Investigating the lignocellulolytic gut microbiome of huhu grubs

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Abstract

New Zealand's endemic longhorned beetle (*Prionoplus reticularis*) larvae, huhu grub, are xylophagous and feed on a broad range of dead trees. Since insects are not believed to have the ability to fully degrade lignocellulose, it is likely that microbes present in the gut assist in degrading lignocellulose to release energy rich sugars. This process is considered the rate limiting step in the utilisation of woody material in biofuel production. To better understand the gut consortium present while feeding on the lignocellulose and cellulose components, huhu grubs were initially collected and reared on non-degraded pine blocks (lignocellulose) or cotton (cellulose). The gut fungal and bacterial community of huhu grubs was investigated using high-throughput sequencing and analysis techniques. Furthermore, the effects of a dietary switch on both fungal and bacterial communities was explored by switching huhu grubs initially reared on pine to cotton, and vice versa. Cellulose- and lignocellulose-reared huhu grubs were expected to host a wide range of bacteria and fungi in their gut, some of which produce lignocellulose degrading enzymes. Assessment of the gut fungal community composition confirmed that cellulose-reared huhu grubs were significantly distinct from lignocellulose-reared grubs. The fungal community of lignocellulose-fed huhu grubs was more functionally diverse, with several yeasts, soft-rot fungi and potential hemicellulose and cellulose degraders present. Whereas, the fungal community of cellulose-reared grubs was mostly abundant in yeasts and potential cellulose degraders. When cotton-reared grubs where switched to pine (lignocellulose), lignocellulose was observed to have a 'bottleneck effect' or selective pressure on the fungal gut community. The gut bacterial community of huhu grubs reared on cellulose or lignocellulose diets seemed to be unaffected by the selective pressure of the diet, and no significant change in the bacterial community was observed following dietary switch. Therefore it was concluded that the bacterial community while still important is playing a secondary role to the fungi. Finally, bioprospecting studies conducted using the frass (excretia) of these huhu grubs resulted in the isolation of a bacterium, *Acinetobacter* H23, capable of producing an extracellular laccase. Overall, the increased understanding of structure and composition of the gut microbiome of huhu grubs reared on cellulose or lignocellulose diets, along with

isolation of novel lignocellulolytic isolates could prove to be significant for the biofuel industry.

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A or group A	Huhu grubs initially grown on cotton diet (batch 3 huhu grubs)
A1 or group A1	Huhu grubs switched to pine from cotton diet (batch 3 huhu grubs)
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AFLs	ARISA fragment lengths
ANOSIM	Analysis of similarity
ARISA	Automated ribosomal intergenic spacer analysis
B or group B	Huhu grubs initially grown on pine diet (batch 3 huhu grubs)
B1 or group B1	Huhu grubs switched to cotton from pine diet (batch 3 huhu grubs)
BLAST	Basic local alignment search tool
BME	2-Mercaptoethanol, or β-mercaptoethanol
CMC	Carboxymethyl cellulose
CTAB	Cetrimonium bromide
DNA	Deoxyribonucleic acid
DP	Degree of polymerization
EDTA	Ethylenediaminetetraacetic acid
ITS	Internal transcribed spacers
LiP	Lignin peroxidase
MC	Main trial on cotton (batch 2 huhu grubs)
MCC	Microcrystalline cellulose
MnP	Manganese peroxidase
MP	Main trial on pine block (batch2 huhu grubs)
MT	Main trial
NMDS	Non-metric multidimensional scaling
OTU	Operational taxonomic unit
PBS	Phosphate-buffered saline
PC	Preliminary trial on cotton (batch1 huhu grubs)
PCR	Polymerase chain reaction
PP	Preliminary trial on pine block (batch1 huhu grubs)
PVP	Polyvinylpyrrolidone

RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulfate
TE	Tris-EDTA
TS	Total sequences
TSB	Tryptic soy broth
YNB	Yeast nitrogen base

Chapter One

Introduction

1.1 General background

Fossil fuels such as crude oils, coal, natural gas and heavy oils are rapidly depleting non-renewable energy sources. This uncertainty about the reserves of non-renewable fuels and an increasing awareness of greenhouse gas emissions has peaked a global interest in alternative energy sources (Höök & Tang, 2013). Among the potentially useful alternative energy sources, biofuel production from biomass is often considered to be a high potential substitute, especially in the liquid transportation sector. Biomass resources mainly include organic matter such as plant material, municipal, industrial and animal waste material, and aquatic plants and algae (Demirbas, 2005; Demirbaş, 2001). The vast amount of unused plant material has huge potential to be transformed into second generation biofuels (Kumar et al., 2008; Sanderson, 2011). Lignocellulosic biomass within plant material provides them with rigidity and structure, and upon breaking down can produce chemical components that could be used in the production of biofuels. The challenge of this process is the extraction of the chemical components from the plant material as the recalcitrant nature of the lignocellulosic biomass means it is very difficult to breakdown. Some components are hard to reach and some insoluble (Kumar et al., 2008; Sanderson, 2011).

In nature, independent of the environment, a consortium of microorganisms is generally involved in the efficient degradation of lignocellulose (Alper & Stephanopoulos, 2009). The members of these consortia possess the ability to tackle challenges including toxic plant defense compounds and inhibitory molecules. Further, the unique metabolic pathways and cellular tolerance to toxic compounds possessed by these microbes are potentially useful in biofuel production (Alper & Stephanopoulos, 2009). However, selecting the relevant members of these microbial consortia involved in lignocellulose degradation in a nutrient rich environment such as tropical soils or pre-degraded wood is challenging. The selective pressure of a nutrient rich environment is very different to that of non-degraded lignocellulose. A wide variety of microorganisms will

thrive in such nutrient rich environments whereas a microenvironment like the gut of a wood-feeding insect provides us with a suitable environment to explore a diet specific (in this case lignocellulose) microbiome (Brune, 2003; Geib et al., 2008; Köhler et al., 2008; Reid et al., 2011).

Several wood-feeding insects are also known to either degrade or circumvent the lignin barrier to meet their nutritional requirements (Brune, 2014; Geib et al., 2008). However, it is widely accepted that the insect gut system alone is unable to degrade lignin (Ohkuma, 2003). The majority of wood feeding insects are either known to thrive on pre-degraded wood (Kukor, 1988), or depend on exo-symbiotic relationships with wood degrading fungi (Johjima et al., 2006; Taprab et al., 2005). However, some beetles access the polymer carbohydrates by circumventing the lignin barrier with the help of a consortium of gut microbes (Geib et al., 2008). Huhu grubs feed solely on wood throughout the larval stage, and are therefore expected to host a consortium of lignocellulolytic microbes (Reid et al., 2011). Therefore by rearing huhu grubs on cellulose or lignocellulose diets we expect to explore their impact on the gut bacterial and fungal community. The selective pressure of the diet will potentially select for a lignocellulolytic gut microbial community. Understanding this microbial consortium is essential for biofuel associated research.

Current knowledge of the gut microbiology is focused on the bacterial diversity of huhu grubs captured from a natural environment (Reid et al., 2011). The use of wild type huhu grubs introduces a range of variables, making it difficult to determine the microbial consortium that is potentially involved in the procurement of polymer carbohydrates. In contrast, we aim to examine the gut microbiome (bacterial and fungal community) of huhu grubs fed on defined diets in a laboratory environment, therefore involving fewer factors influencing the gut microbiome. We will utilise solid pine blocks and cotton to explore the huhu grub gut microbial community on lignocellulose and cellulose diets. We also aim to interchange the diets to understand any observed shift in the gut community, followed by bioprospecting for lignocellulolytic enzymes using culture techniques and enzymatic assays.

1.2 Lignocellulose

Lignocellulose is the major structural component of all plants making it the most abundant renewable resource (Dashtban et al., 2009; Lee, 1997; Sanderson, 2011). Lignocellulose comprises three major polymers namely lignin, hemicellulose and cellulose (Figure 1.1), which can be taken apart to produce ethanol, butanol and furans (Sanderson, 2011) and minor components like ash, pectin, salt and minerals (Van Dyk & Pletschke, 2012).

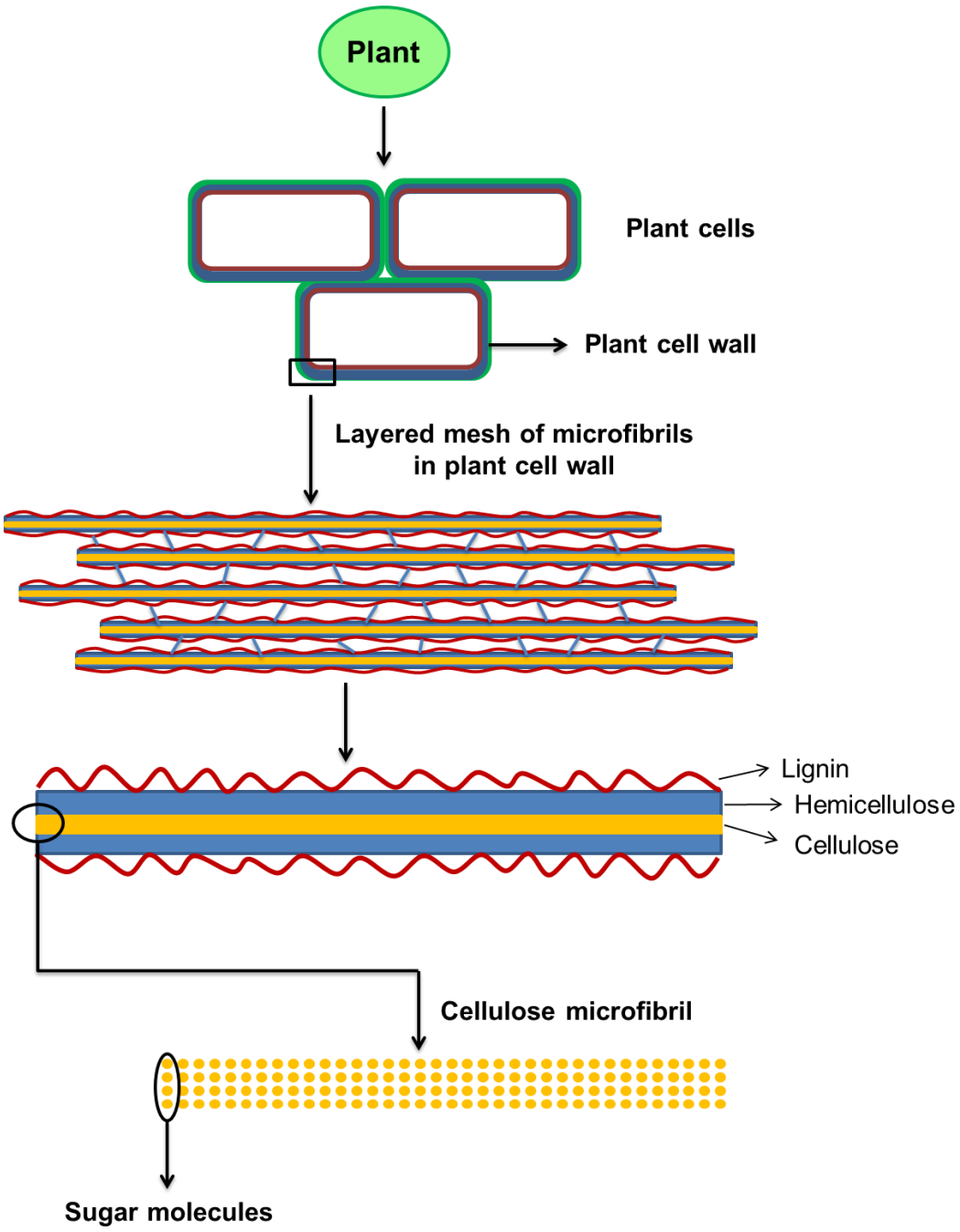

Figure 1.1: Structure of lignocellulose

1.2.1 Cellulose

Cellulose, the most abundant organic molecule on Earth, is a linear biopolymer of anhydroglucopyranose-molecules, connected by β-1,4 glycosidic bonds. It is a structural component of the cell walls that provide plants with mechanical strength and chemical stability (Brown, 2004). Cellulose is found in both crystalline and the non-crystalline forms. Microfibrils are crystalline structures formed by the coupling of adjacent cellulose chains by hydrogen bonds, hydrophobic interactions and VanderWaal's forces. The inter polymer linkages between cellulose and lignin occurs using ether and hydrogen bonds, and between hemicelluloses using hydrogen bonds (Brown, 2004).

The majority of the properties of cellulose are dependent on its degree of polymerization (DP), i.e. the number of glucose units that make up one polymer molecule. The most common range for the DP of cellulose is between 800-10000 units, however it may extend up to 17000. In certain cases ranges lower than 800 are also found. For instance cellulose from wood pulp has a DP ranging between 300 and 1700 (Klemm et al., 2005; Reese et al., 1950; Yamashiki et al., 1990). The degree of hydrolysis is strongly related to the solubility of cellulose. Cellulose is water insoluble and hygroscopic in nature, and remains stable in dilute acid solutions and low temperatures. Cellulose becomes soluble at elevated temperatures and highly acidic conditions. However severe degradation of polymer by hydrolysis is noticed while using concentrated acids. Generally extensive swelling of cellulose occurs in alkaline solutions (Krassig & Schurz, 2002).

1.2.2 Hemicellulose

The second most abundant component of lignocellulosic biomass, hemicellulose, are heterogeneous polymers of pentoses, hexoses and sugar acids (Pérez et al., 2002). The structure and composition of hemicelluloses varies in accordance to their source and extraction method. The highly branched structure of hemicelluloses along with the presence of acetyl groups connected to the polymer chains results in the absence of a crystalline structure. Xylan is the most abundant polysaccharide that belongs to the hemicellulose family. It mainly comprises of five carbon sugar monomers, xylose and six carbon sugars like glucose. Hemicellulose obtained from hardwood slightly differs from that of softwood.

Galactoglucomannans is the main type of hemicelluloses (20% of the dry material) present in the soft woods and is divided into two types with varying galactose content. The glucomannan fraction contains a comparatively lower amount of galactose. Arabinoglucuronoxylan is the second main type of hemicelluloses in the soft woods comprising of about 5-10% of the dry material. The main constituent of the hardwood hemicelluloses is glucuronoxylan. The total percentage of glucoronoxylan varies from 15-30% depending on the species of the hardwood. About 2-5% of hemicelluloses in hardwood are glucomannan. Hemicellulose utilises ether, ester and hydrogen bonds to form inter-polymer linkages with lignin, and hydrogen bonds to form the linkage between cellulose. The intra-polymer linkages are provided by ether and ester bonds (Aspinall, 1959; Ebringerová, 2005; Scheller & Ulvskov, 2010; Talmadge et al., 1973). The other characteristics of hemicelluloses include insolubility in water, increased solubility in acidic conditions and elevated temperatures (Ebringerová, 2005).

1.2.3 Lignin

The heterogeneous polymer lignin is the most recalcitrant component of lignocellulosic material and mainly consists of phenylpropane units such as *p*-coumaryl, guaiacyl, coniferyl, syringyl and sinapyl alcohol (Hendriks & Zeeman, 2009). Lignin composition differs significantly between hardwood and soft wood. Hardwood mainly consists of different proportions of coniferyl and sinapyl alcohol units. On the contrary softwoods consist of more than 90% of coniferyl alcohol with the remaining dominant constituent being p-coumaryl alcohol units. The carbohydrate polymers are tightly bound to lignin by hydrogen and covalent bonding where lignin is interspersed with hemicelluloses forming a matrix which surrounds the cellulose microfibrils and results in a rigid structure (Kirk & Farrell, 1987; Lee, 1997). Lignin prevents the penetration of lignocellulolytic enzymes to the interior lignocellulosic structure by acting as a barrier for any solutions or enzymes by linking to both hemicellulose and cellulose (Dashtban et al., 2009; Himmel et al., 2007; Sánchez, 2009).

1.3 Lignocellulose degradation

Unlike the first generation biofuels that are derived from starch, sugars and plant oils (food crops), new generation biofuels are derived from lignocellulosic biomass such as soft/hard wood and agricultural wastes (non-food

crops)(Chaturvedi & Verma, 2013; Sims et al., 2008, 2010). Lignocellulosic biomass, once broken down, releases sugars and other organic compounds which are further processed to produce biofuels (Kumar et al., 2009; Sanderson, 2011). The conversion of lignocellulosic biomass to biofuel, especially ethanol, is generally carried out in three broad steps: pretreatment of the lignocellulosic material, hydrolysis of cellulose and hemicellulose and fermentation of sugars to ethanol.

The complex cross-linked polymers constituting lignin are tightly bound surrounding cellulose and hemicellulose, making it very difficult to reach them for hydrolysis to sugars and other compounds. Therefore pretreatment technologies are used to alter or remove the tough lignin barrier in order to access cellulose and hemicellulose for their successful hydrolysis into fermentable sugars (Mosier et al., 2005). Effective pretreatment methods need to be low cost, should be applicable to a broad substrate range and have low energy demands (Agbor et al., 2011; Chaturvedi et al., 2013). Various physical, chemical and biological pretreatment methods are employed to expose cellulose and hemicellulose to hydrolysis. Some of the techniques include grinding, milling, pyrolysis, treatment with alkali, treatment with aqueous ammonia at high temperatures, use of oxidizing agents (such as hydrogen peroxide) and biological treatments with the help of various rot fungi (Chaturvedi et al., 2013; Kumar et al., 2009).

There are three major hydrolysis processes that produce sugars suitable for further steps in ethanol production: dilute acid, concentrated acid and enzymatic hydrolysis. Dilute-acid hydrolysis with 2-5% acid concentration are carried out at high temperatures (160 °C – 200 °C) and pressures (~10atm) (Himmel et al., 2007; Iranmahboob et al., 2002; Kumar et al., 2009). With longer retention, enzymatic hydrolysis of lignocellulosic material can also be achieved to produce ethanol (Kumar et al., 2009). As the above mentioned physical and chemical processes have high energy requirements, biological processes which are environmentally friendly and which do not require such high energy for lignin removal, are increasingly being studied (Chaturvedi et al., 2013; Kumar et al., 2009; Wan & Li, 2012).

1.4 Lignocellulolytic fungi and bacteria

Several fungi and bacteria are well known to be involved in lignocellulose degradation, and understanding the enzymatic cocktail and processes utilised by them to degrade lignocellulose has been a topic of immense interest. Fungi causing wood decay are typically classified into brown, white or soft rots, according to the decay patterns observed in the wood. Brown rot fungi are basidiomycetes that utilise a series of cellulases and hemicellulases to attack the wood while modifying lignin to a minimal extent. White rot fungi belonging to Basidiomycota and higher Ascomycota are capable of completely degrading the wood by utilising cellulose and lignin degrading enzymes. Additionally, ligninolytic enzymes of white rots have high oxidative activity and low substrate specificity (Sánchez, 2009). Soft rot fungi belonging to phyla Ascomycota and Deuteromycota are known to attack wood structures with high moisture content and low amounts of lignin, creating diamond-shaped cavities. However, knowledge about the wood degradative enzymatic system employed by soft rots is limited (Goodell et al., 2008). Usually, the lignocellulose degradation by fungi is carried out by two types of extracellular enzymatic systems: oxidative and hydrolytic (Chaturvedi et al., 2013; Sánchez, 2009). Several moulds and staining fungi are also found in decaying wood. With the exception of a few blue stain fungi known to cause soft rot (Käärik, 1974), these fungi are generally incapable of degrading lignified cell walls. A few bacterial species belonging to the *Pseudomonadales* (Pseudomonas) and *Actinomycetales* (*Cellulomonas*, *Streptomyces*) are known to produce lignocellulolytic enzymes (Cheng & Chang, 2011; Goyal et al., 1991; Lynd et al., 2002; McCarthy, 1987; Pérez et al., 2002). However, they generally work as a part of a consortium to achieve lignocellulose degradation. Both wood-degrading fungi and bacteria utilises lignin mineralising enzymes such as laccases, lignin peroxidases, manganese peroxidases, and versatile peroxidases, along with a combination of synergistically acting cellulases and hemicellulases to degrade lignocellulose (Kumar et al., 2008).

1.5 Lignocellulolytic enzymes

1.5.1 Cellulose degrading enzymes

Cellulose is the most abundant carbohydrate produced by plants. Although cellulose is a great source of energy, it is challenging to completely hydrolyse this substrate. Individual enzymes are not capable of completely degrading cellulose. All organisms known to degrade cellulose efficiently produce a combination of sequentially and synergistically acting enzymes to fully hydrolyse cellulose (Béguin & Aubert, 1994). Collectively, these enzymes are referred to as cellulases, and the process of degradation is known as cellulosis. Degradation of cellulose from plants is achieved by the combination of glycosyl hydrolases, endoglucanases and β-1,4-glucosidases (Béguin et al., 1994; Eriksson et al., 1990; Voříšková & Baldrian, 2013). Initially, the long cellulose chains are broken down into shorter glucose polymers and eventually into glucose chains. Additionally, oxidative enzymes such as cellobiose dehydrogenase are also required to fully degrade the cellulose. These enzymes reduce phenoxy radicals and quinones in the presence of cellobiose, which is then oxidised to cellobiono-δ-lactone (Ander et al., 1990).

1.5.2 Hemicellulose degrading enzymes

Due to their heterogeneous structure, hemicellulose molecules are difficult to degrade. Furthermore, the complete degradation of hemicellulose requires synergistic actions of a wide range of hydrolytic enzymes. These enzymes that contribute towards the biodegradation of hemicellulose are generally referred to as hemicellulases. Hemicellulases are classified according to their action on different substrates and include; xylanases, endoglucanases, endomannases, β-xylosidase, β-mannosidases β-mannanases, arabinofuranosidases, α-L-arabinanases and α-galactosidases (Karboune et al., 2009; Pérez et al., 2002). Additionally, an enzymatic cocktail involving these enzymes was shown to be more efficient than their individual activity (Romero et al., 1999). Xylanases have been previously isolated from microorganisms thriving in lignocellulose degrading environments and studied for their biotechnological applications (Pérez et al., 2002)

1.5.3 Lignin degrading enzymes

Degradation of lignin has been extensively studied in basidiomycete fungi. The white-rot fungi *Phanerochaete chrysosporium* is a model organism for lignin-degrading enzyme production (Singh & Chen, 2008). The lignin structure presents some challenges including requiring extracellular enzyme systems to degrade large lignin polymers, requiring an oxidative enzymatic system to degrade C-C and ether bonds and stereo-irregularity of lignin polymer requires reduced specificity of ligninolytic enzymes (Kirk & Cullen, 1998; Souza, 2013).

The most well-characterised enzymes able to degrade lignin include lignin peroxidases (LiP; EC 1.11.1.14), manganese peroxidases (MnP; EC 1.11.1.13), laccases (EC 1.10.3.2), versatile peroxidase (VP; EC 1.11.1.16), dye-decolorizing peroxidases (DyP; EC 1.11.1.19) and hydrogen peroxide (H_2O_2) generating enzymes such as glyoxal oxidase (GLOX) and aryl alcohol oxidase (AAO) (Fisher & Fong, 2014; Souza, 2013).

Lignin peroxidase (LiP) (EC 1.11.1.14) from *Phanerochaete chrysosporium* was the first ligninolytic enzyme to be isolated (Hammel & Cullen, 2008). The LiP catalytic reaction is similar to that of classical peroxidases with a heme group reacting with H_2O_2 forming a two electron oxidised oxo-ferryl intermediate (Hammel et al., 2008; Isroi et al., 2011). However, while classical peroxidase can only act on strongly activated aromatic substrates, LiP can oxidise even moderately activated aromatic rings. Furthermore, the strong redox potential of LiP allows them to oxidise major non-phenolic components of lignin (Abdel-Hamid et al., 2013).

Manganese peroxidases (MnP) contains one molecule of heme, and catalyses an oxidative reaction that is Mn and H_2O_2 dependent (Wong, 2009). The catalytic cycles result in the formation of Mn^{3+} which mediates the oxidation of organic substrates. Mn^{3+} is released from the active site given the presence of appropriate chelators to stabilise it (Banci et al., 1998; Hatakka & Hammel, 2011). Thus, the oxidising power of MnP is then transferred to Mn^{3+}, allowing it to diffuse into the lignified cell wall and attack it from the inside (Hammel et al., 2008). However, unlike LiP, MnP does not naturally oxidise non phenolic components of lignin.

Versatile peroxidases (VP) possess the capability to catalyse both manganese-dependent and manganese-independent reactions. It oxidises phenolic, high redox-

potential nonphenolic aromatic compounds and Mn^{2+} (Fisher et al., 2014; Isroi et al., 2011). Dye-decolorizing peroxidases (DyP) are also heme-peroxidases occurring in fungi and bacteria capable of oxidising high redox potential anthraquinone dyes and lignin-type compounds (Abdel-Hamid et al., 2013). The extracellular H_2O_2 generating enzymes such as glyoxal oxidase (GLOX) and aryl alcohol oxidase (AAO) provide H_2O_2 necessary to support the oxidative turnover of other H_2O_2 dependent (LiP, MnP) enzymes responsible for ligninolysis during lignin degradation (Isroi et al., 2011; Souza, 2013).

Laccases are multi-copper oxidase enzyme which are widely distributed in fungi, bacteria, plants and even in insects (Fisher et al., 2014; Mogharabi & Faramarzi, 2014; Sharma & Kuhad, 2008; Yatsu & Asano, 2009). These blue copper proteins have the ability to oxidise aromatic compounds using oxygen as the terminal electron acceptor, thus falling under the broader category of polyphenol oxidases (Madhavi & Lele, 2009; Mayer, 1986). Laccases oxidise a wide range of compounds including polyphenols, methoxy-substituted phenols and diamines (Thurston, 1994). Some widely used substrates are hydroquinone, catechol, guaiacol, 2,6-dimethoxyphenol (DMP), para-phenylenediamine and syringaldazine (unique substrate for laccase) (Thurston, 1994). It is however difficult to categorise laccases based on their substrate specificity as the range of substrates oxidised varies from one laccase to the other and laccases are known to be nonspecific to its inducing substrate (Kunamneni et al., 2007). The substrate range also varies from one organism to another (Madhavi et al., 2009).

The multitude of laccases is also demonstrated by the formation of laccase isozymes from either the same or different genes encoding for the laccase enzyme (Archibald et al., 1997; Madhavi et al., 2009). Multiple functions have been identified to be associated to laccase enzymes depending on the cell type and cellular conditions the enzyme or isozyme are expressed in. For instance, laccase is associated with breakdown of cellulose in fungi, as fungal virulence factors, lignification of plant cell walls, melanin synthesis in midgut of insects as immune defence and pigmentation process of fungal spores (Assavanig et al., 1992; Mayer & Staples, 2002; Sharma et al., 2008; Thurston, 1994). The presence of multiple binding sites in the enzyme structure concurs with its multifunctional activity (Sharma et al., 2008).

Laccases are glycosylated proteins with mannose being one of the major carbohydrates attached to laccase. Glycosylation of laccase is believed to play a role in laccase activity, thermal stability, secretion, proteolytic susceptibility and copper retention (Finkelstein et al., 2012; Madhavi et al., 2009; Maestre-Reyna et al., 2015).

Laccases are known to typically have three active sites occupied by 4 copper atoms: Type 1 (blue site), Type 2 (normal site) and Type 3 (binuclear site). The reaction mechanism involves the reduction of oxygen to water via a four electron transfer process (Jones & Solomon, 2015; Mogharabi et al., 2014).

The attack of phenolic subunit of lignin by laccases is believed to take place as follows (Kunamneni, Ghazi, et al., 2008; Madhavi et al., 2009; Mogharabi et al., 2014): The reducing substrate reduces the type 1 copper, leading to electron transfer from type 1 copper to the trinuclear cluster made up of type 2 and type 3 copper. Molecular oxygen which acts as the terminal electron acceptor gets reduced to water at the type 2 and type 3 copper sites. The transfer of electron from the substrate to the type 1 copper site may be controlled by redox potential difference, and hence higher its redox potential, higher is the rate for substrate oxidation. This step of electron transfer to the type 1 copper site is proposed to be the rate-determining step (Solomon et al., 1996). The redox potential of type 1 copper site governs the electron release from the oxidised substrate and the reaction efficiency and hence facilitates the substrate oxidation (Mogharabi et al., 2014).

Laccase oxidation was believed to be restricted to phenolic group only. The substrate range of laccases can be extended from the phenolic group of lignin to non-phenolic groups of lignin with the help of a mediator system. Laccase mediators oxidise substrates by either producing stable radicals or via electron transfer. ABTS, the first mediator shown to be effective in delignification and lignin transformation by laccase (Bourbonnais & Paice, 1990) is used in bio-bleaching of wood pulps, and delignification in the pulp industry.

Spectrophotometric approaches are most commonly used to determine laccase activity by measuring the absorbance of the coloured products formed as a result of the oxidation reaction (Mogharabi et al., 2014). Chemicals such as azide, thioglycolic acid and diethyldithiocarbamic acid inhibit laccase activity (Bollag &

Leonowicz, 1984; Johannes & Majcherczyk, 2000a) and could be used as controls for spectrophotometric assays. The smaller anions such as azide and hydroxide ions inhibit laccase reaction by binding to type 2 and 3 copper and interrupting the internal electron transfer.

Some of the other methods used to characterise laccases include polarographic methods to directly measure the oxygen consumption rate (Kulikova et al., 2013) as well as techniques to analyse the distribution of RNA expression such as reverse transcription-polymerase chain reaction (RT-PCR), northern blotting and *in situ* hybridisation (Bustin, 2000).

Previously, laccases have been mostly characterised from ascomycetes, deuteromycetes and basidiomycetes fungi (Madhavi et al., 2009; Mogharabi et al., 2014). However, recent information suggests that these enzymes are widespread in bacteria with only a few studied so far (Ausec et al., 2011; Claus, 2003). An example of one such studied bacterial blue multi-copper protein with laccase like activity is the highly thermostable CotA protein from *Bacillus subtilis* (Hullo et al., 2001; Martins et al., 2002). The higher thermostability of such laccases and the highly efficient bacterial expression systems are advantageous over fungal laccases (Sharma et al., 2007; Singh et al., 2014). Bacterial laccases, in spite of having numerous advantages over fungal laccases, are yet to be used efficiently in industrial or biotechnological applications due to their low yields (Endo et al., 2003; Singh et al., 2014). Therefore, further studies need to be carried out to identify new bacterial laccases with advantageous properties as well as improved yields for efficient industrial applications.

1.6 Wood-feeding insects

While the extent of insect species diversity remains uncertain, the identified species comprise more than half of all known species of organism (Thomas, 2005). Insects are critical in several aspects, directly impacting humans and the environment. For example, insects act as scavengers (Basset & Arthington, 1992), consumers, decomposers of organic matter, and are crucial for biogeochemical cycling of nutrients (Belovsky & Slade, 2000). Furthermore, they are the source of economically important products such as silk (Shao & Vollrath, 2002), honey (Crane, 1975) and certain venom (Ratcliffe et al., 2011; Ruëff et al., 2013). Insects act as pollinators, parasites and predators (Obrycki & Kring, 1998),

playing an integral part in maintaining ecosystem balance, preventing population growth from overrunning natural resources (Belovsky et al., 2000).

The most abundant insects are found within the order Coleoptera (beetles), Diptera (flies, mosquitoes), Hymnoptera (bees, ants and wasps) and Lepidoptera (butterflies and moths) (Hammond, 1992). Insects could arguably be considered as the most adaptive form of life, as illustrated by the plant-insect relationships throughout the evolutionary history of life on Earth (Bennett & O'Grady, 2013). Beetles are estimated to constitute 40% of all described arthropods (Hammond, 1992), with a recent study estimating the number of beetle species to be 1.5 million (Stork et al., 2015). The myriad relationships with beneficial microorganisms have been the foundation of this evolutionary success. Among these beneficial microorganisms, the contributions from gut associated microbes are considered to be highly relevant to the growth (Fukatsu & Hosokawa, 2002; Kaufman & Klug, 1991; Santo Domingo et al., 1998), survivability (Poulsen & Sapountzis, 2012) and adaptability (Kaufman et al., 1991) of insects. Of particular relevance to this thesis are wood feeding insects and their gut microbiome.

Several studies have been conducted on wood feeding insects, including termites, bark beetles, longhorned beetles, cockroaches, and shipworms, to understand the role of their gut microbiome in lignocellulose degradation (Boucias et al., 2013; Breznak, 2000; Brune, 2003, 2014; Geib et al., 2008; Ni & Tokuda, 2013; Watanabe & Tokuda, 2001, 2010). Among these wood-feeding insects, termites and their associated gut microbes have been extensively studied, with a focus on finding novel enzymes with biotechnological potential (Brune, 2003, 2014). Studies conducted on basidiocarp-dwelling beetles have identified about 300 species of yeasts, with some possessing cellulolytic and hemicellulolytic capabilities (Suh et al., 2003; Suh & Blackwell, 2005; Zhang et al., 2003).

Bark beetles are thought to employ yeasts, bacteria and filamentous fungi to gain access to the required nutrients from wood (Adams & Six, 2008; Six, 2013). Geib et al., (2008) analysed the total gut DNA of the wood-feeding cerambycid beetle *Anoplophora glabripennis* and identified a soft rot fungus, *Fusarium solani,* to be potentially involved in lignin degradation. This was further supported by metatranscriptomic data revealing the presence of active fungal and prokaryotic transcripts. Additionally, when the frass (excretia) lignin structure of *A.*

glabripennis was compared with undigested wood, significant lignin side chain oxidation was observed. Importantly the depolymerisation of lignin was believed to have occurred at a higher rate than observed in the white rot fungi. These studies suggest that the insect gut microbiome is a specialised environment with an increased potential for finding economically important microorganisms that might be significant to the biofuel industry.

Cellulose in wood is a rich source of carbohydrate that could generally meet the energy requirement of wood-feeding insects. However, to gain access to this insects have to circumvent the lignin barrier (Geib et al., 2008; Kirk & Highley, 1973). They achieve this by either feeding on decayed wood with freely available carbohydrates, or by utilising the gut microbial consortium to bypass the lignin barrier to gain access to hemicellulose and cellulose. Furthermore insects utilise a mixture of cellulolytic and hemicellulolytic enzymes originating from their gut symbionts, the insect itself, wood-degrading microbes already present on the wood, or a combination of all three sources (Brune, 2003, 2014; Geib et al., 2008; Watanabe et al., 2010).

1.7 Huhu

The huhu beetle (*Prionoplus reticularis*), a member of the longhorned beetle family (Coleoptera: Cerambycidae), is the largest endemic beetle found throughout New Zealand (Edwards, 1959; Hosking, 1978). Huhu grub larvae (Cerambycids) are xylophagous and believed to be dependent on the gut microbial community to aid in lignocellulose degradation (Reid et al., 2011). The life cycle of the huhu beetle involves holometabolism (complete metamorphosis) comprising of four stages (Edwards, 1961a)(see Figure 1.2).

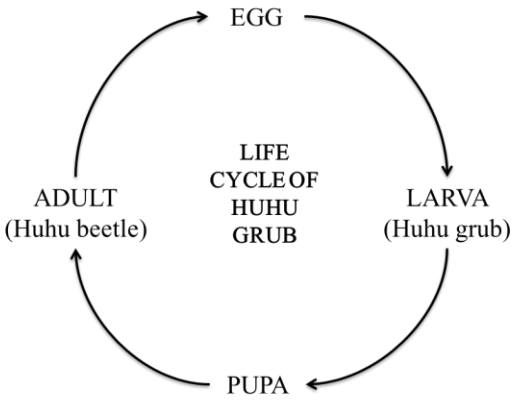

Figure 1.2: Life cycle of huhu (from Edwards, 1961a)

Traditionally, ‘huhu’ is the Māori name for the larval stage (scarabaeiform grub-like), also known as *tunga haere* or *tunga rakau*; however, currently huhu is often used as the name for all life stages of the beetle. Generally, huhu grubs play a beneficial ecological role by assisting in decomposition and nutrient cycling. However, huhu beetles can lay eggs on freshly felled timber, and hence are detrimental to the timber export industry (Rogers et al., 2002). Mature huhu beetles have a life expectancy of two weeks, during which they abstain from consuming any food. During this period they mate and the female beetles oviposit approximately 250-300 eggs onto crevices of cut stumps, dead trees, fallen logs and the dead parts of live trees. The eggs hatch after an incubation period of 16-25 days, followed by a larval stage that extends over 2-3 years in the natural environment (Edwards, 1961a; Hosking, 1978; Morgan, 1960). Fully grown huhu grubs can reach 70 mm in length before they enter an overwintering, non-feeding final instar, followed by a 25 day pupation period to emerge as mature flying huhu beetles (Rogers et al., 2002).

In nature throughout the larval stage huhu grubs solely feed on wood, leaving a trace of sheared wood and egested faecal frass (Edwards, 1959, 1961a; Hosking, 1978). Generally, during this process they tunnel longitudinally (along the grain) through fresh wood and arbitrarily in rotten wood (Edwards, 1959). Huhu grubs have previously been successfully reared in a laboratory environment on a well-supplemented artificial pine sawdust diet resulting in a shortened larval stage of 250 days (Rogers et al., 2002). Huhu grub larvae are known to feed on a broad range of dead trees. This adaptability to various diets (Edwards, 1959; Rogers et al., 2002) makes them an ideal candidate for dietary-manipulation studies that could help in identifying an individual or group of microorganisms potentially involved in lignocellulose degradation.

1.8 Diet-induced gut microbiome alterations

Considerable research has been undertaken on diet-induced gut microbiome alterations in mice and humans (David et al., 2014; Graf et al., 2015; Ji et al., 2014; Wu et al., 2011). Shifts in the gut microbiota were seen to be representative of variations in diet (Ji et al., 2014). Consistent alterations were reported in the microbial community structure of humans, upon short-term consumption of a diet consisting exclusively of plant or animal products (David et al., 2014). The

animal-based diets reduced levels of Firmicutes responsible for metabolizing dietary plant polysaccharides and resulted in a plethora of bile-tolerant microbes (David et al., 2014). Furthermore, a long-term single substrate diet is known to guide the structure and activity of the gut microbial community, leading it to a reduction in inter-individual differences (David et al., 2014; Graf et al., 2015; Wu et al., 2011). Recently, the gut bacterial composition of the cockroach *Periplaneta americana* reared on sugarcane bagasse (pulp residue) and crystalline cellulose was compared with that caught in the wild (Bertino-Grimaldi et al., 2013). The study was conducted using sequence analysis of 16S rRNA clone libraries and the results suggested that the gut bacterial composition was modulated by the diet composition. Furthermore, an abundance of Firmicutes in the cockroach guts on both diets was suggested to be indicative of their involvement in lignocellulose digestion in cockroach guts. These studies show the significance of diet-alteration on the gut community. Therefore, a carefully designed experimental model could provide information about any potential involvement of the huhu grub gut microbial consortium in lignocellulose degradation.

1.9 Significance of high-throughput sequencing

Traditionally, culture-dependent and culture-independent methodologies have been utilised to explore the composition of a microbial community. Culture-dependent studies, while allowing direct observations of microorganisms present in a community, often produce biased results depending upon the experimental conditions and techniques used. Additionally, the majority of microorganisms present in a community cannot be isolated using culture-dependent techniques (Béjà, 2004). Presently, several well established and advanced culture-independent techniques form the basis for community analysis. Among these techniques, and of relevance to this study, is high-throughput sequencing. High-throughput sequencing techniques, like Ion Torrent sequencing, when coupled with the improving capability for data analysis forms an ideal combination to explore the microbial community in an environment. The datasets obtained through high-throughput sequencing can be compared, and shared through several public databases including GenBank (Benson et al., 2005), the Ribosomal Database Project (Cole et al., 2005) and Unite (Abarenkov et al., 2010). Toolsets provided through Open-source software such as Mothur (Schloss et al., 2009) and

QIIME (Caporaso et al., 2010) helps to process and visualise the large amount of data produced through high-throughput sequencing.

1.10 Objectives

Huhu grub larvae have been previously suggested to host a gut microbiome that potentially aids in degrading wood (Reid et al., 2011). However, the information available on the gut microbiome of the huhu grub is limited, and is based on huhu grubs that fed on partially degraded wood in a nutrient rich environment (Rogers et al., 2002). In order to explore the huhu grub microbial diversity on defined cellulose and lignocellulose diets, cotton and non-degraded pine blocks were utilised. Since the later stages of this study involves interchanging the defined diets, it was essential to ensure that the huhu grubs can be reared healthily on these diets. Therefore, the aim of the first chapter (Chapter 2) is to ensure that the huhu grubs can establish themselves on these provided diets and complete its lifecycle. We hypothesise that a difference in the huhu grub gut bacterial and fungal community will be observed according to the diet provided.

In previous experiments a difference in the gut fungal community was found between cellulose and lignocellulose reared huhu grubs. However, the resolution of automated ribosomal intergenic spacer analysis (ARISA) was not enough to distinguish the specific members of the fungal community. In the second chapter (Chapter 3), our goal was to utilise Ion Torrent sequencing and analysis techniques to evaluate the huhu grub gut fungal community diversity in huhu grubs reared on cellulose and lignocellulose diets. We also aimed to switch the cellulose fed grubs to lignocellulose, and vice versa, to understand the implications of a dietary shift on the gut fungal community. We hypothesise that we would be able to see a significant difference in the abundant gut fungal community members between the cellulose and lignocellulose reared huhu grubs. We presume that since the huhu grubs are collected from their natural environment (degraded wood), their transfer to cotton would create a reduction in the functional diversity than that observed in lignocellulose reared grubs. Similarly when the diets are interchanged we expect to see a community shift with the gut fungal community of lignocellulose reared grubs losing its specialist members (that might be involved in breaking down lignin and hemicellulose). However, when the cellulose reared grubs are transferred to pine we might not see

an equivalent gain in diversity since some of the specialist members selected for lignocellulose degradation might have been eliminated from the gut and could not therefore re-establish.

The effects of the dietary shifts on the bacterial community were also examined (Chapter 4). We hypothesised that changes in the gut bacterial communities of huhu grubs reared on cellulose and lignocellulose diets will be seen. The preliminary data from the chapter 2 failed to see a difference. However we expect to observe a shift in the gut bacterial community when the diets are interchanged as the bacterial community adapts to a change in diet.

Results from chapters 3 and 4 indicated the presence of potentially lignocellulolytic bacterial and fungal species inside the huhu grub guts. The aim of chapter 5 was to isolate possible lignocellulolytic microbes and bioprospect for their lignocellulose degrading capabilities with a specific focus on lignin degrading enzymes.

1.11 Summary

Overall, compared to other wood-feeding insects, very little is known about the gut microbial diversity associated with the xylophagous cerambycid huhu grub, fed on non-degraded cellulose and lignocellulose diets. We aim to address this knowledge gap. We sought to establish an experimental model for growing huhu grubs on defined cellulose and lignocellulose diets, and comparing the gut fungal and bacterial community by utilising huhu grub frass (excretia) as a proxy for gut contents. We interchanged these defined diets to understand the impact of dietary switch on the huhu grub gut community. Finally, bioprospecting studies were performed to isolate and identify microbes and enzymes of potential economic importance (Figure 1.3).

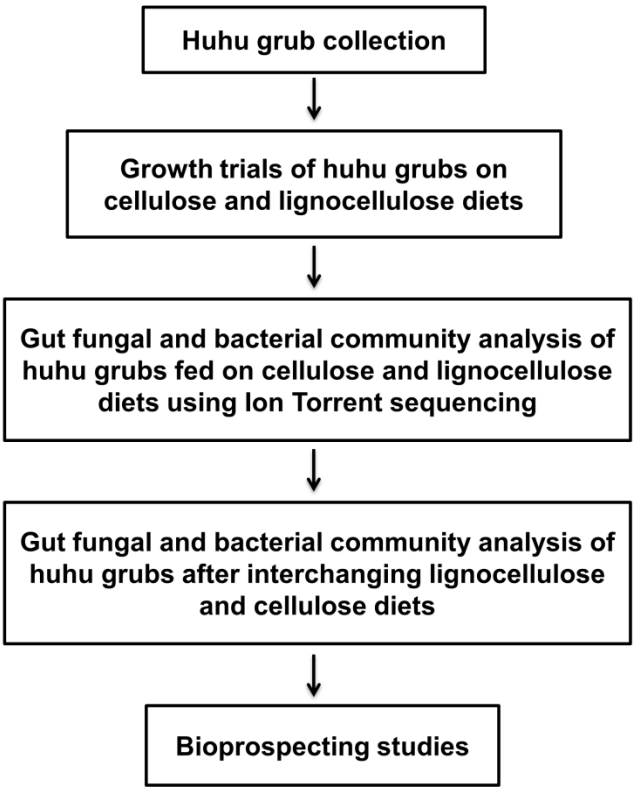

Figure 1.3: Work flow summary

Chapter Two

The effects of defined diets on huhu grubs and their gut microbiome

Abstract

In this study a laboratory rearing method for huhu grubs, *Prionoplus reticularis* (Cerambycidae) on defined cellulose (cotton) and lignocellulose (pine) diets has been developed. Huhu grubs were shown to both gain weight and eventually undergo metamorphosis on the diets, both indications of successful use of the substrates for healthy growth. Since insects typically rely on their gut microbiome to assist in nutrient assimilation this rearing method was used to study the gut microbiome of *Prionoplus reticularis*, using the frass produced as a proxy for the gut. Patterns of fungal community diversity in frass suggest that their distribution is significantly influenced by diet, demonstrating the potential of the experimental model for studying shifts in the gut microbiome induced by diet change. This approach could prove essential in identifying microorganisms, or consortia, involved in the breakdown of lignocellulose.

2.1 Introduction

For this study it was important to minimise the variables experienced by the huhu grubs during their lifecycle in order to study variation in the gut microbial community induced by lignocellulose or cellulose laboratory diets. To establish a dietary model experiment, it was necessary to ensure that the grubs can establish and complete their life cycle on the selected diets while producing enough study material (frass – insect excreta). Huhu grub have previously been successfully reared in a laboratory environment on a well supplemented artificial pine sawdust diet (Rogers et al., 2002). There are no published studies of huhu grubs completing their life cycle on a cellulose or lignocellulose diet in a laboratory environment. We therefore examined the feasibility of rearing huhu grubs on cellulose and lignocellulose diets, by using cotton and solid pine blocks. To this end we analysed the microbial content of the frass of huhu grubs grown on lignocellulose or cellulose using automated ribosomal intergenic spacer analysis (ARISA), as a proxy of the effect of specific diets on the gut microbial

community. We hypothesise that differences in the immediate availability of polymer carbohydrates in the specific diets, lignocellulose being a comparatively more complex substrate than cellulose, will have an impact on the huhu grub gut microbial community.

Results from this study provide preliminary insight about the growth requirements of huhu grubs on defined diets, along with any diet-driven differences in bacterial and fungal communities.

2.2 Materials and methods

2.2.1 Sampling site and grub collection

The huhu grubs used in this study (Table 2.1 and Table 2.2) were collected from two different kahikatea logs from Claudelands Reserve (6.5 ha) in the summers of 2012 (batch 1) and 2013 (batch 2) (Figure 2.1). Claudelands Reserve is located in the Hamilton Ecological District on a floodplain bisected by the Waikato River, and receives a mean annual rainfall of 1200 mm (Singleton, 1991). The average air temperature is 13 °C throughout the year, with a winter average of 9 °C and a summer average of 18 °C (Singleton, 1991). The vegetation mostly consists of native trees, namely kahikatea, tawa, pukatea, titoki, rewarewa and mahoe (Gudex, 1955). The prevailing westerly winds, along with a combination of low rainfall, high temperatures and a low water table, results in considerable wilting and felling of vegetation.

Figure 2.1: A map of Claudelands Reserve
The red spot was the site where batch 1 huhu grubs were collected and the yellow spot where batch 2 grubs were collected.

Fallen logs, along with tree stumps and dead parts of live trees, make an ideal location to search for huhu grubs (Figure 2.2) (Edwards, 1959). The decayed state of the wood and presence of holes running throughout the log were used as preliminary criteria for identifying huhu infested logs. Sections of chosen logs were split longitudinally (along the grain of the wood) using an axe, and were further examined for the presence of tunnels or fresh huhu grub frass, both of which are indicative of the presence of huhu grubs deeper in the wood.

Figure 2.2: Huhu grub inside a log of wood in field

When the huhu grubs were found their length, weight and condition were examined to determine their suitability for the study. Extremely small or injured huhu grubs were rejected, while healthy, average sized (3-5 cm) huhu grubs were carefully collected using forceps and placed individually into plastic containers for transportation to the laboratory. The use of separate containers was necessary to prevent damage through biting (Rogers et al., 2002). Whenever possible, an effort was made to collect enough grubs from the same section of the log, at the same sampling time to reduce variability between grubs.

2.2.2 Preliminary trial

Fourteen huhu grubs were used for preliminary studies to examine the viability of the experimental model. Initial research focused on ensuring survivability and weight gain of the grubs, while maintaining adequate frass production. This was necessary as frass is used as the study material (gut proxy) to understand variations in the gut microbiota of individual grubs on different dietary conditions.

The grubs were transferred in equal numbers to sterile dry pine blocks (PP: Batch 1 huhu grubs (preliminary trial) on pine block) and test tubes filled with cotton (PC : Batch 1 huhu grubs (preliminary trial) on cotton).

Grubs were weighed and their length measured every week. Visual inspection checked their health (hydrated, dehydrated, motility, tunnel formations, and weight loss), frass production and condition of the frass (pellet form or liquid state). Grubs grown on the cotton diet were provided with a fresh batch of cotton at least once a month, or earlier if necessary. The grubs reared on pine were transferred to fresh blocks when collection of frass became difficult due to the intricate tunnels drilled by the grubs. While extracting grubs from pine blocks, extra caution was taken not to injure the grubs whilst slicing through the wood. Frass was collected daily, aseptically inside a sterile laminar flow hood using sterile forceps, sieved to separate the frass from wood or cotton debris, and frozen in microcentrifuge tubes (Figure 2.3) at -20 °C until further use. Frass collected before weight gain (from initial weight during collection) was achieved was not used for this study, as this early weight gain was used as an indication of habituation of the grub to the specific diet. The use of frass as a proxy for the gut ensured a non-invasive collection of gut material for DNA extraction.

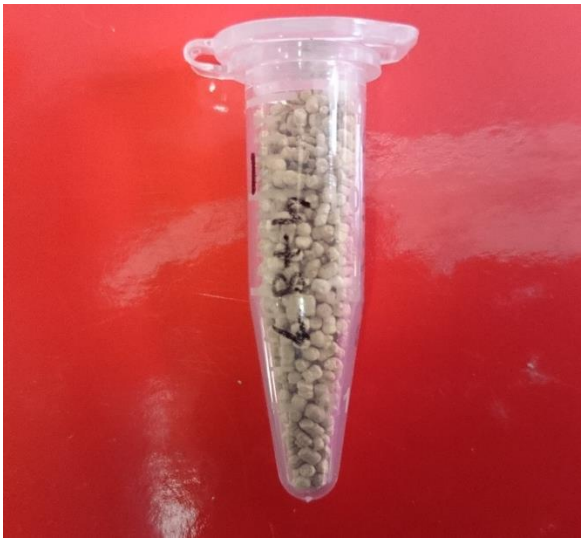

Figure 2.3: Frass collected from pine-reared huhu grub

2.2.3 Modified trial

The second batch of grubs (batch 2) were grown in adapted conditions namely 'Main trial' (MT), remodelled and optimised based on observations from the

preliminary trial. Adjustments involved transferring grubs to sterile, water logged pine blocks, accomplished by submerging the blocks in sterile autoclaved water for a week, maintaining the humidity in the containers storing the pine blocks and the cotton tubes, and adjusting the presentation of the cotton diet to structurally replicate the natural habitat of the huhu grub. The ten huhu grubs used were obtained from a single log of kahikatea from Claudelands Reserve. The collected grubs were kept in individual petridishes for 24 hours, away from light, to ensure previously consumed food was excreted.

The huhu grubs were weighed, and their lengths measured, and were then divided into two groups to be reared individually on separate defined diets. Five grubs were introduced to the lignocellulose diet (MP: Batch 2 huhu grubs (main trial) on pine block) which involved feeding on sterile untreated solid pine blocks with a hole drilled in one end into which the grub was inserted (Figure 2.4). Prior to the introduction of the grubs, pine blocks were immersed in autoclaved water in a sterile container for a couple of days and wiped clean using 100% ethanol inside the laminar flow hood.

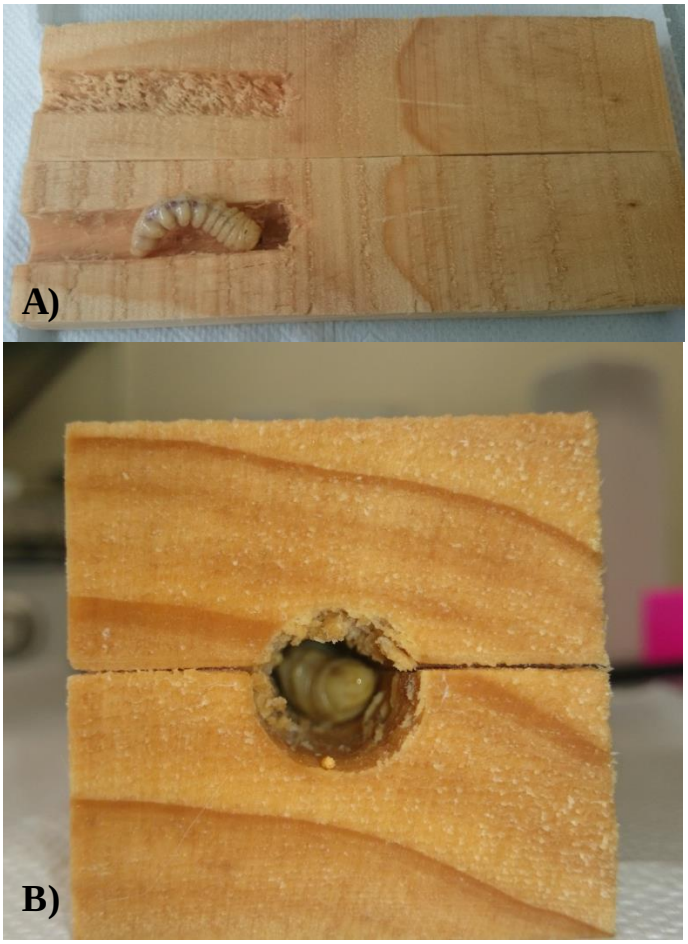

Figure 2.4: Pine-block dietary set up of huhu grub
A) Open view of huhu grub inside the tunnel B) Cross-sectional view of the dietary set up

The remaining five grubs were reared on the cellulose diet, in autoclaved glass tubes containing water-soaked compressed cotton wool (MC: Batch 2 huhu grubs (main trial) on cotton). The grubs were provided with a cylindrical hole in the open end of glass tube (Figure 2.5) and the entire tube was covered with aluminium foil before being placed in a sealed plastic container. The collection and storage of frass were as described in preliminary trial.

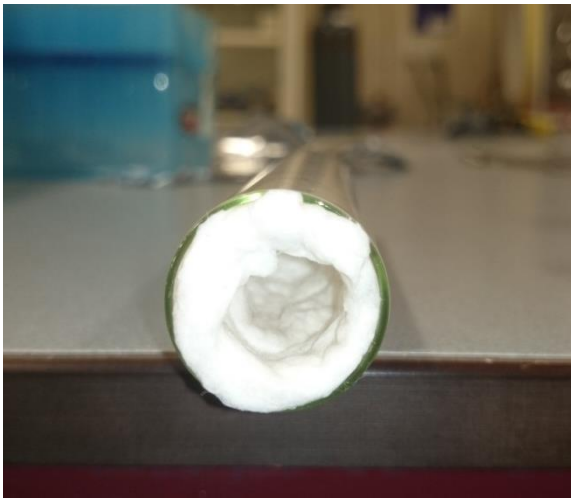

Figure 2.5: Entrance hole in main trial for cotton-reared grubs

2.2.4 DNA extraction and quantification

DNA extraction from frass was achieved using the modified protocol from Barrett et al., (2006). Using sterile forceps, 0.2 g of frass sample was weighed and transferred to individual microcentrifuge tubes. A mixture of 270 µL phosphate buffer (100 mM NaH_2PO4), 270 µL SDS lysis buffer (500 mM Tris pH 8.0, 100 mM NaCl and 10% SDS), and 180 µL CTAB extraction buffer (100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, 2% CTAB, 1% PVP, 0.4% BME) were added to each sample. The frass samples were then mixed thoroughly for five minutes using a Vortex Genie 2 with a 24-tube vortex adapter (Mo Bio Laboratories Inc., Carlsbad, CA, USA). Samples were then simultaneously shaken at 300 rpm and incubated at 55 °C for 40 minutes. Subsequently, samples were centrifuged at 16000 x g for 1 minute followed by the addition of 350 µL chloroform:isoamyl alcohol (24:1) and 35 µL 10 M ammonium acetate, The samples were vortexed and centrifuged at 16000 x g for 15 minutes using a refrigerated centrifuge at 14 °C. The aqueous phase of each sample was transferred to a fresh microcentrifuge tube followed by the addition of an equal amount of isopropyl alcohol. The microcentrifuge tube was inverted a few times to ensure a homogeneous mixture and incubated at -20 °C for two hours. The samples were then centrifuged at 16000 x g for 20 minutes. The resultant pellet was washed with 1 ml of cold 100% ethanol. The ethanol was then discarded and the samples were air dried inside a sterile biological cabinet. The DNA was then resuspended in 20 µL of sterile TE buffer (10 mM Tris-HCl, 0.5 mM EDTA) and stored at -20 °C until further use. Negative controls, with no input of frass samples were processed along with the samples as

described above. DNA extracts were quantified using Qubit dsDNA HS assay kit and Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and checked for quality using NanoDrop ND-1000 (NanoDrop technologies, Montchanin. DE).

2.2.5 Automated Ribosomal Intergenic Spacer Analysis (ARISA)

Polymerase chain reaction (PCR) targeting the bacterial intergenic spacer region between 16S and 23S rRNA genes and the fungal intergenic spacer region containing the two internal transcribed spacers (ITS) and the 5.8S rRNA gene was performed on the DNA samples (obtained from frass) from batch 2 huhu grubs (including the negative controls) (Tiao et al., 2012). The amplifications were performed using primer set ITSF (5' GCCAAGGCATCCACC-3') and ITSR eub-Hex (5' GCCAAGGCATCCACC-3') (Integrated DNA Technologies, Auckland, New Zealand) (Cardinale et al., 2004) for Bacteria and ITS1FHEX (5' CTTGGTCATTTAGAGGAAGTAA-3') (Gardes & Bruns, 1993) and ITS4 (5' TCCTCCGCTTATTGATATGC-3') (White et al., 1990) for fungi. PCR reactions were completed for each extraction in triplicate to reduce stochastic PCR bias (Wintzingerode et al., 2006). Each 30 µL reaction mixture contained 1X PCR buffer, 2.5 mM $MgCl_2$, 0.25 µM primers, 0.2 mM dNTPs (Life Technologies) and 2 µL of template DNA. Thermal cycling was performed in accordance to Cardinale et al., (2004) for bacteria, and Kennedy et al., (2006) for fungi using a Bio-Rad DNA Engine Peltier Thermal Cycler 200 (Bio-Rad, Hercules, CA, USA).

The PCR products were verified using a 1% agarose gel to be of the expected size. Amplicons from the triplicates of each sample were pooled and diluted 1:10 in de-ionized water. A mixture containing 2 µL of each fluorescently labelled PCR diluent, 0.13 µL of Liz-1200 internal size standard (Applied Biosystems, New York, USA) and 8 µL of HiDi formamide (Applied Biosystems) was heat shocked at 95 °C for ten minutes and cooled to 4 °C for 2 minutes, prior to being analysed on an ABI 3130 Genetic Analyzer (Applied Biosystems) at the University of Waikato DNA Sequencing Facility.

2.2.6 ARISA data analyses

ARISA fingerprints were processed using a modified informatics pipeline from Abdo et al., (2006). Resulting chromatograms were primarily analysed using Peak Scanner Software (Applied Biosystems). Peaks between 50 and 1200 base pairs

exceeding 200 fluorescence units were accepted as true peaks. The total number of true peaks was considered to be representative of bacterial or fungal taxon richness for each sample. Peaks within five base pairs of one another in a pairwise comparison between fingerprints were binned together. The resulting data matrix was analysed using a combination of Primer 6 (Clarke & Gorley, 2006) and R for statistical analysis (Team, 2013). In Primer 6, beta diversity was investigated using a resemblance matrix created based on the Bray-Curtis community similarities and dendrograms were created. Analysis of similarities (ANOSIM) and Tukey's honest significance test were used to investigate microbial heterogeneity between and within the huhu grubs reared on different diets.

2.3 Results

2.3.1 Preliminary studies

After a week on the preliminary trial set up (Table 2.1), six of the fourteen huhu grubs (batch 1) collected from Claudelands Reserve died. Among the seven grubs provided with a cotton diet four perished, a mortality rate of 57.1%. However only two of the seven grubs introduced to the pine diet failed to survive, a mortality rate of 28.6%.

It was noticed that there was dehydration (visual observations of dryness and colour), low motility, minimal frass production and a lack of weight gain on the pine grown huhu grubs in the first week. Upon observation, it was concluded that the pine blocks were extremely dry, and therefore were sprayed occasionally with autoclaved milliQ water. This modification had a positive effect on frass production and motility of huhu grubs, however two grubs A8P and A10P experienced weight loss and failed to survive after a month of transfer to the new dietary regime. Visually it was perceived that hydration levels of all pine-reared huhu grubs improved considerably after addition of water, however all but one of the pine reared grubs (A6P) appeared slightly dehydrated in comparison to observations made on the first day of transfer to pine. Adequate frass production and appreciable weight gain were observed for three of the pine reared grubs namely A6P, A7P, A9P (Table 2.2) in the preliminary trial.

Table 2.1: Preliminary details of survival and weight gain of batch 1 huhu grubs

Grub	Diet	Initial length(cm)	Initial weight(g)	Survival after one week	Weight gain after a month
A1C	PC	2.5	0.69	D	-
A2C	PC	3.4	1.4	A	+
A3C	PC	2.6	0.86	D	-
A4C	PC	3.4	1.52	A	+
A5C	PC	3.1	1.12	D	-
A12C	PC	3.8	1.86	A	+
A13C	PC	3.3	1.48	D	-
A6P	PP	3.7	1.67	A	+
A7P	PP	3.5	1.48	A	+
A8P	PP	2.8	0.84	A	-
A9P	PP	3.6	1.77	A	+
A10P	PP	3.3	1.42	A	-
A11P	PP	2.7	0.9	D	-
A14P	PP	3.2	1.24	D	-

PC: Preliminary trial on cotton diet
PP: Preliminary trial on pine diet
D indicates that the grub died and A indicates that the grub was alive after two weeks on specified diet
- indicates a weight loss or an absence of weight gain from the initial weight and + indicates a positive weight gain

In the first week of transfer, cotton-reared grubs did not record a weight gain. However, frass production was comparatively higher than from pine grown grubs. In contrast to pine reared grubs, cotton grown grubs did not present any signs of immediate dehydration. Two weeks after initial transfer there was a noticeable drop (visual observation) in the consumption of cotton followed by indications of dehydration (Figure 2.6). It appeared that the grubs were only able to consume the top loosely arranged part of the cotton and were less successful in tunnelling into the compressed cotton at the bottom of the test tube (Figure 2.6), and had to be provided with fresh cotton tubes every week. Sufficient frass production and acceptable weight gain were observed for all of the surviving cotton-reared huhu grubs (Table 2.2), and was considered as an indication that grubs were adapted to their new diet.

Figure 2.6: Comparison of cotton dietary set ups
A) Preliminary trial on cotton (PC) with visual evidence of dehydration and lack of tunnelling B) Main trial on cotton (MC) showing a healthy grub, excessive frass production, tunnelling and evidence of moulting.

All of the grubs weighing less than one gram failed to adapt to the defined diets, along with four other grubs which either failed to recover from dehydration (Table 2.1) or the initial change in habitat (i.e. from wild to the laboratory diet). These factors were taken into consideration, and in the main trial the selection of grubs from the wild was modified to ensure maximum survival and adaptation of the grubs.

2.3.2 Main trial

Of the ten grubs (batch 2) collected from a single kahikatea log in Claudelands Reserve in February 2013, two grubs died before being transferred to defined diets. The remaining eight grubs were transferred to the improved experimental dietary set up (Section 2.2.3) and registered superior weight gain (Figure 2.7) than seen in the preliminary trial (Table 2.2).

Table 2.2: Growth of huhu grubs on different dietary regimes

Grub	Diet	Initial length (cm)	Initial weight (g)	Weight gain/ month (g)	Mean weight gain/ month ±SD	Visual observation	Frass production
A6P	PP	3.7	1.67	0.06		Healthy motile	L
A7P	PP	3.5	1.48	0.02	0.03± 0.02	Dehydrated, less motile	VL
A9P	PP	3.6	1.77	0.01		Dehydrated, less motile	VL
A2C	PC	3.4	1.4	0.24		Healthy motile	L
A4C	PC	3.4	1.52	0.21	0.24± 0.02	Healthy less motile	L
A12C	PC	3.8	1.86	0.26		Healthy motile	L
06P	MP	3.6	1.6	0.18		Healthy motile	L
07P	MP	3.8	1.82	0.1	0.16± 0.04	Slightly dehydrated, motile	L
09P	MP	3.6	1.61	0.17		Healthy motile	H
10P	MP	3.9	1.84	0.19		Health motile	H
01C	MC	3.5	1.42	0.63		Healthy motile	L
02C	MC	3.5	1.48	0.49	0.62± 0.12	Healthy motile	L
03C	MC	3.4	1.4	0.78		Healthy motile	H
04C	MC	3.7	1.6	0.59		Healthy motile	H

PC: Batch 1 huhu grubs (preliminary trial) on cotton
PP: Batch 1 huhu grubs (preliminary trial) on pine block
MC: Batch 2 huhu grubs (main trial) on cotton
MP: Batch 2 huhu grubs (main trial) on pine block
VL: very low frass production (frass present once a week), L: low frass production (frass present at least twice a week but not every day), H: high frass production (frass present every day)

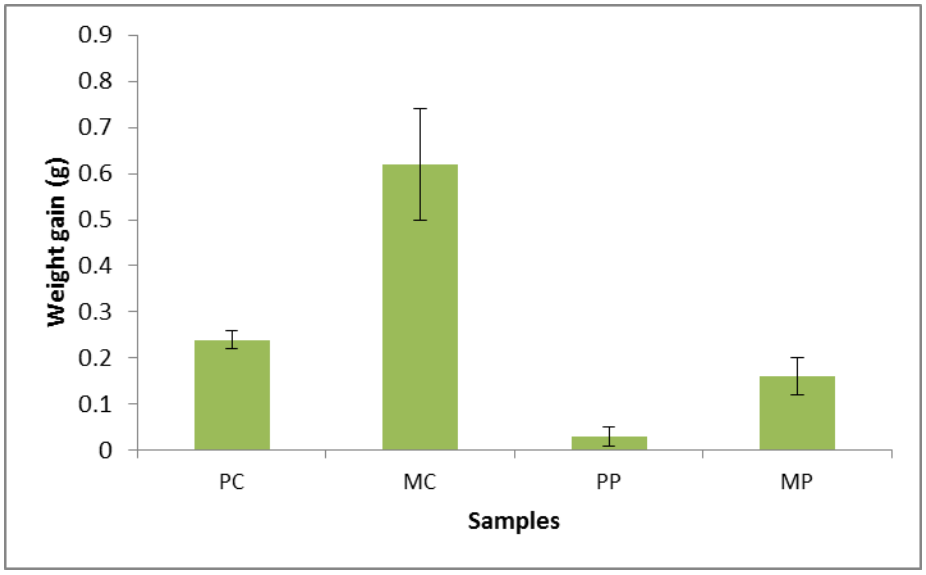

Figure 2.7: Mean huhu grub weight gain per month, with error bars showing standard deviations.
PC: Batch 1 huhu grubs (preliminary trial) on cotton
PP: Batch 1 huhu grubs (preliminary trial) on pine block
MC: Batch 2 huhu grubs (main trial) on cotton
MP: Batch 2 huhu grubs (main trial) on pine block

Another important indicator of grub health is its ability to complete its lifecycle, including pupation, on a given diet. Growth of grubs on a cotton diet indicated a possible shortening of the larval stage compared to the pine diet. One of the huhu grub larvae (Supplementary Figure 7.1) grown on cotton diet in the preliminary trial (batch 1) (Table 2.3), and three in the main trial (batch 2) metamorphosed into huhu beetles (Figure 2.8) after a time period ranging from seven to nine months (Table 2.3). The pupation period of larvae ranged from 20 to 25 days (Table 2.3). The grubs A2C (PC) and 02C (MC) entered a dormant state with minimal frass production, after approximately four and seven months of initial transfer, and failed to establish themselves on a fresh batch of cotton diet. After 4 months the established pine reared grubs from the preliminary experimental set up entered into extended periods of diapause with minimal feeding, lack of tunnelling, and even loss of weight rendering them ineffective for further studies. Furthermore these grubs failed to survive after approximately six to seven months of entering into the diapause (Table 2.3). Two of the four pine-reared grubs (09P and 10P) from the main trial metamorphosed after 480 and 436 days (Figure 2.8), resulting in a metamorphosis rate of 50%. Prior to the metamorphosis these grubs underwent a diapause state for ten (09P) and eleven (10P) months. The three grubs on preliminary trial survived and adapted to the pine diet for a period

ranging from eight to eleven months before entering dormancy followed by mortality.

Table 2.3: The life cycle of huhu grubs on different dietary regimes in the laboratory environment

Name	Dietary trials	Time on diet (Days)	Physical state	Pupation period (Days)
A2C	PC	128	Dead	-
A4C	PC	272	Metamorphosed	25
A12C	PC	281	Dead	-
01C	MC	224	Metamorphosed	25
02C	MC	219	Dead	21
03C	MC	229	Metamorphosed	23
04C	MC	241	Metamorphosed	23
A6P	PP	328	Dead	-
A7P	PP	320	Dead	-
A9P	PP	308	Dead	-
06P	MP	402	Dead	-
07P	MP	386	Dead	-
09P	MP	480	Metamorphosed	Uncertain
10P	MP	436	Metamorphosed	Uncertain

PC: Preliminary trial involving batch 1 huhu grubs on cotton
PP: Preliminary trial involving batch 1 huhu grubs on pine block
MC: Main trial involving batch 2 huhu grubs on cotton
MP: Main trial involving batch 2 huhu grubs on pine block

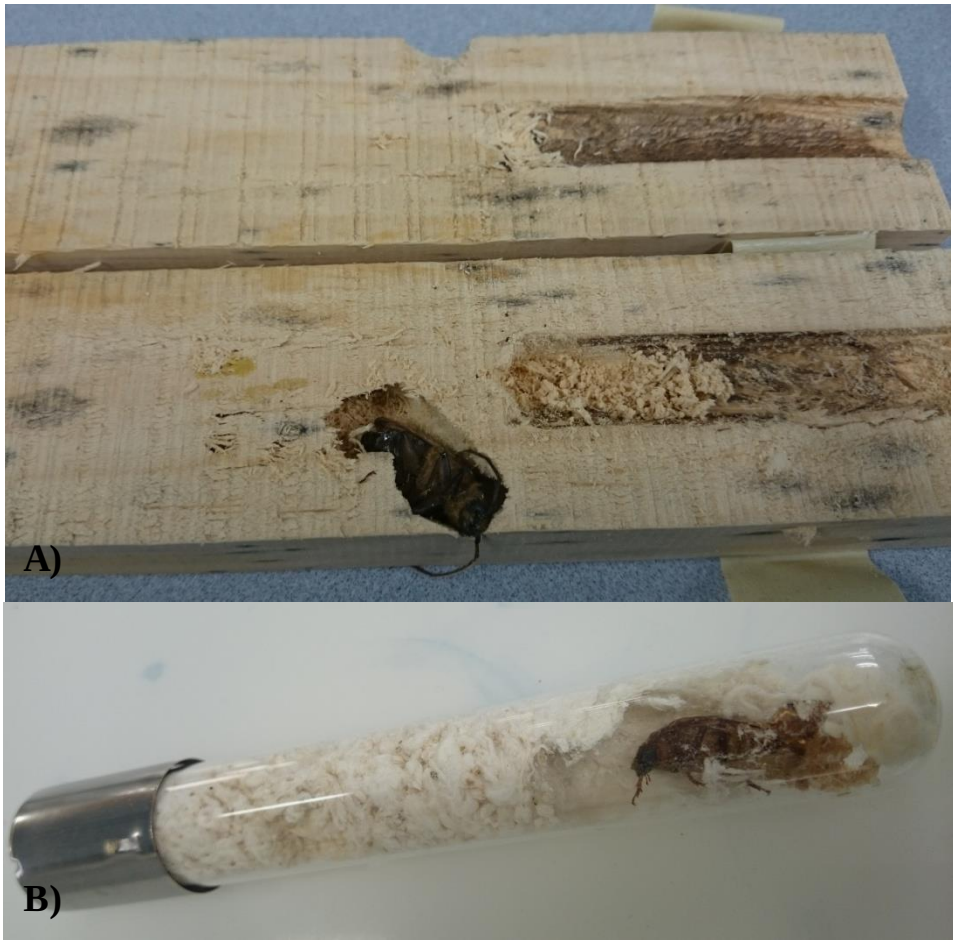

Figure 2.8: Metamorphosed huhu beetles on main trial.
A) Huhu beetle on pine-block B) Huhu beetle on cotton diet.

2.3.3 ARISA data analyses

Beta diversity comparisons were visualized using dendrograms for fungal (Figure 2.9) and bacterial communities (Figure 2.10) from eight huhu grubs (batch 2) grown on pine and cotton diets, and the number of ARISA fragment lengths (AFLs) was used as a proxy for alpha diversity. Bacterial communities were identified to be moderately different between individual grubs. Analysis of similarity (ANOSIM) $R = 0.219$, $P = 8.6\%$, with no specific grouping according to diet. Tukey's Honest Significance test did not indicate any significant differences between *Bacteria* from the huhu grubs grown on different diets.

Fungal communities were dissimilar between pine and cotton diets, ANOSIM $R = 1$, $P = 0.03$, indicating perfect separation. However pine reared grubs showed a significantly increased fungal diversity (7-16 AFLs) when compared with cotton reared grubs (2-4 AFLs) according to Tukey Honest significant test (P=0.003).

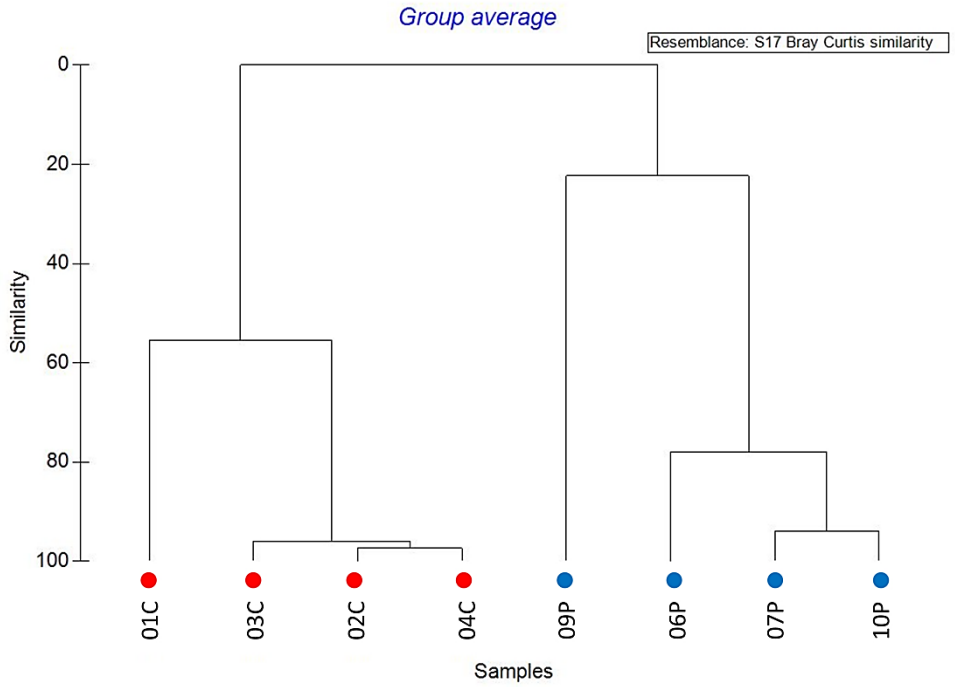

Figure 2.9: Fungal community structure from the frass of huhu grubs grown on cotton and pine diets based on Bray-Curtis Similarity

- Huhu grubs reared on cotton
- Huhu grubs reared on pine

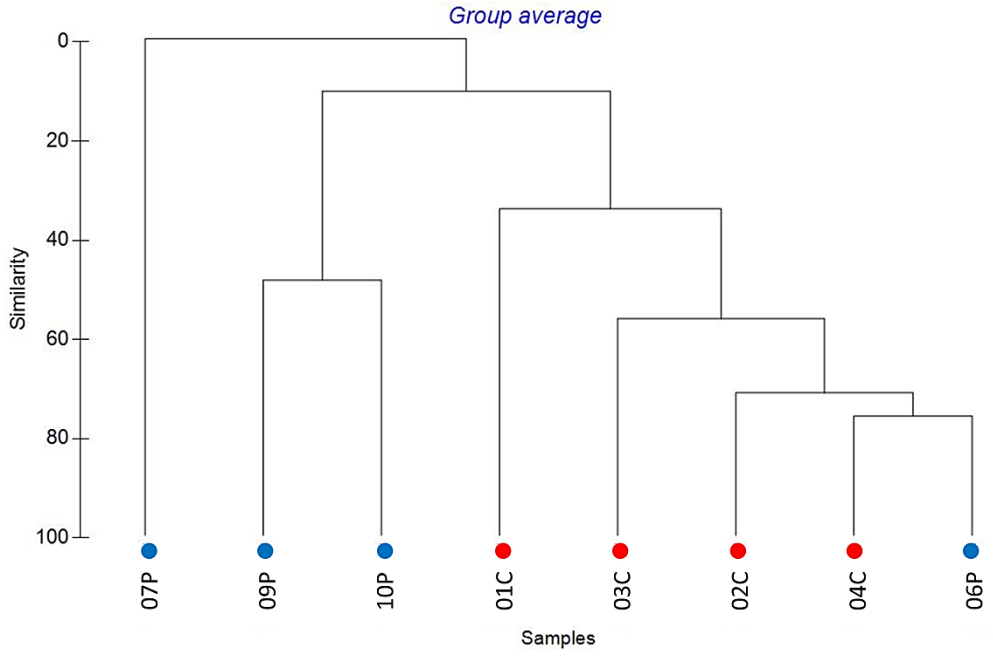

Figure 2.10: Bacterial community structure from the frass of huhu grubs grown on cotton and pine diets based on Bray-Curtis Similarity

- Huhu grubs reared on cotton
- Huhu grubs reared on pine

2.4 Discussion

Previous studies have shown that huhu grubs could feed and gain weight in a laboratory environment while provided with well supplemented artificial diets (Rogers et al., 2002). In this study we have shown the ability of huhu grubs to establish on defined diets such as cotton and non-degraded pine blocks. This work is the first to demonstrate the completion of the huhu grub lifecycle on a cellulose diet. By utilising the frass obtained from the grubs reared on these defined diets, we have successfully established a methodology to analyse the diet-influenced gut microbiome of huhu grubs. In contrast, previous studies were based on wild huhu grubs (Reid et al., 2011) involving a considerable number of variables and assumptions around the grub diet and microbiome. In this study, two dietary arrangements were trialled. Adequate amounts of frass production, significant weight gains and metamorphosis (Table 2.2) were seen for huhu grubs grown on the main trial, in comparison to the preliminary trial, clearly demonstrating the success of changes made to the delivery of the substrate set-up. The importance of moisture in maintaining the metabolism and survival of the grub was seen in the increased weight gain of pine-reared huhu grubs, in contrast to the weight loss seen in the preliminary trial before water was applied to the pine blocks.

Additionally, enhanced weight gain and metabolism were also seen in the main trial involving the cotton-reared grubs in comparison to the preliminary trial. The successful establishment of huhu grubs on the cotton-diet could be positively associated with the increased amount of available moisture facilitated by the modifications made, including an entrance hole (Figure 2.5) similar to their natural habitat (Edwards, 1959; Rogers et al., 2002)) which aided in tunnelling, and the increased shielding from light (Shintani & Ishikawa, 1997). It has also previously been shown that cerambycids are more likely to accept their diet if provided with pulverized plant materials (Gardiner 1970), therefore the compressed arrangement of the cotton itself may have played an important role in diet acceptance.

Visual observations played a crucial role in determining the physical condition of the grubs throughout the study. The absence of moisture in the diet, or the failure of grubs to adapt, could be determined from their appearance (Figure 2.6). Occasionally, the appearance of the grub prior to moulting (shedding skin) was

misunderstood as dehydration. However, grubs were likely to actively produce frass during moulting while the same is not true for dehydration. Another characteristic of an unhealthy grub was minimal frass production leading to diapause or death. In contrast, tunnelling, frass production and weight gain were suggestive of a healthy grub.

The second batch of grubs reported a noticeable increase in the survival and establishment rate (Table 2.3). Other than the modifications of the main trial, the degree of establishment could be due to the selection of grubs within an ideal weight range. The smaller grubs from the first batch failed to adapt to the laboratory diets possibly due to less optimal growth conditions, a lack of significant gut microbiome to support transition to non-degraded pine blocks or cotton diet, a lack of nutrient reserve to persist through the diet change, shock from a sudden change to a new substrate or from handling.

The metamorphosis of huhu grubs (Table 2.3) on the described laboratory diets is another affirmation of the suitability of the chosen diets and their respective arrangement. Huhu grubs are reported to be generalist feeders (Rogers et al., 2002) that have a wide host range, including a variety of New Zealand native and non-native tree species (Edwards, 1959). This could partially explain the successful rearing of huhu grubs on pine blocks. Although the pine blocks used as the dietary source were non-degraded, the huhu grubs successfully tunnelled (Supplementary Figure 7.2) through and consumed the pine blocks, leaving just an outer shell in most instances, replicating their natural behaviour (Edwards, 1961b). Huhu grubs grown on the pine diets in laboratory conditions metamorphosed in less than two years, in contrast to the 3 to 5 years estimated for the completion of the life cycle of the huhu grub on *Pinus radiata* in the natural environment (Edwards, 1961b; Hosking, 1978; Morgan, 1960). The results observed in this study are consistent with previous findings that laboratory reared grubs take less than half the time to complete their life cycle than in the environment (Rogers 2002). Though it is impossible to precisely ascertain the age of the huhu grub larvae when collected, from their weight and length it could be argued that they were in the early stages of development (Rogers et al., 2002). The completion of their lifecycle on a diet of non-degraded pine block is an indication of the presence of a gut microbiome capable of freeing polymer carbohydrates from a lignocellulose complex (Reid et

al., 2011).

Generally, grubs grown on both laboratory diets had a tendency to enter into diapause while being stressed. The pine-reared grubs registered an increased occurrence of unexplainable diapause similar to the results observed on artificial diets (Rogers, 2002). Despite going through diapause, some of the grubs grown on pine diet successfully underwent metamorphosis into beetles. However, the percentage of grubs grown on cotton undergoing metamorphosis was higher than that of pine-reared grubs. Also, the time period between initial transfers of grubs to the defined diet until metamorphosis was significantly lower for cotton-reared grubs (Table 2.3). This is possibly due to the homogeneous nature of the cellulose diet when compared to the heterogeneous nature of lignocellulose. Additionally, cellulose might be a major substrate for the huhu gut microbiome and that when cellulose is supplied in a pure form it is more readily degraded, and the products assimilated, than when typically complexed with lignin and hemicellulose.

It should also be noted here that the timing of metamorphosis is indicative of grubs being in similar stages of their lifecycle at time of collection, there is therefore a high possibility that these grubs may have had the same parental origin. Thereby reducing variability in the gut microbiome that could occur from vertical transmission from different parents (Geib & Jimenez-Gasco, 2009).

2.4.1 Patterns of bacterial and fungal richness

Fungal richness was measured as the number of peaks in ARISA profiles from the frass of each grub. In comparison to other fragment length polymorphism techniques, ARISA allows semi-quantitative comparison of taxon richness within and between samples (Brown & Fuhrman, 2005; Cardinale et al., 2004; Danovaro et al., 2006; Kovacs et al., 2010; Ranjard et al., 2001, 2003). Using this method, we detected a significant variation in fungal taxon abundance between cotton-reared and pine-reared grubs (section 2.3.3). Firstly, the fact that there is a statistically significant difference in the alpha diversity between pine-reared and cotton-reared huhu grubs according to Tukey honest significance test ($P = 0.003$) is supportive of the initial hypothesis of the active involvement of fungi in lignocellulose degradation. The diversity and the taxon richness of fungi from the cotton (cellulose) diet is lower than the pine (lignocellulose) diet, and could be indicative of the involvement of fungi in making polymer carbohydrate available

from a lignocellulose substrate. An alternative explanation is that the fungal population is restricted by the competition from the bacterial population in cotton diets, resulting in the lower abundance of fungi in cotton compared to pine reared grubs. However the dendrogram depicting the beta diversity of fungal taxonomy between the two laboratory diets clearly shows that pine-reared and cotton-reared grubs cluster separately (Figure 2.9). This illustrates that the initial hypothesis, that in the absence of external variables diet could significantly influence the gut microbiome of the host, is valid for the huhu grubs.

2.5 Conclusions

The defined diets and the main trial developed in this chapter are suitable for rearing huhu grubs for the diet influenced microbial studies necessary for this research. This is evident from the weight gain and eventual metamorphosis of the grubs. Similar and optimal sized larvae should be obtained from a smaller log, preferably small tree stumps to reduce the variability involved by vertical transfer of microbes. The patterns of fungal community suggest that the distributions are significantly influenced by diets. Therefore the experimental model could be successfully utilized to study shifts in the gut microbiome induced by diet change and could prove essential in identifying the individual microorganisms or consortia involved in the breakdown of lignocellulose. Further work using high-throughput sequencing (see chapters 3 and 4) is essential to examine the diet-influenced diversity of the bacterial and fungal communities. Additionally, isolation and characterization of microbes with lignocellulolytic activity could provide significant insight into their role in the gut community.

Chapter Three

Effects of defined diets and dietary switch on the gut fungal community composition of huhu grubs

Abstract

In this study, high-throughput sequencing was used to investigate the gut fungi of huhu grubs initially reared on cellulose (cotton) and lignocellulose (pine) diets. Additionally, the effects of a dietary switch on the huhu grub gut fungal community was explored by switching the huhu grubs reared on pine to cotton and vice versa. The gut fungal community of cellulose-reared huhu grubs was abundant in yeasts and potential cellulose degraders, and was shown to be significantly different from lignocellulose-reared huhu grubs which were more functionally diverse, with soft-rot fungi, yeasts, potential hemicellulose and cellulose degraders. Lignocellulose-reared huhu grubs shared greater community similarity than their cotton-reared counterparts, suggesting a filtering or 'bottleneck effect' by lignocellulose. The dietary switch resulted in a significant change in the abundant gut fungal community, indicating a role for fungi in wood degradation in the huhu grub gut.

3.1 Introduction

Previously, using ARISA we demonstrated a variation in fungal communities between defined dietary groups. In this study we aim to better our understanding by utilising high-throughput sequencing to provide taxonomic identification and relative abundance information of huhu grub gut fungi under different treatments. Generally, factors like pH, host-specificity (individual nature of grubs and co-evolutionary effects), life stage, host environment and diet are known to influence the gut community structure (Behar et al., 2008; Mohr & Tebbe, 2006; Santo Domingo et al., 1998; Schmitt-Wagner et al., 2003; Yun et al., 2014). Although evidence from previous studies suggests that the crucial factors that shape the gut community in insects are diet and taxonomy (Colman et al., 2012), we plan to minimise the effects of uncontrolled variables by rearing the grubs in a controlled

environment. We expect this to provide an understanding of the specific effect of diet on the gut fungal community of huhu grubs.

The study was composed of three components (Figure 3.1) and hypotheses. First, we hypothesise that the heterogeneous nature of the pine diet, and the homogeneous nature of the cotton diet, will directly impact the gut fungal community, and a distinct difference between diets will be observed. Second, huhu grubs initially grown on the cotton diet would have encountered a more dramatic substrate shift than those transferred to pine (lignocellulose). Therefore we hypothesise that huhu grubs initially transferred to cotton (cellulose) will have a less diverse community than that of the huhu grubs transferred to pine (lignocellulose). Finally by interchanging the diet we intend to understand the gut fungal community following the substrate shift. We hypothesise that when the diets are interchanged, the gut fungal community of pine-reared grubs will lose the specialist members (members that might be involved in breaking down lignin and hemicellulose). However, when cellulose reared grubs are transferred to pine we might not see an equivalent increase in diversity since some of the specialist members selected for lignocellulose degradation might have been eliminated from the gut and could not therefore re-establish.

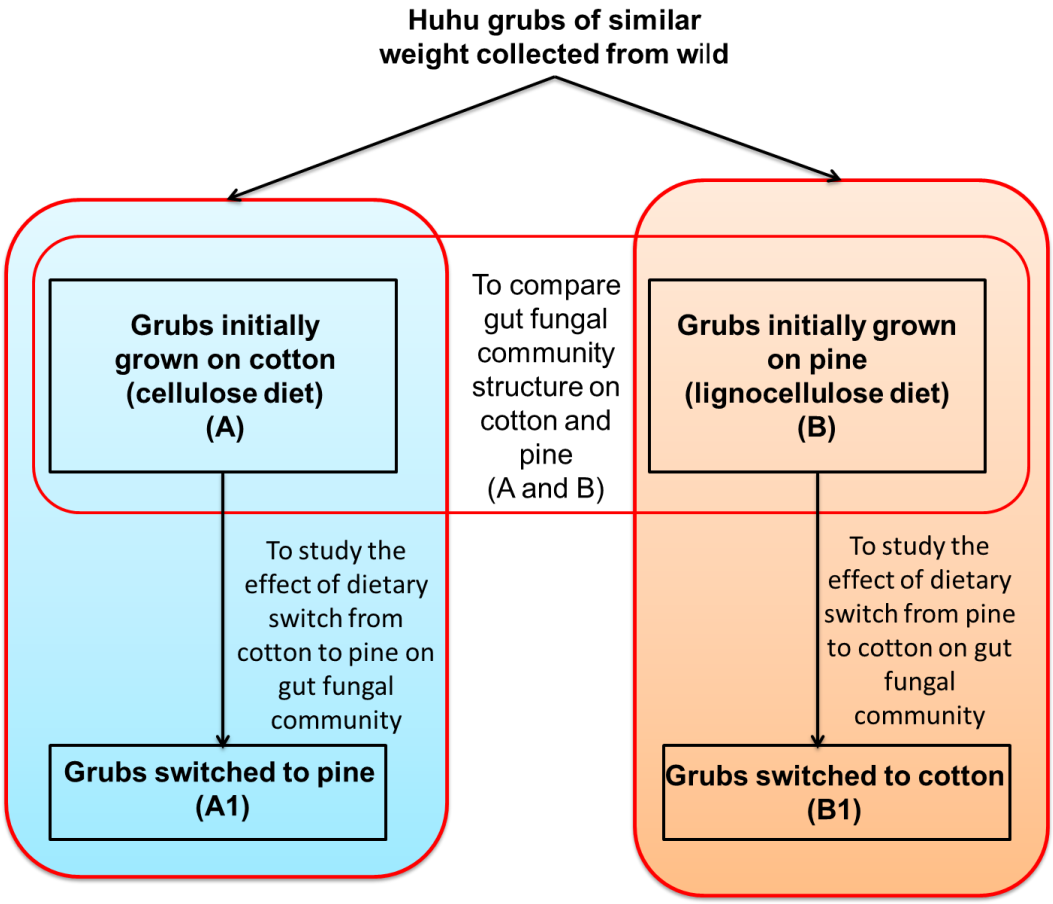

Figure 3.1: Experimental plan
Three red boxes represent the comparisons being investigated

3.2 Materials and methods

3.2.1 Sample site and collection of huhu grubs

Nine grubs (batch 3) were used in this study, grub TAC was collected from a tree stump in Hillcrest Park, and the rest were collected from a single tree stump in Claudelands Reserve, both in Hamilton, New Zealand in January 2014. The grubs were grown on either a pine or cotton diet as previously described (chapter two). Five grubs were grown on the cotton diet, 01C, 06C, 37C, 38C and TAC, collectively group 'A'. The remaining four grubs were grown on the pine diet, 20P, 24P, 41P and 42P, collectively group 'B'. Frass was aseptically collected from grubs and stored at -20 °C. Weight gain and other physical characteristics of each grub were monitored to ensure optimal growth conditions were maintained. Huhu grubs were allowed to grow and gain weight on the defined diets for about four months before a change in diet was made. The grubs initially grown on cotton were switched to pine, and grubs grown on pine were switched to cotton

(Figure 3.2). These grubs were then closely monitored to ensure that there were no adverse physical effects induced by the dietary switch. All the grubs were grown on their new diets for four months and their frass collected.

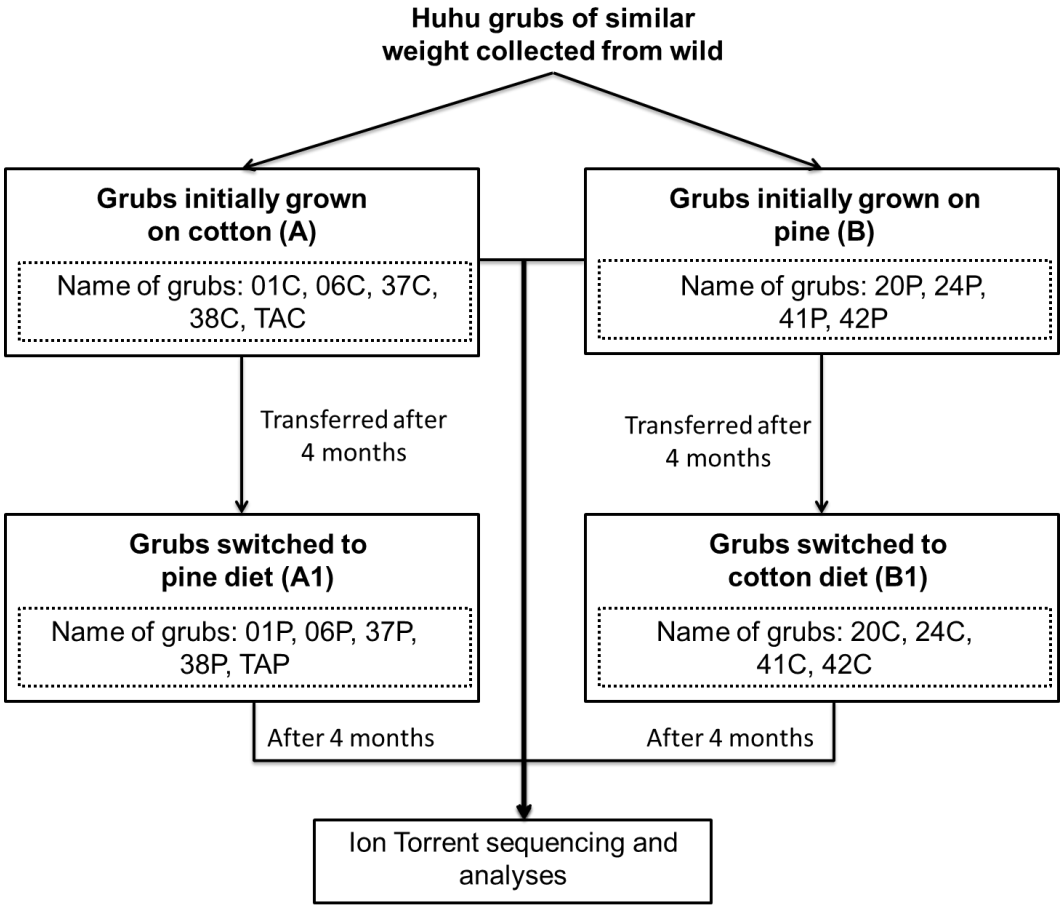

Figure 3.2: Methodology used to study the gut microbial community of huhu grubs

3.2.2 DNA extraction, PCR and Ion torrent sequencing

The frass collected during the third and fourth month after transfer to a specific diet was used as the study material for extracting DNA (chapter two). The ITS region (Figure 3.3) was utilized to identify variation in fungal community diversity and structure in huhu grub frass. PCR was performed using the adapted primer set ITS1F (5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-unique IonXpress barcode-GAT-CTTGGTCATTTAGAGGAAGTAA-3') (Integrated DNA Technologies, Auckland, New Zealand) (Gardes et al., 1993) and the reverse primer ITS 2 with label sequence (P1 sequence)(5'-CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGAT-GCTGCGTTCTTCATCGATGC-3') (White et al., 1990). Each reaction contained 1x PCR buffer (Life Technologies, New Zealand), 0.2 mM dNTPs (Roche Diagnostics, New Zealand), 0.02 mg/mL Bovine serum albumin (BSA), 0.5 µM of each primer, 1 U Platinum Taq (Life Technologies), 0.5 µM $MgCl_2$, 1 ng of DNA, and the reaction was made up to 25 µL with milli-Q H_2O. PCR reactions were completed for each extraction in triplicate to reduce stochastic PCR bias (Ihrmark et al., 2012; Polz & Cavanaugh, 1998). Thermal cycling conditions were: 94 °C for 3 min, then 30 cycles of 94 °C for 45 sec, 58 °C for 1 min, 72 °C for 90 sec, and a final extension of 72 °C for 10 min. The PCR products were verified on a 1% SYBR safe stained agarose gel for visualisation at 75 V for 30 min, with 5 µL of 1 Kb plus ladder (Life technologies).

Figure 3.3: Diagram of fungal ribosomal RNA genes and their ITS regions
Position of forward (ITS1F) and reverse (ITS2) primers are shown.

To clean the PCR products the triplicates were pooled and 60 µL of SPRI select beads was added. This mixture was vortexed and incubated at room temperature for 1 min to ensure the binding of amplicons to the magnetic beads in SPRI select. The beads were separated by placing the microcentrifuge tube on a magnetic rack for 2 min. The supernatant was discarded without disturbing the beads. The beads were then washed twice with 180 µL of 85% ethanol followed by incubation at

room temperature for 30 sec. The washed beads were then dried and the amplicons eluted in 20 µL of 1 X TE. The residual beads were then separated using the magnetic rack and the eluted amplicons were separated and stored in a microcentrifuge tube at -20 °C. The cleaned amplicons were quantified using Qubit dsDNA HS assay kit and Qubit 2.0 Fluorometer (Life Technologies). Negative extraction and PCR controls were cleaned up and analysed along with the samples. Each sample was diluted to 26 pM and mixed together equally to make an equimolar library. Sequencing was performed using Ion PGM sequencing 400 Kit, Ion 318 chip and Ion Personal Genome Machine System (ThermoFisher Scientific).

3.2.3 Ion Torrent analyses

Due to the variable length of the ITS region, reads from the Ion Torrent PGM sequencing were sorted into two groups for processing. Both groups were trimmed and quality filtered using Mothur (Schloss et al., 2009). The group with longer sequence length ranged between 250 bp and 379 bp length, and the shorter sequences were between 130 bp and 250 bp. In both groups, sequences with more than seven consecutive homopolymers were removed (-maxhomop 7). The sequences were then stripped of barcodes and primer sequences. The longer sequences were then truncated to 220 bp and the shorter sequences to 100 bp. Sequences with expected error rate higher than 1% was excluded. OTUs were then clustered to ≥97% pair-wise identity with UPARSE-OTU (Edgar, 2013). Chimeras and singletons were also discarded in this process. Original reads were then mapped onto the representative OTU sequence in order to establish OTU abundance. For taxonomic affiliations, the representative OTU sequences were classified using the BLAST–based method implemented in QIIME (Caporaso et al., 2010) with the UNITE reference database (Abarenkov et al., 2010). The taxonomic assignment threshold was set at 97%.

3.2.4 Data analyses

To visually represent sample community similarity based on sequencing data, a non-metric multidimensional scaling (NMDS) plot was created in PRIMER 7 (PRIMER-E Ltd, Ivybridge, UK) using a resemblance matrix based on Bray-Curtis community similarities. Heat maps and bar plots were used to explain fungal community structure patterns between samples and groups (A, B, A1 and

B1). Goods coverage was calculated to ensure adequate sequencing depth. Furthermore, Tukey's honest significance test and ANOSIM were performed to test specific hypotheses formed from the interpretation of NMDS plots.

3.3 Results

3.3.1 Growth of huhu grubs

All nine huhu grubs exhibited a healthy rate of frass production and positive weight gains while on their initial diet and after dietary switch (Table 3.1). All grubs used in this study initially weighed between 1.61 and 1.7 g and were between 3.5 and 3.9 cm in length (Supplementary Table 7.1). Subsequent to the Ion Torrent sequencing, quality checks and trimming, a total of 151,261 sequences were retained for further analysis. Negative controls yielded no DNA, confirming the adequacy of the extraction and sequencing processes. Goods coverage scores greater than 98% for each grub sample confirmed adequate sequencing depth (Table 3.2)

Table 3.1: Weight gain of huhu grubs while on defined diets

Name	Diet	Mean weight gain/month (g)	Mean weight gain/month ±SD (g)	Group
01C	Cotton	0.52		
06C	Cotton	0.54		
37C	Cotton	0.59	0.59±0.07	A
38C	Cotton	0.68		
TAC	Cotton	0.64		
20P	Pine	0.21		
24P	Pine	0.28	0.28±0.05	B
41P	Pine	0.34		
42P	Pine	0.29		
01P	Pine	0.36		
06P	Pine	0.32		
37P	Pine	0.29	0.34±0.04	A1
38P	Pine	0.41		
TAP	Pine	0.34		
20C	Cotton	0.39		
24C	Cotton	0.51	0.53±0.14	B1
41C	Cotton	0.72		
42C	Cotton	0.5		

'A' grubs collected from wild and reared on cotton diet
'B' grubs collected from wild and reared on pine diet
'A1' grubs switched from cotton diet to pine diet
'B1' grubs switched from pine diet to cotton diet

3.3.2 Gut fungal community composition in initial cotton- (A) and pine-reared (B) huhu grubs

The fungal communities of cotton-reared grubs (A) were mostly Ascomycota (57.5% of total sequences) and Basidiomycota (42.5%). A total of 171 fungal OTUs were present in this group, with 14 abundant OTUs (those representing more than 1% of total sequences in the group).

At the phylum level, there was noticeable variation between the individual grubs in this group (Figure 3.4). Grubs 01C and TAC were dominated by Ascomycota with over 99% of sequences, while grub 06C was 54.7% Ascomycota and 44.7% Basidiomycota. Phylum Basidiomycota overshadowed Ascomycota in grubs 37C and 38C. Sequences grouping to the order *Hypocreales* alone accounted for 99.6% of the total sequences of grub 01C, while accounting for 32.2% of sequences in grub 06C and 14% in grub TAC. At an order level, grub 06C was mostly represented by sequences belonging to *Tremellales*, *Hypocreales*, *Saccharomycetales*, *Sporidiobolales* and *Trichosporonales*. Together these accounted for 97.6% of sequences in grub 06C (Figure 3.5). The orders *Tremellales* and *Trichosporonales* accounted for 95.5% of sequences in grub 37C and 97% in grub 38C. The composition of grub TAC was different from all the other grubs in this group with the order *Helotiales* accounting for 78.2% of all the sequences. Along with *Helotiales* and *Hypocreales*, grub TAC was also dominated by sequences belonging to *Eurotiales* and *Saccharomycetales*. These four orders constituted 99.9% of all sequences in grub TAC (Figure 3.5).

Table 3.2: Ion Torrent sequencing summary statistics

Groups	Total OTUs	Grub	No: of OTUs	Abundant OTUs	Pooled abundant OTUs (%)	Goods coverage (%)
A	171	01C	43	8	99.0	99.7
		06C	102	15	80.7	98.3
		37C	64	16	95.5	99.2
		38C	77	14	93.7	99.2
		TAC	48	8	92.6	99.4
B	151	20P	111	16	86.2	98.8
		24P	131	20	84.6	98.6
		41P	136	21	82.0	98.6
		42P	121	12	81.3	98.6
A1	190	01P	118	22	81.0	98.8
		06P	120	20	84.1	98.6
		37P	90	13	88.7	98.6
		38P	168	18	79.9	98
		TAP	96	19	83.2	99.1
B1	149	20C	90	15	85.9	98.5
		24C	74	12	82.7	99.0
		41C	105	16	78.7	98.7
		42C	90	10	82.4	98.5

'A' grubs collected from wild and reared on cotton diet
'B' grubs collected from wild and reared on pine diet
'A1' grubs switched from cotton diet to pine diet
'B1' grubs switched from pine diet to cotton diet

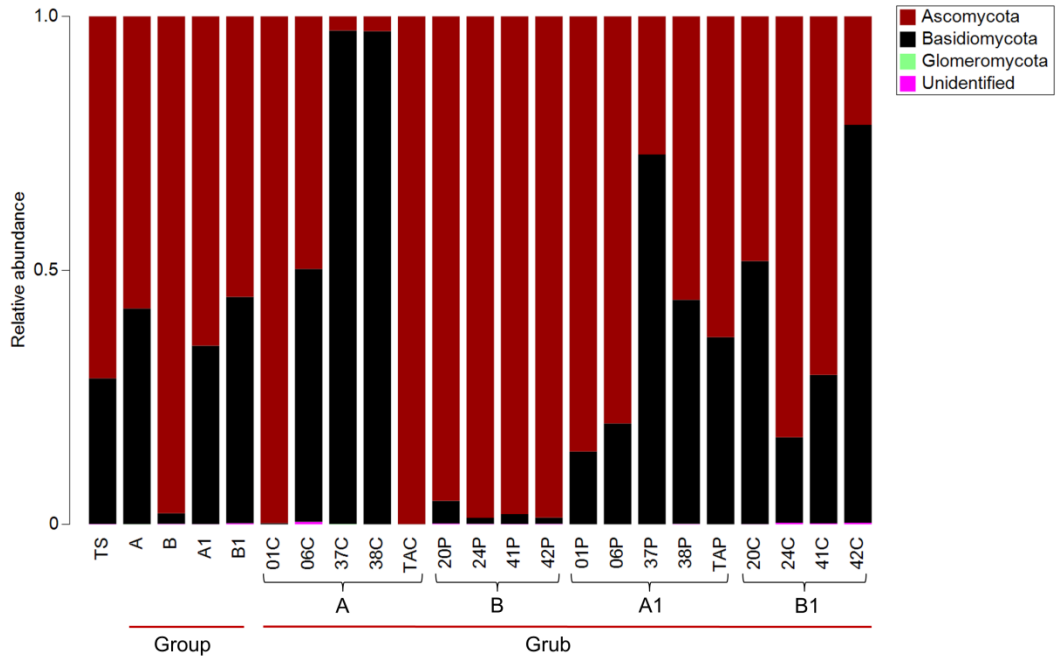

Figure 3.4: The composition of fungal phyla in gut of huhu grubs
TS – total sequences, A - grubs reared on cotton, B - grubs reared on pine, A1 - grubs switched to pine, B1 -grubs switched to cotton

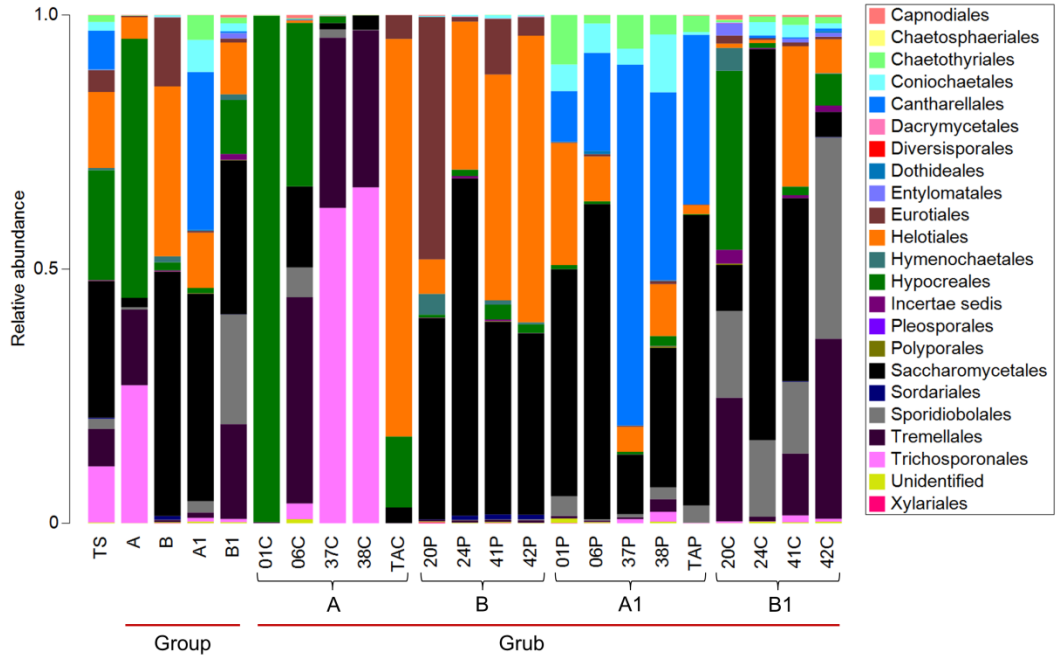

Figure 3.5: The composition of fungal orders in gut of huhu grubs
TS – total sequences, A - grubs reared on cotton, B - grubs reared on pine, A1 - grubs switched to pine, B1 -grubs switched to cotton

As expected, the pine reared grubs had a completely different community structure and population (Figure 3.6 and Figure 3.7). At 151 OTUs these grubs had a lower number of OTUs than the cotton reared grubs. However all four grubs (B) in this group shared 86 OTUs (Supplementary Table 7.3). The Phylum

Ascomycota dominated the fungal community accounting for 97.8% of all sequences, with Basidiomycota representing 2.1%. All four grubs had a significantly higher percentage of Ascomycota than Basidiomycota (Figure 3.4). The five abundant orders (>1% of sequences in the group) *Saccharomycetales* (48%), *Helotiales* (33.4%), *Eurotiales* (13.5%), *Hypocreales* (15.9%) and *Hymenochaetales* (1.2%), together accounted for 97.8% of total sequences of this group (Figure 3.5). Overall, seven dominant genera constituted 95.4% of all the sequences in this group. The most dominant genus *Scytalidium* while being abundant in all the pine-reared grubs had a 32.7% representation in the group (Figure 3.6). The most abundant OTUs (20 OTUs) in pine reared grubs accounted for 78.8% of total sequences of the group, with *Scytalidium*, *Schwanniomyces* and *Scheffersomyces* abundant in all four grubs (Supplementary Table 7.7).

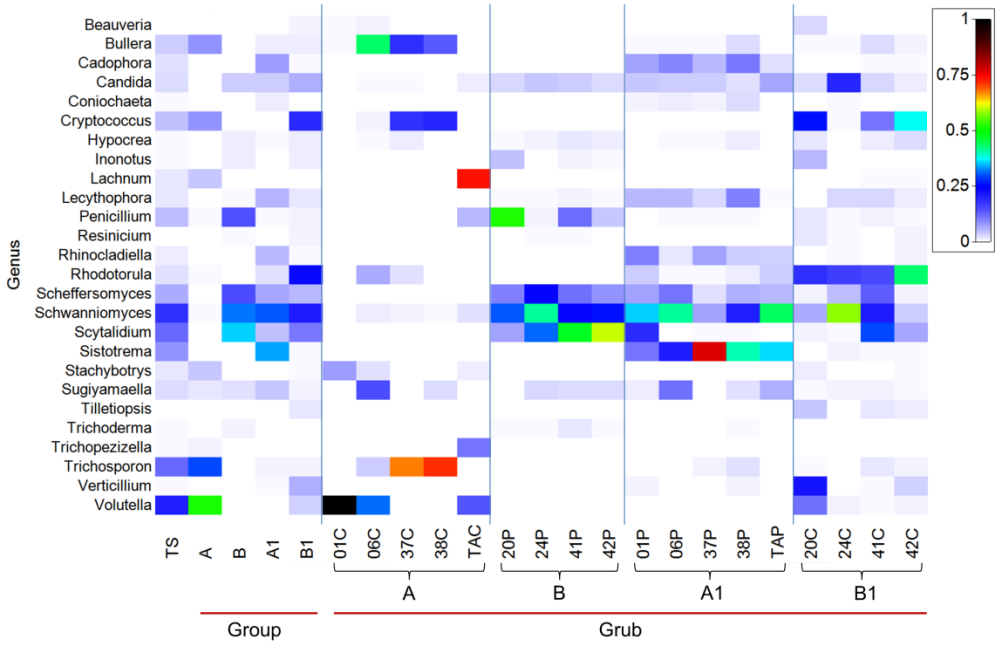

Figure 3.6: Heat map representing the distribution of abundant fungal genera in gut of huhu grubs

Genera comprising more than 1% of sequences in any of the grubs are shown.
TS – total sequences, A - grubs reared on cotton, B - grubs reared on pine, A1 - grubs switched to pine, B1 - grubs switched to cotton The colour gradient represents the relative abundance: Black(high) to white (low)

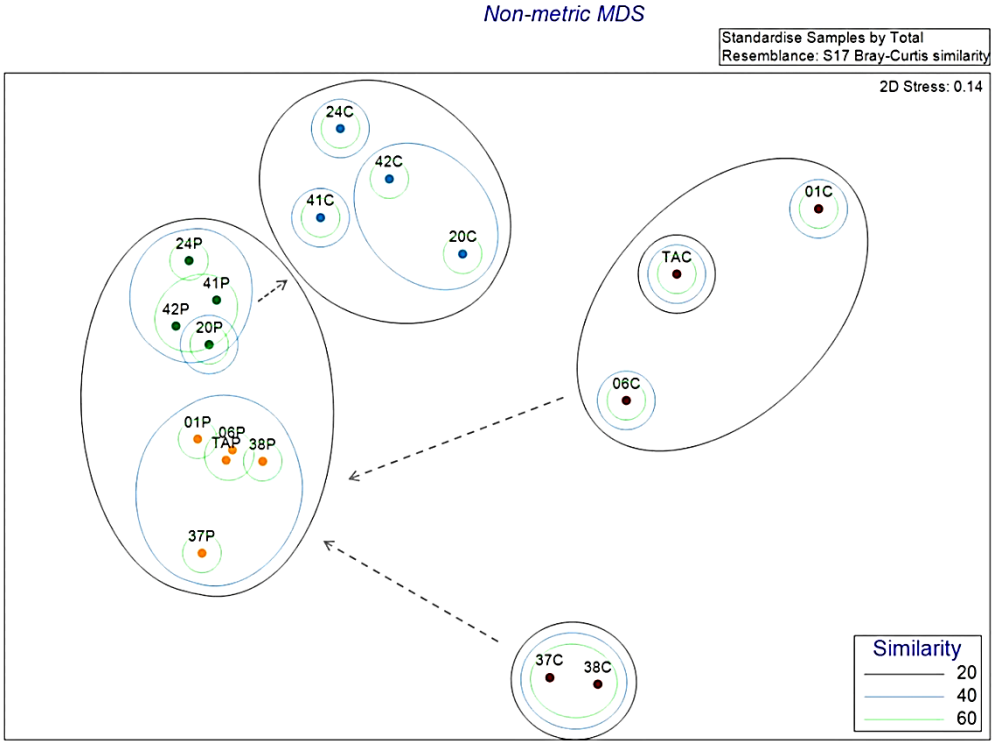

Figure 3.7: Non metric multidimensional scaling (NMDS) ordinations of gut fungal community compositions based on Bray Curtis distances.

- Grubs grown on cotton (A)
- Grubs grown on pine (B)
- Grubs switched from cotton to pine (A1)
- Grubs switched from pine to cotton (B1)
- Direction of clustering of the samples after dietary switch

A total of 111 OTUs were present in the fungal community of grub 20P. Sixteen abundant OTUs accounted for 86.3% of sequences in the grub. *Penicillium* dominated the fungal community in 20P (47.6%), and was also present in high numbers in grubs 41P (10.9%) and 42P (3.6%). However it was not abundant in grub 24P. Grubs 24P, 41P and 42P had 131, 136 and 121 OTUs respectively. Grub 24P contained 20 abundant OTUs that accounted for 79.6% of all its sequences. The 21 abundant OTUs in grub 41P and 12 abundant OTUs in grub 42P represented 82% and 81.3% of their total sequences respectively. The most abundant genus in both of these grubs was *Scytalidium* (Figure 3.6).

3.3.3 Gut fungal community composition of huhu grubs after diet switch

Across the five grubs switched to the pine diet (A1), 190 OTUs with 58 shared OTUs were identified (Supplementary Table 7.4). The library was again dominated by two phyla, Ascomycota represented 64.9% of all sequences and

Basidiomycota accounted for 35.1%. Seven abundant orders made up 97.2% of all the sequences in this group (Figure 3.5). At 48% *Saccharomycetales* were the most dominant order followed by *Cantharellales* (31%), *Helotiales* (10.9%), *Coniochaetales* (6.3%), *Chaetothyriales* (4.9%), *Sporidiobolales* (2.3%) and *Tremellales* (1%). Further, sequences grouping to 11 major genera constituted 95.1% of all the sequences in this group. *Sistotrema* while being abundant in all the grubs in this group also had the most representation at 31%. This was followed by *Schwanniomyces* at 27.7%, *Cadophora* at 7% and *Scheffersomyces* at 6%. Further, *Candida* and *Rhinocladiella* were also abundant in all the grubs switched to pine diet (Figure 3.6). In grub 01P, 22 abundant OTUs represented by eight genera accounted for 81% of its sequences. The abundant fungal community in grubs 06P, 37P, 38P and TAP was represented by 20, 13, 18 and 19 OTUs, and made up 84.1, 88.7, 79.9 and 83.2% of all sequences in these grubs (Table 3.2). The genera *Sistotrema*, *Schwanniomyces*, *Cadophora*, *Scheffersomyces*, *Rhinocladiella* and *Candida* were abundant in all of these grubs (Figure 3.6).

A total of 149 OTUs with 37 shared OTUs were identified across the four grubs (B1) switched to cotton from pine (Supplementary Table 7.5). The phylum Ascomycota represented 55.2% of total sequences and Basidiomycota 44.6%. Nine dominant orders in this group constituted 96.3% of all sequences. Although, *Saccharomycetales* at 30.3% remained the most dominant order after dietary switch the abundance decreased from 48% while these grubs were on pine diet (Figure 3.5). The dietary switch also resulted in an increased representation of *Sporidiobolales* (21.6%) and *Tremellales* (18.6%). Twelve abundant genera in this group accounted for 91.9% of all sequences. Among the dominant genera, *Rhodotorula*, *Schwanniomyces* and *Candida* were abundant in all grubs in this group. Abundant OTUs accounted for a minimum of 78.7% of the sequences in this group, 42C had the lowest number of abundant OTUs at 10 with grubs 20C, 24C and 42C represented by 15, 12 and 16 OTUs respectively.

3.3.4 Comparison of gut fungal community of huhu grubs initially reared on cotton (A) or pine (B) diets

Analysis of Ion Torrent sequencing data indicated a clear separation between the gut fungal communities of grubs reared on cotton or pine diets. Grubs grown on the pine diet clustered together with a Bray Curtis similarity greater than 40%

(Figure 3.7). However the grubs reared on the cotton diet had more heterogeneous gut fungal communities. ANOSIM identified variation between the dietary groups (A and B) with a statistical significance at R = 0.64 and P = 0.01. This was further supported with Tukey's honest significant difference between grubs grown on different diets (P = 0.007).

Variation in the abundant fungal OTUs across the diet groups was examined to provide insight into the effects of diet on gut microflora. The 14 abundant OTUs (Supplementary Table 7.6) in cotton reared grubs represented 84.4% of total sequences, while these were either minor OTUs or absent in pine reared grubs, and the 20 abundant OTUs in pine reared grubs represented 78.8% of total sequences but accounted for only 0.2% in cotton reared grubs (Supplementary Table 7.7). Furthermore, these 20 abundant OTUs were present in all of the pine reared grubs (B). At a genus level the seven abundant genera that made up 97.5% of sequences in group A only accounted for 1.9% in group B with 1.8% of this representation being *Sugiyamaella*. Similarly the seven abundant genera with a representation of 95.4% sequences in group B constituted only 2% of sequences in group A, of which genus *Sugiyamaella* accounted 1.3%.

3.3.5 Comparison of gut fungal community of huhu grubs switched to pine (A1) from cotton (A) diet

A phylum-level community shift was observed after switching initially cotton-reared grubs to the pine diet (Figure 3.4). A two-dimensional NMDS ordination, representing similarities of fungal community compositions between diets (Figure 3.7) showed that the grubs switched to pine clustered together with a similarity above 40%. The number of OTUs common to all grubs in each group increased from 4 in cotton-reared grubs to 58 after being switched to pine. Fungal communities were significantly dissimilar between grubs grown on cotton (A) and those switched to pine (A1), identified by ANOSIM at R = 0.64, P= 0.008. Tukey's honest significance test between these groups confirmed a difference in community structure (P=0.01). The 14 major OTUs that accounted for 84.4% of total sequences in group A were reduced to 0.6% (Supplementary Table 7.8) when switched to pine. Similarly, the 21 major OTUs representing 79.6% of the total sequences of grubs in group A1 accounted for only 1.3% of the total sequences in the grubs while on cotton diet (Supplementary Table 7.9). Also the seven major

genera that accounted for 97.5% of sequences in group A was reduced to 5.4% after dietary switch (A1).

3.3.6 Comparison of gut fungal community of huhu grubs switched to cotton (B1) from pine (B) diet

After undergoing dietary switch to cotton diet, grubs differed considerably in their fungal community structure (Figure 3.4, Figure 3.5 and Figure 3.6). The number of shared OTUs to all the grubs dropped from 86 while on pine to 37 OTUs on cotton. However the total number of OTUs remained similar with 149 OTUs after the dietary switch and 151 OTUs while on pine. The two-dimensional NMDS ordination, representing similarities of fungal community compositions between diets (Figure 3.7) showed that even after the switch to cotton the grubs were clustered together with a minimum similarity above 20%. A variation between the fungal community of the two groups (B and B1) was identified by ANOSIM, R = 0.78 and a significance of P = 0.03. Tukey's honest significance test between the two dietary groups had a significance P = 0.16. A drastic shift in the population was noticed after the dietary switch with the abundant OTUs accounting for 78.8% of sequences in the group (B) decreasing to 25.2% (B1) (Supplementary Table 7.10). Similarly the 21 abundant OTUs that made up 79.6% of sequences in group B1 accounted for 36.9% in group B. On being switched to cotton, all the abundant genera decreased to a great extent (Figure 3.6). Subsequent to the dietary switch, the most dominant genus in group B *Scytalidium* underwent a drastic reduction in their abundance from 32.7% to 9.9%. The most noticeable change was the rise of *Rhodotorula* as the most abundant genus covering 21.6% of sequences in the group (B1) while prior to the dietary switch it accounted for only 0.08%.

3.4 Discussion

In this study, Ion Torrent sequencing offered unprecedented insights into the gut fungal community of huhu grubs. The ITS region of the *rrn* operon was amplified and used to identify fungi as it provided enough variation to be taxonomically informative (Lindahl et al., 2013). Furthermore, the ITS region was recently proposed as the formal barcode for fungi (Schoch et al., 2012). The ITS is highly variable as it does not code for ribosome components (Lindahl et al., 2013), and is widely preferred to distinguish between closely related species (Gazis et al., 2011).

Another crucial factor that contributed to choosing the ITS region was the availability of reliable reference databases, and the fact that to date Dikarya are best represented at the species level by ITS sequences (Begerow et al., 2010).

Wood-feeding insects are known to either feed on previously degraded wood, or utilize symbionts to circumvent the lignocellulose barrier to extract essential nutrients (Geib et al., 2008). In particular, fungi are known to be involved in lignocellulose degradation, both in nature and inside the gut of several insects (Brune, 2014; Geib et al., 2008; Gibson & Hunter, 2010; Hyodo et al., 2000, 2003). Since we provided huhu grubs with sterile non-degraded wood, the fact that the grubs used in this study remained healthy throughout is indicative of an active involvement of its gut microbiome in lignocellulose degradation. The gut fungal community patterns reported in this study further supports our hypothesis that defined diet is a significant driver for the selection and retainment of the community responsible for the degradation of the relevant substrate (diet). The grubs grown initially on pine (B) shared a similar fungal community structure, indicating a prominent dietary influence. This determinant effect of diet on the fungal community is similar to that of bacterial community structure in the guts of higher termites (Mikaelyan et al., 2015).

The clear disparity between the abundance of Ascomycetes and Basidiomycetes in all huhu grubs initially grown on pine (B) was a strong indicator of a selective pressure. The greater abundance of Ascomycetes compared to Basidiomycetes could be attributed to their ability to survive under stressful conditions (Blanchette et al., 2004; Worrall & Wang, 1991). The sudden change in diet from the decayed wood environment in nature, to a strict non-decayed wood in the laboratory could explain the comparatively uniform distribution of Ascomycetes observed in the grubs reared on pine (group B). Furthermore, the recalcitrant nature of lignocellulose may result in the suppression or elimination of fungi that cannot play a role in lignocellulose degradation along with competitive exclusion. While several fungi can co-exist, some are known to have an antagonistic effect towards other microbes (Brimner & Boland, 2003; Fokkema, 1973; Francis & Read, 1994; Schirmböck et al., 1994; Whipps, 1987). The large number of OTUs shared between pine grown grubs was indicative of the significant effect of diet. Furthermore, dominance of the abundant OTUs is indicative of their potential role

in lignocellulose degradation and/or suggestive of their ability to thrive in a harsher, lower nutrient environment. It is also possible that these fungi only extract small components of the lignocellulose without really degrading it. The grub aids this by shredding the wood to increase surface area and expose more of the easily extracted polymers. The largely undigested bulk of the wood is deposited undegraded as frass.

The frass of pine reared grubs (B) was mostly dominated by *Scytalidium*, *Penicillium*, *Schwanniomyces* and *Scheffersomyces*. Noticeably, the abundance of *Scytalidium* in the gut of huhu grubs initially reared on pine was indicative of a soft-rot-based wood degradation. Soft-rot fungi are known to thrive in conditions unfavourable to white and brown rots (Blanchette et al., 2004). The sudden transfer of huhu grubs from their natural environment to a harsh non-degraded lignocellulose diet would have aided in flourishing of the soft-rot fungi. Since the pine wood used in this study was water-logged it could be argued that the conditions provided were favourable towards a soft rot based wood decay. Soft-rot fungi are well known for their preference towards cellulose and hemicellulose with decay patterns indicating cavities within secondary walls of wood cells (Hamed, 2013). Even though all cell wall components can be degraded during soft rot of wood, generally these fungi are reported to have minimal preference towards lignin degradation. This is consistent with the existence of limited lignin modification seen in the frass of huhu grubs (Reid et al., 2011). Additionally, soft rot fungi are also known for their involvement in lignocellulose degradation in the wood feeding Asian longhorned beetle *A. glabripennis* (Geib et al., 2008).

Penicillium spinulosum is a mould that falls under the category of saprotrophic fungi (degrades dead organic matter). In a previous unpublished study using clone libraries (Williams, 2011), *P. spinulosum* was identified as the most abundant taxa in the pine reared gut fungal community of huhu grubs. This reinforces our notion of the most crucial factors influencing the gut fungal community structure to be either the taxonomy of the host (being huhu grub), or the diet itself (pine block) (Colman et al., 2012). However, while *P. spinulosum* was abundant in three of the pine reared grubs, it had only minimal representation in another (grub 24P), indicating that its role potentially has been taken over by other species. This is known as competitive exclusion either the grub might not have hosted the specific

taxa before being reared in the laboratory or antagonistic reactions by other fungi present in the gut. Other abundant fungi observed in this group are also known to play major roles in degradation of plant cell walls, with *Schwanniomyces occidentalis* known for its amylolytic enzymes (Alvaro-Benito et al., 2010) and *Scheffersomyces shehatae* are known to possess plant cell wall degrading enzymes (Martiniano et al., 2013; Scordia et al., 2012; Tanimura et al., 2015). Furthermore, the fermentative ability of the *Scheffersomyces* clade is demonstrated by several members that exhibit high rates of xylose fermentation under various conditions (Suh et al., 2003). The fungal community structure observed in huhu grubs grown on pine (B) almost exclusively consisted of members from Ascomycota belonging to the sub phyla Pezizomycotina and Saccharomycotina (Gibson et al., 2010).

Dietary switch resulted in a shift in the gut fungal community structure and the dominant population in the studied scenarios. The fungal community of grubs switched from pine to cotton diets were dominated by yeasts, especially *Rhodotorula cycloclastica*. The other abundant species included *Cryptococcus laurentii, Candida chilensis, S. occidentalis, S. henanensis* and *Volutella* sp. These yeasts are well known for their capabilities to ferment 5-carbon sugars such as D-arabinose and D-xylose in the guts of plant tissue-feeding insects (Barbosa et al., 2009; Cadete et al., 2009; Calderon & Berkov, 2012; Santos et al., 2011). Although, the yeast-insect relationships are not completely understood, they are presumed to be involved in detoxification of plant metabolites, acting as a source of digestive enzymes and vitamins (Vega & Dowd, 2005). The effect of diet on the gut fungal community was evident when comparing the abundant genera of grubs grown on pine (lignocellulose) to when reared on cotton (Cellulose). The abundant OTUs in one group were either absent or at low abundance in the other. However, the grubs even after being switched to cotton diet seem to share a moderate similarity between each other, explained mostly by the retained abundance of *S. occidentalis* and *S. henanensis*. The wide range of fungal species that might have been able to thrive on cellulose if transferred directly to a cellulose diet from wild, is assumed to have been eliminated while on a comparatively stringent lignocellulose diet, resulting in a less diverse community.

Our hypothesis of the fungal population being driven by diet seems to fit well with the patterns of fungal community observed in grubs grown initially on

cellulose diet (A). The abundant fungi observed were yeasts and *Stachybotrys chartarum*, which are either known to exist in an environment with high cellulose content or for their cellulose degrading capabilities (Murtoniemi et al., 2003; Suh et al., 2003). Further, several cerambycids are already known to have close associations with yeast endosymbionts (Berkov et al., 2007). Although the dominant genera in this group differed between the grubs, they appear to share a degree of functional similarity. The dominant genera included *Volutella*, *Stachybotrys*, *Trichosporon*, *Bullera* and *Cryptococcus* with most being known to be capable of cellulose degradation. In addition to their cellulolytic abilities, *Trichosporon* sp. are also well documented for their xylan degrading capabilities (Stevens & Payne, 1977). Furthermore, phylogenetic studies with broader taxon samplings have illustrated that some species in the genera *Bullera* and *Cryptococcus* are closely related to yeasts in the genus *Trichosporon* (Middlehoven et al., 2004; Okoli et al., 2007). Even though these grubs were collected from decayed wood in nature, after a few months on the cotton diet, wood rot fungi were almost completely absent from the gut fungal population (A). This is indicative of a less complex energy source, suiting fungi capable of competing for nutrients with other microbes.

Grubs transferred to pine from cotton diet shared a greater similarity in their gut fungal community. This greater clustering is indicative of a similar group of fungi being present in all the grubs grown on pine. Subsequent to the dietary switch, the grubs that clustered individually while on cotton diet, clustered closer with the grubs that were already on pine diet (B). This suggests the involvement of a group of fungi in degradation of lignocellulose. Genera *Schwanniomyces, Scheffersomyces* were present in abundance in all of the pine-reared grubs (A1 and B), hence it could be speculated that these three genera could potentially be involved in lignocellulose degradation. Furthermore the presence of *Sistotrema brinkmanii* of order *Cantharellales*, a well-known brown rot and a wood degrader, as the most dominant fungi in this group indicates that the defined diet used in this study has selected for a consortium of fungi that could potentially be involved in lignocellulose degradation in huhu grubs. Studies have shown that *Cantharellales* are abundant in ligninolytic enzymes (Chen et al., 2001; Eisenlord et al., 2013). Although *S. brinkmanii* is debated to have a very slow rate of wood degradation (Son et al., 2010), it is possible that the sheer number of these fungi in the gut

along with the help of other members of the gut fungal community may have accelerated the lignocellulose degradation process. Another possibility is that the production of lignocellulose degrading enzymes by fungi are regulated to ensure expression only under conditions in which the fungi requires plant polymers as a carbon source (Ruijter & Visser, 2006). Also, transfer to a cotton diet from the wild would have resulted in the loss of most of the ligninolytic fungal population as the cellulolytic fungi would have out competed them. The subsequent switch to pine diet may have created an environment with limited free carbon and nitrogen resulting in an activation of ligninolytic enzymes in this taxon.

The mechanism of wood degradation used remains poorly understood as the grubs that were grown on pine diet initially had an abundance of soft rot fungi, whereas the grubs that were switched to pine from a cotton diet were abundant in a well-known wood degrading basidiomycete. Therefore, it could be presumed that the huhu grub gut fungal consortium is potentially capable of using different individual wood rot mechanisms (white rot, brown rot or soft rot) or a combination of many mechanisms dependent on the provided diet and environment. Another dominant fungi was the soft rot fungi *Cadophora melinii*, (Blanchette et al., 2004) known to degrade wood to a limited extent. The presence of the genus *Sugiyamaella* in all the dietary groups was consistent with their presence in wood and frass of lignicolous beetles (Houseknecht et al., 2011; Kurtzman & Robnett, 2007).

3.5 Conclusions

At the outset of this work, three hypotheses were proposed to explain the possible effects of defined diets on the gut fungal community of huhu grubs. We confirmed our first hypothesis that the gut fungal community of pine-reared huhu grubs were significantly different from the gut fungal community of cotton-reared huhu grubs. The large population of soft rot fungi and yeasts capable of producing cell wall degrading enzymes, found in huhu grubs collected from the wild and reared on pine, could be potentially involved in making polymer carbohydrate available from lignocellulose. The gut fungal community of huhu grubs collected from the wild and reared on cotton, and grubs switched to cotton from pine, were mostly abundant in yeasts and some cellulose degrading fungi. This supports the second hypothesis that the substrate shift of huhu grubs from a wood-feeding

environment in the wild to a cotton-diet would result in a less functionally diverse gut fungal community. Finally, defined diets were observed to have a selective pressure on the gut fungal community (NMDS Figure 3.7). Lignocellulose appears to have a filtering or bottleneck effect on the gut fungal community structure, as grubs grown on pine shared a greater community similarity than their cotton-reared counterparts. Since, we could not examine the gut fungal community when huhu grubs were initially collected from the environment we couldn't prove that switching the diet actually changes the abundant community. However, results do confirm that following the dietary switch there is a significant change in the abundant gut fungal community of huhu grubs, indicating a role for fungi in wood degradation in the huhu grub gut.

Chapter Four

Effects of defined diets and dietary switch on the gut bacterial community of huhu grub

Abstract

Huhu grubs, the wood-feeding larvae endemic to New Zealand, have been suggested to host bacteria that potentially aid in lignocellulose degradation. Currently, the information available on the gut bacteria of huhu grubs is limited, and from huhu grubs fed on partially degraded wood in a nutrient rich environment. To address this, high-throughput sequencing was used to investigate the gut bacterial diversity and composition of huhu grubs reared on defined cellulose and lignocellulose diets. The effects of dietary shift on the gut bacterial community were also examined by shifting the cellulose-reared huhu grubs to lignocellulose and vice-versa. This study has identified that at the phylum level the gut bacterial community of huhu grubs was reasonably similar regardless of the nature of the diet provided. There was no shift in the abundant bacterial community following dietary switch, confirming a lack of selective pressure by the diet on the gut bacterial community. *Proteobacteria* and *Acidobacteria* were found to be abundant in all the grubs regardless of diet, suggestive of their role as secondary degraders to fungi.

4.1 Introduction

Dietary source is known to play a crucial role in shaping the bacterial gut community within organisms (Colman et al., 2012). However, the implications of diet on the complexity of the gut bacterial community in huhu grubs are unknown. In this chapter, we aim to examine the gut bacterial community of huhu grubs reared on pine or cotton diets.

We have shown that the gut fungal community of huhu grubs is influenced by diet and hypothesise that the gut bacterial communities of huhu grubs reared of cellulose or lignocellulose diets will also be influenced by the diet. We also expect a shift in the gut bacterial community when the diets are interchanged, as the bacterial community adapts to a change in diet. This study is conducted using the

same frass samples from the lab-reared huhu grubs discussed in chapter 3, and will utilise recent advances in high-throughput sequencing to conduct an in-depth analysis of the gut bacterial community composition using several thousands of short sequences generated using Ion Torrent sequencing. The experimental design and the grubs used are explained in chapter 3 section 3.2.

4.2 Materials and methods

The sampling site, huhu grubs used, Ion Torrent technique and data analyses used (Ion Torrent analyses, NMDS ordination and ANOSIM) are detailed in chapter 3, section 3.2. The frass collection, rearing technique and DNA extraction methodologies are detailed in chapter 2, section 2.2. Any variations in methodology or analyses are detailed here.

4.2.1 Ion Torrent sequencing

The V4 region of the 16S rRNA gene was amplified to identify the gut bacterial community in huhu grubs. Amplifications were performed using the adapted primer set 515F (5'CCATCTCATCCCTGCGTGTCTCCGACTCAG-unique IonXpress barcode-GATGTGCCAGCMGCCGCGGTAA-3') and 806R (5'CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATGGACTA CHVGGGTWTCTAAT-3') (IDT, New Zealand). Each reaction contained 1x PCR buffer (Life Technologies, New Zealand), 0.2 mM dNTPs (Roche Diagnostics, New Zealand), 0.02 mg/mL Bovine serum albumin (BSA), 0.5 µM of each primer, 1 U Platinum Taq (Life Technologies), 0.5 µM $MgCl_2$, 1 ng of DNA and the reaction was made up to 25 µL with milli-Q H_2O. PCR reactions were completed for each extraction in triplicate under the following thermal cycling conditions: 94°C for 3 min, then 30 cycles of 94°C for 45 sec, 55°C for 1 min, 72°C for 90 sec, and a final extension of 72°C for 10 min. The PCR products were verified on a 1% SYBR safe stained agarose gel, cleaned using SPRI select and sequenced using Ion Torrent PGM (ThermoFisher Scientific) at the DNA sequencing facility, University of Waikato, as described in section 3.2.2.

4.2.2 Ion Torrent analyses

Reads from the Ion Torrent PGM were trimmed and quality filtered using Mothur (Schloss et al., 2009). The sequences ranged between 275 and 359 bp in length. The sequences with more than seven consecutive homopolymers were removed (-

maxhomop 7). The sequences were then stripped of barcodes and primer sequences, and were truncated at 250 bp. Sequences with expected error rate higher than 2.5% were excluded. The OTUs were then clustered to ≥97% pair-wise identity with UPARSE-OTU (Edgar, 2013). Chimeras and singletons were discarded in this process. Original reads were then mapped onto the representative OTU sequence in order to establish abundance. For taxonomic affiliations of prokaryotic 16S rRNA gene sequences, the representative OTU sequences were classified using the RDP-classifier (Cole et al., 2007) implemented in QIIME (Caporaso et al., 2010) with the Greengenes reference database (DeSantis et al., 2006). The taxonomic assignment threshold was set at 97%.

4.3 Results

The grubs utilised remained healthy throughout the study, as indicated by their respective weight gain while on the different diets (Table 3.2). After quality checks, trimming and removal of sequences that occurred less than 3 times in the entire library, a total of 396,668 sequences were retained for analysis. Controls yielded no DNA confirming the efficacy of the extraction and sequencing processes. Across all the samples, a total of 376 OTUs were identified. Group A (grubs initially grown on cotton) had the largest number of OTUs at 306, while groups B (grubs initially grown on pine), A1 (grubs switched to pine from cotton) and B1 (grubs switched to cotton from pine) were represented by 237, 220 and 240 OTUs respectively. The number of OTUs in individual grubs varied between 89 and 197 (Table 4.1), and Goods coverage scores (mean = 99.8%, SD = 0.1%) indicated that all major bacterial phylotypes in the huhu gut were identified. Consequently, rarification of reads across samples was not carried out prior to downstream analyses. Tukey's Honest Significance test did not indicate any significant differences between *Bacteria* from the huhu grubs grown on different diets.

Table 4.1: Ion Torrent sequencing summary statistics including Goods coverage

Group	Total OTUs	Grub	No: of OTUs	Goods Coverage (%)
A	306	01C	197	99.7
		06C	194	99.7
		37C	116	99.9
		38C	108	99.8
		TAC	89	99.9
B	237	20P	175	99.7
		24P	177	99.8
		41P	169	99.8
		42P	163	99.8
A1	220	01P	119	99.8
		06P	140	99.8
		37P	141	99.8
		38P	169	99.8
		TAP	102	99.8
B1	240	20C	163	99.7
		24C	155	99.7
		41C	179	99.7
		42C	168	99.7

A - Grubs reared on cotton, B - grubs reared on pine, A1 - grubs switched to pine, B1 -grubs switched to cotton

A total of 11 bacterial phyla were represented in the gut community of huhu grubs grown on both diets (Figure 4.1) with eight of these phyla being represented by > 1% of sequences in at least one huhu grub (Figure 4.2). Phyla *Proteobacteria* (mean 42.5%, SD 12.8%), *Acidobacteria* (mean 27.2%, SD 20.3%), *Actinobacteria* (mean 6.1%, SD 7.0%), *Bacteroidetes* (mean 22.4%, SD 23.7%) and *Planctomycetes* (mean 1.1%, SD 1.3%) accounted for 99.5% of total sequences (i.e., sequences from all grubs, hereinafter referred to as TS). *Proteobacteria*, the most abundant phylum (42% of TS), was dominant in all the dietary groups (Figure 4.3), and was the most abundant phylum in nine grubs in groups A, B and A1 (Figure 4.4). *Acidobacteria* (27.6% of TS) was the most

abundant phylum in five grubs, four of which belonged to group B1 (20C, 24C, 41C and 42C) and one from group B (42P) (Figure 4.4). *Bacteroidetes* was the most abundant phylum in four huhu grubs (06C, 37C, 01P and 06P) and constituted 22.6% of total sequences, but had minimal representation (<1%) in groups B and B1, with the exception of grub 20P. Phyla *Actinobacteria* and *Planctomycetes* accounted for 6.3% and 1% of total sequences respectively. The less abundant phyla included *Aquificae, Candidatus Saccharibacteria, Firmicutes, Verrucomicrobia, Armatimonadetes* and *Gemmatimonadetes* (Figure 4.1).

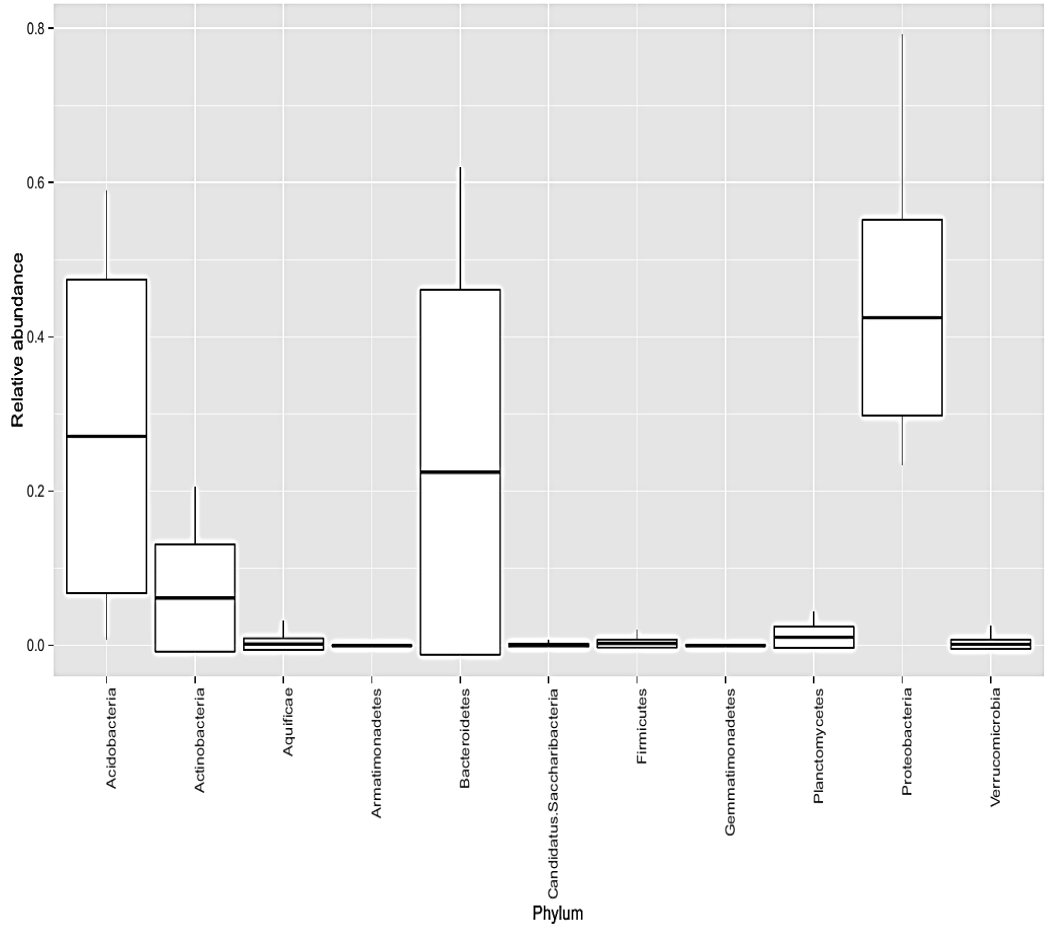

Figure 4.1: Average phylum representation of sequences observed in the 18 huhu gut samples
Horizontal lines within each box indicate mean representation of each phylum with the vertical extent of these boxes indicating the standard deviation of the mean and the extent of the vertical bars indicate the maximum and minimum representation of each phylum.

Sixteen classes were represented across all the dietary groups and differences in their abundance in the groups and individual grubs were analysed (Figure 4.5 and Figure 4.6). Phylum *Proteobacteria* was dominated by sequences from classes *Alphaproteobacteria* (23.3% of TS), *Gammaproteobacteria* (14.1% TS),

Betaproteobacteria (4.5% TS), and a minor presence of *Deltaproteobacteria* (0.01% TS). *Acidobacteria* was comprised of classes *Acidobacteria_Gp1* (27.5% TS) and *Acidobacteria_Gp3* (0.1% TS). Other abundant classes included *Sphingobacteriia* (22.6% TS), *Actinobacteria* (6.3% TS) and *Planctomycetia* (1.0% TS).

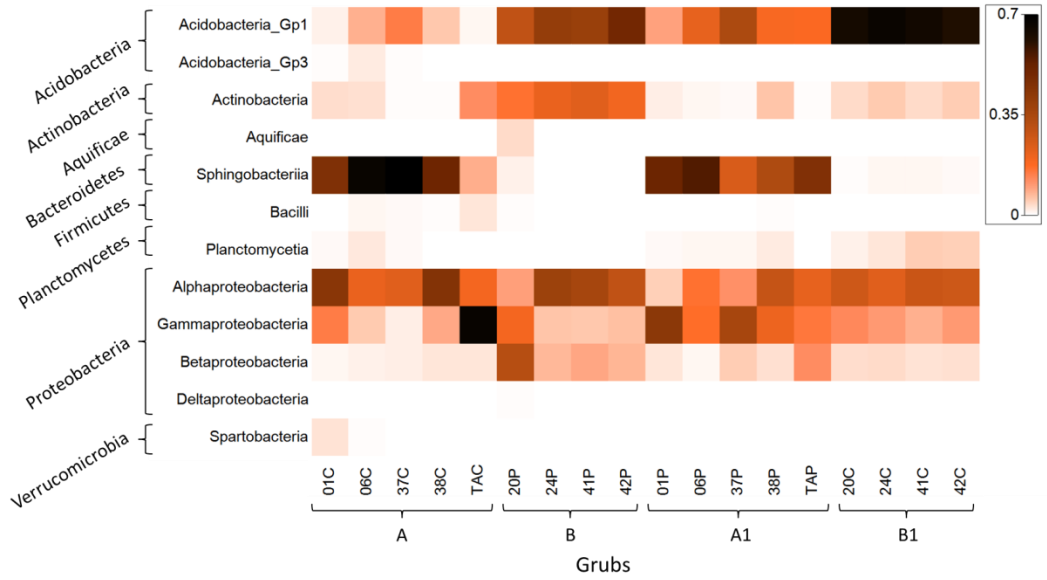

Figure 4.2: Distribution of abundant phyla and classes in the gut of individual huhu grubs
All phyla comprising more than 1% of sequences in any of the grubs are shown.
A - Grubs reared on cotton, B - grubs reared on pine, A1 - grubs switched to pine, B1 - grubs switched to cotton. Colour gradient: Black (high abundance) to white (low abundance)

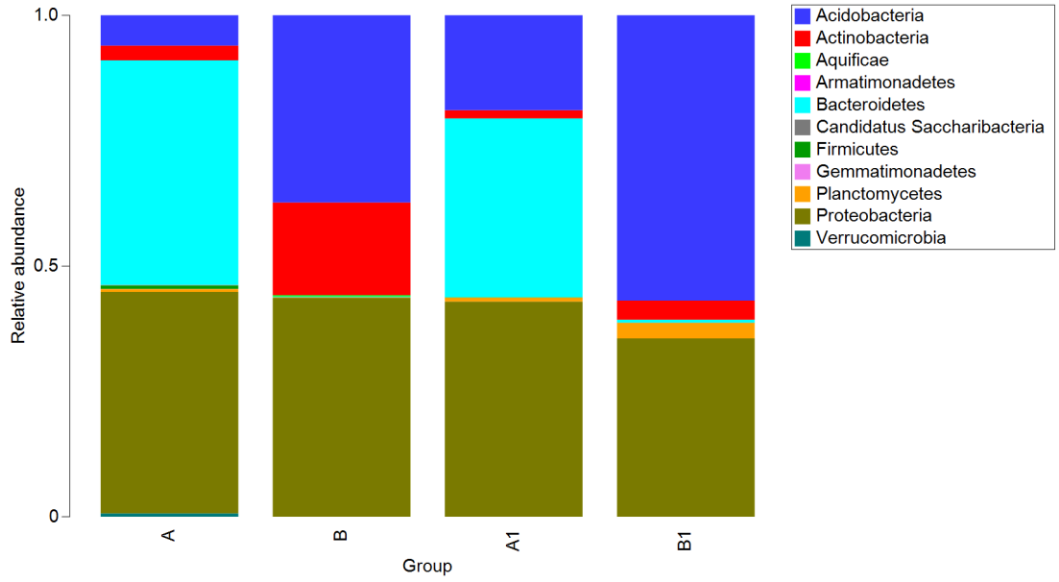

Figure 4.3: The composition of huhu grub gut bacterial phyla in dietary groups
A - Grubs reared on cotton, B - grubs reared on pine, A1 - grubs switched to pine, B1 - grubs switched to cotton

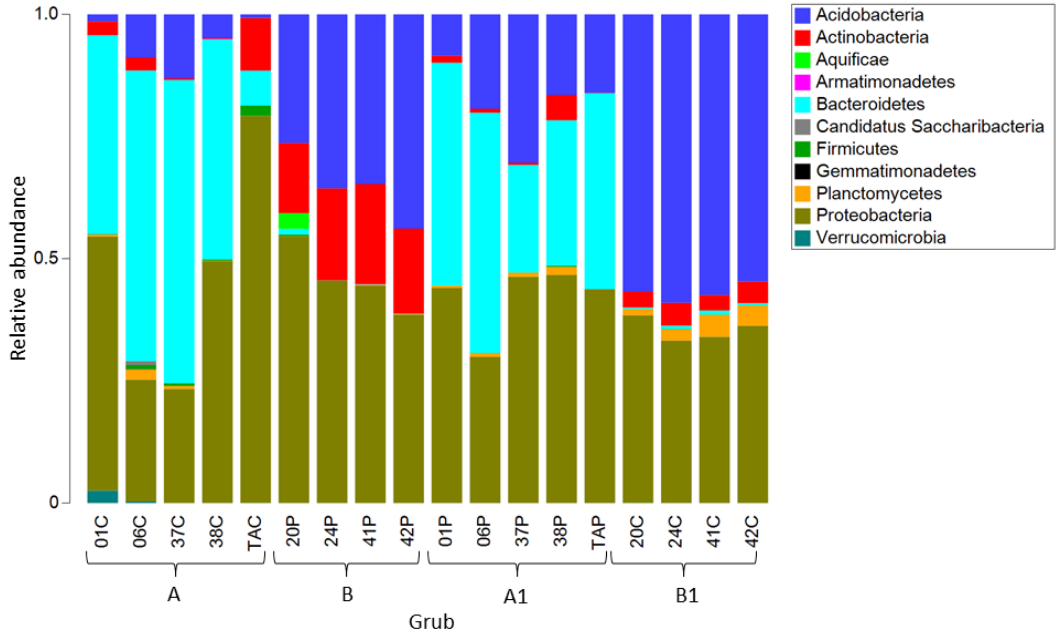

Figure 4.4: The composition of gut bacterial phyla in individual huhu grubs
A - Grubs reared on cotton, B - grubs reared on pine, A1 - grubs switched to pine, B1 - grubs switched to cotton

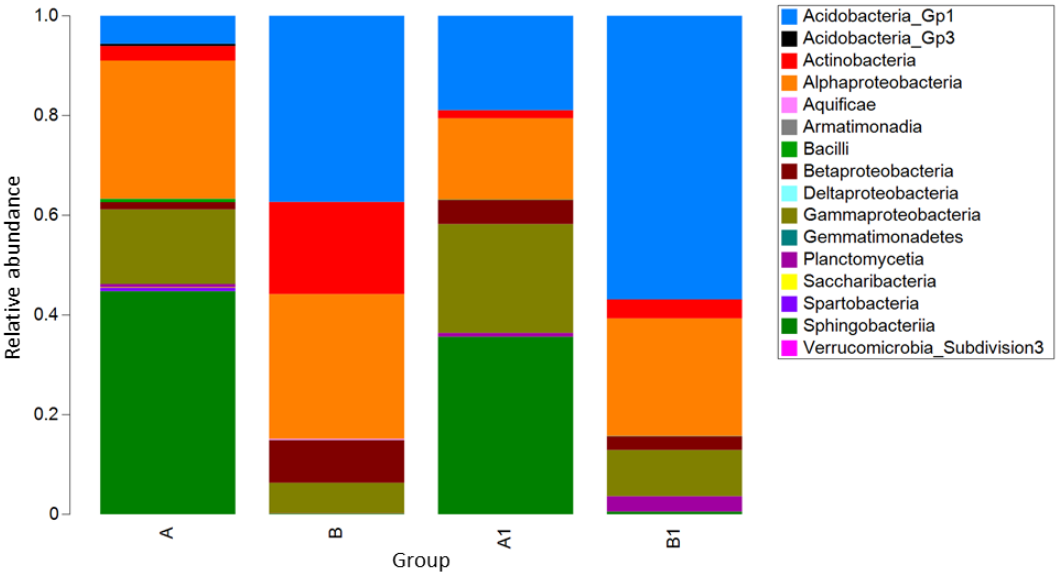

Figure 4.5: The composition of huhu grub gut bacterial classes in dietary groups
A - Grubs reared on cotton, B - grubs reared on pine, A1 - grubs switched to pine, B1 - grubs switched to cotton

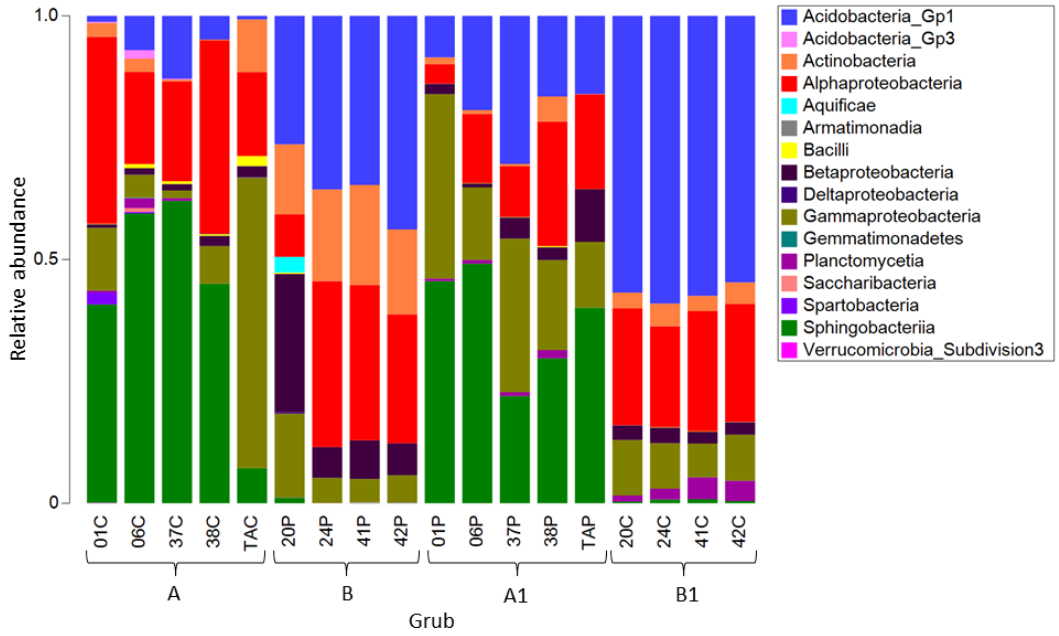

Figure 4.6: The composition of gut bacterial classes in huhu grubs
A - Grubs reared on cotton, B - grubs reared on pine, A1 - grubs switched to pine, B1 - grubs switched to cotton

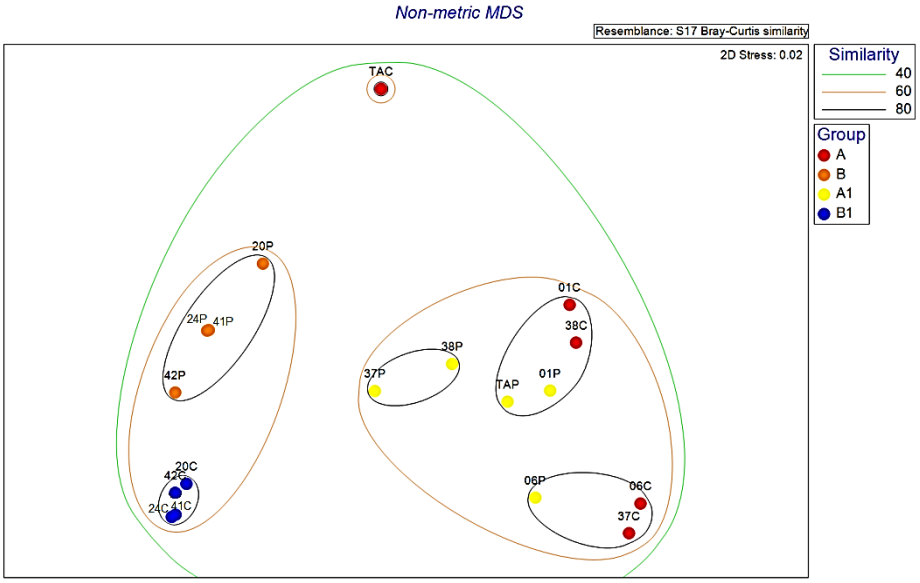

Figure 4.7: Non-metric multidimensional scaling (NMDS) ordination of bacterial community composition in the gut of huhu grubs based on Bray-Curtis similarity
A - Grubs reared on cotton, B - grubs reared on pine, A1 - grubs switched to pine, B1 - grubs switched to cotton. Grubs 24P and 41P overlap.

After switching to pine (A1) from cotton (A), the huhu grubs retained the same phylum-level composition (abundant phyla); however the community structure (phyla abundance) changed. The abundant phyla accounted for 97.8% of all the sequences in group A and 99.1% in group A1 (Figure 4.2). *Bacteroidetes* was the

most abundant phyla in group A representing 44.8% of all sequences, but was reduced to 35.6% after dietary switch. This phylum was represented by a single class of *Sphingobacteriia*. At class level, subsequent to the switch, there was a reduction in the abundance of *Alphaproteobacteria* in all of the grubs (Figure 4.6), from 27.7% in group A to 16.3% in group A1. Further, the switch resulted in an increase in the abundance of *Gammaproteobacteria* (21.8% from 14.9%) and *Betaproteobacteria* (4.9% from 1.5%) (Figure 4.5). Additionally, the dietary switch facilitated an increase in *Acidobacteria* from 6.0% (A) to 18.9% (A1) (Figure 4.3) with the increase almost being entirely accounted by class *Acidobacteria_Gp1* (Figure 4.5).

An increase in the abundance of *Acidobacteria* was noted in all of the grubs when transferred from cotton to pine, while. Subsequent to the dietary switch, a decrease in abundance of *Actinobacteria* was seen in grubs 01P, 06P and TAP. Again prior to the transfer, *Actinobacteria* comprised 10.8% of sequences in grub TAC which was reduced to only 0.1% of its sequences when transferred to pine. The phyla *Verrucomicrobia*, *Firmicutes*, *Planctomycetes*, *Candidatus Saccharibacteria*, *Armatimonadetes*, *Gemmatimonadetes*, and *Aquificae* together comprised 2.2% of total sequences in group A (Figure 4.3). However, after dietary switch phyla *Verrucomicrobia*, *Armatimonadetes* and *Gemmatimonadetes* were completely absent in group A1 and the other phyla accounted for only 0.9% of sequences in group A1 (Figure 4.3).

Huhu grubs grown on pine (B) and switched to cotton (B1) were collectively represented by nine phyla (Figure 4.3). *Proteobacteria*, *Acidobacteria* and *Actinobacteria* were abundant in both group B (pine) and B1 (cotton), with *Planctomycetes* only abundant in group B1. The four grubs switched to cotton (B1) from pine (B) underwent a reduction in abundance of *Proteobacteria* (Figure 4.4), with total group abundance reduced to 35.6% from 43.7% (Figure 4.3). At class level, this change could be accounted by a reduction in *Alphaproteobacteria* from 29.0% to 23.4% and *Betaproteobacteria* from 8.5% to 2.8%, and an increase in the abundance of *Gammaproteobacteria* from 6.1% to 9.3%. The abundance of *Actinobacteria* decreased in all four grubs, resulting in a total abundance of 3.8% on cotton compared to 18.5% when on pine (Figure 4.4). Whereas, *Acidobacteria* (represented almost entirely by class *Acidobacteria_Gp1* in all four grubs)

increased to 56.9% of total sequences on cotton while on pine they constituted only 37.3%. There was also an increased abundance of *Planctomycetes* in all four grubs and an overall abundance of 3.1% in group B1. *Aquificae* represented 3.2% of sequences in grub 20P, but was absent in the other group B grubs. Subsequent to the switch to cotton diet, this phylum was completely eliminated from the gut microbiome. There was minor representation (<0.5%) of phyla *Candidatus Saccharibacteria* and *Firmicutes* in a few grubs from both groups (B and B1) and *Verrucomicrobia* were only present in grub 20C (B1) representing a marginal 0.02% of its sequences.

The NMDS ordination plots at class level exhibited a minimum 40% similarity between the individual grubs all of the four groups (Figure 4.7). This was further supported by ANOSIM identifying some similarity between the groups with statistical significance R = 0.69, P = 0.01. Pairwise ANOSIM identified a high degree of similarity (R=0.14) between groups A (Cotton) and A1 (switched to pine) with low statistical significance P=0.2 and variation between groups B (pine) and B1 (switched to cotton) R=0.9, P=0.03. The variation between groups B and B1 is attributed mainly due to the rise of *Planctomycetia* as an abundant class after the dietary switch (Figure 4.2).

Abundance of the major unique OTUs (>1% TS) across the data set was examined to understand the dominant bacterial components and their distribution in each dietary group (Table 4.2). In total, 25 dominant OTUs grouped into nine families with most of them shared between all the dietary groups. The most abundant OTU *Dyella japonica* was found to be dominant (>1% of total sequences in a group) in all of the dietary groups. A considerable abundance of *Betaproteobacteria* (Figure 4.6) was observed in the grubs grown on the pine diet (including the switched ones, B and A1), mostly represented by OTUs belonging to *Burkholderia*.

Table 4.2: The identity and distribution of the most abundant OTUs (based on total sequences across all samples) in each dietary group

OTU	A	A1	B	B1	Family	Organism	Bit Score	% ID
1	D	D	D	D	*Xanthomonadaceae*	*Dyella japonica*	462	100
2	-	P	D	P	*Chitinophagaceae*	*Chitinophaga soli*	379	94
3	P	D	D	D	*Sphingomonadaceae*	*Sphingomonas polyaromaticivorans*	446	99
4	P	D	D	D	*Acidobacteriaceae*	*Terriglobus* sp. TAA 48	457	99
5	D	-	D	P	*Sphingobacteriaceae*	*Mucilaginibacter sabulilitoris*	429	98
6	P	P	P	D	*Acidobacteriaceae*	*Terriglobus roseus*	431	98
7	D	D	D	D	*Sphingomonadaceae*	*Sphingomonas* sp. HP9M	446	99
8	P	P	P	D	*Acidobacteriaceae*	*Terriglobus roseus*	420	97
9	D	-	-	P	*Chitinophagaceae*	*Chitinophaga cymbidii*	429	98
10	D	P	D	P	*Acidobacteriaceae*	*Edaphobacter modestus*	462	100
11	-	D	D	D	*Acetobacteraceae*	*Acidisoma sibiricum*	440	98
12	P	D	P	P	*Microbacteriaceae*	*Gryllotalpicola daejeonensis*	440	98
13	-	D	D	-	*Acidobacteriaceae*	*Acidipila rosea*	429	98
14	-	D	P	P	*Acidobacteriaceae*	*Acidobacteriaceae* bacterium 277	462	100
15	P	D	D	D	*Acidobacteriaceae*	*Terriglobus roseus*	444	99
16	-	P	D	P	*Xanthomonadaceae*	*Luteibacter* sp. CC13G	451	99
17	D	D	P	D	*Sphingomonadaceae*	*Sphingomonas* sp. HP9M	451	99
18	D	-	P	-	*Sphingobacteriaceae*	*Mucilaginibacter jinjuensis*	457	99
19	D	-	P	-	*Sphingomonadaceae*	*Sphingomonas sediminicola*	407	96
20	-	D	D	P	*Acidobacteriaceae*	*Acidipila rosea*	418	97
21	D	P	P	-	*Enterobacteriaceae*	*Enterobacteriaceae* bacterium 121A	457	99
22	-	-	D	-	*Chitinophagaceae*	*Chitinophaga soli*	374	94
23	P	P	P	D	*Acidobacteriaceae*	*Terriglobus roseus*	444	99
24	P	D	P	P	*Nocardioidaceae*	*Nocardioides oleivorans*	424	97
25	D	-	D	-	*Sphingobacteriaceae*	*Mucilaginibacter mallensis*	462	100

A - Grubs reared on cotton, B - grubs reared on pine, A1 - grubs switched to pine, B1 - grubs switched to cotton
D=dominant (>1% of total sequences in respective dietary group), P=present, - =absent

4.4 Discussion

This study comprehensively examined the complexity of bacterial communities in the gut microbiota of huhu grubs reared on cellulose or lignocellulose diets. Interestingly, in comparison to the gut fungal community of huhu grubs, the dietary switch had minor effects on the gut bacterial community structure. This could indicate that bacteria may not play a major functional role, and that fungi exert limited influence on bacterial community structure in this experimental setting. Previously it was suggested that diet was important in structuring the gut bacterial community, particularly for insect hosts that ingest lignocellulose-derived substances (Colman et al., 2012). However, the findings of our study contradict this existing notion. Bacterial diversity of lab-reared huhu grubs in this study was low, consistent with previous studies of the bacterial community of lab-reared insects (Geib et al., 2008). However, the lab-reared huhu grubs (pine and cellulose diets) shared the same abundant gut phyla (*Proteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes* and *Planctomycetes*) with previously characterised wild huhu grubs (Reid et al., 2011). Retention of the dominant phyla after dietary transfer is suggestive of a collective role of these phyla either in the degradation of cellulose (as cellulose is a common factor in both diets) or in survival of grubs by providing essential nutrients and amino acids required for the degradation of polymer carbohydrates. The absence of *Firmicutes* as an abundant phyla was unexpected, as previous studies on insect gut bacterial diversity have reported *Firmicutes* to be dominant (Yun et al., 2014). Additionally, *Firmicutes* were previously found to be dominant and metabolically active in the guts of wild huhu grubs (Reid et al., 2011) and was reported to be heavily involved in lignocellulose degradation in cockroaches (Bertino-Grimaldi et al., 2013). Since we had adequate sequencing coverage and the minor representation of *Firmicutes* was consistent in all the huhu grubs used in this study, it could be assumed that this phylum is not crucial for gut related metabolism when growing on cotton or pine diets.

One of the abundant phyla in this study, *Actinobacteria*, has been shown to be actively involved in forest litter decomposition (Snajdr et al., 2011), and has been suggested to play a role in hemicellulose degradation in cerambycids (Park et al., 2007). Further, the presence of *Actinobacteria* in insect guts has been widely

reported (Geib et al., 2009; Kaltenpoth, 2009; Lefebvre et al., 2009), and several studies seem to be suggestive of their role as defensive mutualists (Scott et al., 2008; Zucchi et al., 2012) providing protection against pathogenic bacteria (Kaltenpoth, 2009).

In this study, several OTUs belonging to *Acidobacteriaceae* (Table 4.2) grouping to genus *Terriglobus* and *Edaphobacter* were found to be dominant in all the dietary groups. Previously, strains of *Terriglobus* and *Edaphobacter* were reported to be capable of degrading xylan (Eichorst et al., 2011). Additionally, whole genome analysis of *Acidobacteria* indicated their capability to utilise plant polymers (Ward et al., 2009). Following dietary switch to pine (A1) from cotton (A) there was a significant increase in abundance of *Acidobacteria* (Figure 4.3) leading to speculation that these bacteria may be involved in plant polymer degradation.

The prevalence of *Bacteroidetes* in group A and A1 is consistent with their known ability to contribute to cello-oligomer degradation in the cellulose-hydrolysing process (Zhang et al., 2014). Further, these *Bacteroidetes* were exclusively of class *Sphingobacteriia* (Figure 4.2) and order *Sphingobacteriale*s. Several genes encoding various glycosyl hydrolases targeting cellulose and hemicellulose degradation have been identified in the *Sphingobacteriales* (Kanokratana et al., 2013). However, *Bacteroidetes* were minimally represented in the other two groups. This may be due to adverse interactions with fungi, or they were simply out competed by other bacteria due to their initial low abundance, while on a lignocellulose diet. The proliferation of *Planctomycetes* in group B1 could be attributed to a change in the pH of the huhu guts, as *Planctomycetes* are known to be prevalent in the alkaline gut of soil-feeding termites (Köhler et al., 2008).

Proteobacteria was one of the most abundant phyla in all of the huhu grubs, and a dominant Proteobacteria phylotype OTU 1 (Table 4.2), belonging to *Xanthomonadaceae*, matched to *Dyella japonica*. Similarly, a *Dyella* species was also the most dominant and metabolically active bacteria in the wild huhu gut (Reid et al., 2011). Furthermore, a *Dyella* species exhibiting cellulose activity has been previously isolated from termite guts (Cho et al., 2010). Their dominance in all four groups of grubs in this study, and the previously reported prevalence in wild huhu guts, makes a strong case for their role in the active degradation of

plant polymers. The microflora of the gut of insects utilising plant-derived sugar sources have been extensively studied. Similar to the results of this study (Table 4.2) the occurrence of *Acetobacteraceae* (*Alphaproteobacteria*) is widely reported in guts of diverse insects (Corby-Harris et al., 2014; Crotti et al., 2010; Robinson et al., 2010). This could be attributed to direct transmission, selective uptake by insects, or adaptation of *Acetobacteraceae* to colonize the gut of plant dependent insects (Engel & Moran, 2013). *Betaproteobacteria*, dominant in all the grubs, mostly grouped within the order *Burkholderiales* which are known nitrogen fixers (Elliott et al., 2007). Furthermore, a symbiotic relationship between *Burkholderia* and fungi has been proposed (Seigle-Murandi et al., 1996), with the bacteria suggested to be capable of utilizing the aromatic compounds liberated during lignocellulose breakdown by fungi (Laurie & Lloyd-Jones, 1999).

Although grubs in the respective dietary groups shared considerable similarity with respect to their gut bacteria, NMDS plot indicated some variation between grubs, with grub TAC being the most dissimilar to other grubs. The grub TAC was collected from a different location than its counterparts in the group and this could be the reason for its dissimilarity. With an exception of TAC, it is interesting to observe the intimate grouping of data from the grubs. Overall, the bacterial diversity in each grub seemed to be influenced by the microflora present during their collection explaining the individual variation in the gut bacterial community. However, transfer to a controlled environment along with quality of diet (defined diet with presence or absence of lignin) seems to drive a reduction in diversity (from that known to be present in wild huhu grubs), selecting for phyla that are adapted for survival, or are important for survival of the grubs under the experimental conditions. *Proteobacteria* and *Acidobacteria* seem to be present in high abundance regardless of diet (Figure 4.2), therefore these groups could be argued to be involved in the degradation of lignocellulose to an extent. However a lack of significant changes in the community after dietary switch, different to that observed in the gut fungal community, suggests that in the huhu grub the services provided by bacteria are mostly related to providing essential nutrients, nitrogen fixation and utilisation of cellulose.

4.5 Conclusions

This study identified the gut bacterial community of huhu grubs reared on defined diets. At the phylum level, the gut bacterial community of huhu grubs shared a reasonable similarity regardless of the nature of diet provided. The abundant phyla were similar to those previously reported in wood-feeding insects and constituted the majority of sequences. The bacterial community seemed to be unaffected by the selective pressure, or the bottleneck effect, evident in the fungal community after dietary switch. This may be because the bacteria play a largely secondary role to the fungi, being responsible for subsequent metabolism of substrate released by the fungi. A second possibility is that they play a different major role, such as nitrogen fixation, and this might mitigate changes in composition due solely to diet. Regardless of the diet provided, *Proteobacteria* and *Acidobacteria* were abundant in the gut of all huhu grubs in this study. This could be indicative of a supportive role of these phyla independent of the diet provided. Also, the abundant genus *Dyella* detected in this study was previously found to be dominant in the gut of wild huhu grubs, and is assumed to potentially play a role in cellulose degradation. Finally it could be argued that the bacterial diversity in each huhu grub gut, while being tolerant to the stress and selective pressures of dietary changes, is influenced mostly by the microflora present during their collection.

Chapter Five

Isolation and bioprospecting of lignocellulolyic microorganisms from huhu grub frass

Abstract

Lignocellulose degradation is the rate-limiting step in biofuel production from lignocellulosic biomass. The absence of an efficient biological pre-treatment method to remove lignin from lignocellulose is the major cause of this inefficiency. Therefore, the discovery of novel lignin-degrading microorganisms and enzymes could prove advantageous to the biofuel industry. In this study 44 lignocellulolytic isolates were isolated, using semi-selective media, from the frass of huhu grubs reared on lignocellulose and cellulose diets. Furthermore, bio-prospecting studies based on the oxidation activity of ABTS, a substrate for the laccase enzyme, resulted in the identification of a bacterium, *Acinetobacter* H23, capable of producing laccase. Laccases are known to degrade lignin, and identification of a bacterium capable of producing a potentially lignin-degrading enzyme could be of economic importance.

5.1 Introduction

Lignocellulose is the major structural component of all plants and an excellent substrate for bioethanol production (Himmel et al., 2007; Schubert, 2006). However, lignocellulose degradation is the rate limiting step in utilising the woody materials in biofuel production (Kumar et al., 2009). Industrially, notwithstanding the cost-effective release of fermentable sugars from plant biomass, the biological routes for lignocellulose degradation hold desirable advantages over current thermochemical ones. The major cause of the inefficiency in biological routes is the absence of an efficient biological pre-treatment method to remove lignin from lignocellulose (Kumar et al., 2009). Presently, several physicochemical pre-treatment technologies are used in disrupting the lignocellulose matrix to increase accessibility to cellulose and hemicellulose. The expensive nature and required energy inputs of these essential pre-treatments

reduces the appeal of using the biological methods for lignocellulose degradation. However, a biological approach to selectively remove lignin could potentially overcome these drawbacks, thereby offering an appealing cost efficient way of biofuel production.

In the previous chapters we presented a detailed view of the presence of a diet-specific microbial community. Hence it is natural to presume that some of the huhu gut microbes identified might possess lignocellulolytic capabilities. Furthermore, isolating and characterizing the microbial strains is necessary to understand the broad range of potentially economically important microbes and their involvement in lignocellulose degradation.

In this chapter we aim to identify and isolate potentially lignocellulolytic microorganisms using selective and minimal media coupled with screening techniques, with a specific focus on strains capable of producing lignin degrading enzymes.

5.2 Materials and Methods

Fresh frass was aseptically collected from healthy huhu grubs grown on cotton and pine diets (as described in Chapter Two). The frass collected from pine and cotton diets was pooled separately. Pooled frass was then homogenized using a disposable micro-pestle and used for culture-dependent gut microbial community analysis under aerobic and anaerobic growth conditions. Subsets of this homogenised frass were used for serial dilutions followed by plating. One gram of frass sample was diluted in 100 ml of sterile PBS solution (10^{-2} dilution) and further serially diluted down to 10^{-6}. For aerobic culturing, 100 µl of each dilution was inoculated onto selective media detailed below and incubated at 20, 25, 30 and 35 °C. For anaerobic culturing, 100 µl of each dilution was plated onto selective media to which 2 mg methylene blue was added as an oxygen indicator. The anaerobic samples were plated in an anaerobic glove box, transferred to an anaerobic jar system (BD GasPak system with GasPak Plus hydrogen plus carbon dioxide generator) and incubated at room temperature.

The following media and methods of preparation were used for the isolation of microbes from freshly collected huhu grub frass samples. Unless indicated otherwise, chemicals were obtained from Sigma Aldrich and all media were

sterilized by autoclaving for 20 minutes at 121 °C. The semi-selective media was composed of 0.5% KH_2PO_4, 0.67% Yeast nitrogen base (YNB), 0.51% $FeSO_4$ (APS AJAX Finechem, Australia), 1 ml/l trace elements solution, 2% agarose in distilled water with 0.2% carbon source, either larchwood xylan, carboxymethylcellulose (CMC), microcrystalline cellulose (MCC), kraft lignin or pine shavings. Two different fungal selective media were also used for isolation studies.

- Fungal selective media 1 (FSM1) - 1.5% malt extract, 1.8% agar, 0.2% yeast extract, 0.2 g/l chloramphenicol, 0.1 g/l streptomycin sulfate, 0.4 g/l cycloheximide (Sigma, USA) in distilled water. The media was sterilised by autoclaving for 20 minutes at 121 °C. The antibiotics chloramphenicol streptomycin sulfate and cycloheximide were filter sterilised through a 0.2 micron filter (Sartorius, Minsart) and added to the medium after it was autoclaved and had cooled to 50 °C.
- Fungal selective media 2 (FSM2) - 1.5% malt extract, 1.8% agar, 0.2% yeast extract, 0.2 g/l chloramphenicol, 0.06 g/l benlate in distilled water. This was autoclaved for 20 minutes and cooled to 45 °C before adding 0.1 g/l streptomycin sulfate, and 2 ml/l lactic acid (APS Ajax Finechem, Australia) in accordance to a modification from Worrall (1991).

5.2.1 Growth, morphological analysis and storage of isolates

Microbial growth on the inoculated agar plates was restreaked on fresh media plates until pure cultures were obtained. This was followed by preliminary morphological identification primarily based on macroscopic features of the isolates such as colony colour and mycelial habit (fungi). Pure cultures were then stored using the following method: Universal bottles containing 10 ml of TSB (tryptic soy broth) were inoculated with the isolate and incubated at their respective isolation temperature with shaking until visible growth was observed. Then, 800 µl of this culture was then transferred to a sterile microcentrifuge tube with 200 µl of glycerol, vortexed to distribute glycerol and stored in a -80 °C ultra-freezer (Thermo Electron Corporation).

5.2.2 DNA extraction and sequencing of isolates

Isolates were grown on TSB at 30 °C, and when adequate growth was confirmed,

the cells were spun down, and DNA extracted using the CTAB protocol detailed in chapter two.

Initially, the fungal ITS region was amplified using the primer set ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS 4R (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). Each reaction mixture contained 1x PCR buffer (Life Technologies, New Zealand), 0.2 mM dNTPs (Life Technologies), 0.02 mg/ml bovine serum albumin (BSA), 0.5 µM of each primer, 1 unit Platinum Taq (Life Technologies), 0.5 µM $MgCl_2$, 1 ng of template DNA, and the reaction was made up to 25 µl with milli-Q H_2O. Thermal cycling conditions were: Initial denaturation at 94°C for 3 min, 30 cycles of 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 90 sec, and a final extension of 5 min at 72 °C. Bacterial 16S rRNA gene fragments from the isolates were amplified using the primer set 27F (5'-GTGCCAGCMGCCGCGG-3') and 1492R (5'-ACGGGCGGTGTGTRC-3') (Lane, 1991). The reaction mixture used was similar to that for ITS amplification. The thermal cycling conditions were; 95 °C denaturation for 3 min, 25 cycles of 95 °C for 30 sec, 55 °C for 1 min, 72°C for 90 sec, with a final extension at 72 °C for 5 min. Agarose gel electrophoresis was used to visualise the amplified DNA. The PCR reactions were conducted in triplicate and quality control was ensured using positive, negative and extraction controls. Following PCR and electrophoresis, the triplicate amplicons of each isolate were pooled and purified using a QuickClean PCR Purification Kit (Genscript Corporation, New Jersey, USA) according to the manufacturer's instructions. Purified PCR products were sent to the University of Waikato DNA Sequencing Facility for Sanger sequencing. The sequences were then compared with the available sequences in GenBank using the basic local alignment search tool (BLAST)

5.2.3 Carbon utilization of the isolates

Isolates were also grown on a range of carbon substrates to determine their carbon utilization profile. Isolates were cultured individually at their respective isolation temperatures on minimal broth with substrates such as larchwood xylan, carboxymethylcellulose, microcrystalline cellulose, pine shavings and kraft lignin. For this experiment, isolates were grown independently in 250 ml flasks containing 50 ml of TSB, inoculated with individual colonies using a sterile loop.

Once adequate growth was confirmed (increase in OD), 30 ml of the culture was transferred from the flask to a 50 ml centrifuge tube and centrifuged at 3000 rpm for 5 min. The supernatant was removed and the pellets were washed twice and resuspended using 50 ml of yeast nitrogen base (YNB). Resuspended cells (50 µl) were used as the inoculum for culturing in 50 ml of each sterilized minimal media. The composition of the minimal media was as described below:
Minimal media: 0.5% KH_2PO_4, 0.67% YNB, 0.2% $FeSO_4$, 1 ml/l trace elements solution and 0.2% of the carbon source.

Minimal media were named M1 to M5 based on the supplemented carbon source: M1 (carboxymethyl cellulose), M2 (crystalline cellulose), M3 (larchwood xylan), M4 (pine shavings), M5 (kraft lignin). The pattern of growth was analysed using visual analysis, spectrophotometer and checking for morphological changes (reproduction, budding, multiplication) using a microscope.

5.2.4 Enzyme activity assays

Results of carbon utilisation of isolates on minimal media were taken into consideration while testing for cellulase, xylanase and ligninase activities. Only one representative of similar isolates were utilised for enzymatic assays. All assays were conducted in triplicate.

5.2.4.1 Screening for cellulose and xylanase activity using Congo red assay

Carboxymethyl cellulase and xylanase activity were tested qualitatively by a Congo red based plate assay. Aliquots of 5µl from a 48h old liquid culture were spotted onto 10% TSB agar containing either 0.5% CMC or xylan. These plates were incubated at 30 °C for 7–10 days before staining with 0.1% Congo red solution (Teather & Wood, 1982) followed by 2 washes in 1 M NaCl for an hour each. Further, plates were stained with 2% HCl for 5 min to develop better contrast and any indication of clearing was considered a positive result.

5.2.4.2 Screening of isolates for ligninolytic enzymes

(i) Laccase assay

Initially, the isolates and *Escherichia coli* (to be used as negative control for enzymatic assays) were grown in 30 ml TSB in 100 ml flasks at 32 °C with shaking at 100 rpm. Following the visual confirmation of growth, supernatants

were collected after centrifugation of 5 ml of cultures at 3500 rpm for 10 min. Supernatants were then used as crude enzyme extracts to screen for various ligninolytic enzyme activities.

Laccase activity assays were performed in a plate format using 96-well plates. During this assay, 50 µl of supernatant from the isolates and *E. coli* were transferred to a 96-well plate. Fresh solutions of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) at pH 3, 4, 5 and 6 were prepared using 0.1 M McIlvaine buffer (McIlvaine, 1921). A final ABTS concentration of 3 mM was achieved by adding 150 µl of the freshly prepared ABTS solution to the wells containing the supernatants (Huang et al., 2013). Absorption at 420 nm (ε_{420} = 36,000 $M^{-1}cm^{-1}$) was recorded every 15 min on a microplate spectrophotometer (Multiskan GO microplate spectrophotometer, ThermoScientific) for 24 h. The activity of positive samples was confirmed by performing the assay in triplicate and the crude extracellular enzyme activity calculated. The laccase activity was further subjected to inhibitory action by addition of sodium azide (0.1 mM) (Johannes et al., 2000a). Finally, putative laccase gene fragments were targeted using the degenerative primer set Cu1AF (5'-ACMWCBGTYCAYTGGCAYGG-3') (Kellner et al., 2008) and Cu4R (5'-TGCTCVAGBAKRTGGCAGTG-3') (Ausec et al., 2011). The reactions were done in triplicate and each reaction mixture contained 2.5 µl of each primer (20 µM), 0.5 µl of dNTP (10 µM), 3 µl of $MgCl_2$, 3 µl of BSA (10 mg/ml), 2.5 µl of PCR buffer, I U of Taq DNA polymerase (Life Technlogies), 80 ng of template DNA, and the reaction mixture was made to 25 µl with milli-Q H_2O. Thermal cycling conditions were 94 °C denaturation for 3 min, 30 cycles of 94 °C for 30 sec, 48 °C for 30 sec, 72°C for 60 sec, with a final extension at 72 °C for 5 min. The triplicates were pooled, run on a 1% agarose gel at 55 V and bands excised and column purified using a PureLink Quick Gel Extraction Kit (Life Technologies). The purified amplicons were sequenced at the Waikato DNA Sequencing Facility, and sequences were then compared with the available sequences using BLAST.

(ii) Manganese peroxidase assay

Manganese peroxidase activity was assayed according to Kuwahara et al., (1984). The standard reaction mixture contained 0.05 ml of 2 mM $MnSO_4$, 0.1 ml of 0.25 M sodium lactate, 0.2 ml of 0.5% bovine serum albumin, 0.1 ml of 0.1% phenol

red, 0.5 ml of culture supernatant and 0.05 ml of 2 mM H_2O_2 in 0.2 M sodium phosphate buffer (pH 8.0). The mixture was left at room temperature for 5 min and the reaction was ended with 0.04 ml NaOH 2 N. The absorbance was read at 610 nm.

(iii) Lignin peroxidase (LiP) assay

LiP activity was assayed by measuring the rate of H_2O_2-dependent oxidation of veratryl alcohol to veratraldehyde spectrophotometrically (Tien & Kirk, 1988). The standard 2 ml of reaction mixture contained 0.8 mM veratryl alcohol in 0.1 M sodium tartrate buffer (pH 3.0) and 1 ml of culture supernatant. The reaction was started by the addition of 150 mM H_2O_2 and the absorbance was monitored at 310 nm for 1 min.

5.3 Results

Investigation of the lignocellulolytic gut microbial community harboured by huhu grub larvae grown on pine and cellulose diets, using aerobic and anaerobic culturing on semi-selective media, resulted in 23 bacterial (Table 5.1) and 21 fungal isolates (Table 5.2). On average, colonies of bacteria and yeast appeared within two weeks, while other fungal strains on fungal selective media took three to four weeks. Single colonies were picked from the selective media and re-streaked a minimum of two times to ensure isolation of pure colonies. Initially, fungal and bacterial isolates were distinguished by checking their morphology by microscopy. Further, the identity of the 44 isolates was determined by sequencing of the 16S rRNA gene of bacterial and ITS of fungal isolates. Sequences were then compared with sequences in GenBank (Benson et al., 2005) using BLAST.

Each of the isolates was individually cultivated on minimal media supplied with a sole carbon source, either lignin, cellulose or hemicellulose. Only isolates belonging to the genus *Raoultella* were successfully grown under anaerobic conditions. Isolates were examined for their lignocellulolytic degrading properties by cultivation in minimal broth using five different carbon sources to reveal their growth patterns (Table 5.3). The 19 isolates that were used for this carbon utilisation study produced similar results for xylanase and cellulose activity with the Congo red assay, with the exception of the fungus *Pithomyces chartarum* which showed no CMCase activity.

Table 5.1: Bacterial isolates from pine and cotton reared huhu grubs

Isolate	Diet	SM	Temp (°C)	Top blast match	% ID	Overlap	Bit score
01B	Cotton	CMC	25	*Paenibacillus* sp	99	100	1242
02B	Cotton	Xylan	25	*Paenibacillus* sp	99	100	1382
03B	Pine	CMC	30	*Bacillus thuringiensis*	100	100	1203
04B	Pine	CMC	25	*Bacillus thuringiensis*	100	100	1061
05B	Cotton	CMC	30	*Bacillus cereus*	97	100	481
06B	Pine	CMC	30	*Bacillus thuringiensis*	100	100	1290
07B	Pine	PS	30	*Bacillus thuringiensis*	100	100	1075
08B	Cotton	CMC	25	*Microbacterium oxydans*	100	100	1463
09B	Cotton	CMC	30	*Microbacterium* sp	100	100	1088
10B	Pine	CMC	30	*Burkholderia cepacia*	98	100	1129
11B	Pine	CMC	35	*Burkholderia cepacia*	98	100	1103
12B	Pine	CMC	35	*Sphingobacterium* sp	100	100	1201
13B	Pine	CMC	30	*Rahnella aquatilis*	100	100	1205
14B	Cotton	CMC	30	*Rahnella aquatilis*	100	100	1280
15B	Pine	PS	30	*Enterobacter* sp	99	100	1212
16B	Pine	Xylan	25	*Enterobacter* sp	99	100	1157
17B	Pine	CMC	30	*Enterobacter* sp	99	100	968
18B	Cotton	CMC	35	*Enterobacter* sp	99	100	905
19B	Pine	CMC	30	*Raoultella ornithinolytica*	100	100	1354
20B	Pine	Xylan	30	*Raoultella ornithinolytica*	100	100	1216
21B	Cotton	PS	35	*Raoultella ornithinolytica*	100	100	1338
22B	Pine	CMC	30	*Raoultella ornithinolytica*	100	100	1343
23B	Pine	Xylan	30	*Acinetobacter* sp	99	100	2575

SM = selective media, CMC = carboxymethyl cellulose, Xylan = larchwood xylan, PS = pine shavings.

Table 5.2: Fungal isolates from pine and cotton reared huhu grubs

Isolate	Diet	SM	Temp(°C)	Top blast match	% ID	Overlap	Bit score
01F	Pine	Xylan	25	*Pithomyces chartarum*	99	100	959
02F	Pine	PS	25	*Penicillium brevicompactum*	100	100	917
03F	Pine	Xylan	30	*Penicillium brevicompactum*	100	100	913
04F	Pine	PS	30	*Penicillium brevicompactum*	100	100	902
05F	Pine	CMC	30	*Talaromyces purpurogenus*	100	100	924
06F	Pine	CMC	25	*Talaromyces ruber*	100	100	920
07F	Pine	Xylan	25	*Talaromyces purpurogenus*	100	100	926
08F	Pine	MCC	25	*Trichoderma cf. harzianum*	100	100	985
09F	Cotton	CMC	25	*Rhodotorula glutinis*	100	100	649
10F	Pine	Xylan	25	*Scheffersomyces shehatae*	99	100	822
11F	Cotton	Xylan	20	*Scheffersomyces shehatae*	100	100	1018
12F	Pine	Xylan	25	*Scheffersomyces shehatae*	100	100	1018
13F	Pine	Xylan	25	*Scheffersomyces shehatae*	100	100	1022
14F	Pine	Xylan	25	*Scheffersomyces shehatae*	100	100	1027
15F	Pine	Xylan	25	*Scheffersomyces shehatae*	100	100	915
16F	Pine	Xylan	25	*Scheffersomyces shehatae*	99	96	1144
17F	Pine	Xylan	25	*Scheffersomyces shehatae*	100	100	935
18F	Cotton	Xylan	25	*Candida shehatae*	100	100	1014
19F	Cotton	Xylan	25	*Candida shehatae*	100	100	1048
20F	Cotton	Xylan	25	*Candida shehatae*	100	100	1022
21F	Pine	Xylan	25	*Candida shehatae*	100	100	1024

SM = selective media, CMC = carboxymethyl cellulose, MCC = microcrystalline cellulose, Xylan = larchwood xylan, PS = pine shavings.

Table 5.3: Growth patterns of isolates in minimal media with different carbon sources

Isolate	Top blast match	CMC	MCC	Xylan	PS	KL
01B	*Paenibacillus* sp	+	W	+	W	-
04B	*Bacillus thuringenesis*	+	-	+	W	-
05B	*Bacillus cereus*	W	-	-	-	-
08B	*Microbacterium oxydans*	W	-	+	-	-
09B	*Microbacterium* sp	+	-	+	W	-
10B	*Burkholderia cepacia*	W	-	+	-	-
12B	*Sphingobacterium* sp	+	-	+	W	-
13B	*Rahnella aquatilis*	+	-	+	-	-
15B	*Enterobacter* sp	+	-	+	W	-
19B	*Raoultella ornithinolytica*	W	-	+	W	-
23B	*Acinetobacter* sp	+	-	+	W	-
01F	*Pithomyces chartarum*	W	-	W	-	-
02F	*Penicillium brevicompactum*	W	W	+	+	-
05F	*Talaromyces purpurogenus*	+	-	+	+	-
06F	*Talaromyces ruber*	+	-	+	+	-
08F	*Trichoderma harzianum*	+	W	+	+	-
09F	*Rhodotorula glutinis*	+	-	W	W	-
10F	*Scheffersomyces shehatae*	-	-	+	W	-
18F	*Candida shehatae*	-	-	+	W	-

CMC = carboxymethyl cellulose, MCC = microcrystalline cellulose, Xylan = larchwood xylan, PS = pine shavings, KL= kraft lignin
- = no growth, W = weak growth, + = strong growth

Of the 44 isolates grown in TSB, the supernatant of isolate 23B became visibly green (Figure 5.1) following exposure to ABTS and an increase in absorbance at 420 nm was observed over time. This was a positive indication of laccase activity. The enzyme was extracellular in nature as the supernatant was obtained without cell lysis. No colour change was observed with other isolates or with negative controls. The secreted extracellular laccase activity of isolate 23B was tested and found to be active at a pH range 3-6. Absence of activity in the supernatant of isolate 23B after the addition of sodium azide as an inhibitor (Johannes & Majcherczyk, 2000b) along with no activity in the isolate control (boiled supernatant of isolate 23) and *E. coli* ruled out a false positive (Figure 5.2). An activity in comparison to the experimental controls was noted and the crude enzyme activity of supernatant from isolate 23B was calculated to be 45.9 nM/min/ml.

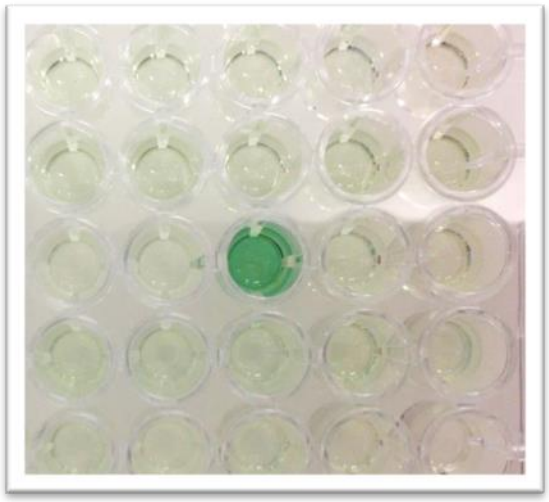

Figure 5.1: ABTS assay showing evidence for laccase activity in bacterial isolate 23B

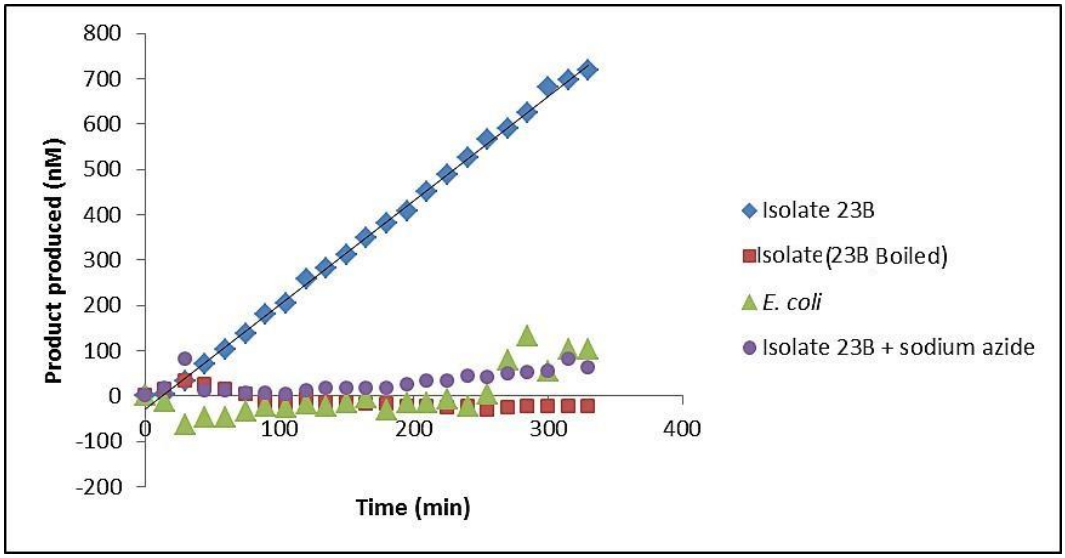

Figure 5.2: Comparison of laccase activity between isolate 23B and negative controls.

A putative laccase gene sequence was amplified from isolate 23B using laccase-specific primers, and the sequence was compared with available sequences in GenBank using BLAST. The sequence closely matched (93% sequence identity) with a region coding for a multicopper oxidase in *Acinetobacter baumannii* D26 (accession number: CP012952.1, Figure 5.3).

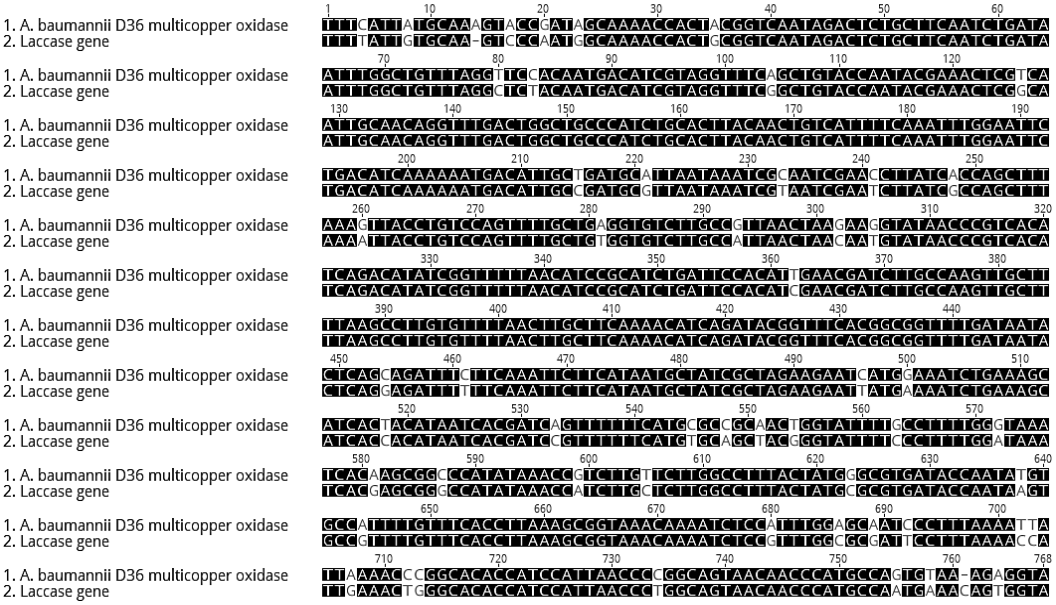

Figure 5.3: Nucleotide pairwise alignment of *Acinetobacter baumannii* D36 multicopper oxidase (accession number: CP012952.1) and putative laccase gene from isolate 23B generated using Geneious 7 (Kearse et al., 2012).
The alignment shows a 93% sequence identity. Black = 100% similar, grey = 65-100% similar, white = less than 65% similar

In order to identify the phylogenetic relationship of the putative laccase-producing isolate 23B to known related species, nucleotide sequences of 16S rRNA genes from the isolate and closely related taxa (identified via blastn), and including 26 representative sequences from family Moraxellaceae (genera *Acinetobacter*, *Alkanindiges*, *Enhydrobacter*, *Moraxella*, *Paraperlucidibaca*, *Perlucidibaca* and *Psychrobacter*) were aligned with MUSCLE (Edgar, 2004) and phylogenetic analysis was conducted using neighbour-joining in Geneious (Geneious R7, Kearse et al., 2012) with the Tamura-Nei distance model (Tamura & Nei, 1993).

The neighbour-joining phylogenetic tree (Figure 5.4) showed high bootstrap support for isolate 23B associating with other *Acinetobacter* species. Particularly, it shared the closest evolutionary distance to *Acinetobacter* sp. isolate S3.MAC.013 (HM063913.1) originating from Brazilian Atlantic forest soil (Bruce et al., 2010). Isolate 23B is also closely related to the characterised species *A.*

baumannii, A. calcoaceticus, and *A. rhizosphaerae*. Since both BLAST and phylogenetic analysis confirm that isolate 23B is an *Acinetobacter* it was renamed *Acinetobacter* H23. None of the fungal and bacterial isolates tested positive for Mn peroxidase or lignin peroxidase activity.

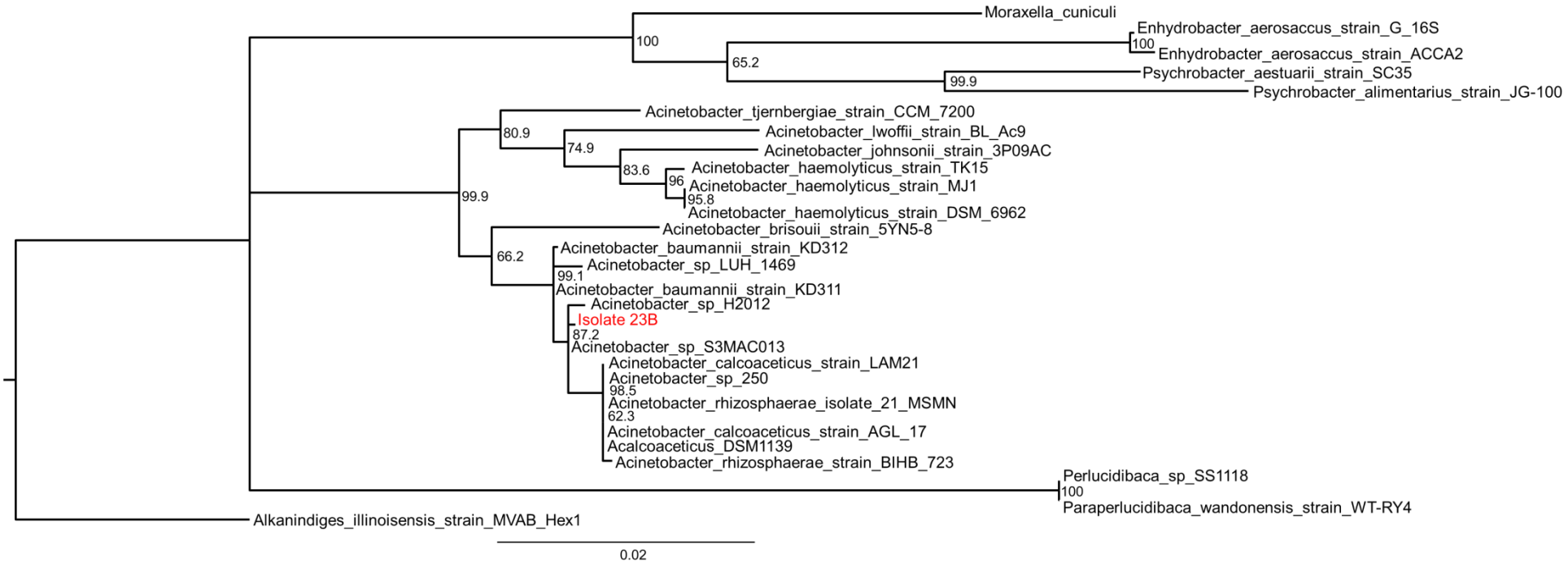

Figure 5.4: The neighbour-joining tree identifying the phylogenetic relationship between bacterial isolate 23B (marked in red) and 26 members of family Moraxellaceae.

Branches corresponding to partitions that were reproduced in less than 60% of bootstrap replicates (1000 replicates) were collapsed and the bootstrap values are indicated at the branch points

5.4 Discussion

In the present study, huhu grub gut bacteria and fungi with lignocellulose degrading capabilities were isolated. Significantly, a bacterial isolate which produced a laccase was identified and isolated. The huhu grubs used in this study were able to grow and survive on lignocellulose diets apparently supported by their gut microbes.

Laccases, due to their huge substrate range and oxidation properties, have always been of immense research interest. However, most research has primarily focussed on fungal laccases, while bacterial laccases are an emerging field of interest. Laccases of fungal origin have been extensively studied due to their abundance, high redox potential, catalytic efficiency towards several substrates and possible biotechnological applications such as transformation or immobilisation of xenobiotic compounds (toxic chemical wastes, pesticides, nitroaromatics etc) (Kunamneni, Camarero, et al., 2008), dye degradation (Abadulla et al., 2000; Kunamneni, Ghazi, et al., 2008) and bioremediation of toxic chemical wastes (Alcalde et al., 2006). However, the glycosylated nature of these enzymes and difficulties in cultivation and genetic manipulation of fungi make production of fungal laccases expensive, difficult, and time consuming (Brijwani et al., 2010). Meanwhile, bacterial laccases are unaffected by these constraints and hence hold several advantages over their fungal counterparts. High levels of enzyme production at reasonable cost is achievable with bacterial laccases as their heterologous expression in *E. coli* is simpler (Francis & Page, 2010). Unlike fungal laccases, bacterial laccases do not require post-translational modifications like glycosylation and are acquiescent to improvements (Santhanam et al., 2011). Additionally, bacterial laccases possess a wider pH range, exhibit higher thermal stability and generally are more tolerant towards high salt concentrations, organic solvents, and common laccase inhibitors (Bugg et al., 2011; Dwivedi et al., 2011; Margot et al., 2013; Reiss et al., 2011; Sharma et al., 2007; Suzuki et al., 2003). Furthermore, the putative functions of bacterial laccases are diverse and could be suitable for several potential industrial and biotechnological applications including bioremediation, degradation of wood to facilitate biofuel production and bleaching (Margot et al., 2013).

This discussion primarily focuses on the laccase expressed by strain *Acinetobacter*

H23, belonging to the order *Pseudomonadales*. The laccase activity was detected using an ABTS assay during bioprospecting studies involving several isolates obtained from huhu grubs. The crude enzyme extract from *Acinetobacter* H23 was analysed and the presence of at least one laccase gene was identified using targeted sequencing. Since *Pseudomonadales* is known to be dominant in early stages of wood degradation in nature (Kielak et al., 2016; Noll et al., 2010), it is tempting to speculate about the possible utilisation of this laccase in the degradation of wood. Furthermore, phylogenetic analysis revealed that the closest reported match of isolate *Acinetobacter* H23 was found in Brazilian Atlantic forest soil considered to be a vast reservoir for lignocellulolytic microbes (Bruce et al., 2010).

In a bioinformatic analysis identifying 1200 putative genes for laccase-like enzymes (Ausec et al., 2011) it was noted that the three domain bacterial laccases could be divided into two groups based on the sizes of individual domains and the whole proteins. The larger group of enzymes consisted of well-studied enzymes like CotA from *Bacillus subtilis,* and the smaller group accounted for 16% of the observed three domain laccases. The genus *Acinetobacter* belonged to the second group with no known characterised representatives.

Although extensive characterisation of *Acinetobacter* H23 was not undertaken, the experiments conducted were adequate to understand the significance of this particular isolate. Important characteristics of strain *Acinetobacter* H23 included the extracellular nature of the laccase, the ease of strain cultivation, and activity in low pH range, all of which are indicative of promising industrial potential and biotechnological application. To date, only a few bacterial laccases have been characterised in detail and most of the identified ones are intracellular or spore-bound (Margot et al., 2013; Sharma et al., 2007). Although most enzymes are substrate-specific, laccases act on a wide spectrum of substrates including, but not limited to, polyphenols, diphenols, benzenethiols and aromatic amines. Therefore, it is important to understand the substrate specificities and characteristics of the laccase produced by *Acinetobacter* H23. Discovery of novel extracellular bacterial laccases with increased pH, substrate range and stability is of high significance to several enzymatic processes involved in pulp, biofuel, textile and paper industries.

Several bacteria and fungi with lignocellulolytic capabilities were isolated and identified during this study. The results observed were expected as these microbes were isolated using semi-selective media from the frass of huhu grubs reared exclusively on lignocellulose and cellulose diets. With the exception of *Acinetobacter* H23, no other microbe capable of producing putative lignin degrading enzymes was isolated. This is understandable as generally the cultivable fraction of microbes present in an environmental sample is very low (Amann et al., 1995). Additionally, the isolated microbes with lignocellulolytic abilities only provide us with an opportunity to characterise the microbe, and the lignocellulolytic characteristic expressed is not an indication of a similar function inside the huhu grub gut. However as shown in this chapter, culture based methods while being selective towards a particular microbial population are essential to identify novel microbes and enzymes. Identification of a bacterial isolate capable of producing putative lignin-degrading enzymes from the frass of these lignocellulose reared huhu grubs is promising for biofuel and wood based industries.

5.5 Conclusions

Bioprospecting studies conducted on the frass of lab-reared huhu grubs resulted in the isolation and identification of a bacterium capable of producing extracellular laccase. The laccase activity of this isolated strain, *Acinetobacter* H23, was confirmed through well-established assays followed by confirmation of the presence of a laccase gene. The limited characterisation performed indicates a potential for industrial applications. However, further characterisation of this laccase as well as identification of the substrate range and viable mediators is necessary to credibly evaluate the potential use and value of this enzyme for biotechnology. Furthermore, the established methodology of growing huhu grubs on defined diets and utilising frass from several stages of their lifecycle could be useful for the isolation of economically important microbes.

Chapter Six

Conclusions and future directions

6.1 Conclusions

Recently, insect-microbe interactions have gained an increased interest through the use of high-throughput sequencing techniques. Previously, the gut components of wild huhu grubs have been studied to understand the gut microbial community (Reid et al., 2011) . However, the research utilised huhu grubs feeding on decayed woods in the wild and was focused on the gut bacterial population. While this provided information on the gut bacterial community, the approach was limited in its explanation of the overall gut microbial population involved in lignocellulose degradation within the huhu gut. Firstly, the use of huhu grubs from their natural habitat (degraded wood) meant that free carbohydrates were available to the grub and they would not necessarily require specific lignocellulose or cellulose degraders in their gut. Secondly, the study focused heavily on bacteria and failed to provide significant details about the gut fungal population and therefore ignored the possibility that a consortium was responsible for lignocellulose degradation. Finally, the study utilised a gut homogenate of several wild huhu grubs and therefore failed to provide any understanding of shared OTUs between grubs which may have provided an insight into functional diversity between individual grubs.

The goal of our study was to address the knowledge gaps identified in previous research exploring the gut microbial community of huhu grubs (Reid et al., 2011). To this end we reared huhu grubs on non-degraded pine blocks (lignocellulose) or cotton (cellulose) diets. Utilising these non-degraded defined diets we aimed to explore the lignocellulose and cellulose diet driven gut fungal and bacterial community in huhu grubs. Additionally, utilisation of frass (excretia) as the study material (gut proxy) allowed us to keep the grubs alive. This facilitated the interchanging of diets to understand the effect of dietary switch on the gut microbiota of individual huhu grubs.

The progress of the experimental model was dependent on several primary questions (answered in Chapter 2): **a)** whether defined diets (cellulose or

lignocellulose) are adequate for huhu grubs to grow healthily and complete their life cycle, this was important as the planned study involved dietary manipulations for which keeping the grubs alive was vital; **b)** Could enough study material (insect frass) be obtained on both defined diets; **c)** Is there a pattern to be observed in the gut microbial community of huhu grub populations grown on different defined diets. Knowledge gained from the preliminary batch of huhu grubs reared on cellulose and lignocellulose diets was used to optimise the sampling and rearing criteria for the second batch of grubs. This optimisation played a crucial role in establishing a healthy group of huhu grubs and ensured that the grubs could grow healthily and metamorphose on both cellulose and lignocellulose diets. An ARISA based community analysis of both the fungal and bacterial huhu gut population was undertaken on the second batch of grubs along with modifications in rearing conditions. The ARISA data supported the hypothesis that at least within the fungal community there is a significant difference between grubs reared on cellulose and those reared on lignocellulose diets. However, no significant difference was seen between the bacterial gut communities of huhu grubs reared on these two diets. While proving crucial in understanding the difference in gut community between the defined diets, ARISA lacked the resolution to determine the specific microbes and their populations while on different diets. The first two batches were essential in streamlining the experimental design and the information gained was used for selection and growth of the third batch of huhu grubs.

High-throughput sequencing allowed taxonomic analysis of gut bacterial and fungal communities of huhu grubs (third batch) grown on cellulose and lignocellulose diets (Chapters 3 and 4). Practically all the grubs used in the study came from the same tree stump (diet) and were of the same age (size and weight), so an assumption could be made that they all entered the experiment with much the same gut flora. After their collection from the wild, the huhu grubs transferred to cotton (cellulose) would have encountered a more dramatic substrate shift than those transferred to pine (lignocellulose), so our original contention was that we would see a reduction in diversity in the cotton grown grubs. Later, when the pine-reared grubs were transferred to cotton we might have seen a similar decrease in diversity. However, when the cotton grubs are transferred to pine we might not see an equivalent gain in diversity since some of the specialist members

selected for lignocellulose degradation might have been eliminated from the gut and could not therefore re-establish.

The assessment of fungal community composition confirmed that the cellulose-reared huhu grubs were significantly distinct from the lignocellulose-reared grubs. This difference was mainly attributed to a bottleneck effect due to the presence of lignin and hemicellulose in the provided diet. This is evident from NMDS (Fig 3.7) where the group of grubs grown on cellulose (A) with different individual gut fungal communities grouped together when shifted to lignocellulose (A1). Additionally, the grubs initially grown on lignocellulose (B) seemed to share reasonable similarity in the gut fungal community. The defined diets had a selective pressure on the gut fungal community of the huhu grubs. The gut community of cellulose fed huhu grubs were dominated by yeasts including members of genera *Trichosporon* and *Cryptococcus* known to be involved in cellulose degradation (Štursová et al., 2012). Similarly, the huhu grubs shifted to cellulose from lignocellulose resulted in *Rhodotorula,* basidiomycete yeast known to be capable of producing endoglucanases (Kasana & Gulati, 2011), accounting for almost 22% of the gut fungal sequences, while previously constituting less than 0.1% when grubs were fed lignocellulose. The gut fungal community of lignocellulose-fed huhu grubs seemed to have a more functionally diverse fungal population with several yeasts, soft rots and potential hemicellulose and cellulose degraders. The gut fungal community observed in huhu grubs collected from the wild and grown on pine (lignocellulose), and that of grubs shifted to pine from cotton (cellulose) was different. This could be due to the elimination from the gut of the specialist members involved in lignocellulose degradation, mainly due to a lack of requirement while on a cellulose diet. Therefore, while shifting to lignocellulose from cellulose an alternative consortium with similar functionality (ability to degrade lignocellulose) takes the place of specialist members. Regardless of the species, if the specific consortium can achieve the requirement for the grub (breaking down of lignocellulose) it will succeed and gain a niche. The same process is thought to occur in the human gut where species diversity is huge but functional diversity is more constrained, that is many species carry out essentially the same job (David et al., 2014; Graf et al., 2015).

At the phylum level, the gut bacterial community of huhu grubs (3rd batch) shared a reasonable similarity regardless of the nature of diet provided. The bacterial community seemed to be unaffected by the selective pressure, or the bottleneck effect, evident in the fungal community after dietary shift. This may be due to bacteria playing a largely secondary role to the fungi, and are responsible for subsequent metabolism of released substrate by the fungi, and as such they "see" a less changed substrate diversity than apparent in that provided to the grubs. A second possibility is that they play a different major role, e.g., nitrogen fixation, and this might mitigate changes in composition due solely to diet. Regardless of the diet provided, *Proteobacteria* and *Acidobacteria* were abundant in the gut of all huhu grubs in this study (Chapter 4). This could be indicative of a supportive role of these phyla regardless of the diet provided. Overall, it could be argued that the bacterial diversity in each huhu grub gut is influenced mostly by the microflora present during their collection rather than selective pressure from the diet.

Bioprospecting studies (Chapter 5) from cellulose- and lignocellulose-reared huhu grub frass resulted in the identification of several lignocellulolytic bacteria and fungi. These results were expected as microbes were isolated using semi-selective media from the frass of huhu grubs reared exclusively on lignocellulose and cellulose diets. However, of particular interest was a bacterium *Acinetobacter H23* identified to be capable of producing an extracellular laccase. The isolation of *Acinetobacter H23* and the properties of the isolate identified during limited characterisation have led to a research initiative in cooperation with the School of Engineering at University of Waikato. In the immediate future we aim to fully characterise the laccase-producing isolate *Acinetobacter H23* followed by complete characterisation of the laccase enzyme produced. We intend to determine the substrate preferences, optimal reaction conditions and molecular cloning of the laccase-encoding gene for heterologous over-expression in *E. coli*. Overall, the work will be aimed at assessing the possible applications of the laccase produced and exploring the viability of cost-effective large scale production of the enzyme.

Collectively, this work has demonstrated that the fungal ecology of the huhu grub gut, when reared on cellulose and lignocellulose, is driven by the nature of the

substrate provided. However, the community patterns suggested that diet has a very limited effect on the gut bacterial community. This demonstrates the need to focus on the whole microbiome to understand insect-microbial interactions, rather than looking at either the bacterial or fungal community in isolation.

6.2 Future directions

While the work has identified consortia of microorganisms that could be responsible for lignocellulose degradation in lab reared huhu grubs, understanding the role of individual microbes and the mechanisms involved in lignocellulose degradation in the huhu grub should be the subject of future research. The rearing technique utilised followed by high-throughput sequencing and isolation studies could be used as a model to understand the substrate relevant communities of other wood-feeding insects, or for further study of huhu grubs. This could be followed by targeted isolation of the most abundant microbes. The isolation and cultivation of desired species is a critical initial step that could pave the way towards proteomic, genomic and transcriptomic analyses. However, cultivation of microorganisms remains a challenging process. Therefore a metagenomics approach could offer a compelling alternative. The recovery of functional genes, especially of the abundant community could reveal the interactions between the huhu grub and the microbiome and shed light on the involvement of microorganisms in lignocellulose degradation.

Additionally, the meta-transcriptome of the huhu grub reared on defined diets could be studied to identify genes that are over-expressed during lignocellulose or cellulose degradation. Several ongoing approaches aim to find an efficient consortium or a single microorganism to be utilised in biofuel production (Alper et al., 2009). Compared to fungi, bacterial consortia are easy to grow and manipulate. Several lignocellulolytic bacteria were isolated during bioprospecting studies. Therefore an interesting approach would be to treat the lignocellulose-reared huhu grubs with a gradually increasing dosage of fungicide with an aim to eliminate the fungal population. If successful, the grubs would retain a bacterial community that could potentially degrade the lignocellulose. Isolating the members of this community will possibly result in the discovery of novel isolates and enzymes.

Another approach would be to rear huhu grubs on a cotton diet and then switch them to a diet which includes extracted pine-lignin mixed with cellulose (cotton). This could further help in analysing the feeding behaviour of the huhu grub, as well as changes in the huhu gut fungal diversity of the huhu grubs to establish if any potentially ligninolytic species will re-appear in the gut when access to their presumed major substrate (lignin) is readily available.

Finally, the isolation of *Acinetobacter H23* and the properties of the isolate identified during limited characterisation have led to a research initiative in cooperation with the School of Engineering at University of Waikato. In the immediate future we aim to fully characterise the laccase-producing isolate *Acinetobacter H23* followed by complete characterisation of the laccase enzyme produced. We intend to determine the substrate preferences, optimal reaction conditions and molecular cloning of the laccase-encoding gene for heterologous over-expression in *E. coli*. Overall, the work will be aimed at assessing the possible applications of the laccase produced and exploring the viability of cost-effective large scale production of the enzyme.

The continued research on the gut microbial community in huhu grubs will increase our understanding of the processes involved in lignocellulose degradation and will help to identify the key microbes and genes involved. This work was crucial in providing a foundation and direction for a comprehensive approach towards understanding the gut microbes of huhu grubs grown on defined diets. These studies are well timed as there is an increasing need for waste recycling and alternative fuel production. Understanding the structure and composition of the microbial consortia involved in lignocellulose degradation inside insect guts, along with their optimal microenvironment, could prove to be significant for the biofuel industry.

Appendices

7.1 Supplementary material for chapter two

Supplementary Figure 7.1: Metamorphosed huhu grub on preliminary trial on cotton

Supplementary Figure 7.2: Tunnels bored by huhu grub on pine block

7.2 Supplementary material for chapter three

Supplementary Table 7.1: The lengths and weights of huhu grubs used in this study prior to their transfer to defined diets

Name	Initial length (cm)	Initial weight (g)
01C	3.7	1.64
06C	3.8	1.65
37C	3.7	1.64
38C	3.7	1.68
TAC	3.8	1.65
20P	3.9	1.7
24P	3.5	1.61
41P	3.6	1.63
42P	3.6	1.67

Supplementary Table 7.2: Taxonomy and distributions of shared gut fungal OTUs in huhu grubs reared on cotton diet

OTU	01C (%)	06C (%)	37C (%)	38C (%)	TAC (%)	Pooled (%)	Species
1	0.00	0.43	0.13	0.12	0.24	0.08	*Schwanniomyces occidentalis*
2	0.00	0.12	0.22	0.02	0.18	0.05	*Candida chilensis*
3	0.02	0.63	0.06	0.01	0.03	0.05	Unidentified
4	0.01	0.04	0.03	0.02	0.18	0.02	*Candida chilensis*
Total	0.03	1.23	0.45	0.17	0.64	0.20	

Supplementary Table 7.3: Heat map detailing the taxonomy and distributions of shared gut fungal OTUs in huhu grubs reared on pine diet (B)

OTU	20P (%)	24P (%)	41P (%)	42P (%)	Pooled (%)	Species
1	0.29	14.73	20.61	24.51	15.04	*Scytalidium* sp.
2	1.73	5.81	9.48	14.20	7.47	*Scytalidium lignicola*
3	25.59	0.31	5.60	2.05	7.16	*Penicillium spinulosum*
4	0.03	16.36	0.08	0.10	5.91	*Schwanniomyces occidentalis*
5	9.47	3.44	4.34	7.88	5.76	*Schwanniomyces occidentalis*
6	1.51	3.36	7.85	11.35	5.60	*Scytalidium lignicola*
7	7.72	3.29	3.87	6.28	4.93	*Schwanniomyces occidentalis*
8	5.13	2.05	2.84	3.79	3.22	*Scheffersomyces henanensis*
9	0.07	7.27	0.14	0.02	2.66	*Schwanniomyces occidentalis*
10	3.76	1.10	2.91	3.45	2.54	*Schwanniomyces occidentalis*
11	2.58	1.52	2.85	3.37	2.42	*Scytalidium lignicola*
12	8.35	0.12	1.71	0.66	2.32	*Penicillium spinulosum*
13	0.18	5.36	1.21	0.25	2.30	*Scheffersomyces henanensis*
14	7.55	0.17	2.12	0.47	2.23	*Penicillium spinulosum*
15	1.15	2.96	1.73	1.56	2.02	*Scheffersomyces henanensis*
16	0.27	0.31	6.20	0.36	1.74	*Schwanniomyces occidentalis*
17	0.07	4.02	0.68	0.06	1.63	*Scheffersomyces henanensis*
18	0.15	3.72	0.85	0.11	1.59	*Scheffersomyces henanensis*

OTU	20P (%)	24P (%)	41P (%)	42P (%)	Pooled (%)	Species
19	2.06	0.62	0.96	1.51	1.17	*Schwanniomyces occidentalis*
20	4.11	0.05	0.86	0.41	1.16	*Inonotus obliquus*
21	3.04	0.07	1.06	0.22	0.95	*Penicillium lividum*
22	0.04	1.51	1.11	0.61	0.94	*Sugiyamaella smithiae*
23	0.05	0.79	1.00	1.35	0.80	*Sugiyamaella smithiae*
24	0.97	0.52	0.71	0.97	0.75	*Candida chilensis*
25	1.18	0.63	0.47	0.70	0.72	*Scheffersomyces henanensis*
26	0.05	1.82	0.15	0.03	0.71	*Scheffersomyces henanensis*
27	0.83	0.38	0.82	0.77	0.66	*Schwanniomyces occidentalis*
28	0.31	0.47	1.35	0.45	0.64	*Trichoderma harzianum*
29	0.51	0.73	0.47	0.55	0.59	*Scheffersomyces henanensis*
30	0.96	0.33	0.32	0.99	0.58	*Schwanniomyces occidentalis*
31	0.04	0.31	1.55	0.08	0.51	*Scheffersomyces henanensis*
32	0.05	0.35	1.42	0.06	0.49	*Candida chilensis*
33	0.02	1.29	0.02	0.05	0.48	*Candida chilensis*
34	0.23	0.25	0.69	0.60	0.42	*Hypocrea virens*
35	1.32	0.02	0.17	0.09	0.34	*Penicillium spinulosum*
36	0.18	0.34	0.40	0.23	0.30	Unidentified
37	0.01	0.39	0.27	0.42	0.29	*Tremella polyporina*
38	0.09	0.20	0.34	0.60	0.28	Unidentified
39	0.05	0.56	0.20	0.01	0.26	*Schwanniomyces occidentalis*

OTU	20P (%)	24P (%)	41P (%)	42P (%)	Pooled (%)	Species
40	0.46	0.14	0.19	0.37	0.26	*Schwanniomyces occidentalis*
41	0.37	0.10	0.32	0.34	0.26	*Candida chilensis*
42	0.42	0.14	0.19	0.34	0.25	*Schwanniomyces occidentalis*
43	0.02	0.24	0.34	0.39	0.25	*Chaetomiaceae* sp.
44	0.89	0.01	0.16	0.06	0.24	*Penicillium lividum*
45	0.16	0.15	0.26	0.34	0.21	*Scytalidium* sp.
46	0.26	0.22	0.13	0.24	0.21	*Scheffersomyces henanensis*
47	0.19	0.18	0.30	0.16	0.21	*Scytalidium* sp.
48	0.13	0.33	0.14	0.13	0.20	*Scheffersomyces henanensis*
49	0.35	0.15	0.12	0.21	0.20	*Candida chilensis*
50	0.01	0.24	0.34	0.11	0.19	*Hypocrea virens*
51	0.22	0.17	0.15	0.25	0.19	*Schwanniomyces occidentalis*
52	0.60	0.13	0.04	0.02	0.18	*Penicillium cinnamopurpureum*
53	0.29	0.12	0.18	0.17	0.18	*Schwanniomyces occidentalis*
54	0.03	0.26	0.21	0.05	0.16	*Schwanniomyces occidentalis*
55	0.27	0.08	0.13	0.22	0.16	*Schwanniomyces occidentalis*
56	0.18	0.04	0.27	0.19	0.15	*Candida chilensis*
57	0.21	0.14	0.07	0.19	0.15	*Candida chilensis*
58	0.16	0.04	0.28	0.10	0.13	*Scytalidium lignicola*
59	0.01	0.07	0.21	0.23	0.12	*Scytalidium* sp.
60	0.01	0.07	0.20	0.22	0.12	*Scytalidium* sp.

OTU	20P (%)	24P (%)	41P (%)	42P (%)	Pooled (%)	Species
61	0.14	0.09	0.03	0.15	0.10	*Schwanniomyces occidentalis*
62	0.02	0.07	0.11	0.21	0.10	*Chaetomiaceae* sp.
63	0.03	0.02	0.20	0.13	0.09	*Chaetomiaceae* sp.
64	0.14	0.01	0.10	0.14	0.08	*Schwanniomyces occidentalis*
65	0.16	0.02	0.09	0.09	0.08	*Candida chilensis*
66	0.12	0.03	0.11	0.10	0.08	*Candida insectorum*
67	0.13	0.04	0.07	0.09	0.08	*Schwanniomyces occidentalis*
68	0.01	0.06	0.08	0.14	0.07	*Chaetomiaceae* sp.
69	0.12	0.02	0.05	0.09	0.06	*Schwanniomyces occidentalis*
70	0.10	0.04	0.05	0.10	0.06	*Schwanniomyces occidentalis*
71	0.04	0.03	0.13	0.06	0.06	Unidentified
72	0.14	0.01	0.05	0.09	0.06	*Scheffersomyces henanensis*
73	0.16	0.04	0.03	0.02	0.06	Unidentified
74	0.11	0.04	0.04	0.07	0.06	*Schwanniomyces occidentalis*
75	0.06	0.05	0.03	0.07	0.05	*Schwanniomyces occidentalis*
76	0.01	0.04	0.07	0.08	0.05	*Hypocrea virens*
77	0.03	0.02	0.09	0.05	0.05	*Bullera* sp.
78	0.03	0.03	0.05	0.03	0.04	*Sugiyamaella valdiviana*
79	0.05	0.02	0.02	0.05	0.03	*Scheffersomyces henanensis*
80	0.03	0.02	0.03	0.03	0.03	*Schwanniomyces occidentalis*

OTU	20P (%)	24P (%)	41P (%)	42P (%)	Pooled (%)	Species
81	0.06	0.01	0.01	0.03	0.02	*Talaromyces marneffei*
82	0.01	0.02	0.05	0.01	0.02	*Trichoderma harzianum*
83	0.02	0.01	0.03	0.05	0.02	*Schwanniomyces occidentalis*
84	0.03	0.01	0.03	0.01	0.02	*Bullera* sp.
85	0.03	0.02	0.01	0.01	0.02	*Scheffersomyces henanensis*
86	0.02	0.01	0.01	0.03	0.02	*Kabatiella* sp.
Total	98.76	91.31	95.19	97.46	94.98	

Colour gradient: Samples: Red (high) to dark green (low), Total: Dark violet (High) to light violet (low), Pooled sequences Dark blue (high) to light blue (low)

Supplementary Table 7.4: Heat map detailing the taxonomy and distributions of shared gut fungal OTUs in huhu grubs switched to pine diet

OTU	01P (%)	06P (%)	37P (%)	38P (%)	TAP (%)	Pooled (%)	Taxonomy
1	6.19	13.01	45.15	24.22	18.12	19.55	*Sistotrema brinkmanii*
2	10.14	11.83	2.07	5.81	9.54	8.07	*Schwanniomyces occidentalis*
3	10.39	9.36	1.90	4.50	14.31	8.01	*Schwanniomyces occidentalis*
4	2.81	4.51	19.16	9.15	8.81	7.96	*Sistotrema brinkmanii*
5	2.26	4.32	2.55	8.59	0.41	4.43	*Lecythophora fasciculata*
6	3.58	7.17	0.90	3.35	1.37	3.52	*Schwanniomyces occidentalis*
7	2.75	3.82	2.25	4.63	0.80	3.18	*Cadophora malorum*
8	6.86	0.59	1.47	1.30	2.44	2.55	*Rhinocladiella similis*
9	0.49	6.09	0.05	1.05	3.58	2.19	*Sugiyamaella lignohabitans*
10	0.66	1.28	4.65	2.28	3.00	2.13	*Sistotrema brinkmanii*
11	2.31	2.16	1.42	2.90	0.60	2.10	*Cadophora malorum*
12	2.04	2.59	0.45	1.14	3.30	1.89	*Schwanniomyces occidentalis*
13	2.18	3.71	0.67	1.10	1.04	1.77	*Scheffersomyces henanensis*
14	1.98	0.88	4.52	1.90	0.26	1.74	*Rhinocladiella* sp.
15	1.60	2.65	1.02	2.24	0.33	1.73	*Cadophora melinii*
16	1.07	1.34	0.45	2.28	0.46	1.35	*Scheffersomyces henanensis*

OTU	01P (%)	06P (%)	37P (%)	38P (%)	TAP (%)	Pooled (%)	Taxonomy
17	0.50	1.14	0.60	2.17	0.13	1.11	*Coniochaeta cateniformis*
18	1.58	1.31	0.27	0.66	1.63	1.10	*Schwanniomyces occidentalis*
19	1.70	0.86	0.37	0.51	2.08	1.07	*Candida chilensis*
20	0.28	3.15	0.02	0.47	1.32	1.03	*Sugiyamaella novakii*
21	0.19	0.26	1.00	0.81	3.17	0.99	*Sistotrema brinkmanii*
22	1.32	1.48	0.20	0.85	0.73	0.98	*Schwanniomyces occidentalis*
23	0.84	1.68	0.15	0.51	0.91	0.83	*Scheffersomyces henanensis*
24	1.01	1.53	0.17	0.37	0.67	0.75	*Scheffersomyces henanensis*
25	0.92	0.48	0.15	0.29	1.01	0.56	*Schwanniomyces occidentalis*
26	0.65	0.50	0.25	0.29	1.03	0.52	*Candida chilensis*
27	0.63	0.04	0.10	0.93	0.03	0.46	Unidentified
28	0.42	0.95	0.05	0.39	0.18	0.43	*Schwanniomyces occidentalis*
29	0.72	0.04	0.17	0.62	0.11	0.40	Unidentified
30	0.10	0.22	1.02	0.51	0.28	0.38	*Sistotrema* sp.
31	0.40	0.36	0.10	0.32	0.68	0.38	*Schwanniomyces occidentalis*
32	0.38	0.39	0.02	0.30	0.62	0.36	*Candida chilensis*
33	0.38	0.17	0.10	0.13	1.04	0.34	*Schwanniomyces occidentalis*
34	0.38	0.65	0.10	0.23	0.13	0.31	*Scheffersomyces henanensis*
35	0.36	0.50	0.02	0.13	0.46	0.29	*Scheffersomyces*

OTU	01P (%)	06P (%)	37P (%)	38P (%)	TAP (%)	Pooled (%)	Taxonomy
							henanensis
36	0.04	0.23	0.17	0.62	0.03	0.28	*Hypocrea virens*
37	0.83	0.12	0.02	0.20	0.02	0.27	Unidentified
38	0.28	0.20	0.07	0.35	0.11	0.24	*Scheffersomyces henanensis*
39	0.15	0.24	0.07	0.12	0.67	0.24	*Candida chilensis*
40	0.59	0.04	0.07	0.10	0.34	0.23	*Exophiala oligosperma*
41	0.26	0.35	0.07	0.20	0.21	0.23	*Schwanniomyces occidentalis*
42	0.03	0.17	0.15	0.47	0.03	0.21	*Hypocrea virens*
43	0.24	0.30	0.10	0.14	0.15	0.19	*Candida chilensis*
44	0.04	0.68	0.02	0.05	0.15	0.18	*Sugiyamaella novakii*
45	0.01	0.37	0.05	0.08	0.42	0.18	*Sugiyamaella lignohabitans*
46	0.18	0.26	0.05	0.12	0.18	0.16	*Candida chilensis*
47	0.09	0.22	0.05	0.08	0.34	0.15	*Schwanniomyces occidentalis*
48	0.13	0.09	0.07	0.06	0.39	0.14	*Schwanniomyces occidentalis*
49	0.20	0.06	0.12	0.07	0.18	0.12	*Rhinocladiella similis*
50	0.05	0.14	0.10	0.21	0.02	0.12	*Scytalidium* sp.
51	0.11	0.12	0.02	0.07	0.23	0.11	*Scheffersomyces henanensis*
52	0.06	0.20	0.02	0.06	0.23	0.11	*Schwanniomyces occidentalis*
53	0.17	0.06	0.02	0.07	0.18	0.10	*Schwanniomyces occidentalis*
54	0.09	0.06	0.02	0.19	0.03	0.10	*Schwanniomyces*

OTU	01P (%)	06P (%)	37P (%)	38P (%)	TAP (%)	Pooled (%)	Taxonomy
							occidentalis
55	0.14	0.13	0.02	0.02	0.11	0.08	*Candida chilensis*
56	0.08	0.12	0.02	0.02	0.13	0.07	*Candida chilensis*
57	0.04	0.14	0.02	0.07	0.03	0.07	*Bullera* sp.
58	0.05	0.01	0.02	0.04	0.07	0.04	*Talaromyces marneffei*
Total	73.91	95.34	95.00	90.39	88.82	88.07	

Colour gradient: Samples: Red (high) to dark green (low), Total: Dark violet (High) to light violet (low), Pooled sequences Dark blue (high) to light blue (low)

Supplementary Table 7.5: Heat map detailing the taxonomy and distributions of shared gut fungal OTUs in huhu grubs switched to cotton diet

OTU	20C (%)	24C (%)	41C (%)	42C (%)	Pooled (%)	Taxonomy
1	14.58	8.81	9.80	33.38	16.78	*Rhodotorula cycloclastica*
2	14.49	0.16	5.44	19.97	10.20	*Cryptococcus laurentii*
3	8.51	0.22	3.15	13.45	6.44	*Cryptococcus laurentii*
4	0.24	1.96	10.30	0.31	3.60	*Schwanniomyces occidentalis*
5	0.05	0.22	9.64	0.22	2.96	*Scheffersomyces henanensis*
6	9.77	0.49	0.23	0.58	2.63	*Volutella* sp.
7	2.19	1.03	1.32	4.65	2.32	*Rhodotorula cycloclastica*
8	0.19	4.84	4.00	0.09	2.27	*Schwanniomyces occidentalis*
9	0.05	9.73	0.19	0.04	2.14	*Candida chilensis*
10	0.24	0.44	3.73	1.39	1.61	*Scytalidium lignicola*
11	0.10	1.63	2.41	0.98	1.33	*Lecythophora* sp.
12	1.99	0.65	0.54	0.80	0.98	*Schwanniomyces occidentalis*
13	0.88	0.05	1.05	1.16	0.83	*Cryptococcus laurentii*
14	0.05	0.98	1.52	0.04	0.68	*Schwanniomyces occidentalis*
15	0.63	0.49	0.51	0.63	0.56	*Schwanniomyces occidentalis*
16	0.24	0.44	1.01	0.27	0.52	*Scheffersomyces henanensis*
17	0.29	0.44	0.89	0.36	0.52	*Cladophialophora* sp.
18	0.24	0.49	0.62	0.67	0.52	*Rhodotorula cycloclastica*
19	0.92	0.27	0.39	0.40	0.49	Unidentified

OTU	20C (%)	24C (%)	41C (%)	42C (%)	Pooled (%)	Taxonomy
20	1.07	0.22	0.27	0.36	0.47	*Schwanniomyces occidentalis*
21	0.44	0.16	0.70	0.40	0.45	*Schwanniomyces occidentalis*
22	0.68	0.05	0.58	0.31	0.42	*Candida chilensis*
23	0.10	0.87	0.58	0.04	0.39	*Scheffersomyces henanensis*
24	0.73	0.05	0.47	0.04	0.33	*Penicillium lividum*
25	0.19	0.44	0.43	0.13	0.30	*Sugiyamaella smithiae*
26	0.10	0.22	0.66	0.09	0.29	*Herpotrichiellaceae* sp.
27	0.05	0.16	0.78	0.04	0.29	*Bullera* sp.
28	0.34	0.49	0.12	0.22	0.28	*Candida chilensis*
29	0.53	0.16	0.12	0.18	0.24	*Candida chilensis*
30	0.05	0.33	0.19	0.31	0.22	Unidentified
31	0.34	0.05	0.08	0.36	0.21	Unidentified
32	0.24	0.16	0.19	0.18	0.20	*Schwanniomyces occidentalis*
33	0.24	0.11	0.12	0.22	0.17	*Candida chilensis*
34	0.05	0.33	0.19	0.09	0.16	*Sugiyamaella lignohabitans*
35	0.10	0.05	0.27	0.13	0.15	*Bullera* sp.
36	0.34	0.05	0.04	0.04	0.11	*Volutella* sp.
37	0.05	0.05	0.04	0.04	0.05	*Trichosporon gamsii*
Total	61.30	37.30	62.60	82.62	62.10	

Colour gradient: Samples: Red (high) to dark green (low), Total: Dark violet (High) to light violet (low), Pooled sequences Dark blue (high) to light blue (low)

Supplementary Table 7.6: Taxonomic assignments and heat map of the most abundant OTUs observed in the gut fungal community of huhu grubs grown on cotton diet and their distribution in the grubs grown on pine diet

OTU	01C	06C	37C	38C	TAC	20P	24P	41P	42P	Pooled A	Pooled B	Phylum	Order	Species
1	86.5%	26.2%	0.0%	0.0%	10.4%	0.0%	0.0%	0.0%	0.0%	44.0%	0.0%	Ascomycota	*Hypocreales*	*Volutella sp*
2	0.0%	1.0%	23.0%	20.7%	0.0%	0.0%	0.0%	0.0%	0.0%	8.9%	0.0%	Basidiomycota	*Trichosporonales*	*Trichosporon gamsii*
3	0.0%	0.6%	16.6%	18.2%	0.0%	0.0%	0.0%	0.0%	0.0%	7.4%	0.0%	Basidiomycota	*Tremellales*	*Cryptococcus fragicola*
4	0.0%	0.6%	14.8%	18.8%	0.0%	0.0%	0.0%	0.0%	0.0%	7.4%	0.0%	Basidiomycota	*Trichosporonales*	*Trichosporon gamsii*
5	0.0%	0.3%	10.2%	6.4%	0.0%	0.0%	0.0%	0.0%	0.0%	3.1%	0.0%	Basidiomycota	*Tremellales*	*Bullera sp VY86*
6	0.0%	0.4%	5.3%	6.7%	0.0%	0.0%	0.0%	0.0%	0.0%	2.7%	0.0%	Basidiomycota	*Trichosporonales*	*Trichosporon gamsii*
7	0.0%	0.4%	4.7%	5.4%	0.0%	0.0%	0.0%	0.0%	0.0%	2.2%	0.0%	Basidiomycota	*Trichosporonales*	*Trichosporon gamsii*
8	0.0%	0.0%	0.0%	0.0%	33.4%	0.0%	0.0%	0.0%	0.0%	1.8%	0.0%	Ascomycota	*Helotiales*	*Lachnum sp*
9	0.0%	0.2%	3.1%	3.5%	0.0%	0.0%	0.0%	0.0%	0.0%	1.4%	0.0%	Basidiomycota	*Trichosporonales*	*Trichosporon gamsii*
10	2.2%	0.4%	0.0%	0.0%	2.3%	0.0%	0.0%	0.0%	0.0%	1.2%	0.0%	Ascomycota	*Hypocreales*	*Volutella sp*
11	2.4%	0.4%	0.0%	0.0%	0.2%	0.0%	0.0%	0.0%	0.0%	1.2%	0.0%	Ascomycota	*Hypocreales*	*Stachybotrys chartarum*
12	2.1%	0.6%	0.0%	0.0%	0.5%	0.0%	0.0%	0.0%	0.0%	1.1%	0.0%	Ascomycota	*Hypocreales*	*Stachybotrys chartarum*
13	0.0%	0.2%	3.0%	2.3%	0.0%	0.0%	0.0%	0.0%	0.0%	1.0%	0.0%	Basidiomycota	*Trichosporonales*	*Trichosporon gamsii*
14	0.0%	0.1%	2.7%	2.4%	0.0%	0.0%	0.0%	0.0%	0.0%	1.0%	0.0%	Basidiomycota	*Trichosporonales*	*Trichosporon gamsii*
Total	93.3%	31.4%	83.3%	84.4%	46.8%	0.0%	0.0%	0.0%	0.0%	84.4%	0.0%			

01C, 06C, 37C, 38C and TAC are grubs grown on cotton diet and is collectively named as 'A'
20P, 24P, 41P and 42P are grubs grown on pine diet and is collectively named as 'B'
Colour gradient: Samples: Red (high) to dark green (low), Total: Dark violet (High) to light violet (low), Pooled sequences Dark blue (high) to light blue (low)

Supplementary Table 7.7: Taxonomic assignments and heat map of the most abundant OTUs observed in the gut fungal community of huhu grubs grown on pine diet and their distribution in the grubs grown on cotton diet

OTU	01C	06C	37C	38C	TAC	20P	24P	41P	42P	Pooled A	Pooled B	Phylum	Order	Species
1	0.0%	0.0%	0.0%	0.0%	0.0%	0.3%	14.7%	20.6%	24.5%	0.0%	15.0%	Ascomycota	*Helotiales*	*Scytalidium sp*
2	0.0%	0.0%	0.0%	0.0%	0.0%	1.7%	5.8%	9.5%	14.2%	0.0%	7.5%	Ascomycota	*Helotiales*	*Scytalidium lignicola*
3	0.0%	0.0%	0.0%	0.0%	0.2%	25.6%	0.3%	5.6%	2.1%	0.0%	7.2%	Ascomycota	*Eurotiales*	*Penicillium spinulosum*
4	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	16.4%	0.1%	0.1%	0.0%	5.9%	Ascomycota	*Saccharomycetales*	*Schwanniomyces occidentalis*
5	0.0%	0.1%	0.2%	0.1%	0.3%	9.5%	3.4%	4.3%	7.9%	0.1%	5.8%	Ascomycota	*Saccharomycetales*	*Schwanniomyces occidentalis*
6	0.0%	0.0%	0.0%	0.0%	0.0%	1.5%	3.4%	7.8%	11.3%	0.0%	5.6%	Ascomycota	*Helotiales*	*Scytalidium lignicola*
7	0.0%	0.1%	0.0%	0.0%	0.1%	7.7%	3.3%	3.9%	6.3%	0.0%	4.9%	Ascomycota	*Saccharomycetales*	*Schwanniomyces occidentalis*
8	0.0%	0.0%	0.0%	0.0%	0.0%	5.1%	2.1%	2.8%	3.8%	0.0%	3.2%	Ascomycota	*Saccharomycetales*	*Scheffersomyces henanensis*
9	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	7.3%	0.1%	0.0%	0.0%	2.7%	Ascomycota	*Saccharomycetales*	*Schwanniomyces occidentalis*
10	0.0%	0.1%	0.0%	0.0%	0.0%	3.8%	1.1%	2.9%	3.4%	0.0%	2.5%	Ascomycota	*Saccharomycetales*	*Schwanniomyces occidentalis*
11	0.0%	0.1%	0.0%	0.0%	0.0%	2.6%	1.5%	2.9%	3.4%	0.0%	2.4%	Ascomycota	*Helotiales*	*Scytalidium lignicola*
12	0.0%	0.0%	0.0%	0.0%	0.0%	8.3%	0.1%	1.7%	0.7%	0.0%	2.3%	Ascomycota	*Eurotiales*	*Penicillium spinulosum*
13	0.0%	0.0%	0.0%	0.0%	0.0%	0.2%	5.4%	1.2%	0.3%	0.0%	2.3%	Ascomycota	*Saccharomycetales*	*Scheffersomyces henanensis*
14	0.0%	0.0%	0.0%	0.0%	0.6%	7.6%	0.2%	2.1%	0.5%	0.0%	2.2%	Ascomycota	*Eurotiales*	*Penicillium spinulosum*
15	0.0%	0.0%	0.0%	0.0%	0.0%	1.2%	3.0%	1.7%	1.6%	0.0%	2.0%	Ascomycota	*Saccharomycetales*	*Scheffersomyces henanensis*
16	0.0%	0.0%	0.0%	0.0%	0.0%	0.3%	0.3%	6.2%	0.4%	0.0%	1.7%	Ascomycota	*Saccharomycetales*	*Schwanniomyces occidentalis*
17	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	4.0%	0.7%	0.1%	0.0%	1.6%	Ascomycota	*Saccharomycetales*	*Scheffersomyces henanensis*
18	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	3.7%	0.8%	0.1%	0.0%	1.6%	Ascomycota	*Saccharomycetales*	*Scheffersomyces henanensis*
19	0.0%	0.0%	0.0%	0.0%	0.1%	2.1%	0.6%	1.0%	1.5%	0.0%	1.2%	Ascomycota	*Saccharomycetales*	*Schwanniomyces occidentalis*
20	0.0%	0.0%	0.0%	0.0%	0.0%	4.1%	0.0%	0.9%	0.4%	0.0%	1.2%	Basidiomycota	*Hymenochaetales*	*Inonotus obliquus*
Total	0.0%	0.4%	0.3%	0.2%	1.3%	81.8%	76.6%	76.9%	82.4%	0.2%	78.8%			

01C, 06C, 37C, 38C and TAC are grubs grown on cotton diet and were collectively named as 'A'. 20P, 24P, 41P and 42P are grubs grown on pine diet and were collectively named as 'B'
Colour gradient: Samples: Red (high) to dark green (low), Total: Dark violet (High) to light violet (low), Pooled sequences Dark blue (high) to light blue (low)

Supplementary Table 7.8: Taxonomic assignments and heat map of the most abundant OTUs observed in the gut fungal community of huhu grubs grown on cotton diet and their distribution on being switched to pine diet

OTU	01C	06C	37C	38C	TAC	01P	06P	37P	38P	TAP	Pooled A	Pooled A1	Phylum	Order	Species
1	86.5%	26.2%	0.0%	0.0%	10.4%	0.0%	0.0%	0.0%	0.0%	0.0%	44.0%	0.0%	Ascomycota	*Hypocreales*	*Volutella sp*
2	0.0%	1.0%	23.0%	20.7%	0.0%	0.0%	0.0%	0.2%	0.6%	0.0%	8.9%	0.2%	Basidiomycota	*Trichosporonales*	*Trichosporon gamsii*
3	0.0%	0.6%	16.6%	18.2%	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%	7.4%	0.0%	Basidiomycota	*Tremellales*	*Cryptococcus fragicola*
4	0.0%	0.6%	14.8%	18.8%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	7.4%	0.0%	Basidiomycota	*Trichosporonales*	*Trichosporon gamsii*
5	0.0%	0.3%	10.2%	6.4%	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%	3.1%	0.0%	Basidiomycota	*Tremellales*	*Bullera sp*
6	0.0%	0.4%	5.3%	6.7%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	2.7%	0.0%	Basidiomycota	*Trichosporonales*	*Trichosporon gamsii*
7	0.0%	0.4%	4.7%	5.4%	0.0%	0.0%	0.0%	0.2%	0.5%	0.0%	2.2%	0.2%	Basidiomycota	*Trichosporonales*	*Trichosporon gamsii*
8	0.0%	0.0%	0.0%	0.0%	33.4%	0.0%	0.0%	0.0%	0.0%	0.0%	1.8%	0.0%	Ascomycota	*Helotiales*	*Lachnum sp*
9	0.0%	0.2%	3.1%	3.5%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	1.4%	0.0%	Basidiomycota	*Trichosporonales*	*Trichosporon gamsii*
10	2.2%	0.4%	0.0%	0.0%	2.3%	0.0%	0.0%	0.0%	0.0%	0.0%	1.2%	0.0%	Ascomycota	*Hypocreales*	*Volutella sp*
11	2.4%	0.4%	0.0%	0.0%	0.2%	0.0%	0.0%	0.0%	0.0%	0.0%	1.2%	0.0%	Ascomycota	*Hypocreales*	*Stachybotrys chartarum*
12	2.1%	0.6%	0.0%	0.0%	0.5%	0.0%	0.0%	0.0%	0.0%	0.0%	1.1%	0.0%	Ascomycota	*Hypocreales*	*Stachybotrys chartarum*
13	0.0%	0.2%	3.0%	2.3%	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%	1.0%	0.0%	Basidiomycota	*Trichosporonales*	*Trichosporon gamsii*
14	0.0%	0.1%	2.7%	2.4%	0.0%	0.0%	0.0%	0.2%	0.2%	0.0%	1.0%	0.1%	Basidiomycota	*Trichosporonales*	*Trichosporon gamsii*
Total	93.3%	31.4%	83.3%	84.4%	46.8%	0.0%	0.0%	0.6%	1.6%	0.0%	84.4%	0.6%			

Grubs 01C, 06C, 37C ,38C and TAC were collected from wild and grown on cotton diet and collectively was named 'A'. These grubs were then switched to pine diet and renamed as 01P, 37P, 06P, 38P and TAP and were collectively called 'A1'.
Colour gradient: Samples: Red (high) to dark green (low), Total: Dark violet (High) to light violet (low), Pooled sequences Dark blue (high) to light blue (low)

Supplementary Table 7.9: Taxonomic assignments and heat map of the most abundant OTUs observed in the gut fungal community of huhu grubs switched to cotton diet and their distribution prior to the dietary switch

OTU	01C	06C	37C	38C	TAC	01P	06P	37P	38P	TAP	Pooled A	Pooled A1	Phylum	Order	Species
1	0.0%	0.1%	0.0%	0.0%	0.0%	6.2%	13.0%	45.2%	24.2%	18.1%	0.0%	19.6%	Basidiomycota	*Cantharellales*	*Sistotrema brinkmannii*
2	0.0%	0.1%	0.2%	0.1%	0.3%	10.1%	11.8%	2.1%	5.8%	9.5%	0.1%	8.1%	Ascomycota	*Saccharomycetales*	*Schwanniomyces occidentalis*
3	0.0%	0.1%	0.0%	0.0%	0.1%	10.4%	9.4%	1.9%	4.5%	14.3%	0.0%	8.0%	Ascomycota	*Saccharomycetales*	*Schwanniomyces occidentalis*
4	0.0%	0.0%	0.0%	0.0%	0.0%	2.8%	4.5%	19.2%	9.2%	8.8%	0.0%	8.0%	Basidiomycota	*Cantharellales*	*Sistotrema brinkmannii*
5	0.0%	0.0%	0.0%	0.0%	0.0%	2.3%	4.3%	2.5%	8.6%	0.4%	0.0%	4.4%	Ascomycota	*Coniochaetales*	*Lecythophora fasciculata*
6	0.0%	0.1%	0.0%	0.0%	0.0%	3.6%	7.2%	0.9%	3.4%	1.4%	0.0%	3.5%	Ascomycota	*Saccharomycetales*	*Schwanniomyces occidentalis*
7	0.0%	0.1%	0.0%	0.0%	0.0%	2.7%	3.8%	2.2%	4.6%	0.8%	0.0%	3.2%	Ascomycota	*Helotiales*	*Cadophora malorum*
8	0.0%	0.0%	0.0%	0.0%	0.0%	14.5%	0.0%	0.0%	0.0%	0.0%	0.0%	3.1%	Ascomycota	*Helotiales*	*Scytalidium sp*
9	0.1%	0.2%	0.0%	0.0%	0.0%	6.9%	0.6%	1.5%	1.3%	2.4%	0.1%	2.6%	Ascomycota	*Chaetothyriales*	*Rhinocladiella similis*
10	0.0%	11.2%	0.0%	1.6%	0.1%	0.5%	6.1%	0.0%	1.0%	3.6%	1.0%	2.2%	Ascomycota	*Saccharomycetales*	*Sugiyamaella lignohabitans*
11	0.0%	0.0%	0.0%	0.0%	0.0%	0.7%	1.3%	4.6%	2.3%	3.0%	0.0%	2.1%	Basidiomycota	*Cantharellales*	*Sistotrema brinkmannii*
12	0.0%	0.0%	0.0%	0.0%	0.0%	2.3%	2.2%	1.4%	2.9%	0.6%	0.0%	2.1%	Ascomycota	*Helotiales*	*Cadophora malorum*
13	0.0%	0.0%	0.0%	0.0%	0.1%	2.0%	2.6%	0.4%	1.1%	3.3%	0.0%	1.9%	Ascomycota	*Saccharomycetales*	*Schwanniomyces occidentalis*
14	0.0%	0.0%	0.0%	0.0%	0.0%	2.2%	3.7%	0.7%	1.1%	1.0%	0.0%	1.8%	Ascomycota	*Saccharomycetales*	*Scheffersomyces henanensis*
15	0.0%	0.0%	0.0%	0.0%	0.0%	2.0%	0.9%	4.5%	1.9%	0.3%	0.0%	1.7%	Ascomycota	*Chaetothyriales*	*Rhinocladiella sp*
16	0.0%	0.0%	0.0%	0.0%	0.0%	1.6%	2.6%	1.0%	2.2%	0.3%	0.0%	1.7%	Ascomycota	*Helotiales*	*Cadophora melinii*
17	0.0%	0.0%	0.0%	0.0%	0.0%	1.1%	1.3%	0.4%	2.3%	0.5%	0.0%	1.3%	Ascomycota	*Saccharomycetales*	*Scheffersomyces henanensis*
18	0.0%	0.0%	0.0%	0.0%	0.0%	0.5%	1.1%	0.6%	2.2%	0.1%	0.0%	1.1%	Ascomycota	*Coniochaetales*	*Coniochaeta cateniformis*
19	0.0%	0.4%	0.1%	0.1%	0.2%	1.6%	1.3%	0.3%	0.7%	1.6%	0.1%	1.1%	Ascomycota	*Saccharomycetales*	*Schwanniomyces occidentalis*
20	0.0%	0.0%	0.0%	0.0%	0.2%	1.7%	0.9%	0.4%	0.5%	2.1%	0.0%	1.1%	Ascomycota	*Saccharomycetales*	*Candida chilensis*
21	0.0%	0.6%	0.0%	0.1%	0.0%	0.3%	3.2%	0.0%	0.5%	1.3%	0.1%	1.0%	Ascomycota	*Saccharomycetales*	*Sugiyamaella novakii*
Total	0.1%	13.0%	0.5%	2.0%	1.0%	75.9%	81.8%	90.0%	80.3%	73.5%	1.3%	79.6%			

Grubs 01C, 06C, 37C ,38C and TAC were collected from wild and grown on cotton diet, and collectively named as 'A'. These grubs were then switched to pine diet and renamed as 01P, 37P, 06P, 38P and TAP, and were collectively called 'A1'. Colour gradient is same as previous tables

Supplementary Table 7.10: Taxonomic assignments and heat map of the most abundant OTUs observed in the gut fungal community of huhu grubs grown on pine diet and their composition on being switched to cotton diet

OTU	20P	24P	41P	42P	20C	24C	41C	42C	Pooled B	Pooled B1	Phylum	Order	Species
1	0.3%	14.7%	20.6%	24.5%	0.0%	0.0%	18.9%	1.6%	15.0%	6.0%	Ascomycota	*Helotiales*	*Scytalidium sp*
2	1.7%	5.8%	9.5%	14.2%	0.0%	0.0%	0.3%	0.0%	7.5%	0.1%	Ascomycota	*Helotiales*	*Scytalidium lignicola*
3	25.6%	0.3%	5.6%	2.1%	0.2%	0.0%	0.1%	0.0%	7.2%	0.1%	Ascomycota	*Eurotiales*	*Penicillium spinulosum*
4	0.0%	16.4%	0.1%	0.1%	0.0%	22.9%	0.2%	0.0%	5.9%	4.9%	Ascomycota	*Saccharomycetales*	*Schwanniomyces occidentalis*
5	9.5%	3.4%	4.3%	7.9%	2.0%	0.7%	0.5%	0.8%	5.8%	1.0%	Ascomycota	*Saccharomycetales*	*Schwanniomyces occidentalis*
6	1.5%	3.4%	7.8%	11.3%	0.2%	0.4%	3.7%	1.4%	5.6%	1.6%	Ascomycota	*Helotiales*	*Scytalidium lignicola*
7	7.7%	3.3%	3.9%	6.3%	0.1%	0.0%	0.1%	0.0%	4.9%	0.1%	Ascomycota	*Saccharomycetales*	*Schwanniomyces occidentalis*
8	5.1%	2.1%	2.8%	3.8%	0.2%	0.4%	1.0%	0.3%	3.2%	0.5%	Ascomycota	*Saccharomycetales*	*Scheffersomyces henanensis*
9	0.1%	7.3%	0.1%	0.0%	0.0%	16.5%	0.0%	0.0%	2.7%	3.5%	Ascomycota	*Saccharomycetales*	*Schwanniomyces occidentalis*
10	3.8%	1.1%	2.9%	3.4%	0.4%	0.0%	0.2%	0.1%	2.5%	0.2%	Ascomycota	*Saccharomycetales*	*Schwanniomyces occidentalis*
11	2.6%	1.5%	2.9%	3.4%	0.4%	0.0%	2.8%	2.6%	2.4%	1.6%	Ascomycota	*Helotiales*	*Scytalidium lignicola*
12	8.3%	0.1%	1.7%	0.7%	0.0%	0.0%	0.0%	0.0%	2.3%	0.0%	Ascomycota	*Eurotiales*	*Penicillium spinulosum*
13	0.2%	5.4%	1.2%	0.3%	0.0%	0.9%	0.2%	0.0%	2.3%	0.3%	Ascomycota	*Saccharomycetales*	*Scheffersomyces henanensis*
14	7.6%	0.2%	2.1%	0.5%	0.1%	0.0%	0.1%	0.1%	2.2%	0.1%	Ascomycota	*Eurotiales*	*Penicillium spinulosum*
15	1.2%	3.0%	1.7%	1.6%	0.0%	0.0%	0.0%	0.0%	2.0%	0.0%	Ascomycota	*Saccharomycetales*	*Scheffersomyces henanensis*
16	0.3%	0.3%	6.2%	0.4%	0.2%	2.0%	10.3%	0.3%	1.7%	3.6%	Ascomycota	*Saccharomycetales*	*Schwanniomyces occidentalis*
17	0.1%	4.0%	0.7%	0.1%	0.1%	0.9%	0.6%	0.0%	1.6%	0.4%	Ascomycota	*Saccharomycetales*	*Scheffersomyces henanensis*
18	0.1%	3.7%	0.8%	0.1%	0.0%	0.8%	0.2%	0.0%	1.6%	0.2%	Ascomycota	*Saccharomycetales*	*Scheffersomyces henanensis*
19	2.1%	0.6%	1.0%	1.5%	0.0%	0.0%	0.0%	0.0%	1.2%	0.0%	Ascomycota	*Saccharomycetales*	*Schwanniomyces occidentalis*
20	4.1%	0.0%	0.9%	0.4%	4.5%	0.0%	0.0%	0.1%	1.2%	1.1%	Basidiomycota	*Hymenochaetales*	*Inonotus obliquus*
Total	81.8%	76.6%	76.9%	82.4%	8.8%	45.4%	39.2%	7.5%	78.8%	25.2%			

Grubs 20P, 24P, 41P and 42P were collected from wild and grown on pine diet, and were collectively called as 'B'. These grubs were then switched to cotton diet, renamed as 20C, 24C, 41C, 42C and were collectively called as 'B1'. Colour gradient same as previous tables.

Supplementary Table 7.11: Taxonomic assignments and heat map of the most abundant OTUs observed in the gut fungal community of huhu grubs switched to cotton diet and their composition prior to the dietary switch

OTU	20P	24P	41P	42P	20C	24C	41C	42C	Pooled B	Pooled B1	Phylum	Order	Species
1	0.0%	0.1%	0.1%	0.1%	14.6%	8.8%	9.8%	33.4%	0.1%	16.8%	Basidiomycota	*Sporidiobolales*	*Rhodotorula cycloclastica*
2	0.0%	0.0%	0.0%	0.0%	14.5%	0.2%	5.4%	20.0%	0.0%	10.2%	Basidiomycota	*Tremellales*	*Cryptococcus laurentii*
3	0.0%	0.0%	0.0%	0.0%	8.5%	0.2%	3.1%	13.4%	0.0%	6.4%	Basidiomycota	*Tremellales*	*Cryptococcus laurentii*
4	0.3%	14.7%	20.6%	24.5%	0.0%	0.0%	18.9%	1.6%	15.0%	6.0%	Ascomycota	*Helotiales*	*Scytalidium sp*
5	0.0%	16.4%	0.1%	0.1%	0.0%	22.9%	0.2%	0.0%	5.9%	4.9%	Ascomycota	*Saccharomycetales*	*Schwanniomyces occidentalis*
6	0.0%	0.0%	0.0%	0.0%	16.4%	0.0%	0.3%	2.1%	0.0%	4.5%	Ascomycota	*Hypocreales*	*Verticillium leptobactrum*
7	0.3%	0.3%	6.2%	0.4%	0.2%	2.0%	10.3%	0.3%	1.7%	3.6%	Ascomycota	*Saccharomycetales*	*Schwanniomyces occidentalis*
8	0.1%	7.3%	0.1%	0.0%	0.0%	16.5%	0.0%	0.0%	2.7%	3.5%	Ascomycota	*Saccharomycetales*	*Schwanniomyces occidentalis*
9	0.0%	0.3%	1.6%	0.1%	0.0%	0.2%	9.6%	0.2%	0.5%	3.0%	Ascomycota	*Saccharomycetales*	*Scheffersomyces henanensis*
10	0.0%	0.0%	0.0%	0.0%	9.8%	0.5%	0.2%	0.6%	0.0%	2.6%	Ascomycota	*Hypocreales*	*Volutella sp*
11	0.0%	0.0%	0.0%	0.0%	2.2%	1.0%	1.3%	4.6%	0.0%	2.3%	Basidiomycota	*Sporidiobolales*	*Rhodotorula cycloclastica*
12	0.1%	0.6%	0.2%	0.0%	0.2%	4.8%	4.0%	0.1%	0.3%	2.3%	Ascomycota	*Saccharomycetales*	*Schwanniomyces occidentalis*
13	0.0%	1.3%	0.0%	0.0%	0.0%	9.7%	0.2%	0.0%	0.5%	2.1%	Ascomycota	*Saccharomycetales*	*Candida chilensis*
14	0.0%	0.0%	0.0%	0.0%	0.0%	4.7%	2.3%	0.9%	0.0%	1.9%	Basidiomycota	*Sporidiobolales*	*Rhodotorula cycloclastica*
15	0.0%	1.1%	0.1%	0.0%	0.0%	7.2%	0.5%	0.0%	0.4%	1.7%	Ascomycota	*Saccharomycetales*	*Candida chilensis*
16	1.5%	3.4%	7.8%	11.3%	0.2%	0.4%	3.7%	1.4%	5.6%	1.6%	Ascomycota	*Helotiales*	*Scytalidium lignicola*
17	2.6%	1.5%	2.9%	3.4%	0.4%	0.0%	2.8%	2.6%	2.4%	1.6%	Ascomycota	*Helotiales*	*Scytalidium lignicola*
18	0.2%	0.2%	0.3%	0.2%	0.1%	1.6%	2.4%	1.0%	0.2%	1.3%	Ascomycota	*Coniochaetales*	*Lecythophora sp*
19	4.1%	0.0%	0.9%	0.4%	4.5%	0.0%	0.0%	0.1%	1.2%	1.1%	Basidiomycota	*Hymenochaetales*	*Inonotus obliquus*
20	0.2%	0.2%	0.7%	0.6%	1.1%	0.0%	1.0%	2.1%	0.4%	1.1%	Ascomycota	*Hypocreales*	*Hypocrea virens*
21	0.0%	0.0%	0.0%	0.0%	3.7%	0.0%	0.0%	0.7%	0.0%	1.1%	Ascomycota	*Hypocreales*	*Verticillium leptobactrum*
Total	9.4%	47.4%	41.5%	41.1%	76.6%	80.8%	76.2%	85.2%	36.9%	79.6%			

Grubs 20P, 24P, 41P and 42P were collected from wild and grown on pine diet, and were collectively called as 'B'. These grubs were then switched to cotton diet, renamed as 20C, 24C, 41C, 42C and were collectively called as 'B1'.Colour gradient same as previous tables.

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