groups Cellulose and Starch is 25

The number of species shared between groups Cellulose and WildType is 71

The number of species shared between groups Pine and Starch is 30

The number of species shared between groups Pine and WildType is 135

The number of species shared between groups Starch and WildType is 30

The number of species shared between groups Cellulose, Pine and Starch is 20

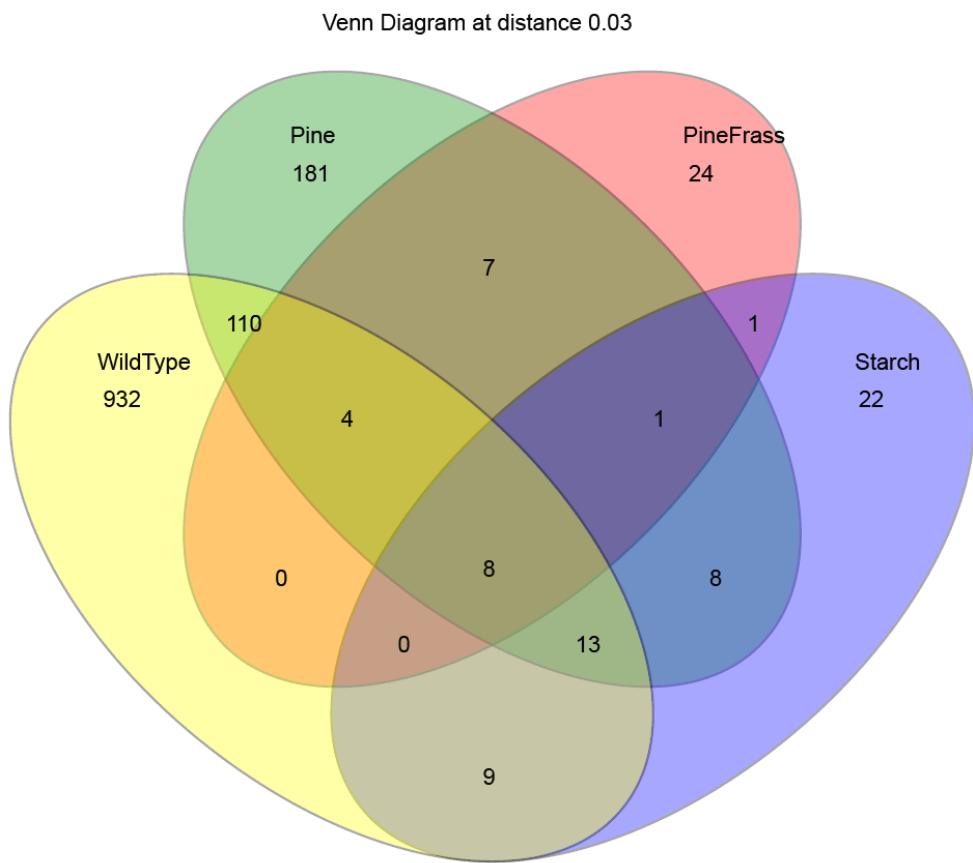
The number of species shared between groups Cellulose, Pine and WildType is 48

The number of species shared between groups Cellulose, Starch and WildType is 20

The number of species shared between groups Pine, Starch and WildType is 21

The total richness of all the groups is 1376

Figure 3-8. Venn diagram showing shared OTUs between Cellulose, Pine, Starch, and Wild Type reared grubs.



The number of species in group PineFrass is 45
The number of species in group Pine is 332
The number of species in group Starch is 62
The number of species in group WildType is 1076
The number of species shared between groups PineFrass and Pine is 20
The number of species shared between groups PineFrass and Starch is 10
The number of species shared between groups PineFrass and WildType is 12
The number of species shared between groups Pine and Starch is 30
The number of species shared between groups Pine and WildType is 135
The number of species shared between groups Starch and WildType is 30
The number of species shared between groups PineFrass, Pine and Starch is 9
The number of species shared between groups PineFrass, Pine and WildType is 12
The number of species shared between groups PineFrass, Starch and WildType is 8
The number of species shared between groups Pine, Starch and WildType is 21
The total richness of all the groups is 1320

Figure 3-9. Venn diagram showing OTUs shared between Pine Frass, Pine, Starch, and Wild Type samples

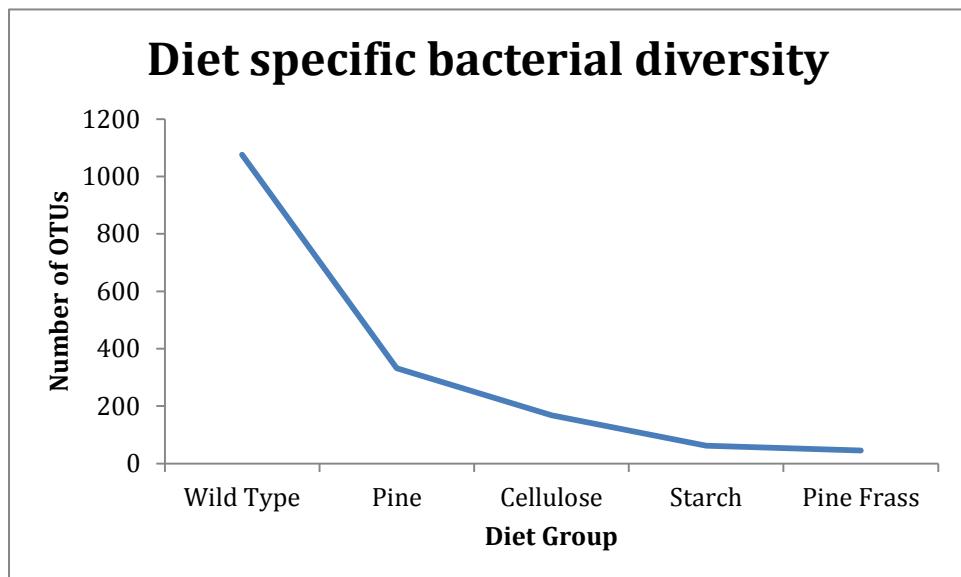


Figure 3-10. Diet specific bacterial diversity

The number of OTUs at the 97% similarity level decreases with decreasing diet complexity.

3.3.3 Phylogenetic analysis

Figure 3-11 shows the Wild Type grub gut bacterial community at the phylum level. The largest groups were the firmicutes (27%), the alphaproteobacteria (17%), the betaproteobacteria (15%), and the bacteriodetes (15%). The large representation from these groups is consistent with other coleopteran gut microbiomes (Schloss, *et al.*, 2006). The tree in figure 3-11 was constructed using only OTUs with greater than 0.1% of the reads from the Wild Type library. Many more phyla were recovered from the data which are part of the “rare biosphere” and are not shown in the figure.

Figure 3-12 represents the Wild Type gut bacterial community members with greater than 1% of the pyrosequencing reads, at the species level. The most abundant species (most reads) were *Lactococcus lactis* (22%), uncultured *Chitinophaga* (5%) *Burkholderia sp* (5%), and *Burkholderia sp.2* (5%). These results are similar to those gained from the full length bacterial 16S rRNA gene clone library analysis (section 3.4.1). Pairwise alignments of cloned (full length 16S rRNA genes) and pyrosequenced (400nt) OTUs had significant similarity (97% level) in the cases of: *Burkholderia sp* and *Burkholderia vietnamiensis*,

Chitinophaga and *Luteifibra arvensicola*, and *Acidisoma tundrae* sequences from both datasets.

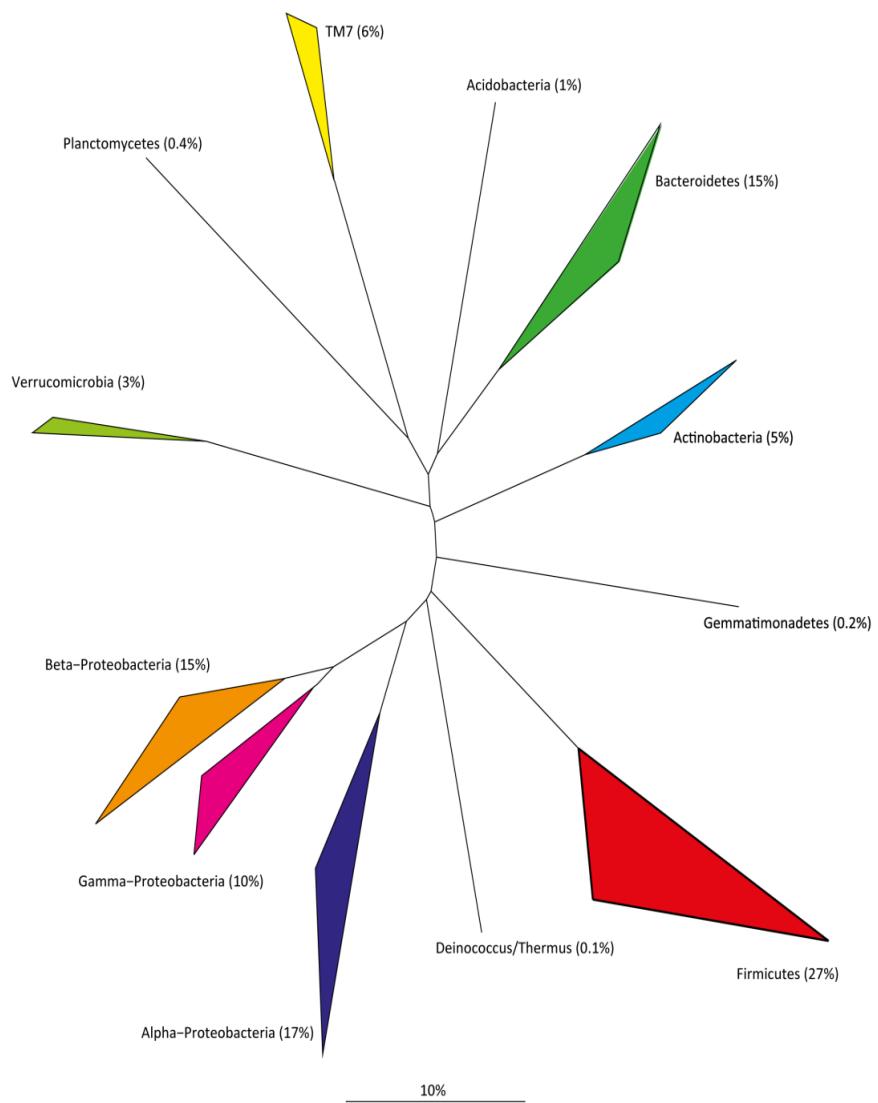


Figure 3-11. Phylum level bacterial community of wild type Huhu grubs derived from pyroseqeuncing reads. Constructed in ARB from OTUs with >0.1% of the total reads.

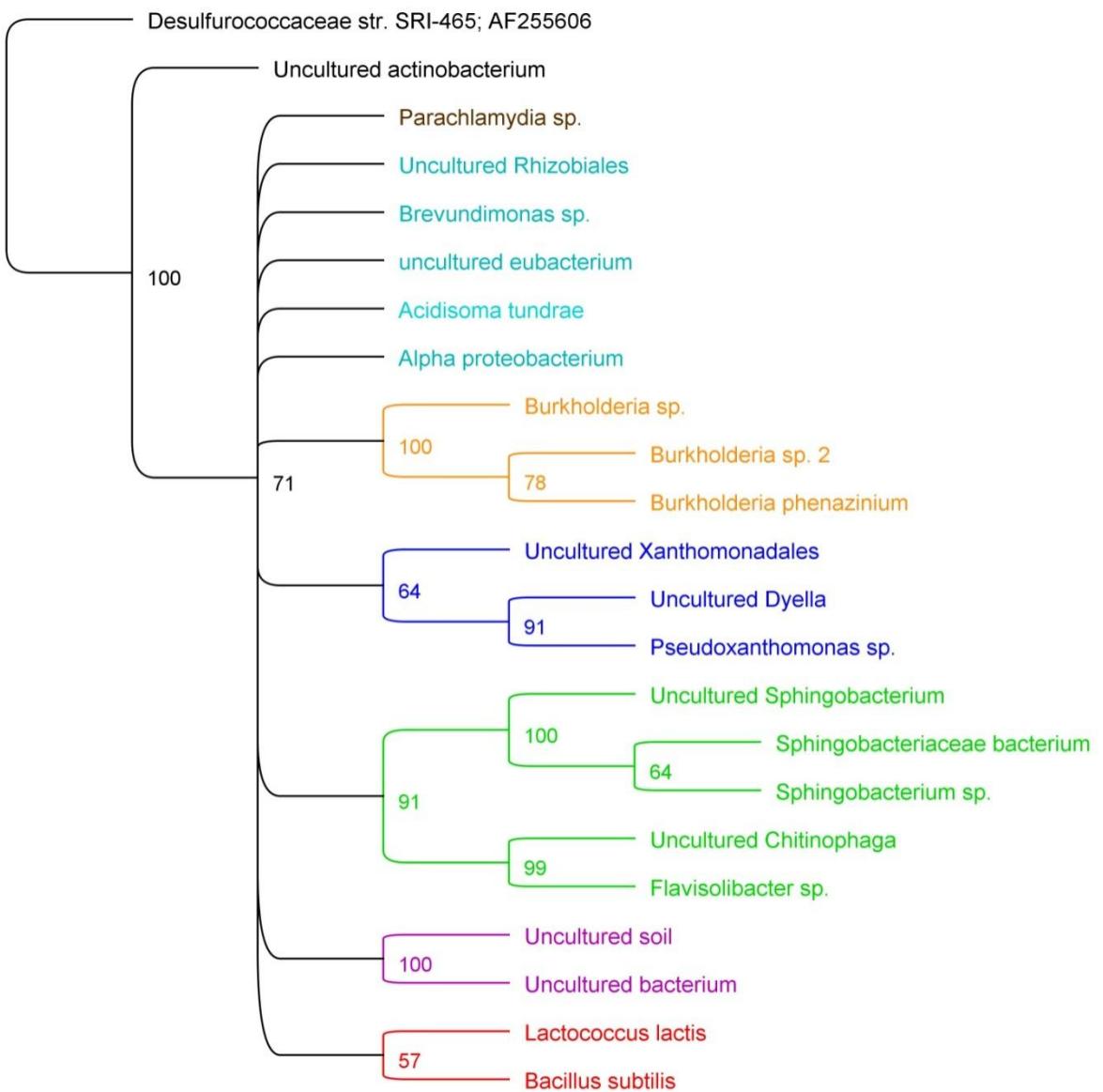


Figure 3-12. OTUs representing >1% of total reads of the Wild Type Huhu grub gut.

Red branches represent the Firmicutes, light blue branches represent the Alphaproteobacteria, green branches the Betaproteobacteria, dark blue branches the Bacteriodetes, and brown branches the Chlamydia, purple the TM7, black the Actinobacteria. AF255606 is a full length *Desulfurococcus* strain 16S rRNA gene sequence and was used as an out-group. Sequences were globally aligned in Geneious Pro (Drummond, *et al.*, 2009) using Jukes and Cantor neighbour joining with 100 bootstrap replicates.

3.4 Clone Library analysis

3.4.1 Bacterial 16S rRNA gene

The 400nt reads generated by current titanium 454 pyrosequencing technology often do not allow for accurate OTU classification below the genus level. In order to address this issue a clone library of full length bacterial 16S rRNA genes was constructed from the same community gut DNA from that used to generate the wild type pyrosequencing data (figure 3-13). In this dataset a strain of *L.lactis* was also the most commonly recovered OTU at 67% of the 94 clones obtained. The second most abundant clone library OTU (8%) which had 98% pairwise identity with *Burkholderia vietnamiensis* in the ncbi BLAST database aligned with 96.4% pairwise similarity to the *Burkholderia sp.1* OTU from the pyrosequencing data. *Burkholderia vietnamiensis* is the one of the species in this genus which is known to be able to fix nitrogen (Gillis, *et al.*, 1995) and this OTU was not detected in the guts of grubs fed on the nutrient rich laboratory media, which are replete with organic nitrogen. Wood is very low in available nitrogen, which is an element essential for life; and xylophagous animals often overcome this dietary limitation via associations with nitrogen fixing gut symbionts. *Burkholderia* has been reported in a wide range of environments (Compan, *et al.*, 2008), including the guts of other cerambycid larvae (Grünwald, *et al.*, 2009) and it is possible that *Burkholderia vietnamiensis* is fixing nitrogen in the Huhu grub gut, thereby alleviating the nitrogen-limitation of the lignocelluloses substrate, allowing the entire system to function. The third most abundant OTU in the clone library, *Acidisoma tundra*, is part of the family of acetic acid bacteria, *Acetobacteraceae* which are capable of oxidising ethanol to acetic acid. This clone library OTU aligned with 98% pairwise similarity to the 14th most abundant OTU in the wild type pyrosequencing dataset (1.3% of reads), which was attributed to the *Acidisoma* genus. The fourth most abundant OTU from the clone library (6%) was 95% similar to an uncultured *Sphingobacteriaceae* bacterium in the ncbi database, closely related to *Luteifibra arvensicola*. The *Luteifibra* sequence originates from a bacterial strain described by Pankratov *et al.* (2006) which grows on xylan and laminarin, but not cellulose, pectin, or chitin. It is therefore possible that the strain detected in the Huhu grub gut might be involved in xylan degradation of wood

hemicelluloses. This clone library OTU aligned with 96.4% pairwise similarity to the second most abundant OTU from the pyrosequencing dataset, which was classified as a *Chitinophaga* species.

Both the pyrosequencing and clone library data for the bacterial members of the Wild type Huhu grub highlighted the absence of any species directly implicated in lignin or cellulose degradation. These results suggest that the Huhu grub gut bacterial community does not play a major direct role in lignocellulose degradation. Instead, their important contribution might be via the provision of fixed nitrogen to the system through a bacterial nitrogen fixation mechanism, or by the removal of metabolic end-products from those organisms directly involved in lignocellulose breakdown.

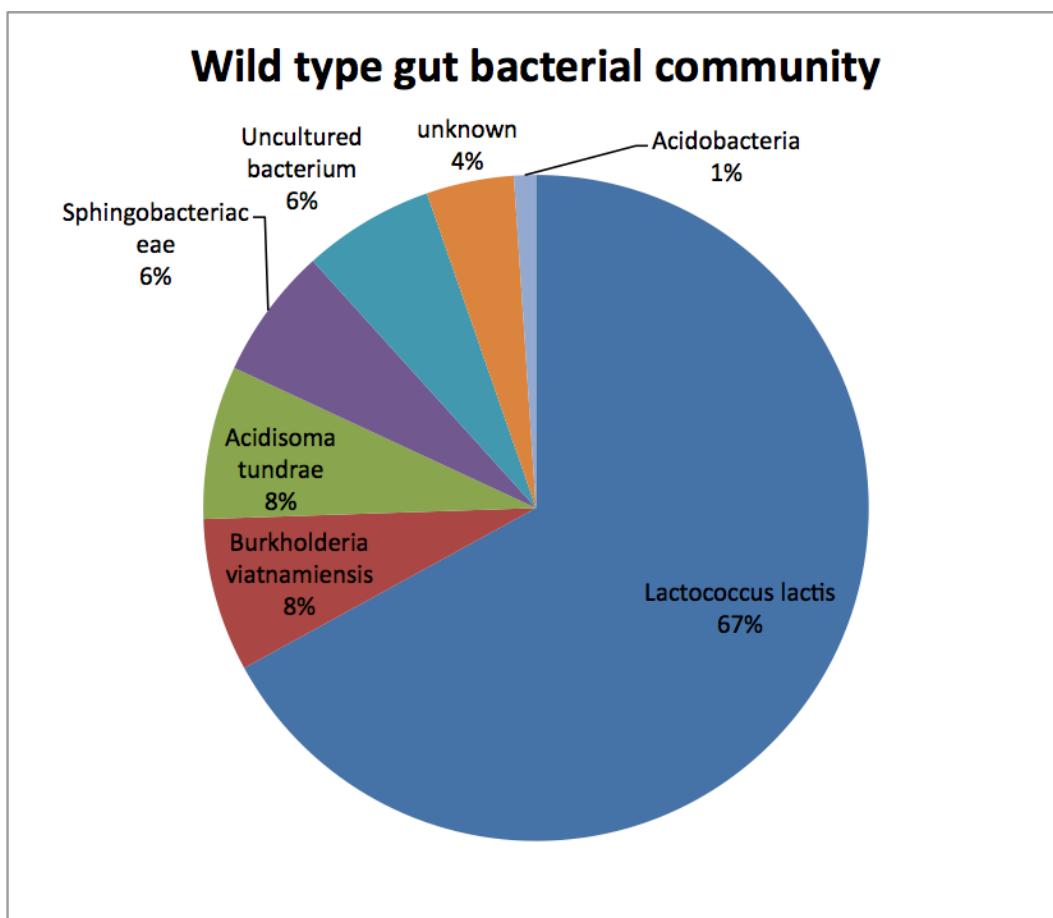


Figure 3-13. Wild type gut bacterial community.

Based on restriction fragment length polymorphism and Sanger sequencing data derived from a library of 94 clones containing full length (1500bp) bacterial 16S rRNA genes.

3.4.2 Fungal ITS region

Figure 3-14 illustrates the diversity of fungi present in the gut of the wild type Huhu grubs, as determined by ITS analysis. At the 97% similarity index 12 different OTUs were documented from a clone library of 109 colonies. The Ascomycetes and Basidiomycetes (higher fungi, or Dikarya) are the predominant groups present in the Huhu gut, with only one lower fungus, an Acaulospora being detected (Figure 3-15). The community is dominated (51% of the library) by an uncultured basidiomycete. Many Basidiomycetes are well documented for their ability to degrade lignocelluloses and both white, and brown rot species are common in this phylum (Martinez, *et al.* 2005). This OTU had 99.4% similarity with an uncultured basidiomycete sequence derived from a pine litter layer (O'Brien, *et al.*, 2005). When only cultured organisms are considered this OTU has highest similarity with *Ganoderma lucidum* (87.4%) and *Stereum rugosum* (87.5%). *G. lucidum* is a white rot fungus which is known to produce a lignin peroxidase enzyme (Asgher, *et al.*, 2010), and *S. rugosum* which is also a white rot fungus has been grown on lignocellulosic material (Hatakka & Pirhonen, 1985). Although the basidiomycete OTU is only distantly related to these species, it is possible that it has similar lignin degrading capabilities. Many of the other fungi present in the wild type gut have also been detected in the guts of coleopteran larva and/or termites. *Xylaria* are known to degrade lignin (Liers, *et al.*, 2006) and have previously been associated with fungus growing termites (Guedegbe, *et al.*, 2009). *Candida lignohabitans* has previously been detected in the guts of passalid and tenebrionid wood boring beetle larvae (Houseknecht, *et al.*, 2010), while *Ophiostoma* are blue stain fungi (which feed on sapwood, but are not known to degrade any lignocellulose components) which are also commonly associated with wood boring beetle larval guts (Jankowiak, 2006). *Candida shehatae* has been reported being continually released from specialised organs called mycetomes in the guts of *R. inquisitor* and *L. rubra* larvae (Grünwald, *et al.*, 2009), and is known to degrade hemicelluloses (xylan and mannan) (Lucas & van Uden, 1986).

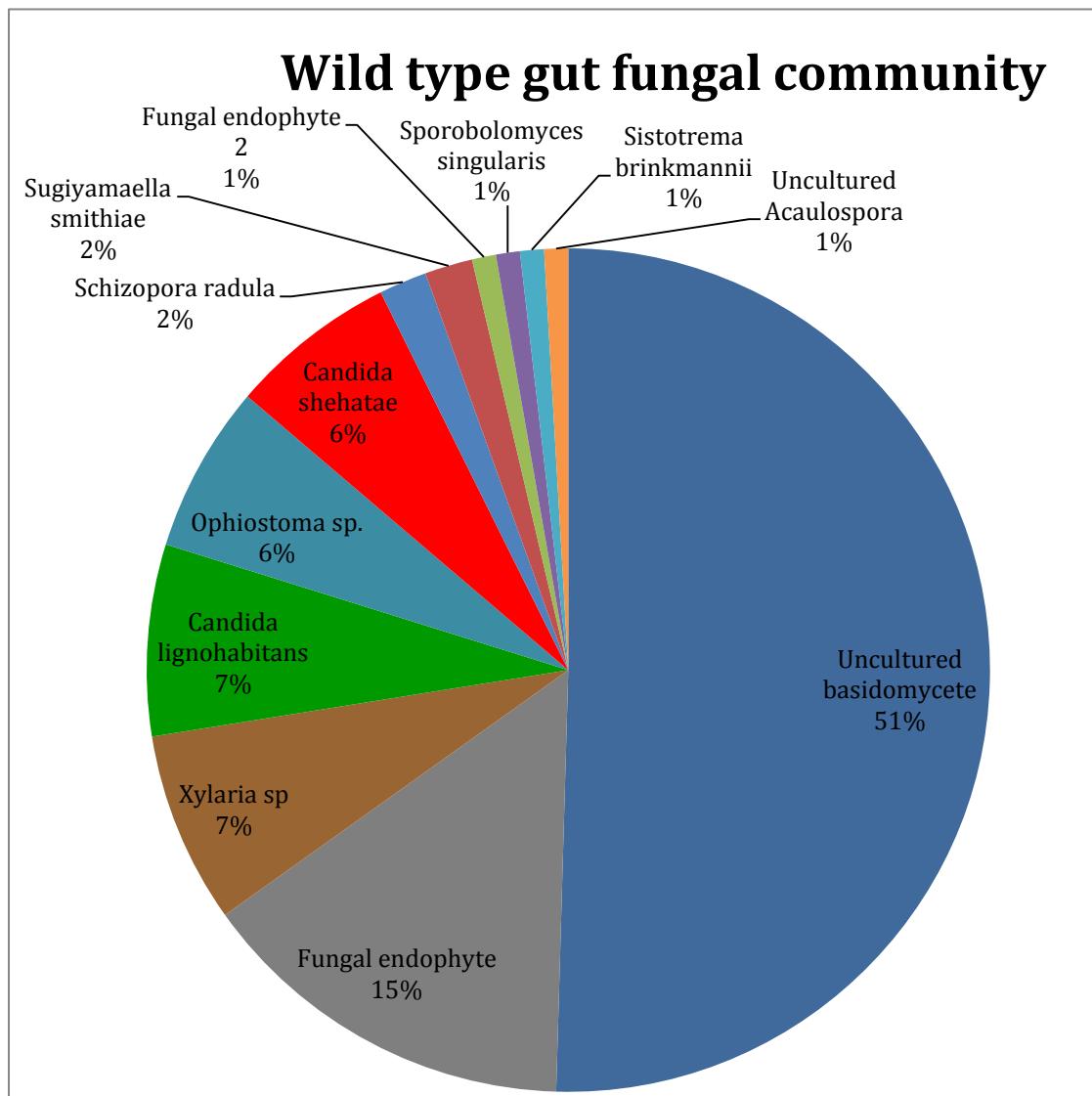


Figure 3-14. Wild type gut fungal community composition.

Based on restriction fragment length polymorphism and Sanger sequencing data derived from a fungal ITS clone library of 109 clones. OTUs were defined at the 97% similarity level.

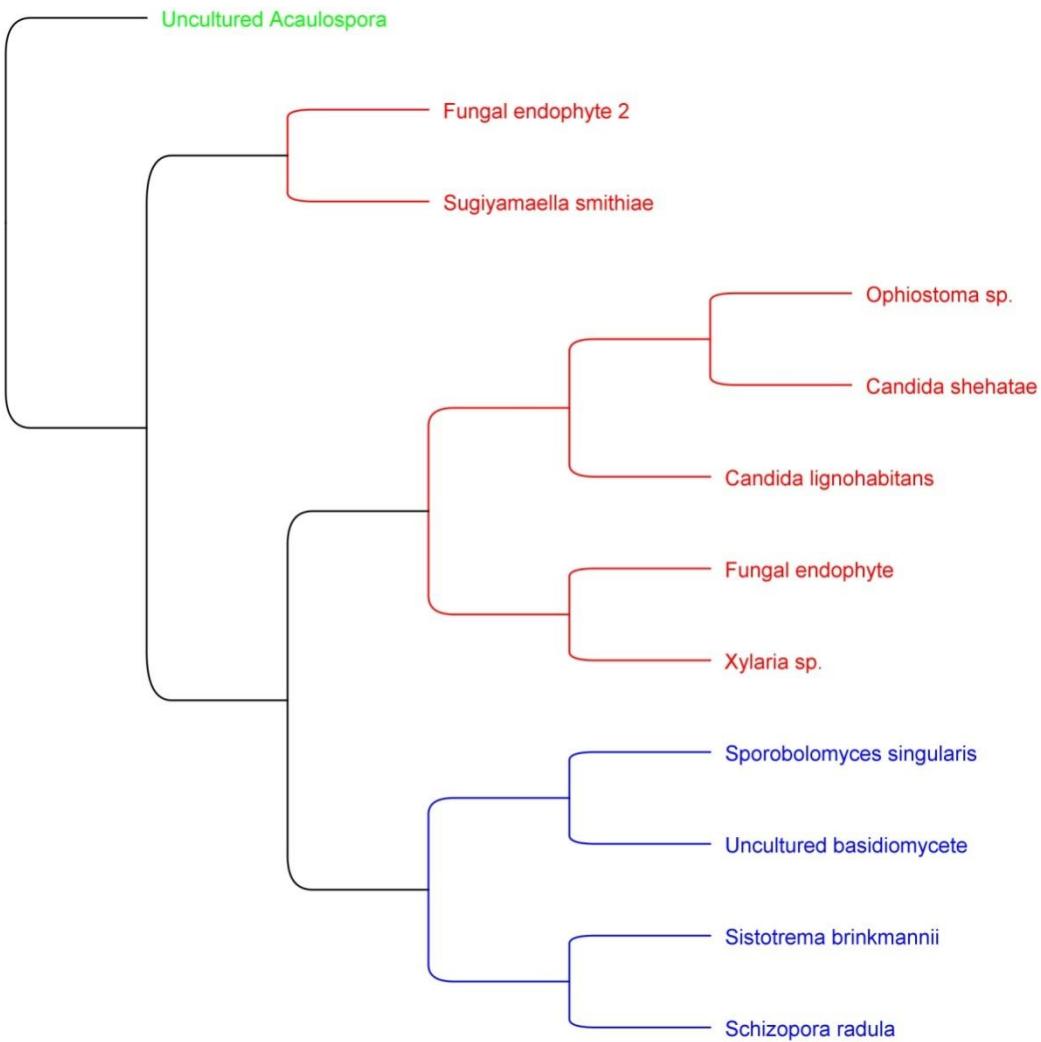


Figure 3-15. Jukes Cantor neighbour joining tree of Wild Type gut fungal ITS sequences

Blue branches represent the Basidiomycetes, red branches the Ascomycetes, the green branch represents the Glomeromycota. Acaulospora was used as an out-group.

The pine block gut fungal community (from grubs fed pine timber, fig 2-4) (figure 3-16) is less diverse than the wild type community, with 6 different OTUs recovered out of 108 clone colonies compared to 12 out of 109 previously. This is unsurprising as it is less likely that a controlled laboratory diet will have microorganisms inhabiting it prior to ingestion by larvae. Both *Candida shehatae* and *Candida lignohabitans* were detected in the gut fungal community grown on the pine block at similar levels to those found in the wild type community. The dominant OTUs in the pine block community were strains from within the *Penicillium* genus, which are commonly known as moulds and are typically saprotrophic fungi (degrade dead organics) which carry out extracellular digestion and transport nutrients through their hyphae. The *Penicillium* sp. sequence had equally high BLAST similarity with *P. crustosum*, *P. expansum*, and *P. commune*. *P. expansum* has been reported as a highly efficient lignocellulose degrader (Yang, *et al.* 2009), *P. crustosum* has been cultured on cellulose and has been isolated from the guts of *S. vestita*, a cerambycid beetle larvae (Delalibera, *et al.* 2005), and *P. commune* has also been shown to have cellulase activity (Zyani, *et al.* 2009). The most commonly recovered ITS sequence from the clone library (37% abundance) had high BLAST similarity with *P. spinulosum* and *P. glabrum* in the ncbi database. *P. glabrum* is known to produce cellulases, but not laccases and is thought not to be capable of lignin degradation (Baldrian, *et al.*, 2010), although it has been reported as a pentachlorophenol (PCP) degrader which is a capability common, but not exclusive to lignin degrading fungi (Carvalho, *et al.*, 2009). There are no reports in the literature of the presence of these strains in the guts of other wood boring beetle larvae. The *Hypocreales* sp. may belong to the species *Beauveria bassiana* which has previously been reported as a Huhu grub pathogen found on numerous dead grubs in the North Island (Edwards, 1959), and belongs to the *Hypocreales* order.

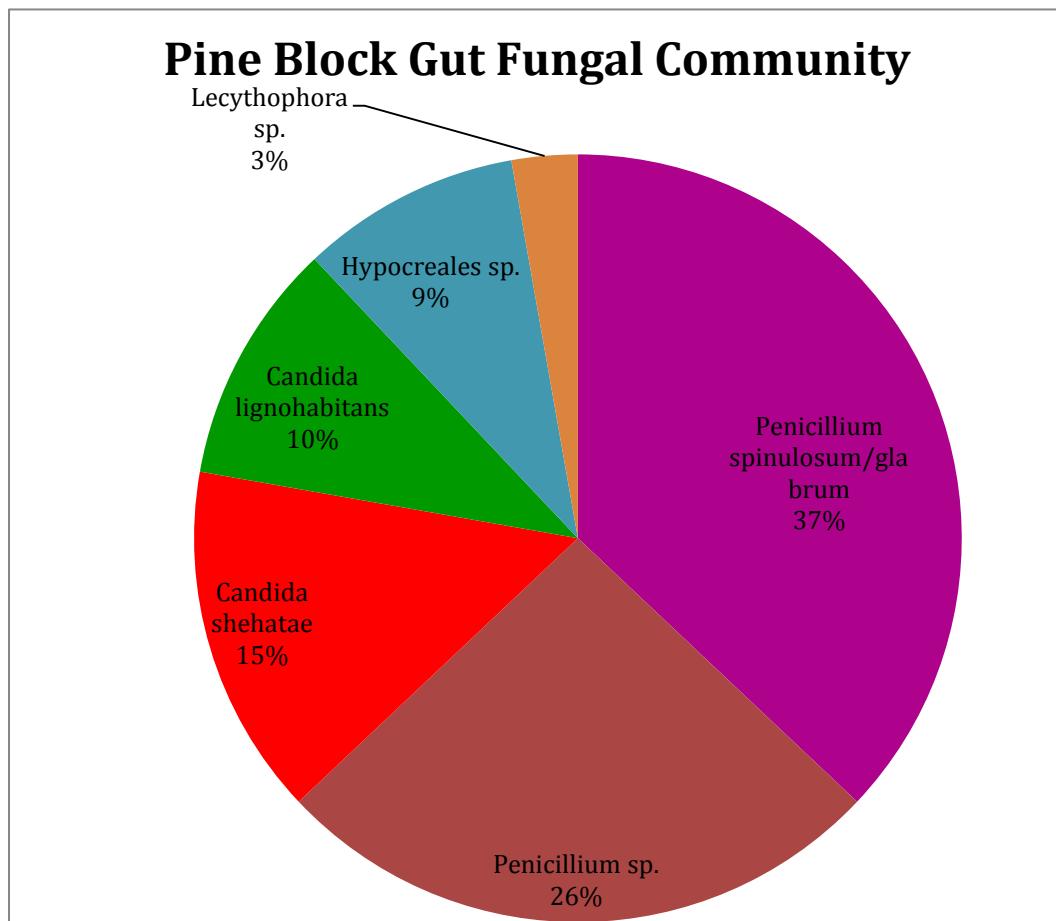


Figure 3-16. Pine block gut fungal community.

Based on restriction fragment length polymorphism and Sanger sequencing data derived from a fungal ITS clone library of 108 colonies. OTUs were defined at the 97% similarity level.

The pine frass fungal community shown in figure 3-17 is of similar but not identical composition to the pine block gut fungal community (figure 3-16). Notable similarities include the presence of *Candida lignohabitans* and *Candida shehatae* at similar levels of abundance (7% and 8%) to those detected in the wild type (7% and 6%) and pine block (10% and 15%) fungal gut communities. The consistent presence of these two strains in otherwise fluctuating fungal communities from the gut and frass of different grubs fed on different diets suggests that they may play an important role in the community and even be conserved commensal or symbiotic organisms for the Huhu grub. It would be interesting to test for vertical transmission of such fungi between Huhu generations. Also similar to the pine block gut fungal community, the pine frass

community has a dominant *Penicillium* species (31%), which has high BLAST similarity (99.7%) to *P.brevicompactum*. The most dominant OTU at 36% had high sequence homology with a *Pichia* sp. in the ncbi database. This uncultured Basidiomycete ITS sequence present in this community is the same as that found in the Wild Type community.

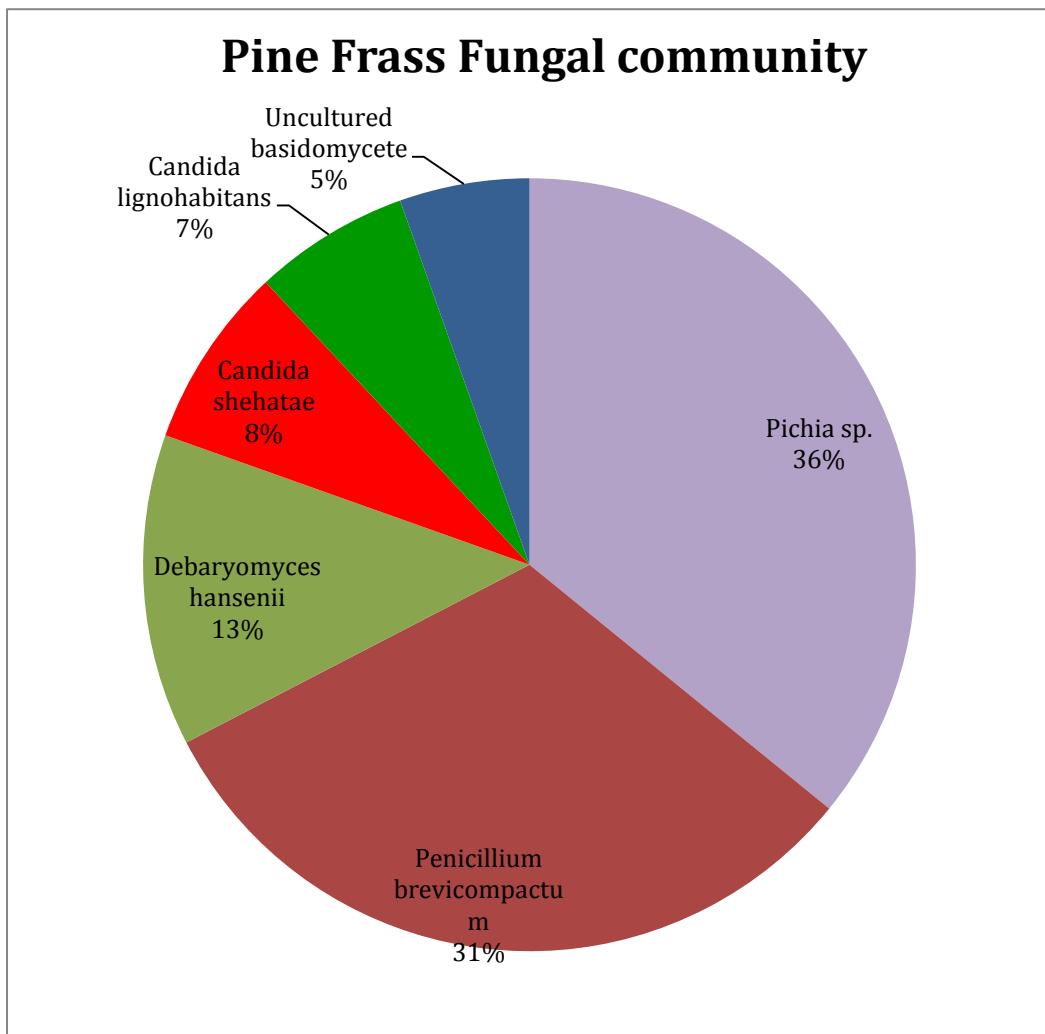


Figure 3-17. Pine block fed fungal frass community composition.

Based on restriction fragment length polymorphism and Sanger sequencing data derived from fungal ITS clone library of 92 colonies. OTUs were defined at the 97% similarity level.

Figure 3-18 shows the fungal community composition of grubs fed only cellulose tissue paper. This community composition contrasts significantly with those observed in both the Wild Type and Pine block fed grubs, comprising of only one

OTU (representing 99% of the fungal community), classified as *Candida shehatae*. This is very interesting when background information on *Candida shehatae* is considered. *Candida shehatae* has been reported being continually released from specialised organs called mycetomes in guts of *R. inquisitor* and *L. rubra* larvae (Grünwald, *et al.*, 2009) which like the Huhu grub are wood boring beetle larvae from the cerambycidae family. *Candida shehatae* is also known to degrade the hemicelluloses, xylan and mannan (Lucas & van Uden, 1986) but has not previously been implicated in cellulose metabolism.

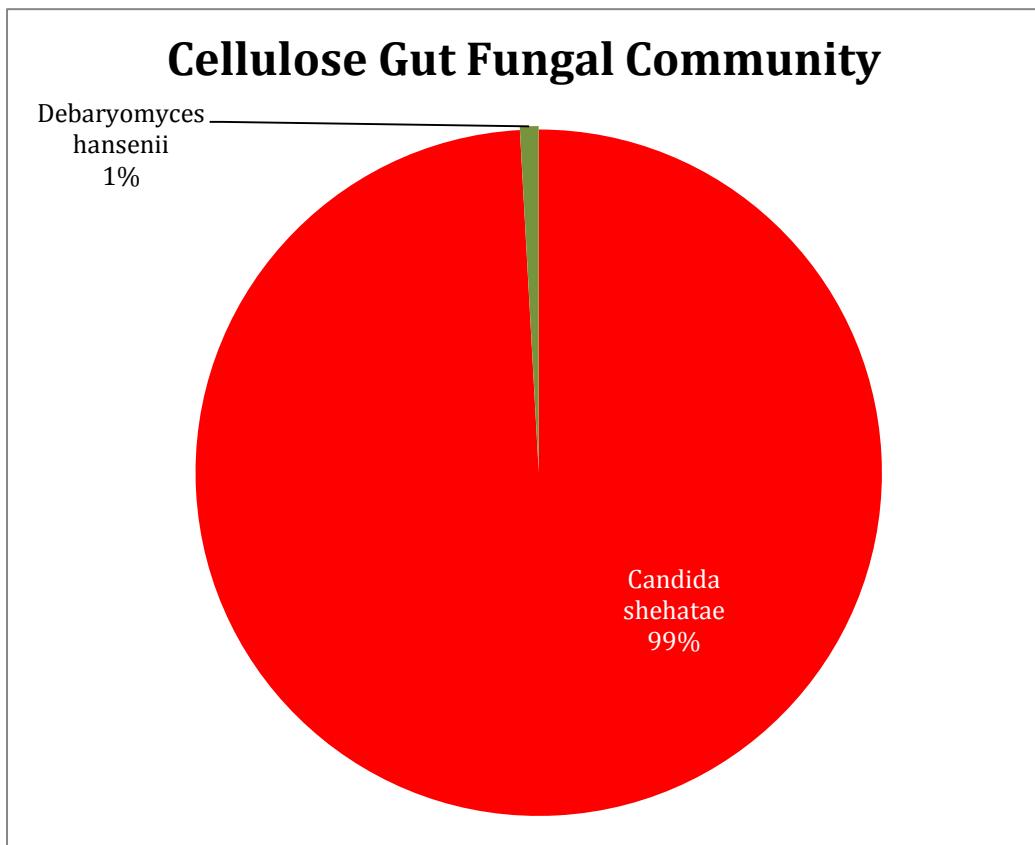


Figure 3-18. Tissue paper cellulose fed fungal gut community composition.

Based on restriction fragment length polymorphism and Sanger sequencing data derived from analysis of 114 colonies in fungal ITS clone library. OTUs were defined at the 97% similarity level.

3.5 Archaeal detection & metabolic end products

No Huhu grub DNA samples used gave positive archaeal 16S rRNA gene amplification with the protocol used. The only band present amongst the repeated experiments was that of the positive control.

The media used to attempt to isolate methanogenic archaea did not yield any growth. This was indicated by the absence of any observable life under the microscope, and no decrease in the pressure of the culture bottles, indicating that the H₂/CO₂ atmosphere was not being utilised.

Methanogenic archaea are commonly found in termites and animal rumens (Liu & Whitman, 2008). H₂ and CO₂ are often the fermentative end products of metabolism for rumen and gut microbial communities, and methanogens use these substrates to anaerobically produce methane. This function serves as an electron sink for such a microbial community, which can continue to function as its end products are continually removed by methanogens. This process appears to be absent from any fermentations in the Huhu grub gut.

Acetogenic bacteria are a taxonomically diverse group capable of utilising H₂ and CO₂ to produce acetate, instead of methane, and are also commonly found in gut systems (Leaphart & Lovell, 2001). No strains known to be capable of acetogenesis were detected in the pyrosequencing analysis of bacterial 16S rRNA genes from the Huhu grub gut (section 3.3.3), and in fact all abundant bacterial and fungal strains detected were known to be either obligately aerobic or facultatively anaerobic in their metabolism. This is concordant with the possibility that neither methanogenesis nor acetogenesis are occurring in the Huhu grub gut, as these processes are obligately anaerobic.

Ethanol is a common metabolic end product of yeast fermentation of sugars and plant material. Accumulated ethanol can be oxidised to acetic acid by acetic acid bacteria. Acetic acid can subsequently be used by a host animal (such as the Huhu grub) for nutrition. The wild type Huhu grub gut has an abundance of both yeasts (figure 3-13, section 3.4.2) and acetic acid bacteria of the family *Acetobacteraceae* (*Acidisoma tundrae*, figure 3-11, section 3.3.3). It is therefore possible that ethanol is the microbial community's primary metabolic end

product, which is converted to acetic acid by bacteria and utilised by the host larvae for nutrition.

3.6 Fungal culturing and identification

When gut contents were smeared on minimal medium agar plates containing pine sawdust as sole carbon source, a single dominant culture was observed, and isolated for DNA extraction. Fungal ITS PCR from this DNA yielded two bands after agarose gel electrophoresis at around 550bp and 620bp. This indicated that there were at least two fungal species/strains present in the original colony. After gel band excision and sequencing, the two bands were BLAST queried in the ncbi database. The smaller band had 90% similarity with *Candida lignohabitans*, which was detected in all clone libraries aside from the cellulose fed gut library. Although this strain remains to be characterised, it is strongly implicated in lignocellulose metabolism. The larger band had 93% similarity to the basidiomycete *Rhodotorula arctica*, a basidiomycete described by Vishniac and Takashima (2010) which has not previously been associated with beetle larvae guts or lignocellulose metabolism.

4 Conclusions

This research has revealed diverse communities of bacteria (1076 OTUs) and fungi (12 OTUs) within the guts of wild Huhu beetle larvae (*Prionoplus reticularis*). Based on OTU classification, the abundant bacterial community does not appear to be responsible for lignin or cellulose degradation, and has limited hemicellulose degrading capacity. Clone library analysis of full length bacterial 16S rRNA genes yielded sequences with high similarity to those generated by pyrosequencing of genes amplified from the same DNA. The detection of an abundant strain of *Burkholderia vietnamiensis* suggests that nitrogen-fixation may be an important bacterial function by providing this essential element to the gut community and the host larvae. The Wild Type fungal community is dominated by an uncultured basidiomycete, a group with many known lignin degraders. This OTU is related to white rot fungal sequences in the ncbi database, members of which are able to grow on lignocellulose and produce lignin peroxidase enzymes. All other fungi present are known to associate with wood boring beetle larvae and are implicated in cellulose and hemicellulose utilisation. These results suggest that fungi are the key members of the gut community likely responsible for lignocellulose degradation in the Huhu grub, while the bacterial community makes contributions to nitrogen supply and fermentative capacity. The absence of methanogenic archaea, acetogenic bacteria or anaerobic fungi suggests the gut environment is predominantly aerobic. The abundance of fermentative yeasts (Figure 3-14) and acetic acid bacteria of the family *Acetobacteraceae* (*Acidisoma tundrae* Figure 3-13) might reflect ethanol production from sugar fermentation and subsequent oxidation to acetic acid as a metabolic route within the gut.

This research has also documented the diet specific nature of the Huhu grub bacterial community by successfully rearing grubs on nutrient rich laboratory media. Dramatic differences were observed when bacterial 16S rRNA pyrosequencing data from the guts of grubs fed on nutrient rich diets were compared to data from Wild Type grub guts. In contrast to the wild type diversity mentioned above, grubs fed on nutrient rich diets (section 2.1) had bacterial gut communities dominated by a single OTU classified as *Weisella*

paramesenteroides, which was not detected in the wild type community.

Burkholderia vietnamiensis sequences thought to be associated with nitrogen fixation in the wild type grubs were absent in the nutrient rich gut communities, which, unlike the wild type communities, had an accessible source of dietary nitrogen. The prevalence of *Weisella paramesenteroides* in the gut flora of grubs fed on nutrient rich media suggests that the host grub was not feeding primarily on the lignocellulose or cellulose components of the respective diets, but instead on the readily digestible sugars. Although the evidence suggests that this is the case, there was still an interesting trend in diversity across the wild type, nutrient pine, nutrient cellulose, and nutrient starch fed gut communities. Bacterial diversity decreased almost linearly with decreasing complexity of the grub diet with a particularly notable decrease in diversity between Wild Type grubs and those adapted to a pine based laboratory medium (figure 3-10). The diversity present in the Wild Type grubs was largely represented by OTUs present in the rare biosphere of the community. That these OTUs are absent when grubs are fed laboratory diets of lower complexity suggests that at least some of them are functional when present. This inference could not have been made without the deep sequencing capabilities afforded by the pyrosequencing method employed.

Huhu grub fungal communities were also shown to be highly diet specific after grubs were reared on controlled laboratory diets of untreated pine blocks and cellulose tissue paper. These diets were used in place of the nutrient rich media because they did not contain any carbon sources other than a) lignocellulose and b) cellulose, meaning that more significant community comparisons could be drawn. The pine block fed gut fungal community was less diverse than the wild type community, and was dominated by *Penicillium* species rather than an uncultured *Basidiomycete*. This difference could be explained by the fact that the diet of the wild grubs would contain ingested fungi, which were degrading the wood but are not obligate gut community members, as well as fungi which colonise the gut, whereas the pine block provided in the laboratory diet was completely undegraded prior to consumption so that only gut-specific fungi would likely be present. These grubs were less prone to colonisation/contamination by

non-specific wood degrading fungi. The *Penicillium* species which dominated the pine block fed gut community are related to known lignocellulose degraders and were not present when grubs were fed a diet of only cellulose. The only OTU present in the cellulose fed fungal gut communitiy (99% of the community) was *Candida shehatae*. This OTU was present at relatively constant levels (around 10% of a given community) in the pine block gut and frass libraries, as was *Candida lignohabitans*. *Candida shehatae* is known to be able to utilise hemicelluloses, but has not been recorded to degrade cellulsoe. Its dominance of the cellulose fed community, and its presence at lower levels when grubs are fed lignocelluloses implicates it in cellulose degradation. Similarly, the prevalence of *Candida lignohabitans* in all diets containing lignocelluloses, and its absence in the cellulose fed grubs implicates it in lignin and hemicellulose degradation.

This thesis has accomplished all of the aims set out in section 1.5 via the use of culture independent methods. It has highlighted a cohort of microorganisms which may be involved in the degradation of all the major components of lignocelluloses, as well as in the fixation of nitrogen and the removal of communitiy metabolic end products. Only culture dependent methods can be used to truly test the hypotheses borne from the conclusions of this study, and many organisms of interest are already being targeted for isolation and cultivation.

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5 Appendices

Appendix A: Bacterial TRFLP

This experiment was fundamentally flawed because the enzyme used (MSP1), had a cut site (**C^ACGG**) in the fluorescently labelled primer 785f (GGATTAGAWACC**CBGG**TAGTC) which was un-noticed due to the presence of the degenerate base B, which denotes equal proportions of C, G, and T.

Methods

The bacterial TRFLP PCR reactions were carried out in triplicate 25 μ L volumes. Each tube contained; 5 μ L of 2 to 10 ng/ μ L template DNA, 0.7 μ M of each primer (Invitrogen Ltd, New Zealand), 785F 6-carboxy-fluoresceine (FAM) labelled (GGATTAGAWACCCBGGTAGTC) and 1391R (GACGGGCRGTGWGTRCA), 3mM of MgCl₂, 2.5 μ L of 10x PCR buffer (Invitrogen Ltd, New Zealand), 2.5 μ L of 2mM dNTPs, 2.5 μ L of 0.2mg/mL bovine serum albumin (BSA), 0.2 μ L of 5U/ μ L Platinum Taq polymerase (Invitrogen Ltd, New Zealand), and autoclaved MilliQ water up to 25 μ L (Millipore, Billerica, MA, USA). Thermal cycling conditions were; Initial denaturation at 94°C for 5 min, 28 cycles of 94°C for 20 sec, 55°C for 20 sec, 72°C for 45 sec, and a final extension of 3 min at 72°C.

Bacterial 16S rDNA PCR products were digested with the MSP1 by adding up to 500ng of DNA to 2 μ L of buffer 4 and 0.8 μ L of 5U/ μ L MSP1 enzyme (NEW ENGLAND BioLabs Inc.) and made up to 20 μ L with autoclaved milliQ water.

Genotypes were resolved along with the internal size standard 1200Liz, using a MegaBACE 500 DNA Analysis System fitted with 40 cm capillary arrays (Amersham Biosciences) loaded with linear polyacrylamide Long Read Matrix (Amersham Biosciences).

Statistical analyses were performed as stated in section 2.3.2.

Results

Gut bacterial TRFLP fragment length data (Table 5-1.) showed a general trend of decreasing diversity from wild type to starch fed larvae, with larvae T being considered an outlier. Greater variation between individuals in the wild and pine groups was observed when compared to the cellulose and starch groups. The three larvae in the starch fed group all shared the same two restriction fragment peaks.

Table 5-1. Gut bacterial TRFLP fragment data

Larvae	Group	Number of restriction fragments	Group mean
81	Wild	17	
82	Wild	7	15
83	Wild	22	
V	Pine	16	
W	Pine	15	10
T	Pine	1	
N	Cellulose	7	
P	Cellulose	3	5
Q	Cellulose	5	
Z	Starch	2	
R	Starch	2	2
X	Starch	2	

Table 5-2. Frass bacterial TRFLP data

Larvae frass	Group	Number of restriction fragments	Group mean ($\pm SD$)
V	Pine	15	
W	Pine	8	10 \pm 4
T	Pine	7	
N	Cellulose	10	
P	Cellulose	11	10 \pm 1
Q	Cellulose	10	
Z	Starch	17	
R	Starch	14	16 \pm 2
X	Starch	-	

Figures 3-1 and 3-2 show distinct clustering of gut communities in regard to larvae diet, especially in the case of the starch fed larvae gut communities which are considered identical to one another and distinct from wild and pine fed larvae guts. These figures show the bacterial frass communities clustering together, but away from their parent larval gut communities. However, when abundance is considered by using untransformed TRFLP data (figure 3-3), gut communities cluster closely with their corresponding frass communities, while differences between wild, pine, cellulose and starch groups are still apparent. This suggests that the main community members are conserved between larvae gut and the frass, while less abundant members change.

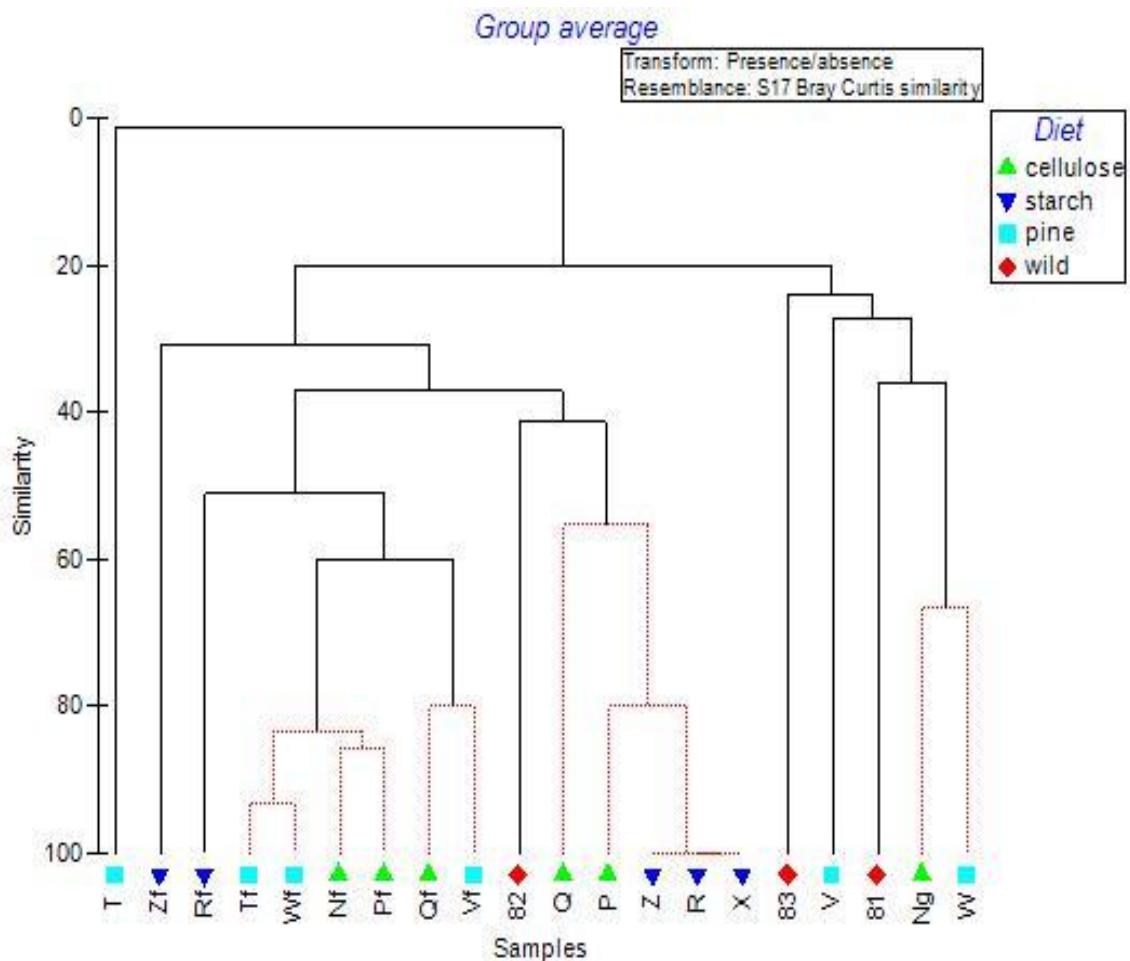


Figure 5-1. Cluster plot showing Bray-Curtis similarity between diet treated bacterial gut and frass TRFLP profiles

Samples connected by dotted red lines were generated using the SIMPROF test, indicating that they cannot be significantly differentiated.

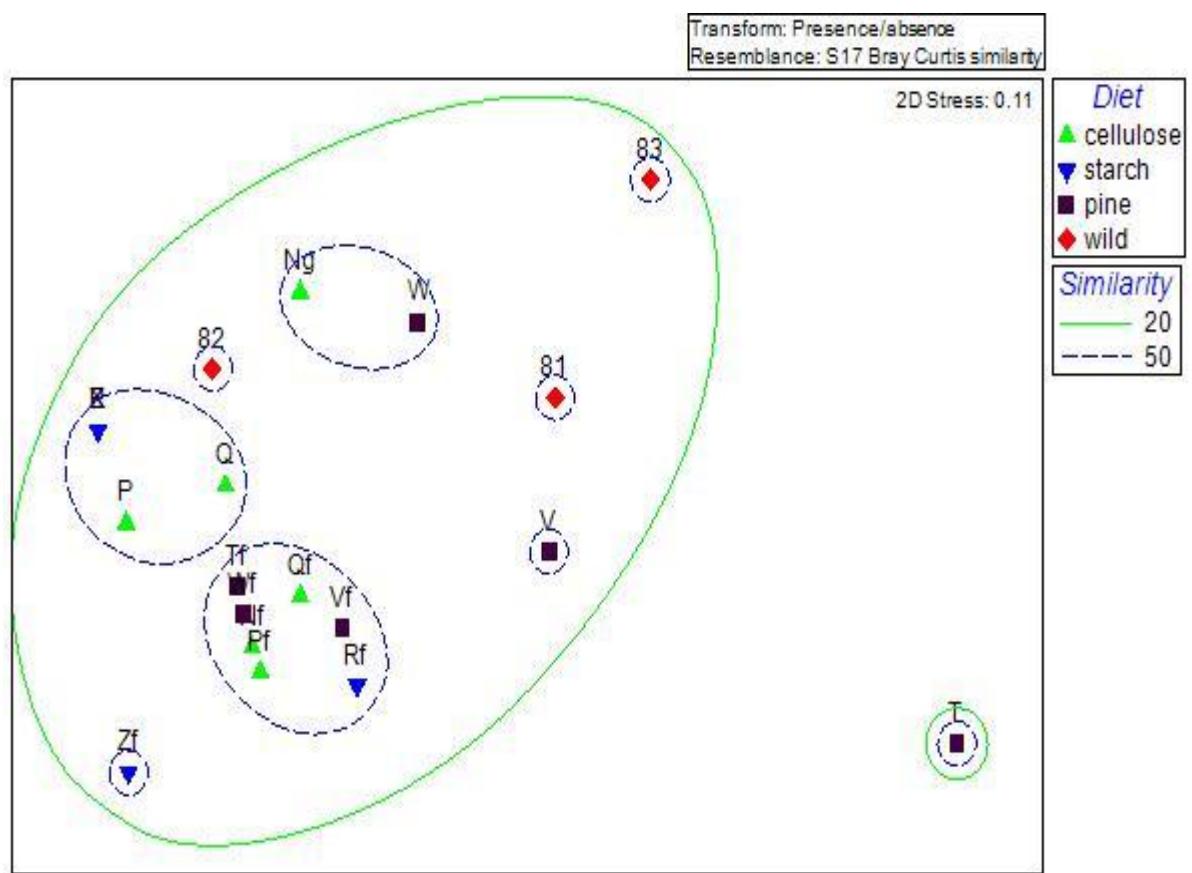


Figure 5-2. Presence/absence transformed Bacterial TRFLP profile MDS

Overall presence/absence transformed two-dimensional, non-metric MDS ordination (stress = 0.11) based on Bray-Curtis similarities of TRFLP profiles of bacterial communities from diet treated Huhu larvae gut and frass. Points within solid green circles cluster at the 20% similarity level, points within broken blue circles cluster at the 50% similarity level.

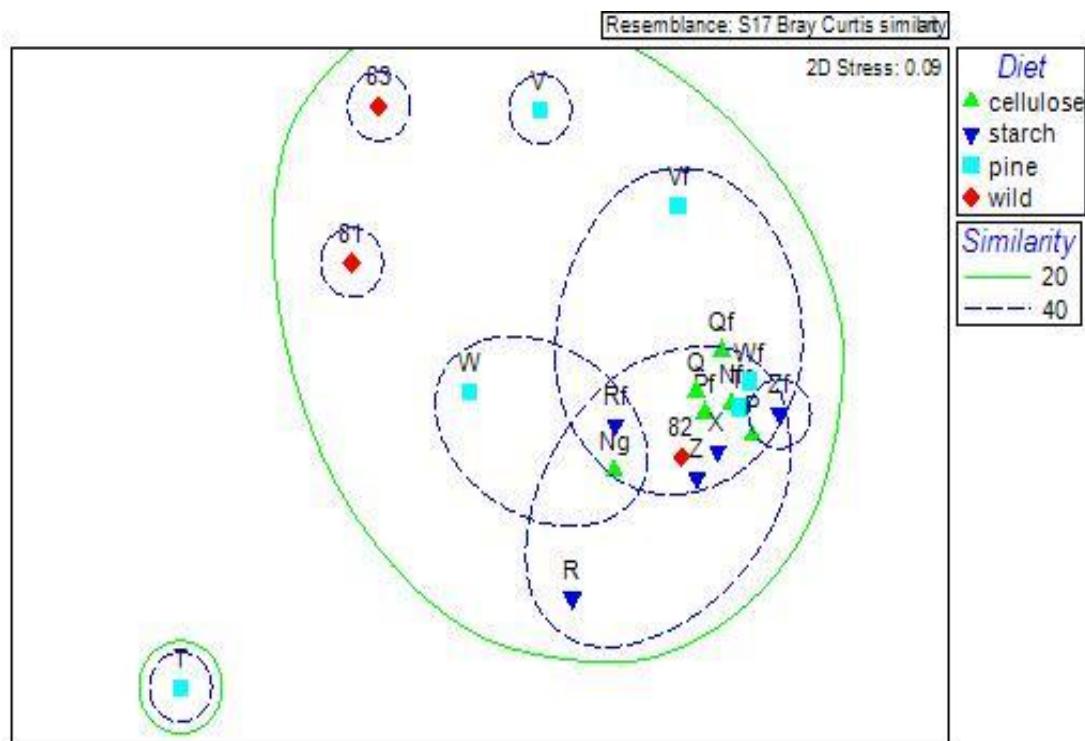


Figure 5-3. Bacterial TRFLP profile MDS, no overall transformation

Untransformed two-dimensional, non-metric MDS ordination (stress = 0.09) based on Bray-Curtis similarities of TRFLP profiles of bacterial communities from diet treated Huhu larvae gut and frass. Points within solid green circles cluster at the 20% similarity level, points within broken blue circles cluster at the 40% similarity level.

Appendix B: DSMZ culture media

734. METHANOBREVIBACTER CURVATUS MEDIUM

NaCl.....	1.00 g
KCl.....	0.50 g
MgCl ₂ x 6 H ₂ O.....	0.40 g
CaCl ₂ x 2 H ₂ O.....	0.10 g
NH ₄ Cl.....	0.30 g
KH ₂ PO ₄	0.20 g
Na ₂ SO ₄	0.15 g
Casamino acids (Difco).....	0.50 g
Yeast extract (Difco).....	0.50 g
Resazurin.....	0.50 mg
NaHCO ₃	5.80 g

Trace element solution SL-10

(see medium 320) 1.00 ml

Selenite-tungstate solution

(see medium 385) 1.00 ml

Vitamin solution (see medium 503) 1.00 ml

Distilled water 1000.00 ml

Prepare medium anaerobically under 80% H₂ + 20% CO₂ gas mixture. Add bicarbonate, trace elements, selenite-tungstate solution, and vitamin solution to the autoclaved medium to sterile, anaerobic stock solutions (flush bicarbonate solution with 80% N₂ + 20% CO₂; other solutions with N₂). If necessary adjust the pH to 7.2 or 7.7 with sterile anaerobic 1M solutions of either HCl or Na₂CO₃. Prior to inoculation add DTT (1mM final conc.) as a reductant from a filter sterilized, anaerobic stock solution.

457. MINERAL MEDIUM (BRUNNER)

Na ₂ HPO ₄	2.44 g
KH ₂ PO ₄	1.52 g
(NH ₄) ₂ SO ₄	0.50 g
MgSO ₄ x 7 H ₂ O.....	0.20 g
CaCl ₂ x 2 H ₂ O.....	0.05 g

Trace element sol. SL-4 (see below) 10.00 ml

Distilled water 1000.00 ml

Adjust pH to 6.9, autoclave.

Trace element solution SL-4:

EDTA 0.50 g
FeSO ₄ x 7 H ₂ O 0.20 g

R2A AGAR

Enzymatic Digest of Casein.....	0.25 g
Enzymatic Digest of Animal Tissue	0.25g
Acid Hydrolysate of Casein	0.50g
Yeast extract	0.50g
Dextrose	0.50g
Soluble starch	0.50g
Dipotassium Phosphate.....	0.30g
Magnesium Sulfate Heptahydrate	0.10g
Sodium pyruvate	0.30g
Agar	15.00g

Adjust pH to 7.2 prior to the addition of agar, autoclave.

Appendix C: Agarose gel electrophoresis

50x TAE Buffer (~pH 8.5)

tris(hydroxymethyl)aminomethane.....242g

Glacial acetic acid.....57.1ml

0.5M EDTA (pH 8.0).....100ml

Make up to 1L with MilliQ water and check pH. Dilute to 1x working concentration with MilliQ water.

All electrophoresis was carried out at a constant voltage of 70V for 40-60 minutes using either 0.8% or 1.2% agarose TAE. Gels were stained with 0.2mg/ml ethidium bromide for 10-30 minutes before being washed with tap water and visualised under ultraviolet light.