Characterisation of the Microbial Communities in the Gastrointestinal Tract of Wood-Eating Organisms

by

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Abstract

Wood recycling is key to biogeochemical cycling and largely driven by microorganisms, with bacteria and fungi naturally coexisting together in the environment. Terrestrial isopods *Oniscus asellus* and *Porcellio scaber* have adaptations to enable them to colonise diverse terrestrial environments and scavenge on dead and decaying organic matter that is rich in cellulose. The Amazonian catfish, *Panaque nigrolineatus* have physiological adaptions enabling the scraping and consumption of wood, facilitating a detritivorous dietary strategy. Substrates high in lignocellulose are difficult to degrade and as yet, it is unclear whether these organisms obtain any direct nutritional benefits from ingestion and degradation of lignocellulose. However, there are numerous systems that rely on microbial symbioses to provide energy and other nutritional benefits for host organisms via lignocellulose decomposition. Whilst previous studies on the microbial communities of *O. asellus*, *P. scaber* and *P. nigrolineatus*, have focused upon the bacterial populations, the presence and role of fungi in lignocellulose degradation has not yet been examined. These studies describe the bacterial and fungal communities within the gastrointestinal tracts using next generation sequencing. The hepatopancreas of *O. asellus* and *P. scaber* was predominantly colonised by one bacterial species and had more fungal diversity. The hindgut was colonised by more diverse bacterial and fungal communities. Due to the woodlouse inhabiting diverse environments, including those with heavy metal pollution, culture methods were used to detect antimicrobial resistance in the gastrointestinal tract of woodlice. The effects of diet on enteric fungal populations were examined in each gastrointestinal tract region of *P. nigrolineatus* and fungal species were found to vary in different regions of the gastrointestinal tract as a function of diet. This is the first study to investigate the bacterial and fungal communities within the hepatopancreas and hindgut from two species of woodlice, using the same individual woodlouse, using next generation sequencing. This is the first study to detect fungi in the digestive tract of any woodlice. This study is the first to examine the fungal community in a xylivorous fish and results support the hypothesis that diet influences fungal distribution and diversity within the gastrointestinal tract of *P. nigrolineatus.* This study provides new insights into the microbial communities that may have a symbiotic role involved in wood degradation in the GI tracts of wood-eating organisms. This study also highlights the need for further research into fungi inhabiting many diverse environments to give more complete and balanced information about the absence and presence of microorganisms.

Declaration

Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.

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List of Abbreviations and Symbols

°C	degree Celsius
β	beta
μg	microgram
μL	microlitre
μm	micrometre
API	Analytical Profile Index
ARG	Antimicrobial Resistance Genes
AMR	Antimicrobial Resistance
BIOM	Biological Observational Matrix
BLAST	Basic Local Alignment Search Tool
bp	base pairs
C	carbon
CAZy	Carbohydrate-Active enZymes database
cm	centimetre
CMC	Carboxymethyl Cellulose
CFU	Colony Forming Unit
CLSI	Clinical & Laboratory Standards Institute
CO_2	carbon dioxide
dH_2O	deionised water
DNA	Deoxyribonucleic Acid
EBs	Elementary Bodies
EDTA	Ethylenediaminetetraacetic Acid
FISH	Fluorescent *in situ* hybridisation
g	gram
G	Guaiacyl unit
GH	Glyosidic Hydrolase
GI	Gastrointestinal
H	p-Hydroxyphenyl unit
HGT	Horizontal Gene Transfer
IBs	Intermediate Bodies
INSDC	International Nucleotide Sequence Databases Collaboration
ITS	Internal Transcribed Spacer
kb	kilobase

L	litre
LiP	Lignin Peroxidase
M	Molar
mg	milligram
$MgCl_2$	Magnesium Chloride
min	minute
mL	millilitre
mM	millimolar
MnP	Manganese Peroxidase
N	North
NCBI	National Centre for Biotechnology Information
ng	nanogram
NGS	Next Generation Sequencing
OTU	Operational Taxonomic Unit
PBS	Phosphate Buffered Solution
PCoA	Principle Coordinate Analysis
PCR	Polymerase Chain Reaction
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved State
pmol	picomole
Psi	pounds per square inch
QIIME	Quantitative Insights into Microbial Ecology
RBs	Reticulate Bodies
rDNA	ribosomal Deoxyribonucleic Acid
RFLP	Restriction Fragment Length Polymorphism
rRNA	ribosomal Ribonucleic Acid
S	Syringyl unit or Svedburg
s	second
SEM	Scanning Electron Microscopy
SSU	Small Subunit
TAE	Tris-Acetate-Ethanol
SDA	Sabouraud Dextrose agar
SSC	Standard Saline Citrate
TE	Tris-EDTA

TEM	Transmission Electron Microscopy
U.K.	United Kingdom
V	Volt
VP	Versatile Peroxidase
W	West
WHO	World Health Organisation
ZOI	Zones of Inhibition

Dedication

This PhD thesis is dedicated to my mum and best friend, who sadly passed away during my PhD. She taught me so much, always supported me and believed in me throughout all my endeavours. From a young child, her life was filled with such adversity, whilst others may have crumbled, she used it to hold onto and support everyone with so much commitment and love.

She was and will continue to be such an inspiration to me.

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I would like to acknowledge the inspirational lecturers at University of Portsmouth during my undergraduate degree, Matt Guille for his tutelage, to Joy Watts and Eric May for igniting my interest in microbiology. I would also like to thank the staff at Agriculture and Agri-food Canada for the opportunity to study microbiology for my 3rd year research project, giving me confidence to work in a laboratory environment.

Thank you to my family and friends, from the sensible, to the knowledgeable, to the coaches, to the crazies! A special thank you to Irene Marden, Claire Lamb, Janine Robinson, Tricia Easterby, Rachel Parry, Alison Robertson, Claire Treacy and Elizabeth Taylor. You have all laughed and cried with me (or at me!), believed in me, celebrated my successes and picked me up in times of need.

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Last but not least, I would also like to acknowledge myself for everything I have achieved during my life, it has been a tough ride at times. I have never given up and have taken the lessons and evolved. My learnings in my personal development work enabled me to stay focussed when balancing my personal and professional life. I am proud of achieving some goals I set many years ago, I have been off course some of the time, but this has been more about enjoying the journey. I have reached my current destination and have new goals that I will pursue in my quest for better self-understanding.

1 General Introduction

1.1 Biogeochemical Cycle and Lignocellulose

Plants capture and store carbon dioxide from the atmosphere during photosynthesis and use energy from the sun and carbon dioxide (CO_2) to create carbohydrates and oxygen. CO_2 is then released back during cellular respiration. In forests, wood degradation is important in biogeochemical cycles, particularly carbon cycling and nutrient cycling, therefore, forests create a natural carbon sink in terrestrial ecosystems (Kauffman *et al.*, 1995; Barford *et al.*, 2001; Weedon *et al.*, 2009). Biogeochemical cycling from degradation of the lignocellulose complex is an important process, with forests representing a globally important stock of carbon, formed of plant polymers. There would be no carbon cycle without microbial decomposition of lignocellulose, for the captured carbon to be available for use by other organisms, microbial ligninolytic activity must occur first (Bardgett *et al.*, 2008). Lignocellulose in living trees is protected from microbial attack but dead wood and its non-woody parts, represent an important source of carbon, energy and other nutrients (Brown and Lugo, 1982; Kauffman *et al.*, 1995; Barford *et al.*, 2001; Tláskal *et al.*, 2017).

1.1.1 Composition of Lignocellulose

Lignocellulose is one of the most abundant forms of organic biomass on the planet and the key components in plants and plant cell walls (Lee, 1997). Lignocellulose biomass is typically composed of cellulose (40-50%), hemicellulose (25-40%), and lignin (15-32%) and the ratio is dependent on the type of plant (Pérez *et al.*, 2002; Weedon *et al.*, 2009; Stokland *et al.*, 2012; Chandra *et al.*, 2015).

1.1.1.1 Cellulose

Cellulose is a carbohydrate composed of β-1,4 linked D-glucopyranose, forming a long chain polymer of 7000 - 15000 repeating glucose monomers. These are 180° alternately rotated forming a highly symmetrical, linear polysaccharide (Chandra *et al.*, 2015). This arrangement allows strong hydrogen bonds to form between the hydroxyl groups forming highly ordered, tight, recalcitrant crystalline microfibrils. Glucose monomers are joined by a condensation reaction at C1 of one pyranose ring and C4 of the next ring, leaving the units joined by a single oxygen atom. As a molecule of water is lost as a hemiacetal and an alcohol reacts to form an acetal, the units are referred to as anhydroglucose units. Cellobiose is formed by two anhydroglucose joining (Fig. 1.1).

In less ordered, amorphous regions, the chains are further apart allowing hydrogen bonding to other molecules, for example, water. This enables cellulose to absorb large amounts of water (Stokland *et al.*, 2012).

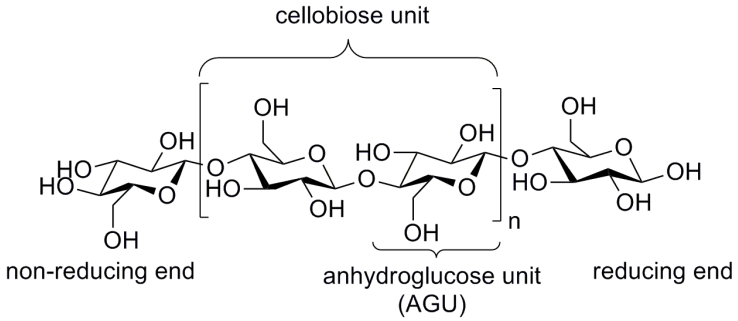

Figure 1:1: The structure of cellulose. The β-1,4 linked D-glucopyranose chain, showing repeating glucose monomers, these are 180° alternately rotated forming a highly symmetrical, linear polysaccharide (Figure from Olsson and Westman, 2013).

1.1.1.2 Hemicellulose

Hemicellulose is a heterogenous biopolymer. It is arranged with a backbone of either homopolysaccharides or heteropolysacharides, particularly xylans and mannans, with branched side chains containing one to three monosaccharaides (Gírio *et al.*, 2010). There is a greater difference of hemicellulose content between plants, than there is cellulose content (Timell, 1967; Sjöström, 1993). The polysaccharides co-crystallise with cellulose filaments and combine with lignin forming a non-crystalline structure that encapsulates cellulose fibrils, strengthening the cell wall (Stokland *et al.*, 2012). This structure increases resistance to enzymatic degradation of the cell wall and the insolubility of cell wall components (Stokland *et al.*, 2012; Chandra *et al.*, 2015).

1.1.1.3 Lignin

Lignin is a complex network of aromatic heteropolymers made from phenylpropanoid pre-cursors, linked together by a variety of ether and carbon bonds. It is the second most abundant organic polymer on Earth, after cellulose, accounting for 30% of organic carbon in the biosphere (Boerjan *et al.*, 2003; Bugg *et al*., 2011a). Lignin is formed by polymerisation of three precursor alcohols, which give rise to guaiacyl (G), syringyl (S) and *p*-hydroxyphenyl (H) units (Fig 1.2). The ratios of G:S:H varies from species to species of plant, with softwood having the highest lignin content made of G units, hardwoods are made of G & S units, and non-woody angiosperms contain G & H units (Martínez *et al.*, 2005; Bugg *et al*., 2011a). The highest concentration of this recalcitrant polymer is in the middle lamella acting as a cement between wood fibres. It is also present in layers of the cell

wall, in particular, the secondary cell wall and together with hemicellulose, form an amorphous matrix, embedded within cellulose fibrils (Fig. 1.3) (Martínez *et al.*, 2005). Lignin composition varies between different wood tissues and is vital for the strength and stiffness of the tissue, as well as cell wall structural integrity, whilst cellulose is more important for the bending or stiffness of the stem (Turner and Somerville, 1997; Chabannes *et al.*, 2001; Jones *et al.*, 2001). Lignin also waterproofs the cell wall, enabling transport of water and solutes through the vascular system and plays a role in protecting plants against microbial pathogens (Campbell and Sederoff, 1996).

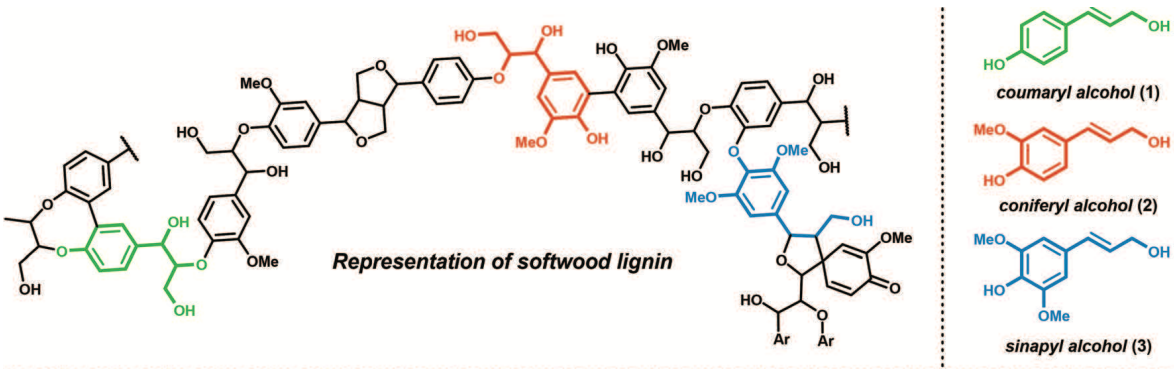

Figure 1:2: The structure of lignin. The building blocks of lignin; p-coumaryl (green), coniferyl (red) and sinapyl (green). Figure from Kärkäs et al. (2016).

1.1.1.4 Wood Cell Structure

The wood cell wall is arranged in layers of different thicknesses (Fig. 1.3B). When cells grow the primary wall is formed consisting of cellulose, hemicellulose and pectin. When they have reached full size, a three-tiered secondary cell wall consisting of three layers; the outer (S1), the middle (S2) and the inner (S3) forms inside the primary wall, containing different ratios of cellulose, hemicellulose and lignin (Fig. 1.3A). The middle lamella is the space between neighbouring cells and is filled with lignin, calcium and pectin, acting as a cement. Lignin formation begins between the primary and secondary wall and extends out into the space between the cells and around the secondary wall, until the cell wall structure is complete, allowing for its resistance to decay from wood decay bacteria and fungi and colonisation of fungal hyphae (Stokland *et al.*, 2012).

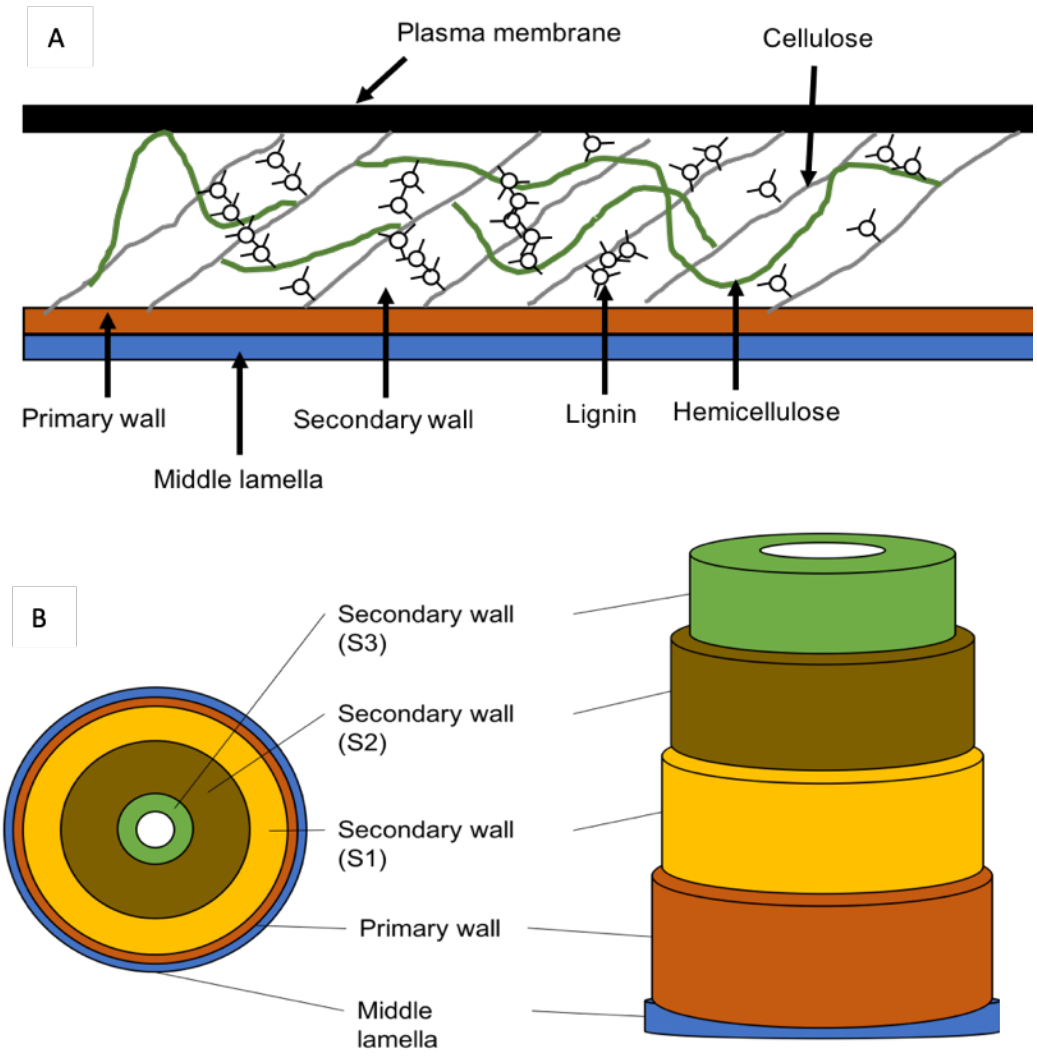

Figure 1:3: Structure of the wood cell wall. (A) Structure of the secondary cell wall containing cellulose, hemicellulose and lignin. (B) Structure of wood cell wall comprised of the middle lamella, primary cell wall and the secondary cell wall. Adapted from (Anwar *et al.*, 2014).

1.1.1.5 Plant Litter

Non-wood components of trees, such as leaf litter, is more readily available to invertebrates, with nutrient concentrations typically higher than wood (Kauffman *et al.*, 1995). Litter decomposition is a complex ecological process and regulated by three main drivers; climate, litter quality and decomposer communities (Couteaux *et al.*, 1995; Worrall *et al.*, 1997; Schwarze, 2007; Chandra *et al.*, 2015). About half of all CO_2 output from the soil is contributed by decomposition of annual leaf litter fall, demonstrating the importance of litter degradation as a direct source of atmospheric CO_2 (Couteaux *et al.*, 1995). Decomposition involves two processes; the synergistic, mineralisation of cellulose, lignin and other compounds by a series of microorganisms; and the leaching of soluble compounds downward into the soil whose carbon and nitrogen are progressively mineralised or

immobilised (Couteaux *et al.*, 1995). These processes are controlled by both abiotic and biotic factors, such as soil and litter chemical composition (Couteaux *et al.*, 1995). Lignin content is an indicator for litter degradability with lignin decomposition a rate limiting step, therefore, rates of decomposition are affected by the activities of ligninolytic enzymes (Gessner and Chauvet, 1994; Steffen *et al.*, 2007; Trum *et al.*, 2010).

1.1.1.6 Lignocellulolytic Enzymes

Enzymes involved in the lignocellulose break down, such as glycosidic hydrolases (GH), are known as Carbohydrate Active Enzymes (CAZymes) (Lombard *et al.*, 2014). Cellulose degradation is catalysed by the most diverse CAZymes; cellulases or GHs which are the primary enzymes, catalysing a single reaction to hydrolyse the β-1,4-glycosidic linkages of cellulose chains (Wilson, 2011). Most cellulolytic microorganisms secrete cellulases outside their cell wall, unable to transport insoluble material across their cell membrane (Wilson, 2008). Some are produced on the cell wall in the form of cellulosomes when growing on cellulosic material and have been found mainly on anaerobic and some aerobic microorganisms (Bayer *et al.*, 1998). Cellulosomes are stable enzyme complexes that are flexible enough to bind to cellulose and hydrolyse the substrate (Lynd *et al.*, 2002). Cellulase digestion produces soluble sugars that are taken into the cell and used for energy (Dimarogona *et al.*, 2012). It is thought there are three types of cellulases involved in cellulolytic activities; endoglucanases which hydrolyse internal bonds in the amorphous region to release new terminal ends, cellobiohydrolases that hydrolyse the existing or chain ends generated by the endoglucanases. Both reactions release cellobiose molecules, that are broken into glucose monomers by the third type; β-glucosidase (Dimarogona *et al.*, 2012). However, recently, it has been discovered that bacteria abundant in litter and forest soil expressed a new uncharacterised means of metabolising cellulose, a glycosidic hydrolase (GH23). It was found to have high structural similarity to known lytic transglucosylases, without expressing the previously known enzymes (López-Mondéjar *et al.*, 2016). Under aerobic conditions complete degradation of cellulose releases CO_2 and water and under anaerobic conditions, CO_2, methane and water is released (Pérez *et al.*, 2002). Products of cellulose hydrolysis are available as carbon and energy sources for microorganisms in the environment, this release of sugar is the main basis of microbial interactions in such environments (Pérez *et al.*, 2002).

Hemicellulose can be a challenging resource to degrade, due to its structural variability requiring many hemicellulases (GHs) working together synergistically (Shallom and Shoham, 2003). The most common hemicelluloses are xylans and mannans. The type and different proportions of

hemicelluloses differ between softwoods and hardwoods. Initially, an endoxylanase or endomannase attacks internal points of the hemicellulose main chain producing smaller fragments. These are hydrolysed by β-xylosidase or β-glucosidase, producing monosaccharides; xylose, glucose and mannose. Lastly, three different enzymes break bonds between the side chains and the main chain (Pérez *et al.*, 2002). Hemicellulose is degraded into monomeric sugars and acetic acid which are available to surrounding microbial communities (Pérez *et al.*, 2002).

Lignin is complex and random in structure making it highly resistant to degradation. Many enzymes are involved in the degradation process and are substrate specific to the wide variety of chemical bonds within lignin, their effectiveness depending on the degree of crystallisation (Li *et al.*, 2009). Initially biodegradation is non-specific, oxidative and mediated by extracellular enzymes referred to as "enzymatic combustion" (Kirk and Farrell, 1987). Wood rotting fungi, in particular white rot fungi are the main lignin degraders, being the only microbes known to completely mineralise lignin (Blanchette, 1991; Mester *et al.*, 2004; Bugg *et al*., 2011a; Bugg *et al*., 2011b). In contrast, brown rot fungi chemically modify lignin but do not utilise it as extensively (Martínez *et al.*, 2005; Pozo *et al.*, 2010; Zhang *et al.*, 2016). White rots have been extensively studied in forest ecosystems, possessing ligninolytic systems consisting mainly of oxidases and peroxidases (Baldrian, 2004; Baldrian and Valášková, 2008).

Laccases are oxidases and contain copper, which catalyse the oxidation of various phenolic substrates, reducing oxygen to water (Kirk and Farrell, 1987; Li *et al.*, 2009). Fungal laccase is important in the initial step of degradation of polymer chains decomposing lignin by splitting aromatic rings. Although laccases can use mediators, it has also been demonstrated that they are capable of oxidising high molecular weight lignin compounds directly through long range electron transfer processes (Shleev *et al.*, 2006).

Peroxidases require the presence of extracellular hydrogen peroxide to oxidise their substrates. In response to nutrient depletion hydrogen peroxide is produced in association with secondary metabolism. Lignin peroxidase (LiP) has high redox potential and efficient at oxidising veratryl alcohol, degrading non-phenolic lignin units, which accounts for up to 90% of the polymer. Manganese peroxidase (MnP) generates Mn^{3+} from Mn^{2+}, acting as a diffusible oxidiser on phenolic or non-phenolic lignin units (Bugg *et al*., 2011a). Versatile peroxidase (VP) combines the catalytic properties of LiP, MnP and plant/microbial peroxidases. VPs oxidise Mn^{2+} similar to MnP and have long range electron transfer pathways, similar to LiP. This suggests different binding sites of MnP are involved in substrate oxidation (Heinfling *et al.*, 1998; Martínez *et al.*, 2005).

1.2 Microbial Degradation of Lignocellulose

Lignocellulose is highly recalcitrant to microbial attack and its degradation is slow. Its natural degradation is a lengthy process, spread over many decades, involving abiotic and biotic environmental factors and the traits of the plant species (Martínez *et al.*, 2005; Cornwell *et al.*, 2009). Wood and its non-woody constituents are high in organic carbon and a rich resource for microbial decomposers who play major roles in biogeochemical cycling in global ecosystems (Tláskal *et al.*, 2017).

Saproxylic organisms include any species that depend, during some portion of their lifestyle, upon decayed woody material from living, weakened or dead trees (Stokland *et al.*, 2012). Saproxylic microorganisms secrete extracellular enzymes, degrading easily accessible substrates such as sugars, starch and amino acids, as well as larger complex substrates, such as, cellulose, hemicellulose and lignin (Staley and Konopka, 1985; Wilson, 2008). The process of wood decay and the microorganisms involved, depend on many factors, such as, the nitrogen content of wood, which is higher in decayed wood than non-decayed wood and water content (Fukasawa *et al.*, 2011a). The dominant agents of wood decomposition are fungi, but it is also known that bacteria inhabit dead wood and plant litter and are involved in the decay process (Greaves, 1971; van der Wal *et al.*, 2007; Hoppe *et al.*, 2015; Kielak *et al.*, 2016; Tláskal *et al.*, 2017). The bacterial community present is dependent on the ecological strategy of the dominant fungus with secondary colonisers driving community shifts, reducing bacterial diversity (Johnston *et al.*, 2019). Wherever bacteria and fungi co-exist, they interact with and influence each other, but little attention has been paid to their interactions in decomposition, focusing more on agriculture, medicine, food and drink (Frey-Klett *et al.*, 2011; Johnston *et al.*, 2016). The bacteria surrounding and interacting with fungi effectively constitutes its microbiome, therefore, they must be considered together (Johnston *et al.*, 2016).

1.2.1 Saproxylic Bacteria

Bacteria are active components in terrestrial and aquatic environments and known for their ability to degrade cellulose, although their mechanism of wood degradation has been less studied in comparison to fungi (Greaves, 1971; Johnston *et al.*, 2016; Rinta-Kanto *et al.*, 2016). There are culture-based studies examining saproxylic bacteria, however, it is unlikely these studies are representative of the natural environment (Murray and Woodward, 2003; van der Wal *et al.*, 2007), It is reported the scarcity of studies surrounding bacterial communities in lignocellulose

degradation in decomposing wood, is due to a large and variable proportion of wood degrading bacteria, presently unculturable in comparison to wood degrading fungi, which has a greater natural tendency for culture (Folman *et al.*, 2008; Johnston *et al.*, 2016). As studies into lignocellulose decomposition by bacteria is not well established, initial experiments have been culture based and setting up artificial environments, rather than assessing their lignocellulose potential in the natural environment (Folman *et al.*, 2008; Hervé *et al.*, 2014; Salvachúa *et al.*, 2015; de Lima Brossi *et al*., 2016). A culture-based study can only indicate part of the bacterial communities present and their role, microbes shown to break down artificial lignin or cellulose substrates is not necessarily applicable to the natural environment. The advent of molecular methods that removed the need for culture has enabled researchers to study saproxylic bacterial communities in more detail (Folman *et al.*, 2008; Hervé *et al.*, 2014; Sun *et al.*, 2014; Hoppe *et al.*, 2015; Kielak *et al.*, 2016; Tláskal *et al.*, 2017).

Bacteria are able to decompose lignocellulose components, in particular cellulose and hemicellulose, with more growing evidence to suggest their ability to degrade lignin too (Crawford *et al.*, 1973; Lynd *et al.*, 2002; Li *et al.*, 2009; Bugg *et al*., 2011a). Although fungi are thought to be the main lignin degraders, there is now emerging evidence that bacteria have ligninolytic enzymes systems and have been recently reported to depolymerise natural lignin (Salvachúa *et al.*, 2015). To date, from soils and wood-feeding insect studies, bacteria that can degrade lignin are represented by Actinobacteria, Alphaproteobacteria and Gammaproteobacteria (Bugg *et al*., 2011b; Huang *et al.*, 2013). Lignin degraders from Alphaproteobacteria are represented by many serotypes from *Brucella*, *Ochrobactrum*, *Sphingobium* and *Sphingomonas* genera (Janusz *et al.*, 2017). The best known ligninolytic bacteria from class Gammaproteobacteria are *Pseudomonas*, *Enterobacter lignolyticus* and *Escherichia coli* (Tian *et al.*, 2014). Some Actinobacteria representatives with abilities to produce catabolic enzymes are from the *Streptomyces* and *Rhodococcus* genera (Tian *et al.*, 2014). Many *Streptomyces* produce laccase, LiP and MnP and some *Pseudomonas* produce LiP and MnP (Tian *et al.*, 2014; Janusz *et al.*, 2017). Some bacteria use the same fungal laccase systems and some are unlike the systems presently known in fungi, with additional enzymes and pathways of lignin degradation in bacteria yet to be identified (Huang *et al.*, 2013; Brown and Chang, 2014; Janusz *et al.*, 2017).

Wood-rot fungi are considered the main wood degraders, however, in anaerobic systems, bacteria may assume this function as they are better adapted to these conditions (Huang *et al.*, 2013). Whereas aerobic microorganisms produce single enzyme components connected to binding molecules, anaerobic bacteria produce a range of enzymes called a cellulosomes, located on the

surface of the bacterial cell, with adhesion to cellulose by a scaffoldin protein (Schwarz, 2001; Lynd *et al.*, 2002). Through degradation of wood, bacteria can produce smaller aromatics that can be used for aromatic catabolism, which is widespread amongst soil bacteria, especially Actinomycetes (Brown and Chang, 2014). Four groups of saproxylic bacteria have been described based on their role in decomposition: bacteria that do not affect the wood strength, but makes it more water permeable; bacteria with decomposition abilities, attacking wood cell walls and affecting wood strength; bacteria which function as an integral part of the total microflora, contributing to the breakdown of wood; and the passive colonisers, not contributing to wood decomposition but influencing other organisms by producing inhibitory compounds (Greaves, 1971). Distinct patterns of bacterial degradation of wood cells have been described as tunnelling, erosion and cavitation (Singh and Butcher, 1991). Tunnelling bacteria degrade lignified elements, such as tracheids, fibres and vessels, penetrating into the cell wall, breaching the S3 layer barrier, after attaching to the lumen face. The cell wall is degraded by bacteria tunnelling through all areas of the cell wall in random directions, until the cell wall, including the lignified lamella is degraded (Fig. 1.4) (Singh, 2012). Bacteria involved in erosion, degrade secondary wall layers, depleting cellulose and hemicellulose from the wood and characterised by a honeycomb pattern (Fig. 1.4) (Blanchette, 2000). They attack the cell wall from the lumen towards the middle lamella and produce erosion troughs in the exposed faces of the cell wall, producing slime and glide along the microfibrils. These can occur with tunnelling bacteria and/or soft-rot fungi and can degrade wood under conditions that may inhibit some wood decay fungi, such as low oxygen and waterlogging (Greaves, 1971; Clausen, 1996; Singh, 2012; Huang, *et al*., 2013). Bacteria involved in cavitation form irregular cavities within the secondary cell wall, orientated perpendicular to the long direction of the fibre (Fig. 1.4). They decompose carbohydrates of the secondary wall leaving residual wall material in the cavities (Blanchette, 2000).

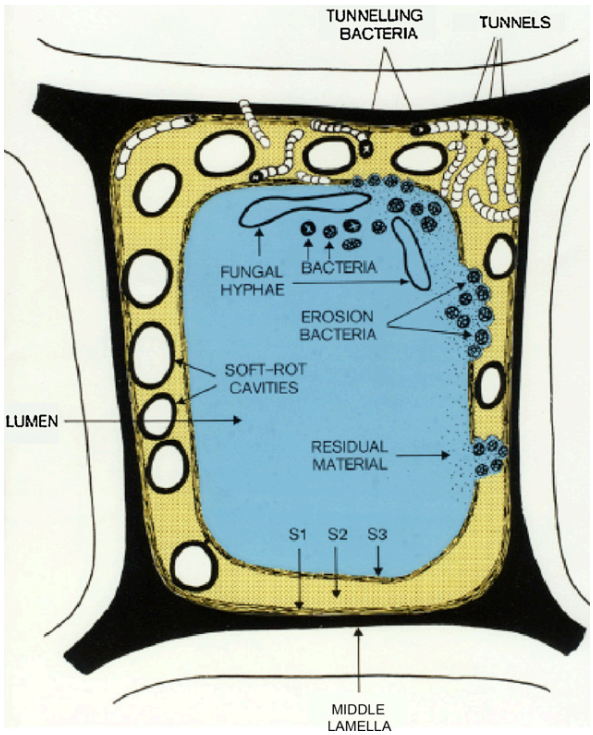

Figure 1:4: A diagram illustrating wood decay patterns produced during cell wall degradation by soft rot fungi and tunnelling and erosion bacteria (Singh, 2012).

1.2.2 Wood Degradation by Fungi

Fungi play a key role in the majority of global carbon cycling as primary decomposers of organic material, such as lignocellulose (Boddy and Watkinson, 1995; Kavanagh, 2011; Voříškova and Baldrian, 2013; Chandra *et al.*, 2015). Some fungal strains, such as *Phanerochaete chrysosporium, Trichoderma reesei* and *Aspergillus brasiliensis* are well studied as model organisms for lignocellulose degradation (King *et al.*, 2010; Xie *et al.*, 2014). Most fungi already discovered are terrestrial, inhabiting soil detrital matter, are parasitic to plants or can cause disease in animals and humans (Moore *et al.*, 2015). Some are aquatic, primarily freshwater with a few marine fungi currently described (Moore *et al.*, 2015). Fungi cause the most serious microbiological deterioration of wood due to their ability to cause rapid structural failure (Green and Highley, 1997). The initial decay process involves fungal communities of endophytes, pathogens and saprophytes present in plant tissues (Boddy, 2001). Wood degradation is thought to be associated with mycelial growth, allowing transport of scarce nutrients such as iron and nitrogen into the nutrient poor lignocellulosic substrate, constituting its carbon source (Sanchez *et al.*, 2005). Fungi that degrade wood possess a wide range of enzymes that break down cell wall polymers, penetrating the cell wall and alter its chemistry. The resulting constituents are taken up by the hyphae for energy and

anabolism (Mohebby, 2005; Schwarze, 2007). Wood decay is dependent on alterations in the wood with three types commonly recognised; white rot, brown rot and soft rot fungi which attack wood, based on their mode of decay (Worrall *et al.*, 1997). These are noticeable by their effects on wood colouration and consistency.

White-rot fungi, in particular Basidiomycota; *Phanerochaete chrysosporium,* are the most studied wood degrading fungi (King *et al.*, 2010; Bugg *et al*., 2011a; Bugg *et al*., 2011b). They are potent degraders due to their ability to degrade or modify all structural wood components; cellulose, hemicellulose and lignin, using a large set of enzymes, which gives rise to cellulose-enriched white material (Blanchette, 1991; Mester *et al.*, 2004; Bugg *et al*., 2011a; Bugg *et al*., 2011b). The white-rots can utilise lignin as a sole carbon and energy source and destroy lignin as a way to gain access to cellulose and hemicellulose (Martínez *et al.*, 2005; Sánchez, 2009). White-rots decompose lignin by secreting enzymes such as laccases, LiPs and MnPs, degrading it to CO_2 (Sánchez, 2009). Two forms of white-rot fungi have been described due to their ability to degrade lignin selectively or simultaneously with cellulose; simultaneous rot and selective delignification (Otjen and Blanchette, 1986). Most species use one form of degradation, although under alternative conditions, some species can cause a combination of both (Blanchette, 1991).

Both white-rot and brown-rot fungi invade the lumen of wood cells (Kirk and Farrell, 1987). It was previously thought all known white-rot and brown-rot wood-degrading fungi were Basidiomycota, however, it is now considered many are Ascomycota. It has been demonstrated that there is inadequacy in classifying fungi as white rots or brown rots, based on their capabilities to degrade lignin, cellulose and hemicellulose (Riley, *et al*., 2014). Riley *et al*., (2014) concluded that the by placing species of fungi into these two categories, does not capture the diversity of fungal wood decay mechanisms, suggesting there is a continuum rather than a dichotomy. All wood decay fungi are essential recyclers of plant biomass in forest ecosystems with many species growing on dead wood or leaf litter, that are rich in lignocellulose (Baldrian and Valášková, 2008; Zhang *et al*., 2016; Buzzini *et al.*, 2017; Vaz *et al.*, 2017). In contrast to white-rot, brown-rots have evolved mechanisms that are faster and more polysaccharide specific, enabling them to circumvent lignin, penetrating hemicellulose to access cellulose, with only minor modifications to lignin (Martínez *et al.*, 2005; Pozo *et al.*, 2010; Zhang *et al.*, 2016). Brown-rots have lost key white-rot genes including many linked to lignin degradation and cellulose hydrolysis (Zhang *et al.*, 2016). Due to the rapid loss of cellulose, the wood undergoes a significant loss of strength (Cowling, 1967). As lignin is the major component of the degraded plant cell wall, the wood shrinks, darkens and is easily broken into crumbly, brick shaped pieces (Green and Highley, 1997).

White-rot and brown-rots are known as macrofungi, in contrast, soft-rot fungi are microfungi (Fukasawa *et al.*, 2009, 2010). Soft-rots are predominantly Ascomycetes that degrade cellulose and hemicellulose in the central layer of the secondary cell wall, with some species decomposing lignin by eroding the secondary cell wall, decreasing the content of acid-insoluble material (Savory, 1954; Liers *et al.*, 2006; Chandra *et al.*, 2015). A spongy consistency arises from the erosion of the central layer, with the removal of cellulose giving a darkened surface. In contrast to white and brown rot fungi, the soft-rot fungi grows inside the cell wall slowly, either by excavation of cavities in the secondary layer or enzymes being released onto the cell wall (Hale and Eaton, 1985). The fungi either branch or continue into the adjacent cell through the cell wall, forming cylindrical and biconical cavities along cellulose microfibrils (Blanchette *et al.*, 2004). The diameter of the cavity increases as it develops and a new cavity is formed by longitudinal growth, with the process repeating several times. The hyphae growing inside the cell walls branch in a T-shaped manner to breakdown the cell wall. After apical extension within the cell wall, hyphae growth stops and the cavity continues to be created by enzymatic activity along the hyphae, which increases in diameter (Hale and Eaton, 1985). The enzymes released from the hyphae do not diffuse into the cell wall, instead they act only on the cell wall surface (Eriksson *et al.*, 2012). Soft rots inhabit soil and can utilise cellulose in fallen wood, however, they are not classed as true wood decay fungi as soil is their primary habitat. However, they have demonstrated to be decay agents of wood with high water content, lowering lignin content and also favouring harsh and extreme environments, such as Antarctica, where ground surface thaws providing moisture for soft-rot fungi to grow (Crawford *et al.*, 1990; Blanchette *et al.*, 2004; Chandra *et al.*, 2015).

Other microfungi, such as moulds (Ascomycota and Zygomycota) and blue-stain fungi (Ascomycota) are both discolouring fungi, growing on the surface of wood and often the initial colonisers (Hukka and Viitanen, 1999; Fukasawa *et al.*, 2009). Examples of mould fungi are *Verticillium* spp., *Trichoderma* spp., *Penicillium* spp., *Fusarium* spp. and *Mucor* spp. (Fukasawa *et al.*, 2009). Wood associated yeast species have been shown to secrete CAZymes. Jiménez *et al*., (1991) screened 51 yeast species associated with wood for enzymatic activities and found *Aureobasidium microstictum* was the only yeast able to grow in carboxymethyl cellulose (CMC), straw hemicelluloses and wood xylan and displayed β-glucosidase and β-xylosidase activities. This suggests it is involved in the process of both cellulose and hemicellulose degradation. Many other yeasts, such as, *Debaryomyces hansenii*, *Middelhovenomyces petrohuensis* and *Tausonia pullulans* were able to grow on CMC and straw hemicellulose and had cellulase and pectinase enzymes to degrade these substrates (Jiménez *et al*., 1991). Xylanolytic activities have been reported to be mainly associated

to genera *Aureobasidium*, *Pseudozyma*, *Scheffersomyces* and *Sugiyamaella,* indicating the potential to degrade hemicellulose (Buzzini *et al*., 2017). Few yeast species are reported to be able to produce enzymes from the cellulolytic complex but many yeasts show β-glucosidase activity, the final step of converting cellobiose to glucose (Buzzini *et al.*, 2017). As aforementioned, fungi, including yeasts are abundant at different stages of the decay process, it has been reported, that the presence and distribution of yeasts in different stages of wood decay, that *Ganoderma applanatum* and *Candida railenensis* were very abundant at the initial stages of decay, whereas, *Sugiyamaella chiloensis* and *Candida sake* were predominant at the final stages. *Naganishia albida* was the only yeast species present at all four stages of degradation, showing an increasing abundance through the decay process (González *et al*., 1989). Yeasts form an important association with Basidiomycetes during the wood decay process, in which bacteria are closely associated (Buzzini *et al*., 2017). The presence of these yeasts during different stages of wood decay highlight the ecological role played by yeast. Saccharomycetes are especially associated with the guts of saproxylic insects involved in lignocellulose degradation (Calderon and Berkov, 2012).

1.3 Symbiosis Systems

Anton de Bary (1879) originally described symbiosis as the association (temporal or spatial) between individuals who do not belong to the same species, independent of the effects on the organisms involved. This encompasses a broad range of interactions, be they negative (parasitism), neutral (commensalism) or positive (mutualism) (Horváthová *et al.*, 2015).

1.3.1 Bacterial and Fungal Interactions in Wood Degradation

The role of fungi in wood degradation has been studied in more depth than bacteria. However, bacteria in wood should also be considered when examining the presence and identity of wood-decay fungi, as current evidence strongly indicates they greatly influence the bacterial community (Folman *et al.*, 2008; Hoppe *et al.*, 2015). The interactions between fungal and bacterial populations can play a critical role in the breakdown of lignocellulose in the environment (Shortle *et al.*, 1978; Benner *et al.*, 1984; Lang *et al.*, 2000). These synergistic interactions can alter microbial community structure, development, and composition (Romaní *et al.*, 2006; Frey-Klett *et al.*, 2011). Wood degradation by fungi can be inhibited or promoted by bacteria depending on the species present and the growth stage at which the association is initiated (Murray and Woodward, 2003; Lugtenberg and Kamilova, 2009). For example, in a microcosm study, white rot fungi; *Hypholoma*

fasciculare and *Resinicium bicolor* were inoculated onto wood blocks. These species were selected as *H. fasciculare* is a common coloniser of beech wood and other broad-leaved tree species and *R. bicolor* is associated with coniferous trees and found in angiosperm woodlands. The fungi colonised the wood blocks for 15 days before the inoculated blocks were placed on soil that contained unidentified mixed bacterial species. The culturable population of bacteria dropped to from 61% to 1% when wood-decay fungi were present, indicating fungi had a big impact on the bacterial population (Folman *et al.*, 2008). Bacteria often adapt to the conditions created by fungi, for example, in one study the decreased pH of wood by white-rot fungi had a deleterious and selective effect on bacteria, whilst in another study the same fungi produced high acidity and produced radical oxygen species and the bacteria adapted to the environment (Kielak *et al.*, 2016). Bacteria can alter the structural integrity of wood, by increasing permeability, providing more favourable attack sites for fungi and increasing overall decomposition rates (Clausen, 1996). Bacteria and fungi inhabiting together can compete for, or provide each other with resources; these interactions can be powerful mutual drivers with positive and negative feedbacks. Bacteria can have a negative effect on the fungal community, competing for sugars released by fungal extracellular enzymes during wood degradation (de Boer and van der Wal, 2008). However, synergistic effects can also occur, bacteria can provide fungi with limiting nutrients or vitamins in exchange for these sugars, or by supplying biologically fixed nitrogen that fungal hyphae transport to the wood degradation site (Seidler *et al.*, 1972; Aho, 1974; Clausen, 1996; de Boer and van der Wal, 2008). These bacterial-fungal interactions can be highly specific with symbiotic associations developing between bacterial cells and fungal hyphae (Frey-Klett *et al.*, 2011).

Fungi work synergistically in lignocellulose degradation. Basidiomycetes are dominant in early stages of decay with many other fungi occurring in the latter stages, such as primary delignification by white-rot fungi which promotes soft-rot fungi activity (Tanaka *et al.*, 1988; Crawford *et al.*, 1990; Fukasawa *et al.*, 2009, 2010). Wood decay is a function of a diverse, interacting ligninolytic fungal community.

1.3.2 Animals with a Diet Rich in Cellulose

As aforementioned, specific microorganisms have adapted to degrade lignocellulose and play a major role in biogeochemical cycling. This symbiosis goes a stage further with three-way symbionts involving bacteria, fungus and a host (Fig. 1.5). The ecosystem of the gastrointestinal (GI) tract is a nutrient rich environment, allowing microorganisms to colonise and grow (Wells and Varel, 2007). This gut microbiota plays an important role in host development, physiology and health, influencing

resistance to infection, immunology, physiology, gut development and digestion of complex carbohydrates, such as lignocellulose (Mueller *et al.*, 2012; Llewellyn *et al.*, 2014; Bouchon *et al.*, 2016). Animals that have a diet rich in lignocellulose play a key role in the carbon cycle as they play an essential role in plant decomposition and recycling of nutrients, accomplished by the host and gut microbe activities (Brauman *et al.*, 2001; Watanabe and Tokuda, 2010; Ni and Tokuda, 2013; Bredon *et al.*, 2018).

Woody plant material is a challenging dietary resource for animals to digest, as plants contain recalcitrant polymers including cellulose, hemicellulose and lignin (Bayer *et al.*, 1998). Employing a wood-based dietary strategy with its low nutritional quality and lack of nitrogenous compounds, xylivores and detritivores must either rely on the activities of endosymbiotic microbes or produce the essential endogenous cellulose and lignin degrading enzymes. As cellulose is the major component of lignocellulose biomass and the most abundant renewable organic compound on Earth, it is not surprising that it is used as a food source by many organisms (Kostanjšek *et al.*, 2010).

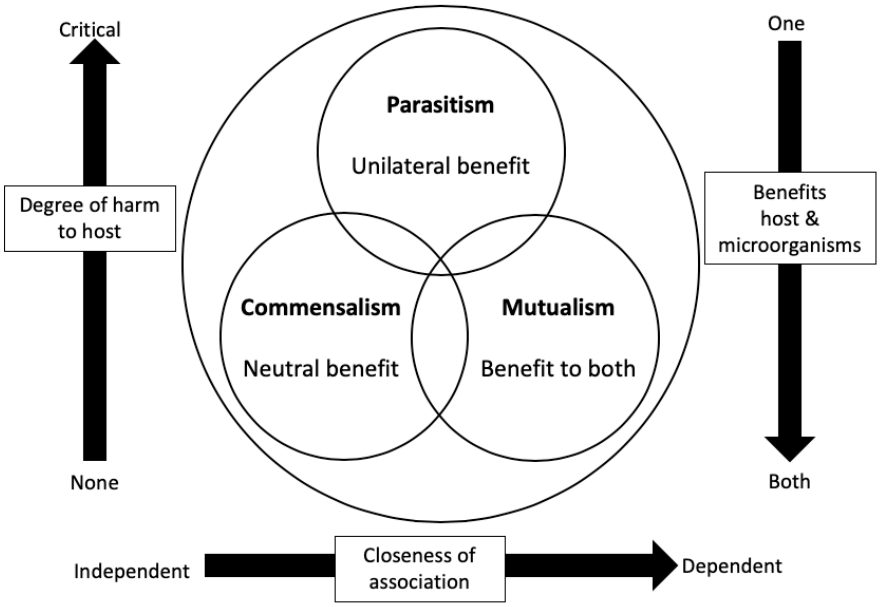

Figure 1:5: Host/microbe interactions. Venn diagram showing inter-relationships of various symbioses and relationship to the host. Adapted from (Wells and Varel, 2007)

1.3.2.1 Insects

It has been reported that wood-eating isopod *Limnoriids* are devoid of microbes in their digestive tract, instead relying on a range of endogenous glycosyl hydrolases that enable greater aptitude for lignocellulose digestion than animals relying on endosymbiotic bacteria (King *et al.*, 2010). In contrast, whilst many insects have endogenous cellulases in their tissues, these work symbiotically

with the consortium of microbes inhabiting the digestive tract (Watanabe and Tokuda, 2010). Several species of saprophagous and xylophagous beetles; *Anoplophora chinensis*, *Amphimallon solstitiale, Oryctes nasicornis, Cucujus cinnaberinus, Schizotus pectinicornis, Rhagium mordax and Rhagium inquisitor,* have been investigated for bacterial and fungal diversity in the digestive tract. Many dominant bacterial and fungal sequences from these GI tracts were found to produce cellulases, however, work has yet to be carried out to characterise the function of the gut associated bacteria (Rizzi *et al.*, 2013; Ziganshina *et al.*, 2018). Bacteria and fungi are known to be important partners to beetles, affecting the metabolism of the host, with symbionts providing the host with a supply of carbon, nitrogen, energy, vitamins and pheromones (Grünwald *et al.*, 2010; Rizzi *et al.*, 2013; Kaltenpoth and Steiger, 2014).

The termite gut; *Reticulitermes flavipes* requires a greater contribution from microbial cellulose digestion, which works synergistically with endogenous cellulases to enable them to degrade 74–99% of cellulose and 65–87% of hemicellulose, making digestion of wood by termites even more efficient than ruminants (Breznak and Brune, 1994; Watanabe and Tokuda, 2010). Lignocellulose digestion in termites involves both the host and its gut microbiota, including protists (Brune, 2014). Lower termites have a large hindgut containing protists and a high abundance of Spirochaetes, with termite species containing between 3 species of protist in *Coptotermes formosanus* and 19 species of protist in *Hodotermopsis japonica* (Brune, 2014; Otani *et al.*, 2014). After ingestion of wood, any glucose is released in the midgut and reabsorbed into the termite epithelium. The partially digested wood enters the voluminous hindgut paunch, where it is phagocytized by cellulolytic flagellate protists, hydrolysing polysaccharides using cellulases and hemicellulases secreted into their digestive vacuoles. Short chain fatty acids, produced by fermentation, are reabsorbed by the termite, with lignin residues excreted as faeces (Brune, 2014). Whereas lower termites are restricted to a wood diet, higher termites have completely lost flagellate protists in their hindgut and have adopted new strategies for cellulose digestion, enabling them to eat a variety of other food sources, such as grass, plant litter and herbivore dung. This allowed them to create a symbiotic relationship with plant biomass-degrading fungus *Termitomyces*, living together as obligate mutualistic symbionts, allowing the fungi to overcome unfavourable seasonal conditions and allowing the termite to exploit lignocellulose (Nobre and Aanen, 2012). The fungus grows on the fungus comb maintained by termites, continually adding pre-digested plant material. The fungus breaks down the woody substrate, converting it into a form the termites can digest, as they lack enzymes that can break down wood efficiently (Nobre and Aanen, 2012). Higher fungus-growing termites; *Macrotermes subhyalinus* and *Microtermes toumodiensis* have a distinct gut microbiota

compared to lower termites; *Coptotermes niger* and *Reticulitermes santonensis* and higher non-fungus-growing termites; *Nasutitermes corniger* and *Amitermes meridionalis*. This indicates a predominance of Firmicutes and Bacteroidetes, which thrive on a fungus rich diet (Otani *et al.*, 2014). A diet rich in fungus will be rich in protein and provide the termite with energy in the form nutrients.

1.3.3 Wood-Eating Catfish *P. nigrolineatus*

Although there are many herbivorous fish, the diet of *P. nigrolineatus* is unique, having evolved to eat wood, a strategy unknown outside the Neotropics. Although this fish is xylophagic, it is debated whether it is xylivorous, using the consumption of wood as an energy source, after the digestion of recalcitrant polysaccharides by endosymbiotic microbial communities (Lujan *et al.*, 2011; Bauer *et al.*, 2014). To date, there is no evidence of xylivory and therefore, *P. nigrolineatus* is more likely to obtain energy from the consortium of endosymbiotic bacteria colonising the GI tract, that have been shown to have cellulose degrading and nitrogen fixing capabilities (Nelson *et al.*, 1999; McDonald *et al.*, 2012, 2015; Di Maiuta *et al.*, 2013; Watts *et al.*, 2013).

Fish represent the greatest diversity of all vertebrates (Clements *et al.*, 2014), however, understanding their gut microbiota and its significance on its host is lacking compared to terrestrial vertebrates. Amazonian Loricariidae is a species rich family of catfish distributed in freshwater ecosystems of the Neotropics (Isbrücker, 1980; Eschmeyer *et al.*, 2018). One member of the Loricariidae, *P. nigrolineatus* (Fig. 1.6A), has been the focus of study by several groups due to its ability to imbibe large amounts of wood, up to 70% of the GI tract contents (German, 2009). *P. nigrolineatus* uses spoon-shaped teeth and a suckermouth to allow ingestion of woody material by rasping (Fig. 1.6B) (German, 2009). Stable isotope studies provide support for the consumption of large amounts of cellulose as part of their diet (German, 2009; German and Bittong, 2009; Lujan *et al.*, 2011), which may offer selective advantage when river nutrients are limited during the dry season (Araujo-Lima *et al.*, 1986). The *P. nigrolineatus* GI tract is approximately ten times its body length, providing a large surface area with many different microenvironments (German, 2009). The tract is coiled and convoluted filling the entire abdominal cavity (Fig. 1.6C), coiling around the auxiliary organ, whose function is unknown, and is located midway between the mouth and anus (German, 2009; McDonald *et al.*, 2012).

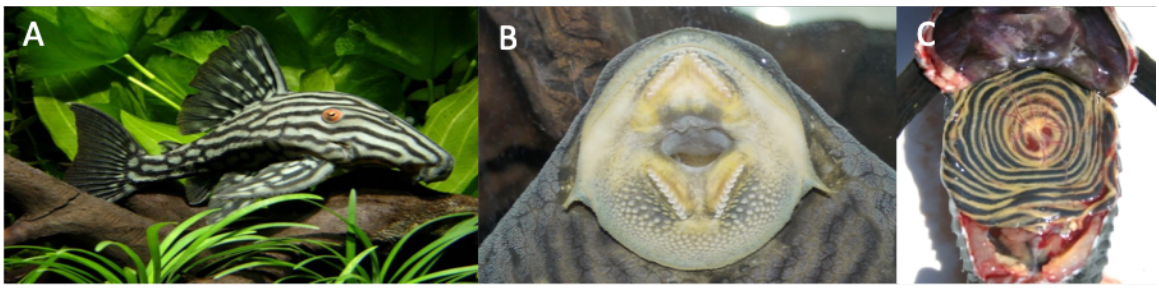

Figure 1:6: Morphology of *P. nigrolineatus.* (A) external view of the fish (B) arrangement of teeth and suckermouth (C) GI tract arrangement in the body's cavity ((B&C) German, 2009a; (A) 1Fish2FishDartmouth, 2019).

1.3.3.1 Bacterial Communities in the GI tract of P. nigrolineatus

Many studies have previously focused on bacterial communities colonising the intestine of fish species, including *P. nigrolineatus* with little known about the fungal communities (Roeselers *et al.*, 2011). Previous studies into *Panaque* have described the isolation of cellulolytic bacteria from the GI tracts and faeces, demonstrating the presence of a consortium of microorganisms performing cellulose breakdown (Nelson *et al.*, 1999; McDonald *et al.*, 2012; Di Maiuta *et al.*, 2013). Using 16S ribosomal ribonucleic acid (rRNA) gene and culture-based analyses, the enteric bacterial community of *P. nigrolineatus* appeared distinct and specialised in each region of the GI tract. The dominant bacteria found were sequences similar to Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria (McDonald *et al.*, 2012, 2015; Watts *et al.*, 2013). The midgut contained phylotypes with high sequence similarity to cellulose degrading bacteria and nitrogen-fixing species were found capable of *in situ* nitrogen fixation (McDonald *et al.*, 2015). The hindgut was dominated by Bacteroidetes (Watts *et al.*, 2013; McDonald *et al.*, 2015). Bacterial species richness has been shown to decrease distally from foregut, through to the midgut and hindgut (McDonald *et al.*, 2012). Research has reported wood-eating catfish are detritivores, specialised in consuming low-quality food and do not have anatomical adaptations of true xylivory (German, 2009; German and Bittong, 2009). German concluded the rapid transit time of digested wood (3 hours) and low activity of digestive polysaccharides, meant little energy was taken up from digested wood with fish losing weight on a wood only diet. His findings indicated cellulolytic activity was caused by microbes living on the wood surface in a biofilm, ingested as part of a detrital diet (German, 2009).

The bacterial microbiome within the GI tracts of other fish have been studied and is dominated by Proteobacteria, which can represent up to 90% of communities, Fusobacteria, Firmicutes and to a lesser extent, Bacteroidetes, Actinobacteria and Verrucomicrobia (Llewellyn *et al.*, 2014). Comparatively, little is known about the diversity, abundance and role of the fungal microbiota in

these systems (Clements *et al.*, 2014). A recent study by Siriyappagouder *et al*., (2018) showed the fungal community of the zebrafish (*Danio rerio*) intestine to be varied according to rearing conditions and diet, with wild zebrafish being colonised by Dothideomycetes and laboratory reared being colonised by Saccharomycetes. Interestingly, the same study found that fish caught in the wild and kept under laboratory conditions for two months had a more similar intestinal mycobiota to laboratory fish than wild fish.

Studies on bacterial communities have shown the importance for host health (Llewellyn *et al.*, 2014; Tarnecki *et al.*, 2017), so it is likely that fungi play an important role in the fish microbiome. Yeasts are known to play a vital role in fish nutrition, as they produce digestive enzymes (Banerjee and Ghosh, 2014) and have been identified as part of the normal microbiota of the fish GI tract and essential for fish health (Waché *et al.*, 2006; Raggi *et al.*, 2014; Ochangco *et al.*, 2016).

1.3.4 The Common Woodlouse

The most successful colonisers of terrestrial habitats among Crustacea are Oniscidean isopods and are distributed widely throughout terrestrial ecosystems, from very wet conditions to desert environments (Sfenthourakis and Taiti, 2015). Isopods invaded land from marine ancestors and evolved some unique morphological adaptations, including a reduced body size, a water-resistant cuticle, a water conducting system, pleopodal lungs, and a brood pouch for the development of juveniles (Bursell, 1954; Hornung, 2011).

Terrestrial isopods have adapted to terrestrial food sources and are detritivores, scavenging on dead and decaying organic matter that is rich in cellulose and other polysaccharides (Wieser, 1966). This allows them to indirectly take part in the decomposition and cycling of energy and organic matter back into terrestrial environments, whilst promoting microbial activity by fragmenting the substrate, proliferating some of the ingesting microorganisms in their gut and distributing them in their faeces (Hassall *et al.*, 1987; Zimmer and Topp, 1997; Zimmer, 2002). Isopods also use the ingested microorganisms as a source of nutrients, vitamins and enzymes (Hassall and Jennings, 1975; Carefoot, 1984; Gunnarsson and Tunlid, 1986; Ullrich *et al.*, 1991). The species being used in this study are the common woodlouse *Oniscus asellus* (Linnaeus, 1758) and the common rough woodlouse *Porcellio scaber* (Latreille, 1804), which live in a temperate climate in damp, dark environments.

1.3.4.1 The Terrestrial Isopod Digestive System

The digestive system of both these species of woodlice is tripartite. It consists of a short, cuticle lined foregut, which includes an oesophagus and stomach, hepatopancreas which consists of two pairs of blind-ending digestive glands which possess a spiral groove and a tube-like, cuticle lined hindgut (Hames and Hopkin, 1989; Kostanjšek *et al.*, 2002) (Fig. 1.7). The hindgut is divided into the anterior chamber and the palliate region which leads to the rectum (Hames and Hopkin, 1989). The food is masticated and compressed in the foregut and mixed with secretions from the hepatopancreatic lobes. The liquids from the foregut are channelled into these lobes and the solids are passed from the foregut into the anterior hindgut (Hassall and Jennings, 1975; Hames and Hopkin, 1989). The anterior hindgut has a pair of dorsal typhlosole channels, which are an adaptation for terrestrial living (Hames and Hopkin, 1989). When the hindgut is full, muscles surrounding the hindgut contract and force liquid back into the foregut via the typhlosole channels (Hartenstein, 1964). This fluid, containing secretions from the hepatopancreas and digesta is filtered in the foregut and passed into the lumen of the hepatopancreas for further digestion and absorption of nutrients. This process may occur several times in one digestive cycle (Hames and Hopkin, 1989). A primary and secondary filter have been reported, that separate solids and fluid components of the ingested food, channelling the solids into the hindgut whilst the fluids are drawn into the hepatopancreas (Griffiths and Wood, 1985; Storch, 1987; Hames and Hopkin, 1989; Storch and Strus, 1989). Hames & Hopkin (1989) reported this filter prevents particles greater than 40 nm from entering the hepatopancreas, but Wood & Griffiths (1988) reported the size of these particles were larger than 1.17 μm. Storch (1987) postulated that when the paired inferolateralia are drawn apart, the resulting gap may allow large particles to enter the atrium of the hepatopancreas and bypassing the secondary filter. The hepatopancreas is hypothesised to be the major site for production and secretion of digestive enzymes and absorption of soluble nutrients, with the hindgut playing a lesser role in this process (Alikhan, 1972; Hassall and Jennings, 1975; Hryniewiecka-Szyfter and Storch, 1986; Hames and Hopkin, 1989; Zimmer, 2002).

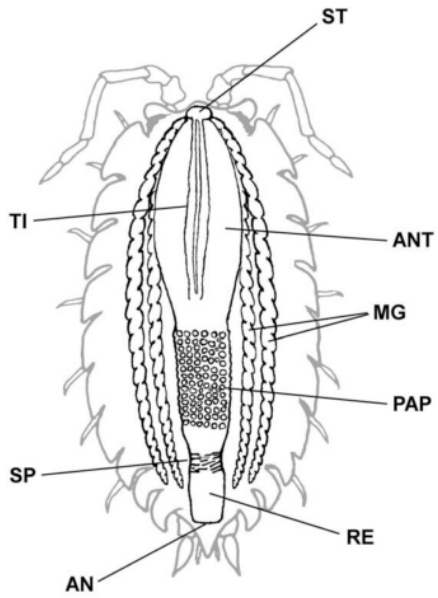

Figure 1:7: Digestive system of *P. scaber*. ST - stomach, TI - typhlosole, ANT - anterior hindgut, MG - midgut glands (hepatopancreas), PAP - papillate region of hindgut, SP -sphincter, RE - rectum, AN - anus (Kostanjšek *et al.*, 2002).

1.3.4.2 The Gut Microbiota in the Hepatopancreas of Woodlice

The lobes of the hepatopancreas has previously been the focus of the majority of studies as they harbour dense bacterial populations in *O. asellus*, *P. scaber*, *Porcellio dilatatus* and *Ligia pallasii* (Wood and Griffiths, 1988; Hames and Hopkin, 1989; Zimmer and Topp, 1998b, 1998a; Zimmer, 1999; Bouchon *et al.*, 2016). The function of these lobes are important as they are suggested to be involved in the hydrolysis of cellulose, oxidative breakdown of lignin and tannins and therefore, contribute to ecosystem degradation processes (Zimmer and Topp, 1998b, 1998a; Zimmer, 1999; Zimmer *et al.*, 2002). Endogenous cellulase and lignocellulase genes have been reported in the tissues of *P. scaber*, *Limnoria* and *A. vulgare* (King *et al.*, 2010; Kostanjšek *et al.*, 2010; Bredon *et al.*, 2018) and these genes have more recently been reported to be produced from the gut microbiota of *A. vulgare* too (Bredon *et al.*, 2018).

In the woodlouse, although dense microbial populations have been detected, the diversity has been shown to be low and thought to provide a mutualistic relationship with the woodlouse, aiding the nutritive utilisation of low-quality food sources (Zimmer, 2002). *Candidatus Hepatoplasma crinochetorum* (hereafter *Hepatoplasma*) and *Candidatus Hepatincola porcellionum* (hereafter *Hepatincola*) are specifically associated with the hepatopancreas and have stalk-like cytoplasmic

appendages inserted into the epithelial brush border of hepatopancreatic cells (Wang *et al.*, 2004a; Wang *et al.*, 2004b).

The hepatopancreas has an unusual structure and the major site for the production and secretion of digestive enzymes and absorption of nutrients and *O. asellus* and *P. scaber* have been shown to harbour dense populations of bacteria (Wood and Griffiths, 1988; Hames and Hopkin, 1989; Zimmer and Topp, 1998b; Zimmer, 1999). A number of bacterial species have been found in the hepatopancreas of isopods, these have been from a range of qualitative visual methods to recent sequencing methods (Table 1.1). More recent studies show the hepatopancreas to be colonised by one of two bacterial symbionts (Wang *et al.*, 2004a; Wang *et al.*, 2004b). Mycoplasma like *Hepatoplasma* were first found to be densely colonising the hepatopancreas of *P. scaber* and described as gram negative, spherical cells (0.5 to 0.8 μm diameter), with no cell wall or outer membrane. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) showed stalk-like appendages at one or two cell poles that attach to the host epithelial microvillous brush border of cells (Wang *et al.*, 2004a). The other symbiont, *Hepatincola* was first described from the hepatopancreas of *P. scaber* by TEM as rod shaped (1.5-3.8 μm x 0.5 μm), with a cell wall structure typical of gram-negative bacteria (Wang *et al.*, 2004b). It was found to be unusual with a number of electron dense inclusions in the cytoplasm and a stalk-like appendage at one cell pole, with its closest but distant relative among the pathogen Rickettsiales. Since this discovery, many studies on *O. asellus* and *P. scaber* have focused on these individual bacteria and not the bacterial community as a whole. Having dense populations of *Hepatoplasma* in the hepatopancreas is known to have a positive correlation with survivorship when fed low quality food, however the presence of pathogenic *Hepatincola* has a negative correlation to host longevity, when fed low- or high-quality food (Fraune and Zimmer, 2008).

Table 1:1: Bacterial taxa from the hepatopancreas of isopods. Terrestrial, freshwater* and marine^.

Host organism	Bacteria detected	Experimental method	Reference
P. scaber, Asellus aquaticus*	*Aeromonas*	Analytical profile index (API) 20 E diagnostic tests Polymerase chain reaction (PCR), cloning, sequencing, Fluorescent *in situ* hybridisation (FISH)	(Ullrich *et al.*, 1993) (Wang *et al.*, 2007)
A. aquaticus*	*Burkholderia*	PCR, cloning, sequencing, FISH	(Wang *et al.*, 2007)
O. asellus, P. scaber, Trachelipus rathkil	*Candidatus Hepatincola porcellionum*	Cloning, sequencing, *In situ* PCR, cloning, sequencing, FISH PCR & sequencing Genome sequencing	(Wang *et al.*, 2004b) (Wang *et al.*, 2007) (Fraune and Zimmer, 2008) (Leclercq *et al.*, 2014)
P. scaber, O. asellus, Philoscia muscorum, A. vulgare, T. rathkil, Alloniscus perconvexus, Ligia oceanica^	*Candidatus Hepatoplasma crinochetorum*	Cloning, sequencing, *In situ* PCR, cloning, sequencing, FISH PCR & sequencing	(Wang *et al.*, 2004a) (Wang *et al.*, 2007) (Fraune and Zimmer, 2008)
P. scaber	*Candidatus Rhabdochlamydia porcellionis*	Cloning & *In situ* PCR & FISH *In situ*	(Kostanjšek *et al.*, 2004) (Sixt *et al.*, 2013) (Kostanjšek and Marolt, 2015)
P. scaber, A. vulgare	*Candidatus Rickettsiella isopodorum*	PCR, sequencing, TEM, phylogeny	(Kleespies *et al.*, 2014)
P. scaber, O. asellus	*Citrobacter freundii*	API 20 E diagnostic tests	(Ullrich *et al.*, 1993)
O. asellus	Enterobacteriaceae	API 20 E diagnostic test	(Ullrich *et al.*, 1991)
P. scaber	*Enterobacter agglomerans*	API 20 E diagnostic tests	(Ullrich *et al.*, 1993)

P. scaber	*Enterobacter intermedium*	API 20 E diagnostic tests	(Ullrich *et al.*, 1993)
A. vulgare	*Halomonas*	qPCR, 454 amplicon pyrosequencing	(Dittmer *et al.*, 2016)
P. scaber	*Klebsiella pneumoniae*	API 20 E diagnostic tests	(Ullrich *et al.*, 1993)
P. scaber, Ligia oceanica^, A. vulgare	*Pseudomonas*	PCR, cloning, sequencing, FISH qPCR, 454 amplicon pyrosequencing	(Wang *et al.*, 2007) (Dittmer *et al.*, 2016)
A. aquaticus*	*Rhodobacter*	PCR, cloning, sequencing, FISH	(Wang *et al.*, 2007)
A. vulgare	*Rickettsiella*	qPCR, 454 amplicon pyrosequencing	(Dittmer *et al.*, 2016)
A. vulgare	*Shewanella*	qPCR, 454 amplicon pyrosequencing	(Dittmer *et al.*, 2016)
A. vulgare	*Wolbachia*	qPCR, 454 amplicon pyrosequencing	(Dittmer *et al.*, 2014, 2016)

Along with previously discussed *Hepatincola*, other pathogenic bacteria are known to colonise the hepatopancreas. *Candidatus Rhabdochlamydia porcellionis* has been discovered in *P. scaber* displaying distinctive white spots on the tissue (Drobne *et al.*, 1999; Kostanjšek *et al.*, 2004; Sixt *et al.*, 2013; Kostanjšek and Marolt, 2015). It has a similar developmental cycle characteristic for Chlamydiae, consisting of replicative reticulate bodies (RBs), intermediate bodies (IBs), and infectious elementary bodies (EBs) (Drobne *et al.*, 1999; Kostanjšek *et al.*, 2004). This bacterium inhibits apoptosis as a defence mechanism in infected insect cell lines (Sixt *et al.*, 2013).

Rickettsiella is another pathogenic bacterium known to infect arthropods (Fryer and Lannan, 1994). Infected isopods exhibit milky white masses (0.2 mm diameter), visible on infected tissue (Abd El-Aal and Holdich, 1987; Drobne *et al.*, 1999) and causes a slow developing and highly contagious disease in isopods, causing death (Bouchon *et al.*, 2011). Microscopy has shown the infected glands changed from its spiral appearance to loose and stretched, with cells of the hepatopancreas densely filled with microorganisms enclosed in vacuoles, that get released into the lumen (Drobne *et al.*, 1999). *Rickettsiella* is able to survive in the soil outside their host for many years, facilitating its natural route of infection via horizontal transfer from ingesting contaminated food (Bouchon *et al.*, 2016).

Wolbachia are members of the Alphaproteobacteria; Rickettsiales order, a diverse group of intracellular bacteria that comprises species with parasitic, mutualistic and commensal relationships with their hosts. Unlike other Rickettsiales that need a vertebrate host to complete the parasitic lifestyle, *Wolbachia* infection is restricted to invertebrates (Sicard *et al.*, 2014). *Wolbachia* is widespread among arthropods and generally behave as obligate intracellular reproductive parasites, manipulating cellular and reproductive processes, such as feminisation and cytoplasmic incompatibility (Werren *et al.*, 2008). It is vertically transmitted via the maternal line, inducing the development of male embryos into functional females by inhibiting the function of the androgenic gland (Vandekerckhove *et al.*, 2003). Cytoplasmic incompatibility has also been demonstrated in isopods, preventing infected males from successfully mating with females that lack the same *Wolbachia* strain (Bouchon *et al.*, 2016). It is also horizontally transferred via cannibalism and predation, being able to cross the intestine barrier and survive in other tissues (Le Clec'h *et al.*, 2013). It has been reported that *Wolbachia* is present in all major tissues of *A. vulgare* and represents the predominant taxon in tissues of infected females with distribution depending on the *Wolbachia* strain and tissue type, not the host genotype (Dittmer *et al.*, 2014).

1.3.4.3 The Gut Microbiota in the Woodlouse Hindgut

The hindgut has not been the focus of many studies as the microbial communities are thought to be transient due to the straight tube-like anatomy, the short retention time of food and the frequent removal of the cuticular lining along with residual material during the ecdysis (Hartenstein, 1964; Hassall and Jennings, 1975; Hames and Hopkin, 1989; Kostanjšek *et al.*, 2007; Mrak *et al.*, 2015). The majority of studies examining the bacterial communities in the hindgut were carried out prior to the identification of the endogenous cellulase gene in the hepatopancreas (Kostanjšek *et al.*, 2010) (Table 1.2). A number of previous studies have been carried out on the faeces, however, they are not included in Table 1.2, as they do not allow for the mucus membrane to be included and not representative of what microbes are resident in the hindgut (Ineson and Anderson, 1985; Hassall *et al.*, 1987; Ullrich *et al.*, 1991).

Table 1:2: Bacterial taxa from the hindgut of terrestrial isopods. Only empty hindguts through flushing or starvation were included.

Host organism	Bacteria detected	Experimental method	Reference
P. scaber	*Achromobacter*	Cloning, PCR for *merA gene*	(Lapanje *et al.*, 2010)
P. scaber	*Alcaligenes*	Cloning, PCR for *merA gene*	(Lapanje *et al.*, 2010)
O. asellus	*Arthrobacter*	TEM & diagnostic tests Cloning, PCR for *merA gene*	(Ullrich *et al.*, 1991) (Lapanje *et al.*, 2010)
P. scaber	*Bacillus*	Cloning, PCR for *merA gene*	(Lapanje *et al.*, 2010)
P. scaber	*Bacteroides*	Cloning, PCR for *merA gene*	(Lapanje *et al.*, 2010)
P. scaber	*Candidatus Bacilloplasma*	Microscopy, cloning, PCR, FISH 454 amplicon pyrosequencing, PICRUSt	(Kostanjšek *et al.*, 2007) (Dittmer *et al.*, 2016)
A. vulgare	*Candidatus Hepatincola*	454 amplicon pyrosequencing, PICRUSt	(Dittmer *et al.*, 2016)
O. asellus	*Corynebacteriaceae*	TEM & diagnostic tests	(Ullrich *et al.*, 1991)
O. asellus	*Corynebacterium*	Culture & biochemical tests	(Ineson and Anderson, 1985)
P. scaber	*Desulphotomaculum ruminis*	Cloning & sequencing	(Kostanjšek et al. 2004)
O. asellus	*Enterobacter agglomerans*	Culture, SEM, diagnostic tests	(Griffiths and Wood, 1985)
P. scaber	*Enterococcus faecium*	Cloning, PCR, restriction fragment length polymorphism (RFLP)	(Kostanjšek et al. 2004)
P. scaber	*E. saccharolyticus*	Cloning, PCR, RFLP	(Kostanjšek et al. 2004)
O. asellus ***Glomeris marginata***	*Klebsiella pneumoniae*	Culture & biochemical tests	(Ineson and Anderson, 1985)
A. vulgare	*Halomonas*	454 amplicon pyrosequencing, PICRUSt	(Dittmer *et al.*, 2016)
A. vulgare	*Hepatoplasma*	454 amplicon pyrosequencing, PICRUSt	(Dittmer *et al.*, 2016)
P. scaber	*Listeria*	Cloning, PCR for *merA gene*	(Lapanje *et al.*, 2010)
O. asellus	*Micrococcaceae*	TEM & diagnostic tests	(Ullrich *et al.*, 1991)
P. scaber	*Neisseria*	Cloning, PCR, RFLP	(Kostanjšek *et al.*, 2002)

P. scaber	*Ochrobactrum anthropi*	Cloning, PCR for *merA gene*	(Lapanje *et al.*, 2010)
P. scaber	*Paracoccus*	Cloning, PCR for *merA gene*	(Lapanje *et al.*, 2010)
O. asellus	*Plesiomonas*	Culture, SEM, diagnostic tests	(Griffiths and Wood, 1985)
O. asellus	*Pseudomonadaceae*	TEM & diagnostic tests	(Ullrich *et al.*, 1991)
O. asellus	*Pseudomonas fluorescens*	Culture, SEM, diagnostic tests	(Griffiths and Wood, 1985)
P. scaber	*Pseudomonas*	Cloning, PCR for *merA gene* 454 amplicon pyrosequencing, PICRUSt	(Lapanje *et al.*, 2010) (Dittmer *et al.*, 2016)
A. vulgare	*Rickettsiella*	454 amplicon pyrosequencing, PICRUSt	(Dittmer *et al.*, 2016)
A. vulgare	*Shewanella*	454 amplicon pyrosequencing, PICRUSt	(Dittmer *et al.*, 2016)
A. vulgare	*Wolbachia*	454 amplicon pyrosequencing, PICRUSt	(Dittmer *et al.*, 2016)

It has been previously thought the hindgut microbiota of terrestrial isopods mainly consists of ingested microbiota (Reyes and Tiedje, 1976; Ineson and Anderson, 1985), with bacteria and fungi serving as a source of food-limited nutrients (Zimmer and Topp, 1998b). However, the existence of a resident microbiota has also been indicated (Ineson and Anderson, 1985; Gunnarsson and Tunlid, 1986; Ullrich *et al.*, 1991). More recently sequences similar to obligately anaerobic bacteria were retrieved by a molecular approach from the hindgut of *P. scaber*, leading the authors to conclude they were autochthonous in the hindgut and not ingested (Kostanjšek *et al.*, 2002; Kostanjšek *et al.*, 2004). Bacteria were detected in the hindgut of *P. scaber* using TEM and described as wall-less, rod shaped and attached to cuticular spines protruding from gut lumen of the papillate region, these were reported to be sequences most similar to Tenericutes; Mollicutes; *Candidatus Bacilloplasma* and has also been more recently detected in *A. vulgare* (Kostanjšek *et al.*, 2007; Dittmer *et al.*, 2016). This observation strongly suggests the presence of bacteria adapted to the gut environment for long periods. It has been proposed that the shed hindgut cuticle is re-ingested to maintain and re-establish the lost minerals and microbial communities, however, to date, it has been reported that *Bacilloplasma* has never been detected in soil or associated with litter or isopod faeces (Bouchon *et al.*, 2016).

1.4 Heavy Metal and Antimicrobial Resistance in the Woodlouse Gut

The communities in the hindgut of terrestrial isopods can change depending on factors including environmental conditions. Metal contamination as a result of industrialisation and waste disposal affect all organisms and ecosystem processes, introducing metal tolerance in microorganisms (Lapanje *et al.*, 2010). Heavy metals in the environment can be moderately to highly toxic to bacteria, however, bacteria have the ability to adapt to environmental changes (Seiler and Berendonk, 2012). It has been shown that when *P. scaber* is exposed to environmental mercury pollution, the bacterial communities of the gut change and adapt (Lapanje *et al.*, 2007, 2010). The total bacterial community is not able to tolerate metals, which may result in differences in species richness in the gut of animals from metal polluted and non-polluted environments (Lapanje *et al.*, 2007, 2010). If metal pollution is chronic, the gut bacterial community may develop population-induced community tolerance via horizontal gene transfer (HGT) (Lapanje *et al.*, 2008, 2010). When looking at the differing communities within the hindgut of *P. scaber* in heavy metal environments, it was found that 80% had the mercury resistance gene; *mer*A gene present on the plasmids (Lapanje *et al.*, 2010).

Heavy metals and antimicrobial resistance (AMR) and can share structural and functional resistance systems (reviewed in Singer *et al.*, 2016 and references therein). These include reduction in membrane permeability, alterations of cellular targets, drug and metal alterations, efflux and sequestration with metal ions, using one or multiple mechanisms, accompanied by one or more antibiotics (Baker-Austin *et al.*, 2006). Co-selection of heavy metals indirectly selecting for AMR has been a concern since the 1970s (Koditschek and Guyre, 1974). Co-selection is achieved in two ways; co-resistance, whereby multiple genes encoding for heavy metal and antibiotic resistance are found on the same mobile genetic elements, such as plasmids, transposons and/or integrons, or cross-resistance describes mechanisms that provide tolerance to one or more antibiotic and/or metal (Summers, 2002; Chapman, 2003; Singer *et al.*, 2016). An example of cross resistance is a single enzyme functioning as an efflux pump for multiple metals and antibiotics (Hayashi *et al.*, 2000). Environmental pollution by heavy metals not only trigger the co-selection process, but due to co-regulation of resistance genes they decrease antibiotic susceptibility, therefore increasing AMR (Baker-Austin *et al.*, 2006). These genes are subject to combined transmission via HGT due to their close arrangement, whereby resistance genes are transferred among different taxonomic or ecological bacterial groups via mobile genetic elements (the mobilome), thus facilitating the spread of AMR in human pathogens (Chamier *et al.*, 1993; Stepanauskas *et al.*, 2006; Siefert, 2009; Seiler and Berendonk, 2012; Bengtsson-Palme *et al.*, 2017). The transfer of genetic material, between

bacterial cells is induced by stressors, such as antibiotics and heavy metals. It is known that woodlice can adapt and survive when exposed to heavy metals in the environment (Lapanje *et al.*, 2010; Jutkina *et al.*, 2016; Zhang *et al.*, 2017).

The O'Neill report predicts AMR may cause 10 million deaths a year by 2050 (O'Neill, 2014). World Health Organisation (WHO) revealed widespread occurrence of AMR among 500,000 people with bacterial infections across 22 countries, with some of the world's most common and most dangerous infections proving drug resistant (WHO, 2018). Humans have created environments with unparalleled mixing opportunities between environmental bacteria and human pathogens, through clinical, industry, aquaculture and agricultural practices and wastewater treatment plant discharge (Singer *et al.*, 2016) (Fig. 1.8). The interplay between different environments, the evolution and widespread dissemination of antimicrobial resistance genes (ARGs) in human pathogens is a constant clinical challenge (Arias and Murray, 2009). It is accepted that resistance genes did not originate in bacterial pathogens, therefore the sources of these genes would be acquired through HGT, which originated in the environment (Waksman and Woodruff, 1940; Martínez, 2012; WHO, 2014). Following selection of resistance genes within humans or animals treated with antimicrobials, resistant bacteria and antimicrobials are excreted in an active form and persist in the environment, creating a complex network of dispersal (Kasprzyk-Hordern *et al.*, 2009). A gene is defined as resistant when its presence allows a bacterium to withstand a higher antibiotic concentration or its absence increases susceptibility of the antibiotic (Martínez *et al.*, 2007).

Bacteria develop resistance either from mutations or by acquisition of antibiotic resistance genes from other bacteria in the environment through HGT via conjugation, transformation and transduction (Martínez, 2012; Hiltunen *et al.*, 2017). The mobilome is key for the spread of novel and known resistance genes, making them available to a larger part of the bacterial community in an environment, spreading beyond species (Martínez, 2012; Gaze *et al.*, 2013; Zhang *et al.*, 2017). These are associated with foreign genetic elements that contain ARGs, such as plasmids, transposons and integrons (Zhang *et al.*, 2017). Unless there is a selection pressure for maintaining ARGs or their fitness cost are negligible, the mobilisation of ARGs occurs continuously with novel resistance genes selected against unless there is a strong pressure to maintain them (Martínez, 2012; Bengtsson-Palme *et al.*, 2017).

AMR is considered one of the greatest challenges to global health threat (WHO, 2014), however little is known about the dispersal of AMR in the natural environment, in particular, highly mobile

species, such as woodlice, acting as AMR dispersers (Wellington *et al.*, 2013; Greig *et al.*, 2015; Huijbers *et al.*, 2015).

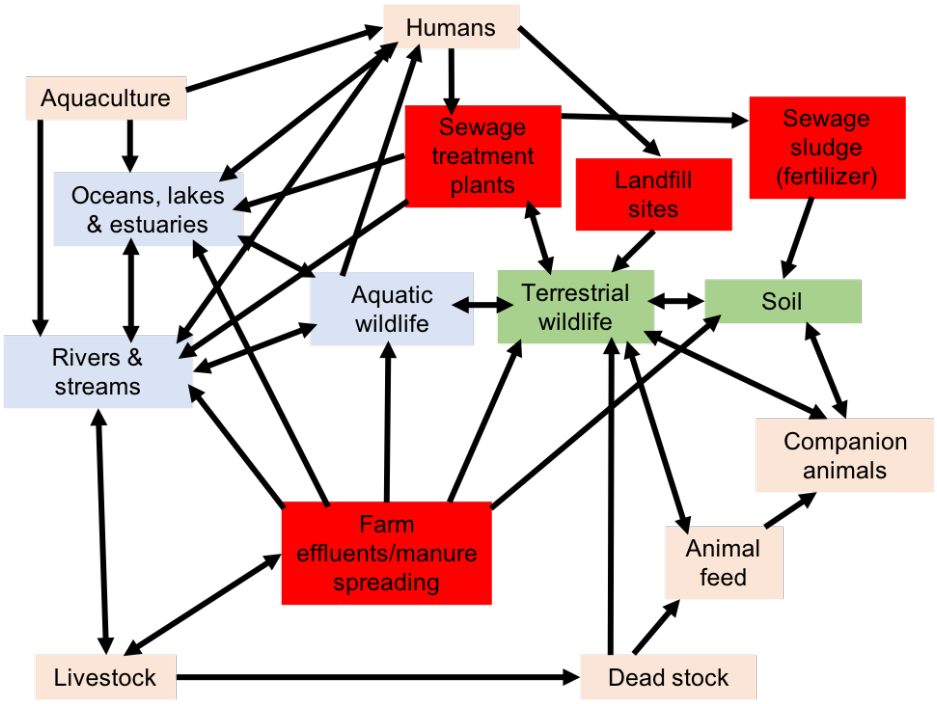

Figure 1:8: Dispersal of AMR across the environment (adapted from Arnold *et al*., (2016)).

1.5 Characterising the Gut Microbiota

A core microbiota is defined as the common members of different microbial consortia residing in a particular habitat (Turnbaugh *et al.*, 2007). It is important to identify core species to unravel the ecology of the microbes within a habitat and to start to understand their role and function in that community (Shade and Handelsman, 2012).

Culture-dependent and culture-independent methods are the two main approaches to characterising microbial populations. Culture-dependent methods rely on growth medium and biochemical tests to identify bacteria and fungi that are isolated under specific conditions. These can include the type of culture medium, presence of antibiotics and/or antifungals, temperature, atmosphere, pressure, incubation time, absence or presence of other microorganisms and sample collection conditions (Lagier *et al.*, 2015). Resulting colonies would be analysed further using biochemical tests. As different culture media are needed to grow specific organisms, all culture media are biased. This approach is time-consuming and laborious and with the majority of

microbiota having unknown growth requirements or cannot, to date, be grown under any conditions (Snyder *et al.*, 2009). 'The great plate count anomaly' describes the difference between the numbers of cells from natural environments that form viable colonies on agar media and the numbers countable by microscopic examination (Staley and Konopka, 1985). An example of this, as discussed earlier, is in the woodlouse hepatopancreas, dense populations of bacteria have been observed via microscopy, however, to date, the most dominant has not been cultured successfully.

Culture independent methods have been developing over the last several decades. Sequence based approaches allowed analysis of microbial populations, that had previously not been accessible due to their inability to be cultured or their rarity. Influential work by Carl Woese, analysed the sequence of the ribosomal small subunit RNA and found it to be conserved across all life, due to its vital role in protein synthesis. Woese adopted the small subunit ribosomal (SSU) sequence referred to as 16S rRNA gene sequence to study phylogeny, enabling differentiation between eukaryotic (animal, plant and fungi) and prokaryotic (bacteria and archaea) organisms (Balch *et al.*, 1977; Fox *et al.*, 1977; Woese and Fox, 1977). Sanger Sequencing was the first Deoxyribonucleic Acid (DNA) sequencing method, developed by Frederick Sanger in 1977 enabling researchers to sequence DNA from single isolates for identification (Sanger *et al.*, 1977). The ability to amplify genomic DNA came in 1983 with the development of the PCR using universal primers, this revolutionised microbiology and launched a new era of molecular identification (Mullis *et al.*, 1986, 1987).

1.5.1 The 16S rRNA Gene

The 16S rRNA gene is widely used as a 'barcode gene' in microbial ecology studies to examine community structure and diversity (Pace, 1997; Hebert *et al.*, 2003). Sequences of some loops form secondary structures and are conserved across almost all bacterial species due to the functions involved, whereas the features of the structural parts are largely variant and specific to one or more classes (Wang and Qian, 2009). The gene is present in all prokaryotes and allows comparison of phylogenetic relationships, with conserved regions enabling universal or specific PCR primers to bind to the DNA whilst variable regions allow differentiation between species (Fig. 1.9) (Vos *et al.*, 2012; Větrovský and Baldrian, 2013). One primary issue for determining abundance using 16S gene sequencing data is copy numbers can vary from one to fifteen, therefore, observed relative abundance can differ from true abundance (Klappenbach *et al.*, 2001; Kembel *et al.*, 2012). Reacting to environmental changes, the existence of multiple rRNA operons may have two potential functions: allowing the bacterium to grow rapidly providing a multiplier effect on translation and

functional differentiation between rRNA operons would allow for differential expression of rRNA operons (Case *et al.*, 2007).

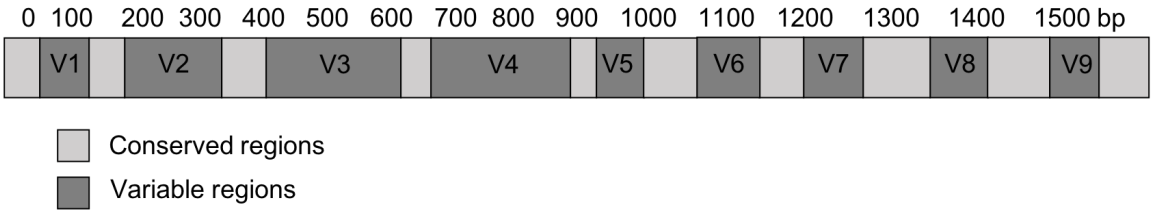

Figure 1:9: The 16S rRNA gene with conserved regions (light grey) and variable regions (dark grey) with length of regions indicated in base pairs.

1.5.2 ITS rDNA Region

The Internal transcribed spacer region (ITS) is considered a suitable barcode region for species-level identification of fungal organisms (Schoch *et al.*, 2012). The SSU 18S rRNA gene was previously used but was found to be less discriminatory for fungi at lower taxonomic levels, compared to the prokaryotic equivalent (Hartmann *et al.*, 2010; Halwachs *et al.*, 2017). The ITS ribosomal deoxyribonucleic Acid (rDNA) region (rDNA is the DNA sequence that encodes the rRNA) contains two variable non-coding regions; ITS1 and ITS2 which are nested within 5.8S gene repeat between the highly conserved 18S rRNA and 23S rRNA genes (White *et al.*, 1990) (Fig. 1.10). The ITS regions are transcribed but sliced away before the ribosome assembly, making them highly variable with closely related species differing in sequence and intraspecific variation low (Schoch *et al.*, 2012; Lindahl *et al.*, 2013). Using universal primers, located at the adjoining conserved ribosomal genes or 5.8S gene, the ITS region can be amplified for a wide range of fungi. Amplification of both ITS rDNA regions and 5.8S gene (600-700 base pairs (bp)) with primers binding to the 18S and 23S rRNA, gives longer stretches of conserved sequence in the amplicon. This increases the occurrence of chimeric sequences and less likely to occur when amplification is restricted to an individual ITS region (250-400 bp) by using the 5.8S gene for primer binding (Ihrmark *et al.*, 2012).

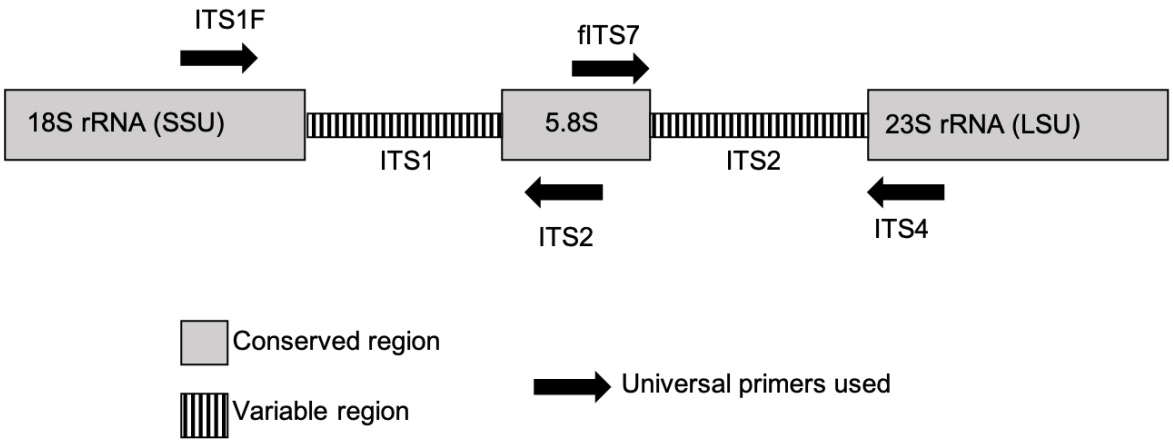

Figure 1:10: The fungal region with conserved regions (light grey) and variable regions (vertical lines) and position of universal fungal primers used in this study.

The use of 16S rRNA and ITS rDNA gene sequencing has its limitations including bias derived from: DNA extraction methods, PCR amplification steps and reagents and sequencing and bioinformatic analysis. In fact, all methods will introduce unique biases (Wu *et al.*, 2010; Schloss and Westcott, 2011; Sergeant *et al.*, 2012; Vos *et al.*, 2012; Edgar, 2017; Usyk *et al.*, 2017).

1.6 NGS and Microbial Ecology

The arrival of next generation sequencing (NGS) revolutionised microbial ecology, enabling complex, mixed microbial communities within an environment to be analysed, previously inaccessible with culturing methods (Snyder *et al.*, 2009; Schloss and Westcott, 2011; Edgar, 2017; Halwachs *et al.*, 2017). The major advantage of NGS is the ability to produce a vast volume of data cheaply and rapidly (Goodwin *et al.*, 2016; Greay *et al.*, 2018). Whilst universal 16S bacterial primers are well established, ITS fungal primers are improving with better understanding of fungal diversity (Ihrmark *et al.*, 2012; Usyk *et al.*, 2017).

This advent of NGS posed a problem of placing sequences, such as 16S rRNA gene and ITS rDNA sequences within a bacterial or fungal taxonomy. The two widely used methods of grouping sequences are to group into operational taxonomic units (OTU) or phylotypes (Schloss and Westcott, 2011). OTUs are defined as clusters of 16S/18S rRNA or ITS rDNA sequences that are similar to each other based on their similarity to other sequences in the community (Schloss and Westcott, 2011). OTUs have been defined with a ≥97% nucleotide identity cut off, with the remaining 3% scattered among the hypervariable regions (Stackebrandt and Goebel, 1994). OTUs are the most widely used units in characterising microbial communities into taxa (Schmidt *et al.*,

2014). However, this only gives a fingerprint of diversity present, as different methods of clustering result in marked differences (Schmidt *et al.*, 2014).

Phylotyping defines reference taxonomic outlines to classify sequences to taxonomic bins, based on their similarity relationship to reference sequences (Schloss and Westcott, 2011). This is problematic when organisms belonging to the same species have different phenotypes or organisms with the same phenotype belong to a different taxonomic lineage (Schloss and Westcott, 2011). For example, sequences belonging to *Pseudomonas* could express pathogenic or beneficial genes in a sample. As phylotyping is based on previously cultured organisms, difficult to culture organisms and members of candidate phyla lack a well-defined taxonomy at genus or species level (Schloss and Westcott, 2011).

There are a wide variety of statistical approaches to analyse and characterise microbial sequences from environmental samples. The microbial diversity can produce a huge dataset; therefore, statistical tests can be applied to allow determination of which multispecies metacommunity has greater diversity and whether the differences are statistically significant (Gorelick, 2006).

Alpha-diversity is used to determine the microbial diversity within the chosen samples and beta-diversity determines the difference in species composition between samples and is important for evaluating trends in a large number of samples (Hamady *et al.*, 2010). Rarefaction curves plot the number of OTUs clustered from the sequences in a given sample. They are used to determine if the sampling depth for that dataset is sufficient to observe all taxa and infer the total diversity of the community (He *et al.*, 2015; Hughes *et al.*, 2016). As well as total observed species found in the samples, alpha diversity metrics used in this study are $Chao_1$ and Simpsons diversity indices, which have advantages and disadvantages. $Chao_1$ estimates species richness based on the number of rare or unique species present in a dataset. The more of these species present, the higher the overestimation of species richness will be (Chao, 1984; Chao and Lee, 1992; Hughes *et al.*, 2016). Simpsons diversity index considers species richness and evenness and used to construct a vector of probability that two sequences randomly selected from the sample belong to the same species. This measure is weighted towards the more abundant species and therefore compliments $Chao_1$ (Simpson, 1949; Hill, 1973; Gorelick, 2006; Tuomisto, 2012).

Beta-diversity determines the distance or dissimilarity between samples (Lozupone and Knight, 2005; He *et al.*, 2015). UniFrac is a phylogenetically aware measure, used to compare OTU structure and diversity. Here, based on the lineages the samples contain, unweighted (qualitative, considering absence or presence) UniFrac measures the distance between samples (Lozupone and

Knight, 2005). These distances are plotted onto a principle coordinate analysis (PCoA) plot to visualise the percentage of variation contained in the dataset.

Using these new improved molecular tools enables new ways to investigate microbial symbiosis and really understand the microbial interactions in an environment.

1.7 Aims and Objectives of this Work

The objectives were to: -

- Provide insight into the resident fungal communities within the GI tract of wood-eating fish *P. nigrolineatus*
- Interpret the diversity of resident bacterial and fungal communities within the GI tract of common woodlice; *O. asellus* and *P. scaber*
- Compare the hepatopancreas and hindgut communities of these woodlice species and discuss possible symbiotic interactions enabling them to eat a diet rich in lignocellulose
- Isolate bacterial species from the hepatopancreas with a particular focus on previously unculturable species
- Examine the AMR species cultured from the woodlouse gut

2 Material and Methods

2.1 Media and reagents

All media was prepared as specified in the manufacturer's instructions and autoclaved at 121°C for 15 mins at 15 psi. All media ingredients were sourced from Oxoid or Fisher Scientific unless otherwise stated (Table 2.1).

Table 2:1: Media solutions for culturing bacteria and fungi.

Media	Concentration/reagents	
Sabouraud Dextrose agar (SDA)	65 g/L	
Malt agar	50 g/L	
Nutrient agar	28 g/L	
Brain Heart Infusion agar	47 g/L	
Mueller Hinton agar	38 g/L	
Minimal salt agar - then supplemented with one of the following carbon sources (Watts *et al.*, 2013)	$NaNO_3$ K_2HPO_4 $MgSO_4$ $7H_2O$ (BDH labs) KCl Bacteriological agar	1.0 g/L 1.8 g/L 0.9 g/L 0.5 g/L 20 g/L
	Carboxymethyl-cellulose (Sigma)	5 g/L
	D(+)-Cellobiose (Agros)	5 g/L
	Lignin (Sigma)	5 g/L
	Sawdust (*Pinus*) (Jollies, Waterlooville)	5 g/L
	Hay (*Lolium*) (Jollies, Waterlooville)	5 g/L

All powdered antibiotics and antifungals were filter sterilised through a 0.22 μm filter (Fisher Scientific) and added to the autoclaved media (Table 2.2).

Table 2:2: Antibiotics used in autoclaved media and disk diffusion.

Antibiotics	Final Concentration	
Chloramphenicol with ethanol (Acros Organics)	100 mg/L	
Gentamicin with dH_20 (Fisher)	50 mg/L	
Ampicillin with dH_20 (Fisher)	100 mg/L	
Tetracycline with dH_20 (Fisher)	20 mg/L	
Cycloheximide with dH_20 (Acros Organics)	50 mg/L	
Diffusion discs (Oxoid)	Penicillin G Ampicillin Erythromycin Tetracycline Gentamicin Streptomycin	10 units 10 μg 15 μg 30 μg 10 μg 10 μg

Buffers and solutions used for all experiments are listed (Table 2.3), any concentrations of reagents are also recorded. All were sourced from Fisher Scientific unless stated otherwise.

Table 2:3: Buffers and solutions used, all were sourced from Fisher Scientific unless otherwise stated.

Buffers and solutions	Concentration/reagents
Ringers solution	
Phosphate buffer solution (PBS)	1X
Ethylenediaminetetraacetic acid (EDTA) (0.5 M, pH 8.0)	186.1 g EDTA (Sigma) 20 g NaOH (Sigma) Deionised water to 1L
Tris-Acetate-Ethanol (50X) (TAE)	242 g tris 57.1 mL glacial acetic acid 100 mL 0.5M EDTA Deionised water to 1L
Ethanol	99.6%
Isopropanol	
Glycerol preservation solution	20%
Bromophenol blue loading buffer	1 mL glycerol 25 mg bromophenol blue Deionised water to 10 mL
Ethidium bromide	0.05%

DNA ladder ExACT gene	100-2000 kb 1000-15000 kb
Primers (Sigma)	3.2 pmol/μL
DreamTaq PCR mix (Thermo Scientific)	2X
Magnesium Chloride ($MgCl_2$) (Thermo Scientific)	25 mM
Tris-EDTA (TE)	10 mM Tris 1 mM EDTA (0.5M)
Saline	0.5%
Carnoy's solution	60% Ethanol 30% Chloroform 10% Acetic acid
Triton X100	0.05%
20X Standard saline citrate (SSC)	NaCl 87.6 g Na Citrate 44.1 g Deionised water to 500 mL
Prehybridisation buffer	12.5 mL formamide (100%) 2.5 mL 20X SSC 10 mL deionised water
Hybridisation buffer	12.5 mL formamide (100%) 2.5 mL 20X SSC 6 mL dextran sulphate (50%)
Tween20	0.05%

2.2 Animal Specimen Acquisition

2.2.1 *P. nigrolineatus* Collection and Husbandry

This experiment was performed in Institute of Marine and Environmental Technology, University of Maryland Baltimore County by collaborators; Ryan McDonald and Harold Schreier. *P. nigrolineatus* (L-190) were imported from South America by the fish wholesaler Aquascapesonline (Belleville, NJ). They were randomly assigned to individual, filtered and aerated tanks kept at 29 ± 1 °C. Fish (40 mm, standard length) were fed a mixed diet of hearts of palm (*Euterpe precatoria*), algae pellets (Hikari Tropical Sinking Algae Wafers, Hayward, CA), and date palm wood (*Phoenix dactylifera*) during an acclimation period of three weeks under conditions specified by IACUC 071509JW-01. For the duration of the experiment, the fish on a mixed diet were provided with palm hearts and algae every second day, while wood was constantly available. Wood was thoroughly soaked in sterile

water and autoclaved three times prior to being provided to the fish. Fish were then moved to a palm wood-only diet or a mixed diet of palm hearts and palm wood. This feeding regimen was maintained for three weeks prior to sacrifice. Due to fish mortality during the feeding experiments, only one fish from each treatment could be analysed, but, due to the rarity of these fish and difficulties in obtaining the fish it was important to analyse the results.

2.2.1.1 P. nigrolineatus Dissection

The dissection was also carried out by collaborators; Ryan McDonald and Harold Schreier (2.2.1). After conclusion of the feeding experiment, one fish from each treatment was sacrificed by anaesthetic overdose in 3-aminobenzoic acid ethyl ester (MS-222, 50 mg/L) as described previously (McDonald *et al.*, 2012; Watts *et al.*, 2013). After removing the ventral body plate, sterile ice-cold PBS was added to the abdominal cavity. The intestine was separated immediately distal to the stomach, removed from the body cavity, uncoiled and measured rapidly in cold PBS. The auxiliary lobe was separated from the intestine, which was then divided into three parts of equal length, defining foregut, midgut, and hindgut regions. Tissue samples were processed using the Qiagen (Germantown, MD, USA) DNeasy Blood and Tissue Kit with pre-treatments for Gram-positive and Gram-negative bacteria according to the manufacturer's instructions. DNA extracted from three samples of each GI tract region was pooled and processed for PCR amplification.

2.2.2 *O. asellus* and *P. scaber* Collection and Husbandry

Woodlice (*Porcellio scaber* and *Oniscus asellus*) were collected from two sites (50° 48' 40.56"N 1° 03' 27.51"W and 50° 47' 32.57"N 1° 05' 49.25"W) between 19-02-17 and 02-03-17. Each species was placed in separate plastic fish tanks (6 L, Ferplast Geo, 30 x 20 x 20.3 cm) (Amazon, U.K.) with soil, wood and leaf litter from the collection sites and stored in a dark incubator (15°C) for at least 14 days to acclimate. Water was placed in the incubator to keep conditions moist and sterile water was sprayed into the tanks every 2 days.

After acclimation *P. scaber* (n=55) and *O. asellus* (n=55) larger than 8 mm were selected from the tanks and placed individually into petri dishes (85 mm) lined with filter paper, moistened with sterile distilled water (1 mL) and sterile carrot shavings. The petri dishes were incubated at 15°C for 3 days in a sealed container, lined with wet tissue paper to keep conditions moist. The woodlice were transferred to fresh petri dishes with 1 cm^2 piece of PVA Ramer® foam sponge (Boots Ltd), wetted with 2 mL dH_20 and returned to the box lined with wet filter paper and incubated for 7 days. During this 7-day period, woodlice were transferred to clean petri dishes daily, to limit coprophagy.

2.2.2.1 Tissue Preparation and Dissection

At the end of the experiment, woodlice were sacrificed by placing individuals at -20°C for 10 minutes and surface sterilised in 70% ethanol for 60 seconds with subsequent washes (x3) in Ringers solution for 60 seconds per wash. Woodlice were dissected using aseptic technique. The hepatopancreas was extracted from the body cavity removed from the lobes. The hindgut was pulled from the posterior end and the anus removed. Tissues were used for culture and anti-microbial experiments and DNA extraction.

2.2.2.2 Fluorescent In situ Hybridisation (FISH)

FISH was used to observe the placement of microbial cells within the lumen of the tissues.

2.2.2.2.1 Fixation and Preparation

O. asellus (n=10) and *P. scaber* (n=10) were sacrificed and hepatopancreas and hindgut tissues removed as stated in 2.2.2.1. Tissues were fixed in Carnoy's (Table 2.3) solution for 2 hours at 4°C, then stored in ethanol (99.6%) at -20°C until required. Tissues were rehydrated in 30% sucrose solution and incubated for 16 hours at 4°C. Tissues were placed in a mould with optimal cutting temperature medium and subjected to liquid nitrogen. Tissues were sliced using a cryostat (5 μm) and placed on poly-lysine coated slides.

2.2.2.2.2 Hybridisation

The tissue slices were washed twice for 15 minutes in PBS (1X), then washed in triton x100 (0.05%). Tissues slices were denatured in prehybridisation buffer (Table 2.3) for 40 minutes at 60°C. The EUB338 cy3 probe (10 μL) was added to hybridisation buffer (Table 2.3) in a 0.2 mL PCR tube and heated to 95°C for 3 minutes in a PCR block and transferred immediately to ice. The prehybridisation buffer was removed from the slides and the hybridisation buffer containing the probe was added to the slides and incubated in a sealed container for 14 hours at 37°C. The hybridisation buffer was removed and slides washed twice in prehybridisation buffer for 15 minutes at 40°C. Slides were washed twice in SSC (2X), then twice in SSC (0.1X) for 15 minutes at 40°C, then six washes in TWEEN20 (0.05%) in PBS (1X) for 15 minutes each at 18°C. Histomount was put onto the sample and covered with a coverslip, sealed and incubated for 24 hours at 4°C. The samples were visualised on a fluorescence microscope.

2.2.2.3 Bacteria Cultured from the Hepatopancreas

O. asellus (n=13) were sacrificed and washed as stated in 2.2.2.1. The hepatopancreas was extracted and homogenised aseptically. Samples were serial diluted with Ringers and plated (10 μL) onto 1:10 nutrient agar, Brain Heart Infusion agar, minimal salt agar (Table 2.1) infused with CMC, cellobiose or lignin and the same infused agar with 2 mL glycerol added. Each agar was infused with cycloheximide (Table 2.2) to prevent fungal growth. Plates were incubated at 23°C, 30°C and 37°C for between 3 and 42 days. Isolates were plated onto the corresponding agar and incubated at the corresponding temperature. (GenBank accession numbers; MK592794-MK592807).

2.2.2.4 Wood Degrading Bacteria and Fungi Cultured from the Hindgut

O. asellus (n=10) and *P. scaber* (n=10) were sacrificed and washed as stated in 2.2.2.1. The hindgut was extracted and homogenised aseptically. Samples were serial diluted with Ringers solution and plated (10 μL) onto agar (Table 2.1) infused with CMC, lignin, hay or sawdust. Inhibitors of bacteria (chloramphenicol, ampicillin, and gentamicin) or fungi (cycloheximide) (Table 2.2) were added. Plates were incubated at 20°C for between 5 and 42 days to allow for growth on different agars and to allow fastidious organisms to grow. Individual colonies were isolated onto corresponding agar and incubated at 20°C. (GenBank accession numbers; MK590239-MK590243 (16S), MK593597-MK593604 (ITS)).

2.2.2.5 Antimicrobial Resistance

To examine the spread of AMR in the environment, *O. asellus* (n=13) were sacrificed as stated in 2.2.2.1. The hepatopancreas and hindgut were serial diluted into Ringers solution in separate tubes and plated (10 μL) onto nutrient agar infused with a fungal inhibitor cycloheximide (Table 2.2) and either ampicillin, chloramphenicol, gentamicin, tetracycline to inhibit bacteria (Table 2.2 and 2.4) or a combination of two, three or four, and incubated at 20°C for 5 days. Different isolates resistant to the antibiotics were selected by their appearance and plated onto nutrient agar and incubated at 20°C for 5 days.

Table 2:4: The mode of action of the antibiotics used in this study.

Antibiotic	Mode of Action	Reference
Ampicillin	Inhibits cell wall synthesis	(Tomasz, 1979; Raynor, 1997)
Penicillin	Inhibits cell wall synthesis	(Tomasz, 1979; Davies, 1994; Raynor, 1997)
Chloramphenicol	Inhibit protein synthesis – binding to 50S ribosomal subunit	(Chopra and Roberts, 2001; Trivedi *et al.*, 2015)
Tetracycline	Inhibit protein synthesis - interfere with the attachment of tRNA to mRNA	(Chopra and Roberts, 2001; Trivedi *et al.*, 2015)
Erythromycin	Inhibit protein synthesis - prevents elongation of the protein and prevents translocation of the ribosome	(Wolfe and Hahn, 1964)
Gentamicin	Inhibit protein synthesis - ribosomal blockade, misreading translation and causes membrane damage	(Hahn and Sarre, 1969; Tangy *et al.*, 1985)
Streptomycin	Inhibit protein synthesis - ribosomal blockade, misreading translation and causes membrane damage	(Davis, 1987)

Nutrient agar was used to examine environmental AMR and to examine the growing crisis within humans, Mueller Hinton agar using the Kirby Bauer method was used (Mueller and Hinton, 1941; Bauer *et al.*, 1966). Isolates previously identified as having resistance, in the previous experiment, were mixed in saline (0.5%) to 10^8 cells using McFarland Standard 0.5. The suspension was plated onto Muller Hinton agar and nutrient agar, in triplicate. Antibiotic discs; Penicillin G, Ampicillin, Erythromycin, Tetracycline, Gentamicin and Streptomycin (Table 2.2) were added on top of the plated sample. Samples on Muller Hinton agar were incubated at 35°C for 16 hours, as stated by the Kirby Bauer method and samples on nutrient agar were incubated at 20°C for 24 hours to emulate environmental temperature. Controls of saline (negative), *Staphylococcus* and *Escherichia coli* (positive) were also plated. Zones of inhibition (ZOI) were recorded using a ruler and reflected light source 3 inches above a black, non-reflecting surface (Barry *et al.*, 1979). ZOI were measured from isolates grown on nutrient agar and were considered susceptible to a particular antibiotic if

the bacteria formed a clear zone around the disc, indicating whether the isolates were resistant or susceptible to each antibiotic. The ZOI measurements from the isolates grown on Mueller Hinton agar were recorded as resistant, intermediate or susceptible according the Clinical & Laboratory Standards Institute (CLSI) guidelines (CSLI, 2015). (GenBank accession numbers; MK590203-MK590215).

2.3 Preparation for Microbial Community Analysis

O. asellus (n=5) and *P. scaber* (n=5) were sacrificed and washed as stated in 2.2.2.1. The hepatopancreas and hindgut used were extracted from the same individual.

2.3.1 DNA Extraction

Hepatopancreas and hindgut tissues extracted from *O. asellus* and *P. scaber* were used to examine the microbial communities and isolates from the hepatopancreas, hindgut and AMR experiments. The tissues were subjected to DNA extraction using FastDNA spin kit for soil (MP Bio). Manufacturer protocol was followed; however, the samples were eluted in Tris-EDTA (TE) buffer (1X) (100 µL) instead of DES water and stored at 4°C.

2.3.2 DNA Quantification

For fungal community analysis to be carried out on *P. nigrolineatus* GI tract and microbial community analysis to be carried out on *O. asellus* and *P. scaber* hepatopancreas and hindgut, total gut DNA was extracted. The concentration of DNA in each sample was measured using a NanoDrop Spectrophotometer ND-1000 (Labtech International, Uckfield, U.K.). The 260/280 nm ratios were recorded.

Table 2:5: Universal bacterial and fungal primers used in PCR reaction to amplify the 16S rRNA region and ITS rDNA region.

Primer	Orientation	Target	Primer sequence	Reference
pA	Forward	16S	AGAGTTTGATCCTGGCTCAG	(Edwards *et al.*, 1989)
pH	Reverse	16S	AAGGAGGTGATCCAGCCGCA	(Edwards *et al.*, 1989)
1392R	Reverse	16S	ACGGGCGGTGTGTAC	(Ferris *et al.*, 1996)
1406R	Reverse	16S	ACGGGCGGTGTGTAC	(Ferris *et al.*, 1996)
ITS1F	Forward	ITS	CTTGGTCATTTAGAGGAAGTAA	(Gardes and Bruns, 1993)
ITS4	Reverse	ITS	TCCTCCGCTTATTGATATGC	(White *et al.*, 1990)
341F	Forward	V3-V4	CCTACGGGNGGCWGCAG	(Herlemann *et al.*, 2011)
785R	Reverse	V3-V4	GACTACHVGGGTATCTAAKCC	(Herlemann *et al.*, 2011)
ITS1F	Forward	ITS1	CTTGGTCATTTAGAGGAAGTAA	(Gardes and Bruns, 1993)
ITS2	Reverse	ITS1	GCTGCGTTCTTCATCGATGC	(White *et al.*, 1990)
fITS7	Forward	ITS2	GTGARTCATCGAATCTTTG	(Ihrmark *et al.*, 2012)
ITS4	Reverse	ITS2	TCCTCCGCTTATTGATATGC	(White *et al.*, 1990)

2.3.3 Polymerase Chain Reaction (PCR)

To confirm the presence of bacterial and fungal DNA in the samples, PCR was used to selectively amplify the 16S rRNA gene or ITS rDNA region using universal primers (Table 2.5). PCR amplification was carried out in a T100 Thermal Cycler (Bio Rad) in 25 µL reactions [0.5 µL template, 11 µL nuclease free water, 0.5 µL of each primer (3.2 pmol), 12.5 µL master mix (2X buffer, 0.4 mM of each nucleotide, 4 mM $MgCl_2$, 0.025 μL^{-1} polymerase (DreamTaq Green, Thermo Scientific)]. PCR conditions using pA and pH (Table 2.5) were 5 min at 94°C; 30 cycles of (60 s at 94°C; 90 s at 62°C; 60 s at 72 °C); 10 min at 72°C (Table 2.6). Amplification of fungal DNA was carried out using ITS1F and ITS4 (Table 2.4) as specified above but with 10 µL nuclease free water and the addition of extra 1 mM $MgCl_2$ per sample to facilitate binding of the primers. PCR conditions were 15 min at 96°C; 30 cycles of (30 s at 94°C; 30 s at 51.6°C; 40 s at 72°C); 10 min at 72°C (Table 2.6). Due to negative results, reamplification of fungal PCR product was conducted using 1 µL diluted PCR product and water (1:10) in 25 µL reactions. PCR conditions for fungal reamplification were as stated in Table 2.5, except 25 cycles were used instead of 30.

Table 2:6: Thermocycling conditions used when amplifying bacterial DNA using pA and pH (Edwards *et al.*, 1989) and fungal ITS rDNA region using ITS1F and ITS4 (White *et al.*, 1990; Gardes and Bruns, 1993).

Primers	Step		Temperature	Time
Bacteria pA/pH	Initial Denaturation		94°C	5 minutes
	30 cycles	Denaturation	94°C	60 seconds
		Annealing	62°C	90 seconds
		Extension	72°C	60 seconds
	Final extension		72°C	10 minutes
	Hold		4°C	∞
Fungi ITS1F/ITS4	Initial Denaturation		96°C	15 minutes
	30 cycles	Denaturation	94°C	30 seconds
		Annealing	51.6°C	30 seconds
		Extension	72°C	40 seconds
	Final extension		72°C	10 minutes
	Hold		4°C	∞

2.3.4 Gel Electrophoresis

The quality of genomic DNA was assessed using 1.5% low melt agarose gel (Fisher Scientific) electrophoresis. Bromophenol blue loading dye (5 µL) and genomic DNA (10 µL) was loaded into the gel and electrophoresed for 2 hours with a 40 Volt (V) current applied with Tris-acetate (TAE) buffer (1X). PCR product was loaded into a 1% agarose gel (Fisher Scientific) and electrophoresed for 1 hour applying 60 V. Results were visualised after staining with ethidium bromide (0.05%) on Gel Doc™ EZ Imager (Bio Rad).

2.3.5 Confirmation of Fungi in the GI tract

PCR results amplifying the fungal ITS rDNA region from total woodlice gut DNA extraction was negative. Therefore, to confirm the presence of fungi and send for NGS, *P. scaber* (n=3) and *O. asellus* (n=3) hepatopancreatic and hindgut tissue were plated onto Malt agar and SDA with bacterial inhibitors (chloramphenicol, gentamicin and ampicillin). Plates were incubated at 20°C and growth observed after 5 days.

2.3.6 Sanger Sequencing

DNA was extracted and subjected to PCR from isolates from the hepatopancreas, hindgut and AMR culture experiments (2.2.2.3-2.2.2.5). The PCR product was cleaned (PCR purification kit, Qiagen) and sent to Sanger (Source Bioscience, Oxford) for 16S rRNA gene and ITS rDNA region sequencing using universal bacterial and fungal primers (Table 2.4). FinchTV and Basic Local Alignment Search Tool (BLAST) were used to determine OTU at ≤97% similarity. The highest result on BLAST with the same max score, query coverage, E-value and identity, was recorded.

2.3.7 Next Generation Sequencing

Regions of the 16S rRNA gene (V3-V4) from the woodlice and ITS rDNA regions (ITS1 and/or ITS2) from *P. nigrolineatus* and woodlice were amplified from metagenomic DNA and prepared for NGS, performed by LGC Genomics (GmbH, Berlin, Germany) using the Illumina MiSeq V3 platform (San Diego, CA, USA). Genomic DNA (30 μL) from each sample (1-10 ng/μL) was supplied for analysis of the 16S rRNA gene (V3-V4), ITS1 rDNA and/or ITS2 rDNA regions were amplified by PCR using universal primers (Table 2.5) using the company's protocol. Each sample was tailed with unique Illumina adapter and barcode to enable identification of each sample after sequencing.

2.4 Bioinformatic Analysis

2.4.1 Data Pre-Processing

This step was carried out by LGC and based on their protocol, pre-processing and analysis of the raw sequencing data of all libraries for each sequencing lane was conducted using Illumina bcl2fastq 1.8.4 conversion software (Illumina). Fastq files were de-multiplexed and sequences were sorted by amplicon inline barcodes allowing for 1 mismatch per barcode and the barcode sequence then clipped from the sequence. Reads with missing barcodes, one-sided barcodes or conflicting barcode pairs were discarded. Sequencing adapters and primers were clipped from sequences allowing for 3 mismatches per primer. If pairs of primers were not present in the sequence fragments, these sequences were removed and if primer dimers were detected, the outer primer copies were clipped from the sequence. The sequence fragments were turned into forward-reverse primer orientation after primer sequences were removed. Sequence fragments were submitted to GenBank (Accession numbers; SRR5808488-SRR5808499 (*P. nigrolineatus*), SRR6435374-SRR6435374 and SRR6440253–SRR6440272 (*O. asellus* and *P. scaber*)).

2.4.1.1 Bacterial 16S rRNA Gene Analysis

The 16S rRNA gene pre-processing and OTU clustering from amplicons was performed by LGC using the Mothur 1.35.1 platform. (Schloss *et al.*, 2009). Sequences were removed containing ambiguous bases, with homopolymer stretches of more than 8 bases or with an average Phred (Ewing *et al.*, 1998) quality score below 33. Sequences were aligned to the 16S Mothur-Silva SEED r119 reference alignment (available as part of Mothur at http://www.mothur.org/w/images/5/56/Silva.seed_v119.tgz). Truncated or unspecific PCR products were filtered out and each sample was subsampled to 50,000 sequences per sample. The subsampling level was chosen according to the total amount of reads, the number of samples and the complexity of the data set. This subsampling level was checked by rarefaction to ensure sufficient subsampling level. Chimeras were eliminated using the UCHIME algorithm (Edgar *et al.*, 2011). Sequences from other domains of life and taxonomic classification was carried out against the Silva reference classification (Quast *et al.*, 2013). OTUs were clustered at the ≥97% identity level, using the Mothur cluster.split method (https://www.mothur.org/wiki/Cluster.split). To obtain counts for each sample, OTU count tables were created by OTU consensus taxonomical calling and integrating the taxonomical classification of the cluster member sequences. Putative species level annotation of OTUs was carried out with National Centre for Biotechnology Information (NCBI) BLAST+ 2.2.29 (Edgar, 2010) with representative sequences of each OTU, with at least 2 observed sequences, queried against the ribosomal database project release 11.4 reference. BLAST+ (Edgar, 2010) parameters set were E <=0.1 with percentage identity >=90%.

2.4.1.2 ITS rDNA Analysis

The ITS rDNA region pre-processing and OTU clustering from amplicons was performed by LGC using the Mothur 1.35.1 platform (Schloss *et al.*, 2009). Short products were filtered, and sequences were subsampled to 20,000 sequences per sample for the woodlouse experiment and 60,000 sequences per sample for the *P. nigrolineatus* experiment, with distances generated using USEARCH (Edgar, 2010). Chimeras were eliminated using the UCHIME algorithm (Edgar *et al.*, 2011). The similarity threshold for ITS sequences belonging to the same OTU was set to ≥97% and clustered by CD-HIT-EST (Fu *et al.*, 2012) with cluster representative sequence to the most abundant species. Taxonomical classification of OTUs was performed against the UNITE v6 database (Koljalg *et al.*, 2013) with sequences assigned to ≥97% identity threshold.

2.4.2 OTU Diversity Analysis

To allow comparison, samples processed by LGC were normalised, to reduce bias introduced by variable library size (Chen *et al.*, 2018), to the lowest number of reads per sample for downstream analysis, by Quantitative Insights into Microbial Ecology v1.9.0 (QIIME) (Caporaso *et al.*, 2010). Diversity analyses were carried out using QIIME utilising Python v3.5 scripts to rarefy the datasets and for performing alpha diversity analysis. OTUs from the same consensus lineage were clustered together into one OTU, for example, sequences similar to *Pseudomonas* at ≤97% but differed slightly were put together into one OTU. After OTUs were clustered, the input file; biological observational matrix (BIOM) required for analysis was made, to further analyse diversity.

2.4.2.1 Alpha Diversity

To observe microbial differences within samples of *P. nigrolineatus, O. asellus and P. scaber,* scripts were used within the QIIME program (http://qiime.org/scripts). Samples were filtered from the OTU table to the sample that contained the least number of sequences (Schloss *et al.*, 2011) and rarefied by random sampling using filter_samples_from_otu_table.py and single_rarefaction.py scripts. Alpha rarefaction plots were made using ten iterations and averaging the results using make_rarefaction_plots.py script. Taxa was summarised into different taxonomic levels (summarize_taxa.py) and bar charts were constructed (plot_taxa_summary.py). To examine the diversity in different groups of data the mapping file was sorted into groups (sort_otu_table.py), collapsed to only use the group required (collapse_samples.py) and the two previous commands used to enable the data to be analysed for different groupings. Compare_alpha_diversity.py was used to compare differences in diversity within each sample using non-parametric (Monte Carlo) method. An OTU network was created using make_OTU_network.py, which was visualized in Cytoscape 3.4.0 (Lopes *et al.*, 2011) using a spring-embedded layout. To investigate the existence of a core microbiome, compute_core_microbiome.py script was used, giving the core microbiome at 50% then at 5% iterations until either 100% or until there was not a core microbiome.

2.4.2.2 Beta Diversity

To examine differences in between microbial communities in samples of *O. asellus* and *P. scaber*, beta diversity was analysed using beta_diversity_through_plots.py script which created weighted and unweighted UniFrac distance 3D PCoA plots. To better understand the data points, 2D PCoA plots were created based on unweighted (qualitative) UniFrac distances using create_2d_plots.py script.

2.4.2.3 Statistical Analysis

Statistical tests examined if there was a significant statistical difference between samples. The clustered OTU table was rarefied ten times and integrated into one OTU table using parallel_multiple_rarefactions.py in QIIME. This script used a minimum sequence of 10, maximum sequences (lowest number of sequences in the samples) and the steps between the minimum and maximum sequences ((maximum-minimum)/10). Alpha diversity was measured using parallel_alpha_diversity.py using observed_species, $Chao_1$ and Simpson diversity metrics. The depth of rarefaction was determined by the lowest number of sequences assigned to a sample. The tests of significance were performed using a non-parametric t-test using 999 Monte Carlo permutations, within compare_alpha_diversity.py and make_distance_boxplots.py scripts.

3 Characterisation of the Fungal Communities in the GI Tract of *P. nigrolineatus*

3.1 Introduction

P. nigrolineatus has evolved to have physiological features that have enabled these fish to eat wood. The resident bacterial communities within the GI tract have already been characterised using 16S rRNA gene and culture-based analyses experiments (McDonald *et al.*, 2012, 2015; Watts *et al.*, 2013). The presence of cellulose degrading and nitrogen fixing bacteria have been demonstrated in the GI tract (Nelson *et al.*, 1999; McDonald *et al.*, 2012, 2015; Di Maiuta *et al.*, 2013). Ongoing studies (McDonald, Watts and Schreier, manuscript in preparation) suggest the enteric bacterial community may lack selected enzymatic activities essential for lignocellulolytic digestion, which may be provided by the fungal community and/or host. Despite these publications, there is no knowledge into the presence of a resident fungal community. It is well known that bacteria and fungi work symbiotically in the degradation of lignocellulose in the terrestrial environment, therefore, it is plausible to suggest this relationship also exists in the GI tract of an organism that eats wood (Shortle *et al.*, 1978; Benner *et al.*, 1984; Lang *et al.*, 2000; Folman *et al.*, 2008; Hoppe *et al.*, 2015; Siriyappagouder *et al.*, 2018). Additionally, it is understood the bacterial communities have a positive impact on the host development and health (Mueller *et al.*, 2012; Llewellyn *et al.*, 2014; Bouchon *et al.*, 2016), yet there is no research to date, investigating the same role of fungi with the host.

Therefore, characterising the resident fungal communities in the different regions of the GI tract, would provide the groundwork for further research into the role fungi has in enabling *P. nigrolineatus* to exist on such a low-quality food.

3.2 Aims

The major aims of this chapter are to explore the diversity of fungi in the fore-, mid- and hindgut of wood-eating catfish *P. nigrolineatus,* to examine if any resident fungal community exists and if these communities differ in different regions of the GI tract. This work builds on previous published work characterising the resident bacterial diversity in the GI tract which has also discovered communities of cellulose degrading and nitrogen-fixing bacteria.

3.3 Preparation for Next Generation Sequencing

The fish were cared for and prepared by Ryan McDonald and Harold Schreier at University of Maryland, Baltimore County as stated in 2.2.1. Genomic DNA was sent to LGC Genomics.

3.3.1 DNA Quantification

Fish fed a diet of wood (n=1) or a mixed diet (n=1) were sacrificed as stated in the materials and methods (2.2.1.1). The low number of replicates was due to the low survival rate of fish bought from the environment to captivity. Total gut DNA was extracted and samples were checked for DNA concentration (260/280) using NanoDrop (wood-diet (ng/μL) - foregut 23, midgut 48, hindgut 360; mixed-diet - foregut 56, midgut 64, hindgut 74).

3.3.2 Confirmation of Fungi in Samples

PCR was used to amplify the ITS rDNA region using universal fungal primers (ITS1F/ITS4 Table 2.5). The PCR product was reamplified to confirm the presence of fungi. Results were visualised on agarose gel electrophoresis showing DNA bands at 650 bp for all samples except wood-fed fish foregut.

3.4 Distribution and Diversity of Fungi in the GI Tract of *P. nigrolineatus*

Samples were sent to LGC Genomics (GmbH, Berlin, Germany) to sequence the ITS rDNA region using Illumina MiSeq V3 platform (San Diego, CA, USA). This enabled analysis of fungal sequences that were found in the enteric regions of a *P. nigrolineatus* fed either a wood-diet or a mixed-diet.

3.4.1 Analysis of ITS rDNA Sequences Detected in *P. nigrolineatus*

Sequences corresponding to the ITS1 rDNA and ITS2 rDNA regions were PCR amplified from the foregut, midgut and hindgut regions of fish fed either a mixed-fed diet or a wood-fed diet. A total of 302,894 reads were obtained from the fish fed a mixed-diet and 401,188 reads obtained from the fish fed a wood-diet. After processing and subsampling sequences to 60,000 reads per sample, a total of 256,280 sequences, clustered into 207 preliminary OTUs, were obtained from the ITS2 amplification and analysed using USEARCH (Edgar, 2010) (Table 3.1). The ITS1 sequence analysis was found to be considerably less sensitive for this study (Table 3.2). ITS2 rDNA OTUs were binned into taxonomic groupings allowing comparison of the alpha diversities across tissues and diet.

Table 3:1: NGS results from *P. nigrolineatus*. Number of raw reads, total clusters and unique OTUs from sequencing ITS2 rDNA region of *P. nigrolineatus* GI tract regions. Sequences were assigned to OTUs with ≥97% sequence identity.

Diet and enteric region	Total raw reads	Total clusters (≥97%)	Unique OTUs (≥97%)
Mixed-fed foregut	117,122	28,298	55
Mixed-fed midgut	64,210	28,090	69
Mixed-fed hindgut	121,562	52,802	95
Wood-fed foregut	152,882	59,490	81
Wood-fed midgut	150,590	59,404	67
Wood-fed hindgut	97,716	28,187	59
All	1,147,382	256,280	207

Table 3:2: Fungal phyla within the GI tract of *P. nigrolineatus*. Total number of preliminary identified OTU clusters from the fungal ITS1 and ITS2 rDNA regions. Unknown sequences could not be identified.

Consensus lineage	Total OTUs	
	ITS1	ITS2
Ascomycota	75	117
Basidiomycota	30	35
Blastocladiomycota	1	0
Glomeromycota	1	0
Zygomycota	1	0
Protista	0	4
Unknown fungi	57	32
Unknown sequences	25	19

3.4.2 Alpha Diversity Analysis

To better understand changes in the microbial community in the different GI tract regions of *P. nigrolineatus*, $Chao_1$ estimations of diversity was applied to OTU distributions (Table 3.3). The data was normalised to the sample containing the lowest number of sequences, 28,090. Fungal diversity of the mixed-diet fed fish, increased distally with the greatest diversity in the hindgut and the lowest in the foregut. The opposite trend was observed in the wood-diet fed fish with the foregut having the most fungal diversity and the hindgut having the least. The mixed-diet fed hindgut had the highest diversity and the mixed-diet fed foregut had the lowest diversity when analysing both fish.

T-tests revealed there was no significant difference (P = 0.4) in the average number of fungal OTUs observed between wood-diet or mixed-diet fed fish. The difference between S_{obs} and S_{Chao1} suggests unique or rare OTUs being present in all samples, the wood-fed foregut (Table 3.3) had a higher number than any other tissue region examined.

Table 3:3: Comparison of ITS2 rDNA region OTU species richness within *P. nigrolineatus* GI tract. A non-parametric estimate $Chao_1$, was used to estimate species diversity in different regions of the GI tract. For phylotype richness estimations, OTUs were binned to species.

	Wood Fed Fish			**Mixed Fed Fish**		
	Foregut	**Midgut**	**Hindgut**	**Foregut**	**Midgut**	**Hindgut**
Observed OTUs	67.8	57.1	59	54.9	69	85.7
$Chao_1$	95.2	79.6	70	76.9	82.3	96.1

These findings were supported by the rarefaction analysis, which demonstrated that the mixed-diet fed hindgut had the highest detectable species richness, while the foregut of mixed-diet fed fish had the lowest. The rarefaction analysis suggests that additional sequencing would allow more novel OTUs to be detected in the wood-fed diet foregut and midgut and mixed-diet fed foregut with further sampling, but the majority of the diversity present had been sampled as rarefaction was reaching asymptote at 28,090 sequences (Fig. 3.1).

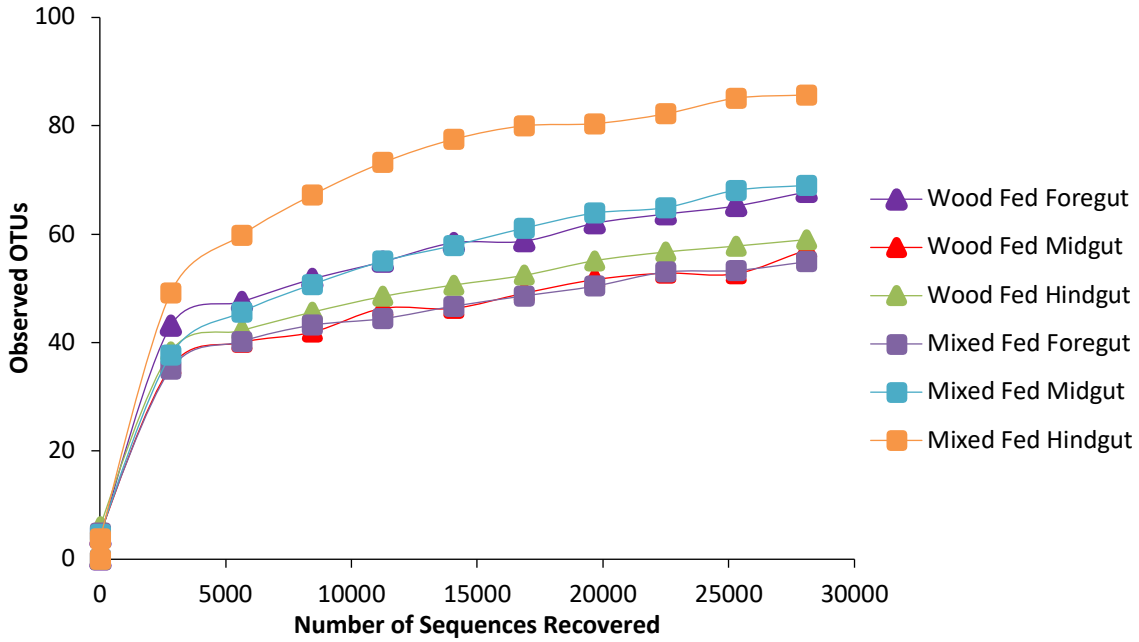

Figure 3:1: Rarefaction graphs with OTUs derived from *P. nigrolineatus* via sequencing of the ITS2 rDNA region. The data was normalised on the sample containing the lowest number of sequences, 28,090 sequences were subsampled from the wood-fed fish and mixed-fed fish.

3.4.3 Fungal Community Diversity in the GI Tract of *P. nigrolineatus*

The taxonomic composition of the microbial community varied across tissue type and diet (Fig. 3.2 and Fig. 3.3). Sequences that were greater than 1% abundant, were reported as these sequences were considered to be abundant than those having less than 1% abundance, which were grouped in unclassified/other. Taxa were grouped and reported at class level with lower taxonomic classifications (>1%) also reported. By using the UNITE v6 database (Koljalg *et al.*, 2013) it was possible to identity sequences similar to two fungal phyla, all GI tract regions in both fish, fed different diets, were dominated by sequences most related to Ascomycota (80.9%), with more in the wood-diet fish (87.7%) than the mixed-diet fish (74.1%), followed by Basidiomycota (4.6%) (Table 3.4). At lower taxonomic levels, the fungal diversity recovered from the GI tract was dominated by sequences similar to Sordariomycetes (50 OTUs); Hypocreales and Microascales and Dothideomycetes (32 OTUs); Pleosporales. Sordariomycetes dominated the mixed-diet fed fish predominantly in the hindgut, whilst Dothideomycetes was prevalent in the wood-diet fed fish. Sequences with high sequence similarity to *Fusarium oxysporum* were prevalent in all tissue regions except the wood-fed diet hindgut and decreased in numbers distally in both fish. The wood-fed diet hindgut was dominated by sequences similar to *Cirrenalia macrocephala* (13.8%) (Fig. 3.3). Other sequences present throughout the GI tracts of both dietary regimens included Ascomycota;

Aureobasidium pullulans and *Debaryomyces prosopidis* (both more abundant in wood-fed fish), and Basidiomycota; *Malassezia restricta*. The three most abundant sequences were *F. oxysporum*, *A. pullulans* and *Botrytis caroliniana,* being found most abundantly in the wood-diet fed foregut (Fig. 3.2).

Different OTUs were detected within each region of each fish, but only Agaricomycetes; genus *Exidia* (1%) was found >1% in just one region; the wood-fed diet midgut, all other sequences were found in multiple tissue regions (Fig. 3.3). The core microbiome of the fish was dominated by sequences with high similarity to Sordariomycetes and Dothideomycetes in all regions, with the former more prevalent in the mixed-diet fed fish and the latter dominated in the midgut and hindgut of the wood-diet fed fish (Fig. 3.2). The mixed-diet fed hindgut indicated the most diversity of OTUs, with many unique to this region, but many are shared with the midgut (Fig. 3.3).

Table 3:4: Percentage of phyla in ITS2 rDNA region of *P. nigrolineatus* fed either a wood or a mixed diet.

Consensus lineage	Total % (Average)	Wood fed fish			Mixed fed fish		
		Foregut %	Midgut %	Hindgut %	Foregut %	Midgut %	Hindgut %
Ascomycota	81	97	82	85	74	56	92
Basidiomycota	5	1	7	8	11	0.3	1
Fungi unclassified	5	2	11	7	5	2	1
Protista	0.1	0	0	0.4	0	0.2	0.01
Unknown	10	0.2	0.6	0	10	43	6

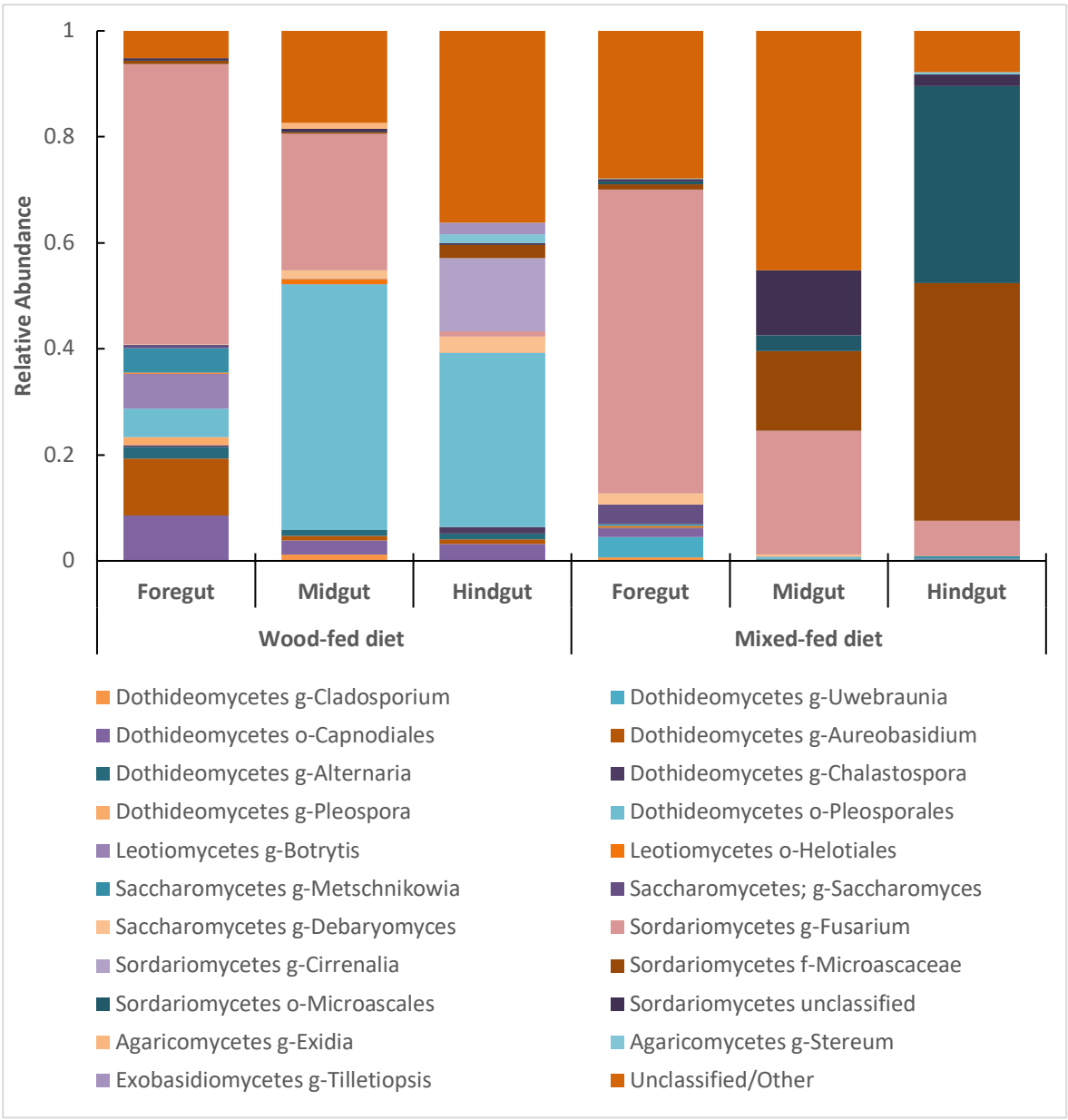

Figure 3:2: Relative abundance of dominant fungal taxa (>1%) detected by sequencing the ITS2 region from the various GI tract regions of *P. nigrolineatus* fed either a wood diet or a mixed diet. Sequences were assigned to OTUs with ≥97% sequence identity. Sequences found in <1% abundance are grouped together in Unclassified/Other.

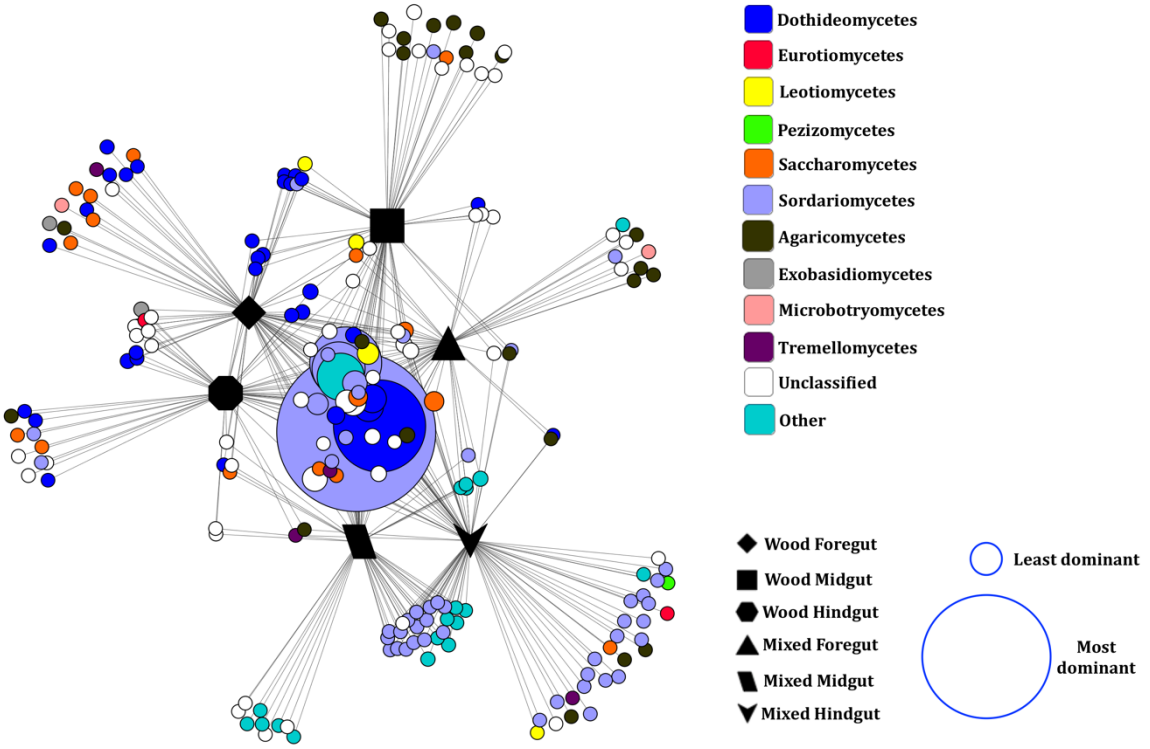

Figure 3:3: OTU network showing distribution of all OTUs identified to class detected via sequencing of the ITS2 region from different regions of the GI tract of *P. nigrolineatus* fed either a wood diet or a mixed diet. Node size indicates the number of reads assigned to an OTU while node colour indicates consensus taxonomy.

3.5 Relative Distribution and Diversity of Fungi Based on Dietary Regimen

OTUs were binned into taxonomic groupings allowing comparison of fungal diversity within the GI tract of wood-fed fish and mixed-fed fish, irrespective of GI tract region. In this analysis, the GI tract of the mixed-fed fish was found to have the most diversity (Fig. 3.4). A t-test indicated there was no significant difference (P = 0.582) in the number of observed OTUs between the wood-fed (61) and mixed-fed fish (70) (Fig. 3.5). $Chao_1$ analysis indicated that the wood-fed fish (82) had a higher number of rare and unique species compared to mixed-diet fed fish (85) as the difference between observed and $Chao_1$ was greater.

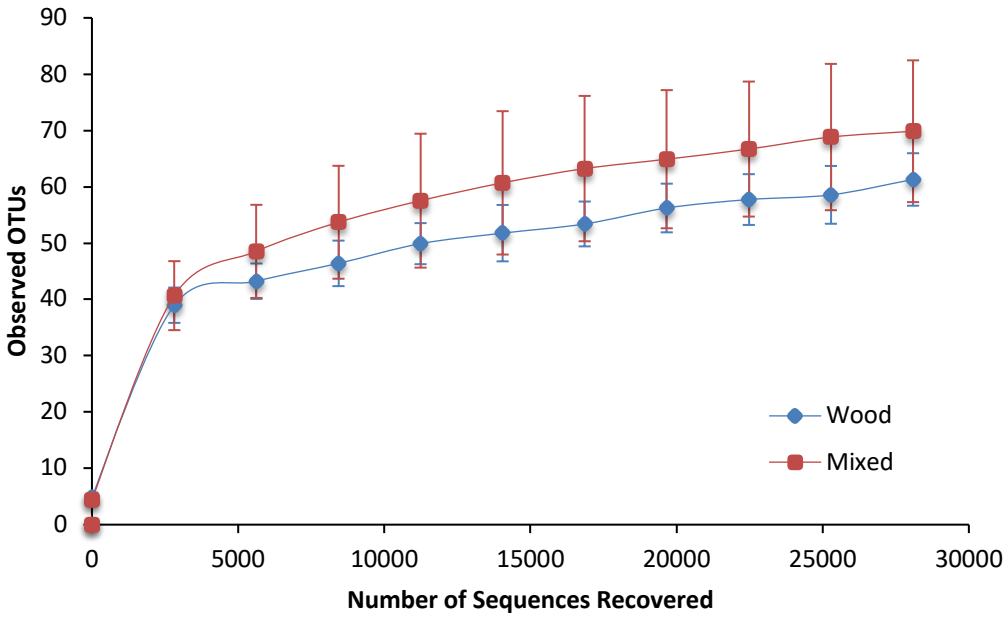

Figure 3:4: Rarefaction graphs showing number of OTUs in the wood-fed fish and the mixed-fed fish. OTUs were derived from sequencing of the ITS2 rDNA region, binned to species. The data was normalised on the sample containing the lowest number of sequences, 28,090 sequences were subsampled from the wood-fed fish and mixed-fed fish.

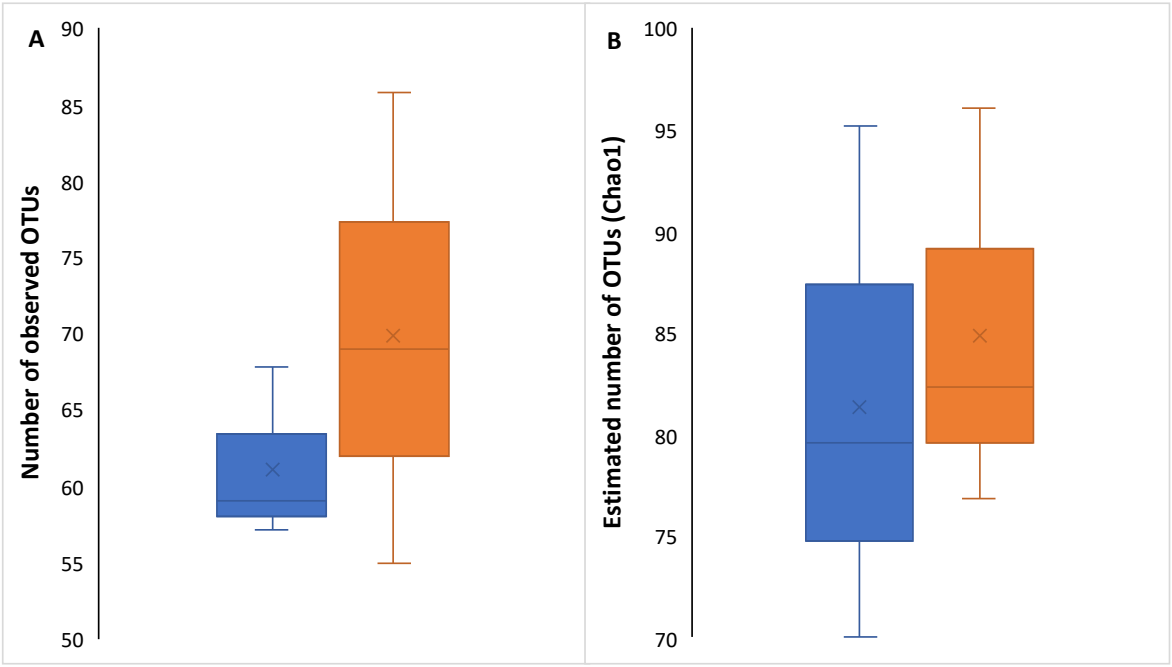

Figure 3:5: Alpha diversity boxplots showing fungal diversity within *P. nigrolineatus* GI tract. The mean (X) median (-) and range of OTUs within the wood-fed fish (n=3) (blue) and the mixed-fed fish (n=3) (orange) at 28,090 sequences per sample. (A) Results from observed species and (B) Results from $Chao_1$ estimations.

The most prevalent classes in the wood-diet fed fish were sequences similar to Dothideomycetes (40%) and Sordariomycetes (36%) and the mixed-diet fed fish were sequences similar to Sordariomycetes (73%) (Fig. 3.6). The wood fed fish was dominated by sequences similar to Sordariomycetes; species *F. oxysporum* and *C. macrocephala*, Dothideomycetes; orders Pleosporales and Capnodiales, species *A. pullulans*, Leotiomycetes; species *Botrytis caroliniana* and Basidiomycota incertae sedis; species *M. restricta.* The mixed-fed fish appears (Fig. 3.6) to be less diverse and dominated by sequences with high similarity to Sordariomycetes; order Microascales, family Microascaceae, species *F. oxysporum,* and Basidiomycota incertae sedis; species *M. restricta*. However, this is due to several OTUs not being assigned to lower taxonomic levels. Three OTUs detected from the Dothideomycetes class; genus *Alternaria* were found in the foregut, midgut and hindgut of the wood-diet fed fish but absent in the mixed-diet fed fish. Dothideomycetes; species *A. pullulans* was found in high abundance in enteric regions of the wood-diet fed fish but in low abundance in the mixed-diet fed fish.

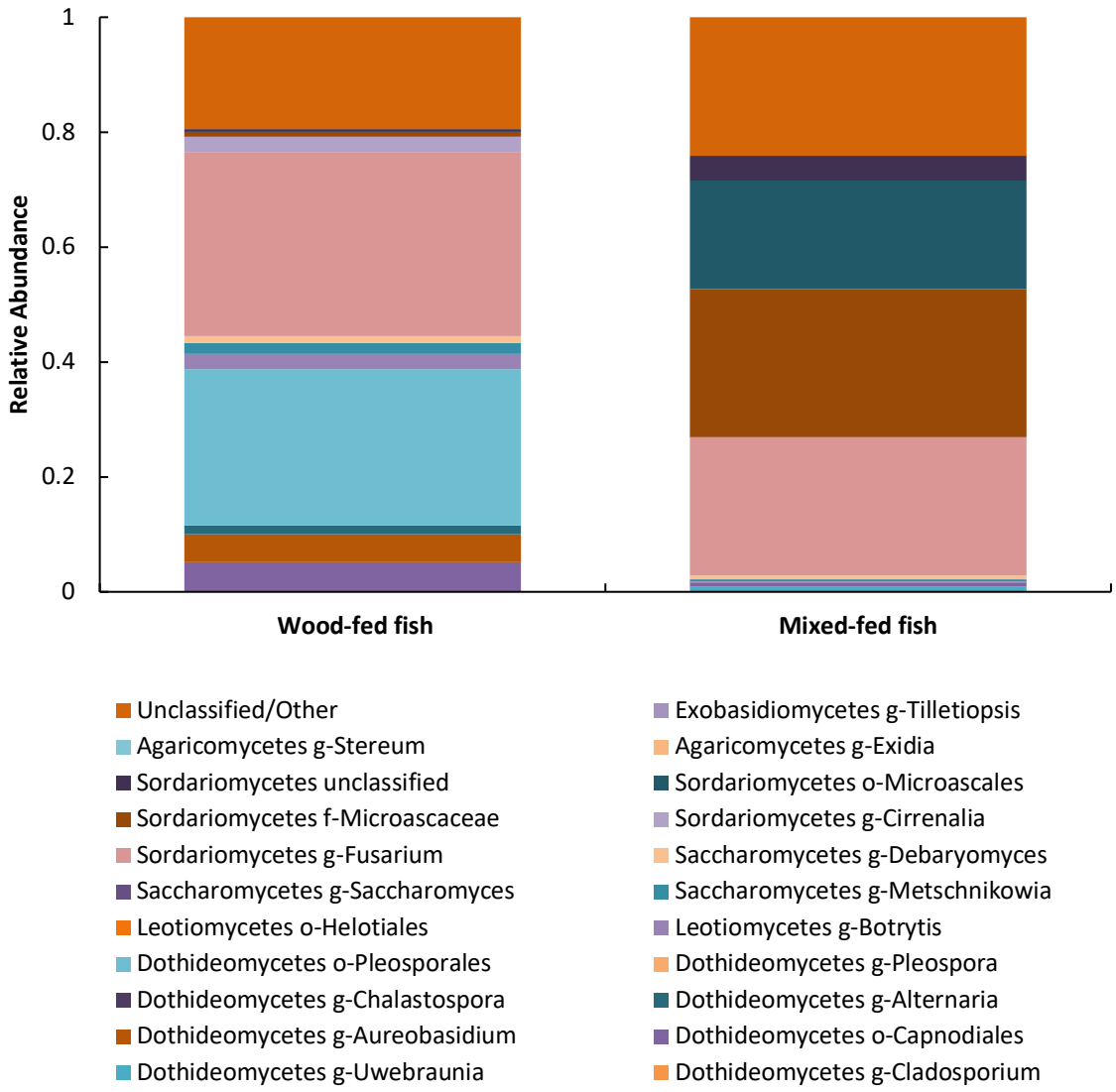

Figure 3:6: Relative abundance of dominant fungal taxa (>1%) detected by sequencing the ITS2 region from the GI tract regions of *P. nigrolineatus* fed either a wood diet or a mixed diet. Sequences were assigned to OTUs with ≥97% sequence identity. Sequences found in <1% abundance are grouped together in Unclassified/Other.

3.6 Relative Distribution and Diversity of Fungi Based on Enteric Region

Each region of the GI tract was analysed for differences in community composition irrespective of diet. Analysis did not reveal any tissue specific fungal communities detected greater than 1% abundance, but sequences similar to Tremellomycetes were found solely in the hindgut and not found in high abundance in either feeding regimen. This suggests it may be conserved in this region and have importance in wood degradation. However, analyses were based on the sequences of one

foregut, midgut and hindgut from each diet and due to the lack of biological replicates, this may not reflect accurately the variability amongst individuals (Prosser, 2010).

3.7 Distribution and Diversity of Fungi in the GI Tract from ITS1 rDNA Analysis

A total of 290,524 sequences were clustered into 190 OTUs, were obtained from the ITS1 rDNA amplification and analysed using USEARCH (Table 3.5). OTUs were binned into taxonomic groupings allowing comparison of fungal community alpha diversities across tissues and diet.

Table 3:5: NGS data of fungal sequences obtained from *P. nigrolineatus* GI tract. Number of raw reads, total clusters and unique OTUs from sequencing ITS1 rDNA region. Sequences were assigned to OTUs with ≥97% sequence identity.

Diet and enteric region	Total raw reads	Total clusters (≥97%)	Unique OTUs (≥97%)
Mixed-fed foregut	164,232	59,906	90
Mixed-fed midgut	195,182	59,665	85
Mixed-fed hindgut	170,170	59,325	60
Wood-fed foregut	76,118	35,515	58
Wood-fed midgut	88,190	40,300	60
Wood-fed hindgut	78,430	35,813	52
All	990,704	290,524	190

3.7.1 Alpha Diversity of Fungi in GI tract of *P. nigrolineatus*

To detect changes in alpha diversity, $Chao_1$ estimations of diversity was applied to OTU distributions (Table 3.6). At 35,515 sequences, fungal diversity was highest in the foregut of the mixed-diet fed fish, decreasing through the midgut to the hindgut (Table 3.6). The wood-diet fed fish shows similar results in all regions with the midgut having the higher fungal diversity than the fore and hindgut. T-tests revealed there was no significant difference ($P = 0.29$) in the average number of fungal OTUs observed between wood or mixed-diet fish. The difference between S_{obs} and S_{Chao1} suggest more unique or rare OTUs being present in the mixed-fed midgut (Table 3.6) than any other tissue region examined.

Table 3:6: Comparison of ITS1 rDNA region OTU species richness. A non-parametric estimate $Chao_1$, was used to estimate species diversity in different regions of the GI tract. For phylotype richness estimations, OTUs were binned to species.

	Wood Fed Fish			Mixed Fed Fish		
	Foregut	**Midgut**	**Hindgut**	**Foregut**	**Midgut**	**Hindgut**
Observed OTUs	58	59.5	52	84.6	74.6	52.4
$Chao_1$	59.2	63.7	52.3	94.2	93.1	78.1

These findings were supported by the rarefaction analysis, which demonstrated that the mixed-diet fed foregut had the highest detectable species richness, while the hindgut of mixed-diet fed fish had the lowest. Sequences obtained from the ITS2 region indicated the mixed-diet hindgut had the highest detectable species richness and the mixed-diet fed foregut had the least (Fig. 3.7).

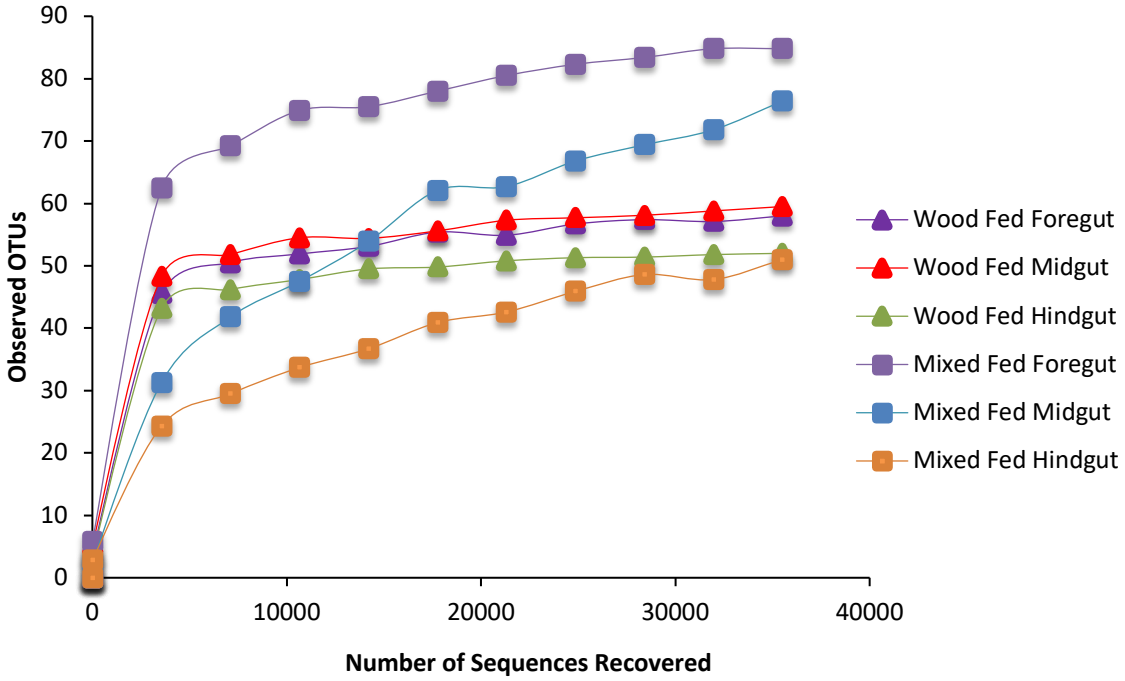

Figure 3:7: Rarefaction graphs with OTUs derived from sequencing of the ITS1 rDNA region, binned to species. The data was normalised on the sample containing the lowest number of sequences, 35,515 sequences were subsampled from the wood-fed fish and mixed-fed fish.

3.7.2 Fungal (ITS1 rDNA) Community Diversity in the GI Tract of *P. nigrolineatus*

The taxonomic composition of the microbial community (>1%) varied across tissue type and diet (Fig. 3.8). By using the UNITE database it was possible to identity sequences similar to five fungal phyla, with all gut regions in both fish fed different diets were dominated by sequences most related to Ascomycota (Table 3.7), followed by Basidiomycota in the wood-diet fed fish and Blastocladiomycota in the mixed-diet fed fish. Sequencing this region was less sensitive than the ITS2 region and the majority of sequences were Ascomycota; unclassified, Fungi; unclassified and unknown sequences. Similar to results from ITS2 analysis, sequences similar to Sordariomycetes; species *F. oxysporum* was most dominant in the wood-diet fed and mixed-diet fed foregut and decreases distally in both fish (Fig. 3.8). There were many OTUs that were unique to one region of one fish, but only *Cryptovalsa ampelina* (2%) and *Exophiala lecanii-corni* (9%) were found >1% in one region; the wood-fed diet midgut. A core microbiome could not be established due to the high number of unclassified and unknown sequences.

Table 3:7: Percentage of phyla from sequencing the ITS1 rDNA region in fish fed a wood or mixed diet.

Consensus lineage	Total % (Ave)	Wood fed fish			Mixed fed fish		
		Foregut %	Midgut %	Hindgut %	Foregut %	Midgut %	Hindgut %
Ascomycota	82.8	87.7	86.1	82.6	52.8	89.4	98.1
Basidiomycota	0.8	0.3	0.9	2.2	1.3	0.1	3.37E-05
Blastocladiomycota	0.7	0	0	0	1.1	3.1	0.1
Glomeromycota	0.01	0.01	0	0	0	0	0
Zygomycota	0.05	0	0.05	0	0	0	0
Fungi unclassified	11.4	11.8	12.5	13.7	28.8	1.3	0.6
Unknown sequences	4.2	0.2	0.4	1.6	15.9	6.1	1.2

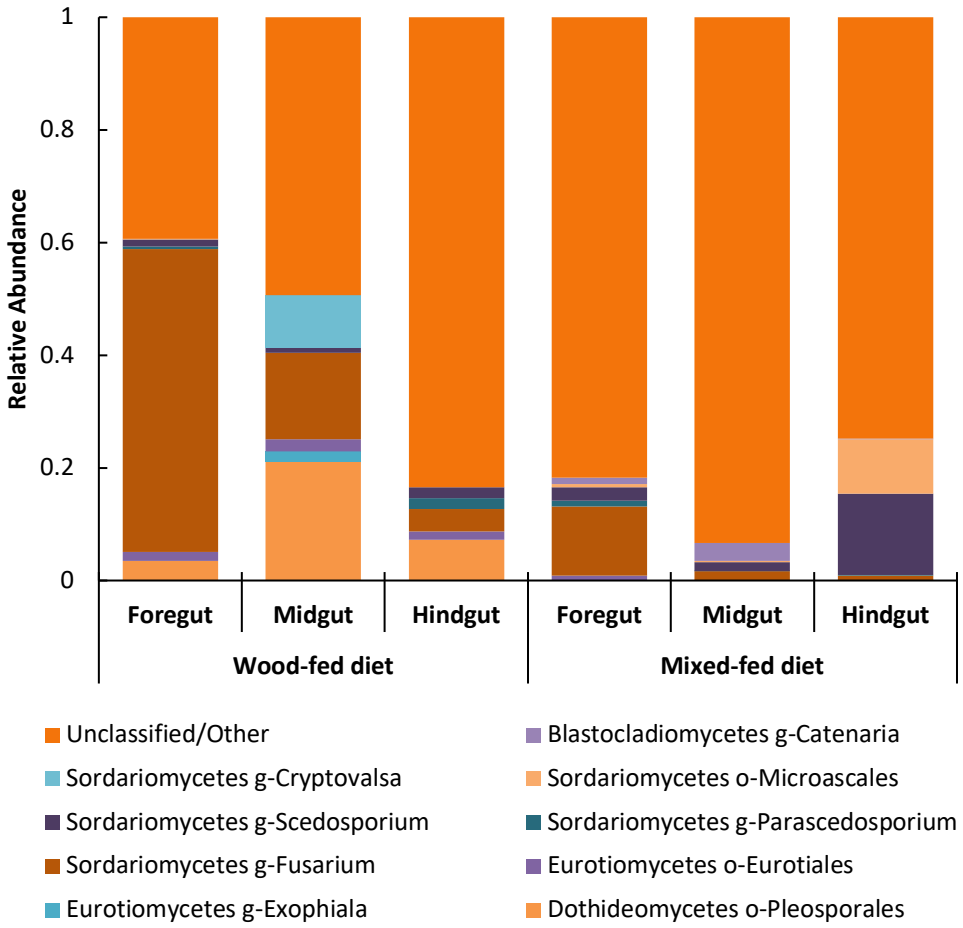

Figure 3:8: Relative abundance of dominant fungal taxa (>1%) detected by sequencing the ITS1 region from the GI tract regions of *P. nigrolineatus* fed either a wood diet or a mixed diet. Sequences were assigned to OTUs with ≥97% sequence identity. Sequences found in <1% abundance are grouped together in Unclassified/Other.

The most dominant classes in the wood-diet fed fish were sequences similar to Sordariomycetes (30%) and Dothideomycetes (11%) and the mixed-diet fed fish were Sordariomycetes (15%) (Fig. 3.8). The remaining sequences in the wood-diet fed fish and mixed-fed diet fish consisted of unclassified Ascomycota, unclassified fungi and unknown sequences (59% and 84% respectively). From the sequences at class level, the wood-diet fed fish had sequences similar to Sordariomycetes; species *F. oxysporum* (24%) and *Cryptovalsa ampelina* (3%), Dothideomycetes; order Pleosporales (11%) and Eurotiomycetes; order Eurotiales (2%). The mixed-diet fed fish had sequences similar to Sordariomycetes; order Microascales (3%), species *F. oxysporum* (5%) and *Scedosporium dehoogii* (6%). Sequences similar to Dothideomycetes; order Pleosporales were in all regions of the fish fed a wood-diet but absent from the mixed-diet fish. However, sequences similar to Blastocladiomycetes; genus *Catenaria* were present in all enteric regions of the mixed-fed diet fish

but absent in the wood-diet fish.

3.8 Discussion

The fungal communities of wood-diet and mixed-diet fed *P. nigrolineatus* GI tracts were investigated by sequencing the rDNA ITS region. The analysis was carried out on only one fish that was raised on each diet, this was due to complications associated with fish acquisition and rearing. It is recognised that a rigorous analysis of the GI tract communities requires the application of several fish. However, this still provides valuable insights into the fungal communities present, as it was found that each GI tract region possessed a distinct community and this is the first report of the presence of fungi throughout a fish GI tract (Marden *et al.*, 2017).

In their natural habitat *P. nigrolineatus* imbibes large quantities of wood in its diet and it is hypothesised to have developed a symbiotic relationship with microorganisms to degrade this resource. Their long GI tract provides microenvironments enriched with lignocellulose that are suitable for microbial colonisation and degradation, providing energy to the microbial community and host. Wood decomposition is dynamic and the rate of decomposition depends on many factors including priority effects and successional changes in microbial communities (Hiscox *et al.*, 2015; Purahong *et al.*, 2016). In forest ecosystems, fungi have been shown to shape bacterial community composition and are thought to be more abundant in the early stages of decay, with fungal mycelia doubling faster than bacterial cells and bacterial-fungal interactions facilitating decomposition (Romaní *et al.*, 2006; Frey-Klett *et al.*, 2011; Bengtsson, 2012; Hervé *et al.*, 2014; Johnston *et al*., 2018). Although bacterial-fungal interactions are vital in plant cell wall digestion, aerobic and anaerobic fungal activity has been shown to be responsible for most cell wall degradation by penetrating into plant tissues not normally available to bacteria (Lee *et al.*, 2000).

The findings from this study indicate that *P. nigrolineatus* that were fed a wood-diet or a mixed-diet had different fungal communities. This is consistent with previous and current research showing similar variations for the bacterial communities in different regions of the GI tract (McDonald *et al*. 2012; McDonald, Watts and Schreier, manuscript in preparation). The hindgut is of particular interest, which has been shown to contain cellulolytic bacteria (McDonald *et al.*, 2012; Di Maiuta *et al.*, 2013; Watts *et al.*, 2013), as well as fungi with sequences similar to *C. macrocephala*, which has been associated with waterlogged wood (Mouzouras *et al.*, 1986; Björdal, 2012; Marden *et al*., 2017). Sequences similar to Tremellomycetes, *Cyberlindnera jadinii, Tilletiopsis, Malassezia sympodialis* and *Trechisporales* were found exclusively to this region and

further research is needed to investigate whether these fungi play a role in wood degradation. Any relationship between these bacteria and fungi and their role in the fish GI tract remains to be established. It has previously been shown that bacterial symbionts in the fish GI tract plays a role in health and metabolism, therefore, it is quite plausible that endogenous fungal communities may play similar roles, whether the fish would benefit from having fungi present or whether they work together symbiotically with the bacterial communities is yet to be determined (Banerjee and Ray, 2017).

Combined with previous studies (McDonald *et al.*, 2012, 2015; Di Maiuta *et al.*, 2013; Watts *et al.*, 2013), these results suggest bacteria and fungi co-inhabit in the GI tract of *P. nigrolineatus*, with the potential of degrading dietary wood. This could provide an explanation to why these fish survive on such a low-nutrient substrate. Ongoing studies (McDonald *et al*. 2012; McDonald, Watts and Schreier, manuscript in preparation) suggest that the enteric bacterial community may lack selected enzymatic activities essential for lignocellulolytic digestion, these enzymes may be provided by the fungal community and/or the host. ITS1/ITS2 sequences for several cellulase-producing fungi have been found in the present study (Marden *et al.*, 2017), including those with sequences similar to *F. oxysporum*, *A. pullulans, Botrytis caroliniana, Metschnikowia, Alternaria* and *Debaryomyces*. *F. oxysporum*, which dominated the foregut and midgut regions, has been shown to excrete enodocellulases, exocellulases and β-glucosidase (Alconada and Martinez, 1996). These cellulolytic activities may form part of a symbiotic relationship, allowing the bacteria to benefit from the primary stage of cellulose degradation. The proposed slow gut passage time (German, 2009) would support this symbiotic relationship. While there are different fungi in different regions of each fish, with many not identified to lower taxonomic levels, many may be carrying out similar functional roles, acting on cellulose to enhance bacterial activities. Future studies would examine whether genes utilised for wood degradation are expressed in different ways.

While this study has identified the fungal microbiota within the GI tract, the type of symbiotic relationship the fungi has with bacteria and host remains unknown. During the breakdown of lignocellulose, microbes may produce volatile fatty acids and amino acids, which are absorbed by the fish and providing them with an energy source. Bacteria facilitate fungal decomposition of lignin by altering wood chemistry, structure and permeability (Clausen, 1996; de Boer *et al.*, 2005; Frey-Klett *et al.*, 2011). They may also provide nutritional benefits to wood degrading fungi via nitrogen fixation, allowing fungi to decompose nitrogen-sparse wood (Aho, 1974). An active nitrogen-fixing community has been identified in the GI tract of *P. nigrolioneatus* (McDonald *et al.*, 2015),

indicating the possibility of a mutualistic symbiosis between fungi and bacteria within the fish GI tract. Further studies would be required to confirm such a relationship.

Previous studies on the microbial community of *P. nigrolineatus* have focused on and characterized the cellulolytic and diazotrophic bacterial communities no studies to date have examined the fungal community profile of this, or any other fish. This present study describes the major fungal populations within the different GI tract regions of fish fed either a wood or mixed diet. Different fungal communities were detected across tissue regions and dietary regimens, indicating diet and tissue type affects fungal diversity in fish (Marden *et al.*, 2017). It is unknown if *P. nigrolineatus* gains energy directly from the digestion of wood (German and Bittong, 2009; Lujan *et al.*, 2010). Therefore, it is possible that the resident fungal communities in the GI tract are important for wood-only diets that lacks readily available carbon and nitrogen by supplying a digestible source of carbon or other micronutrients.

This study is the first to examine the fungal community in a wood-eating fish and these results indicate the presence of a diverse fungal population that may play critical roles in cellulose degradation with potential nutritional benefits for the fish. Additionally, these previously under-studied fungal species may have novel cellulolytic and lignin degrading capabilities that could have implications for the generation of biofuels. Understanding the role of the fungal communities in lignocellulose degradation and their interaction with GI tract bacteria in this process is the focus of future studies.

4 Characterisation of the Bacterial and Fungal Communities in the Hepatopancreas from *O. asellus* and *P. scaber*

4.1 Introduction

Isopods such as *O. asellus* and *P. scaber* have successfully colonised diverse terrestrial environments and feed mainly on decaying plant material such as leaf litter, wood and grass (Zimmer, 2002). This initial study compared two different species of woodlice that are ubiquitous in the U.K. The hepatopancreas of woodlice consist of two pairs of tubular midgut caeca (Hames and Hopkin, 1989), that secrete digestive enzymes and reabsorb digestively released nutrients (Zimmer, 2002). This area of the gut is thought to play a role in the hydrolysis of cellulose and the oxidation of lignin (Zimmer and Topp, 1998c; Zimmer, 1999; Zimmer *et al.*, 2002). It is proposed that the bacteria located in the hepatopancreas may have facilitated the colonisation of terrestrial habitats and the ability to utilise recalcitrant food sources (Zimmer and Topp, 1998b; Zimmer *et al.*, 2001, 2003). Other researchers found dense colonisation of bacteria visualised by electron microscopy in the hepatopancreas of several isopods (Wood and Griffiths, 1988; Hames and Hopkin, 1989; Zimmer and Topp, 1998b; Zimmer, 1999). The bacterial diversity within these glands was reported to be low, colonised by predominantly one or two, currently unculturable bacterial species; *Hepatoplasma* or *Hepatincola* (Wang *et al.*, 2004a; Wang *et al.*, 2004b). It has been previously reported that a filter between the foregut and hepatopancreas prevents particles, such as fungi and large bacteria from entering and colonising (Storch, 1987; Wood and Griffiths, 1988; Hames and Hopkin, 1989).

Therefore, this will be the first study to investigate the presence of fungal species in woodlice. This will also be the first study to compare the bacterial and fungal microbiota from the same hepatopancreas using NGS from two species of woodlice. This is also the first study to compare this microbiota in the hepatopancreas and hindgut (Chapter 5) from the same individual woodlouse.

4.2 Aims

The major aims of this chapter are

- To confirm the presence of fungi in the hepatopancreas of two species of woodlice; *O. asellus* and *P. scaber*

- To explore the diversity of bacteria and fungi in the hepatopancreas of two species of woodlice, *O. asellus* and *P. scaber* using NGS.

4.3 Overview of the Microbiota Within the Hepatopancreas of *O. asellus* and *P. scaber*

All DNA samples were subjected to PCR to ensure the presence of bacteria and culture methods for confirmation of fungi. DNA samples were sent to LGC Genomics (GmbH, Berlin, Germany) to amplify the 16S rRNA gene (V3-V4) and ITS rDNA region (ITS2) using Illumina MiSeq V3 platform (San Diego, CA, USA). This enabled analysis of bacterial and fungal sequences observed in the hepatopancreas of *O. asellus* (n=5) and *P. scaber* (n=5).

Illumina MiSeq sequencing platform generated a total of 6,566,594 reads by sequencing the 16S rRNA gene compared to 822,178 reads from sequencing the ITS2 rDNA region of *O. asellus* and *P. scaber* hepatopancreas (Table 4.1). After processing and subsampling, the resulting abundance of unique bacterial OTUs was considerably higher than fungal (Table 4.1). The 16S rRNA gene data was subsampled at 50,000 reads (see 2.4.1.1) and 100% of samples generated that number of reads and the ITS2 rDNA data was subsampled to 20,000 reads and 30% generated that number. This initial analysis was performed by LGC and showed over inflation of diversity, therefore the analysis was repeated with different parameters and the number of OTUs was greatly reduced (Table 4.1). Therefore, subsequent analysis of OTU abundance took place on the subsequent processing.

Table 4:1: NGS results from sequencing bacteria and fungi in the hepatopancreas of woodlice. Number of raw reads, total clusters, unique OTUs and own analysis of OTUs from sequencing the 16S rRNA gene and ITS2 rDNA region of *O. asellus* and *P. scaber* hepatopancreas. Sequences were assigned to OTUs with ≥97% sequence identity.

Woodlice species	**No of samples**	**Total raw reads**		**Total clusters (≥97%)**		**Unique OTUs (≥97%) LGC analysis**		**Unique OTUs (≥97%) Own analysis**	
		16S	**ITS2**	**16S**	**ITS2**	**16S**	**ITS2**	**16S**	**ITS2**
O. asellus	5	3,706,672	586,290	249,966	63,616	633	41	137	23
P. scaber	5	2,859,922	235,888	249,996	60,392	644	39	92	20
All		6,566,594	822,178	499,962	124,008	1,277	80	229	46

4.4 Bacterial 16S rRNA Gene Sequence Analysis Detected in the Hepatopancreas of *O. asellus* and *P. scaber*

Bacterial sequences corresponding to the 16S rRNA gene (V3-V4) were PCR amplified from the hepatopancreas of *O. asellus* and *P. scaber.* OTUs were binned into taxonomic groupings to allow for comparison of bacterial communities within this tissue. Sequences were assigned to 137 unique OTUs (*O. asellus*) and 92 OTUs (*P. scaber*) and Simpson's diversity indices indicates higher diversity in *O. asellus* (Table 4.2).

Table 4:2: Comparison of V3-V4 16S rRNA gene OTU species richness with in woodlice hepatopancreas. Non-parametric estimates $Chao_1$ and Simpson's diversity indices, were used to estimate species diversity in the hepatopancreas of *O. asellus* and *P. scaber*. For species richness estimations, OTUs were binned to genera.

Woodlice species	Total observed OTUs	$Chao_1$ estimation	Simpson's diversity index - D
***O. asellus* (n=5)**	137	222	0.42
***P. scaber* (n=5)**	92	114.9	0.33

4.4.1 Alpha Diversity Analysis of Bacterial Communities in the Hepatopancreas of Woodlice

To enable comparison of the microbial community within the other samples in the study, the data was normalised to the sample containing the lowest number of sequences (49,940 sequences) (Schloss *et al.*, 2011). A t-test revealed there was no significant difference ($P = 0.051$) in the number of observed bacterial OTUs between the hepatopancreas of both species of woodlice, but the diversity of bacterial OTUs was higher in *O. asellus* (Fig. 4.1A). QIIME analysis discounted two anomalies (shown on Fig. 4.1A) for *P. scaber*, however if anomalies were retained the range would be similar for both woodlouse species. To capture all bacterial diversity, $Chao_1$ estimations of diversity were applied to OTU distributions (Fig. 4.1B). These estimations indicate more OTUs may be found with additional sampling, this would suggest more unique and rare bacterial species were found, especially in *O. asellus* (Table 4.2). A significant difference ($P = 0.008$) in OTU distribution and diversity was detected using $Chao_1$ with the predicted spread of OTUs being less diverse in *P. scaber* than *O. asellus* (Fig. 4.1B).

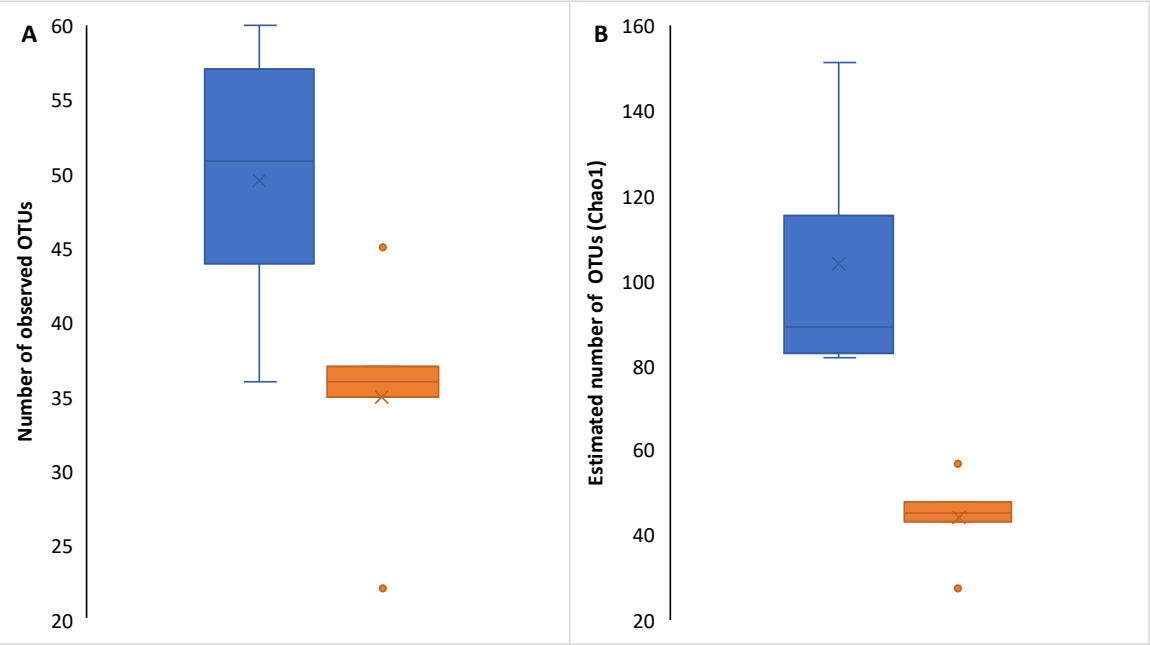

Figure 4:1: Alpha diversity boxplots showing bacterial diversity within woodlice. The mean (x), median (-) and range of bacterial OTUs within the samples of *O. asellus* (blue) and *P. scaber* (orange) hepatopancreas at 49,940 sequences per sample. (A) Observed species (B) $Chao_1$ estimations of diversity.

These findings were supported by the rarefaction analysis which demonstrated *O. asellus* had higher average detectable species richness than *P. scaber*; 49.6 OTUs and 35 OTUs respectively (Fig. 4.2). In both species of woodlice, the majority of 16S rRNA diversity had been sampled as observed species had reached asymptote. However, differences in S_{obs} and S_{chao1} (Fig. 4.2 and Table 4.2) suggests additional sampling would allow more novel OTUs to be detected in *O. asellus*.

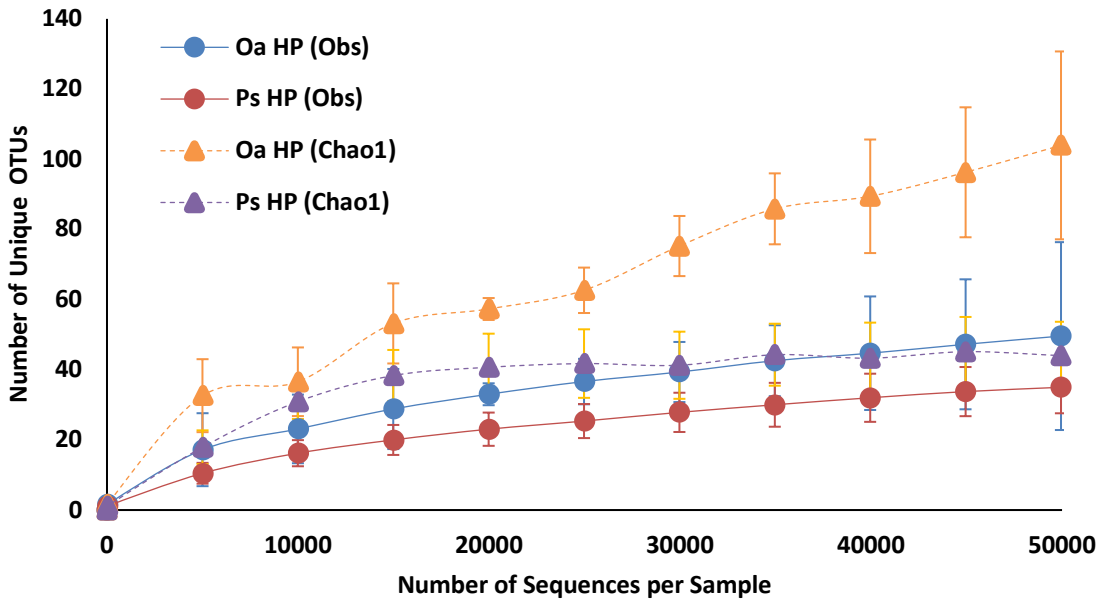

Figure 4:2: Rarefaction graphs with OTUs derived from sequencing the 16S rRNA region, binned to genera. The data was normalised on the sample containing the lowest number of sequences, 49,940 sequences were subsampled from the hepatopancreas of *O. asellus* (Oa) and *P. scaber* (Ps) and reported for observed species (Obs) and $Chao_1$ estimations.

4.4.2 Beta Diversity Analysis

To better understand the presence of bacterial diversity within the woodlouse samples, taxonomic profiling was applied and showed two distinct clusters corresponding to bacterial OTUs, one cluster representing *O. asellus* and one *P. scaber* (Fig. 4.3). Two samples of *O. asellus* and two samples of *P. scaber* were clustered together indicating these samples were similar in communities.

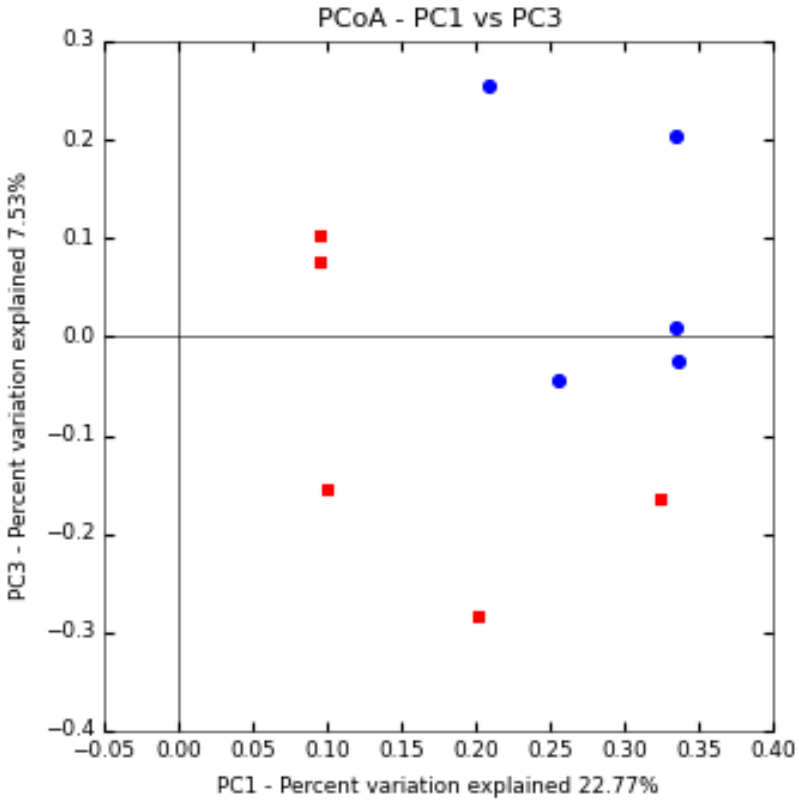

Figure 4:3: Similarities of bacterial communities in the hepatopancreatic lobes of woodlice; *O. asellus* (red square) (n=5) and *P. scaber* (blue circle) (n=5). PCoAs shown were generated from unweighted UniFrac distances. Axes represent the two synthetic variables explaining the greatest proportion of variation in the data set. Figure supplied by LGC Genomics.

4.4.3 Bacterial Diversity in the Hepatopancreas

The majority of reads found in greater than 1% abundance were assigned to sequences similar to eleven phyla. There was limited diversity in the hepatopancreas, with the majority of OTUs assigned uniquely to sequences similar to phylum; Tenericutes (76.9%) in both *O. asellus* and *P. scaber* (Fig. 4.4). The remaining diversity was different between woodlice, with *P. scaber* having more unclassified bacteria, compared to *O. asellus*, which had sequences similar to phylum; Proteobacteria, family Incertae sedis and Coxiellaceae.

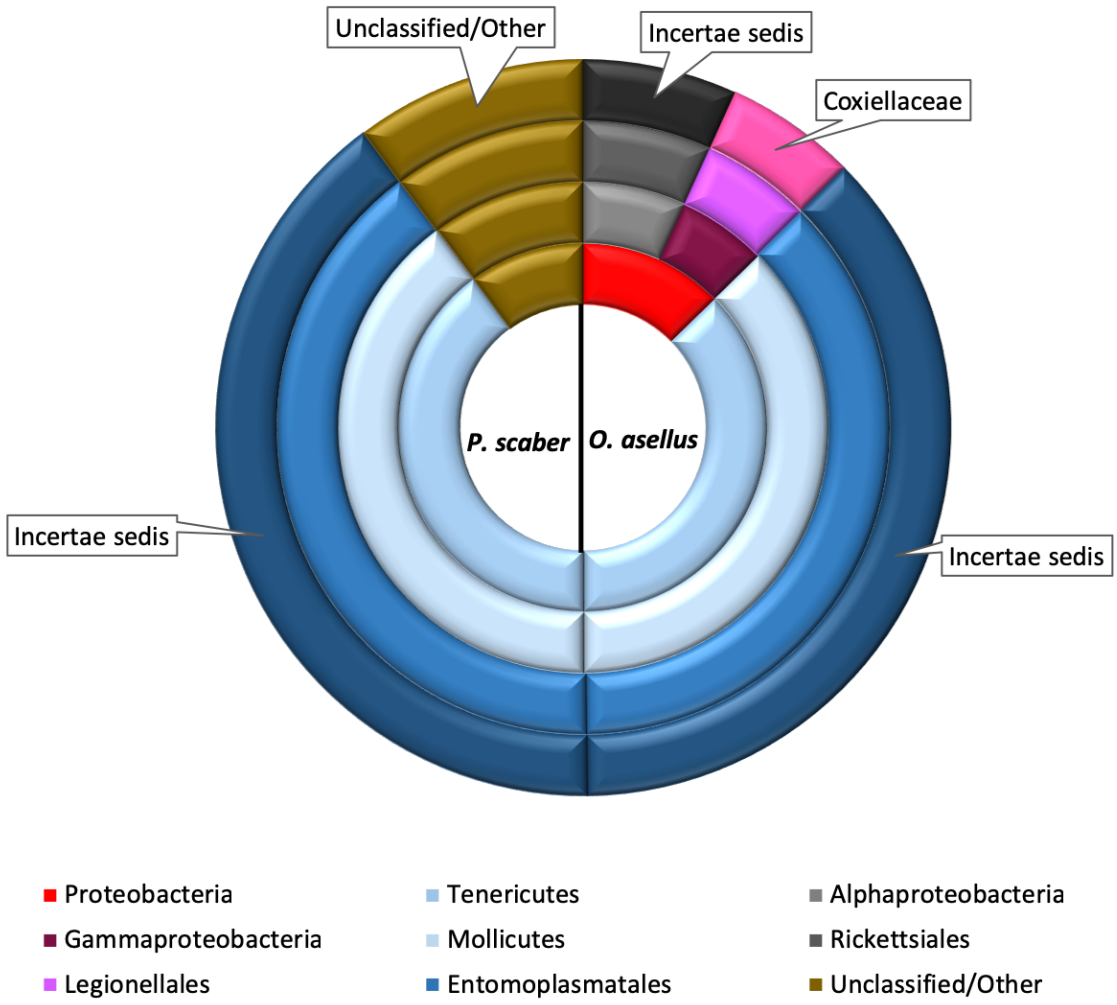

Figure 4:4: The relative abundance of bacterial taxa from *O. asellus* and *P. scaber* hepatopancreas detected by amplifying the 16S rRNA gene. Sequences detected greater than 1% abundance are shown.

The bacterial communities in the hepatopancreas of *O. asellus* and *P. scaber* were not diverse. Taxa were grouped and reported at class with lower taxonomic classifications also reported if greater than 1% abundant. Sequences found less than 1% abundance and unclassified bacterial sequences were grouped in unclassified/other (Fig. 4.5). Sequences similar to *Hepatoplasma* were dominant in both woodlice species (*O. asellus* 74% and *P. scaber* 79%). Sequences similar to *Rickettsiella* (11%) and *Hepatincola* (14%) were also present in *O. asellus* (Fig. 4.5A). One individual sample of *O. asellus* had a different composition and was dominated by sequences similar to *Hepatincola* (68%) and *Rickettsiella* (32%) (Fig 4.5B).

In this study a core microbiome element was defined as an OTU that has appeared in 80% of samples, this level was chosen due to there being 5 replicates in the study. Fourteen OTUs were found in over 80% of samples, OTUs that were found greater than 1% abundance were found in all

samples, were sequences similar to *Hepatoplasma* (>1% in all samples) and *Rickettsiella* (>1% in 3 samples, <1% in 7 samples) (Fig. 4.5B). Some OTUs were present in individual woodlice of the same species and some were present between woodlice species (Fig. 4.5B & 4.6). The OTUs most predominantly shared between all samples were from phylum Tenericutes, with sequences similar to Proteobacteria and Actinobacteria OTUs having less relative abundance. Individual replicates had communities that were unique, with samples of *O. asellus* having more diverse and unique OTUs than *P. scaber* (Fig. 4.6).

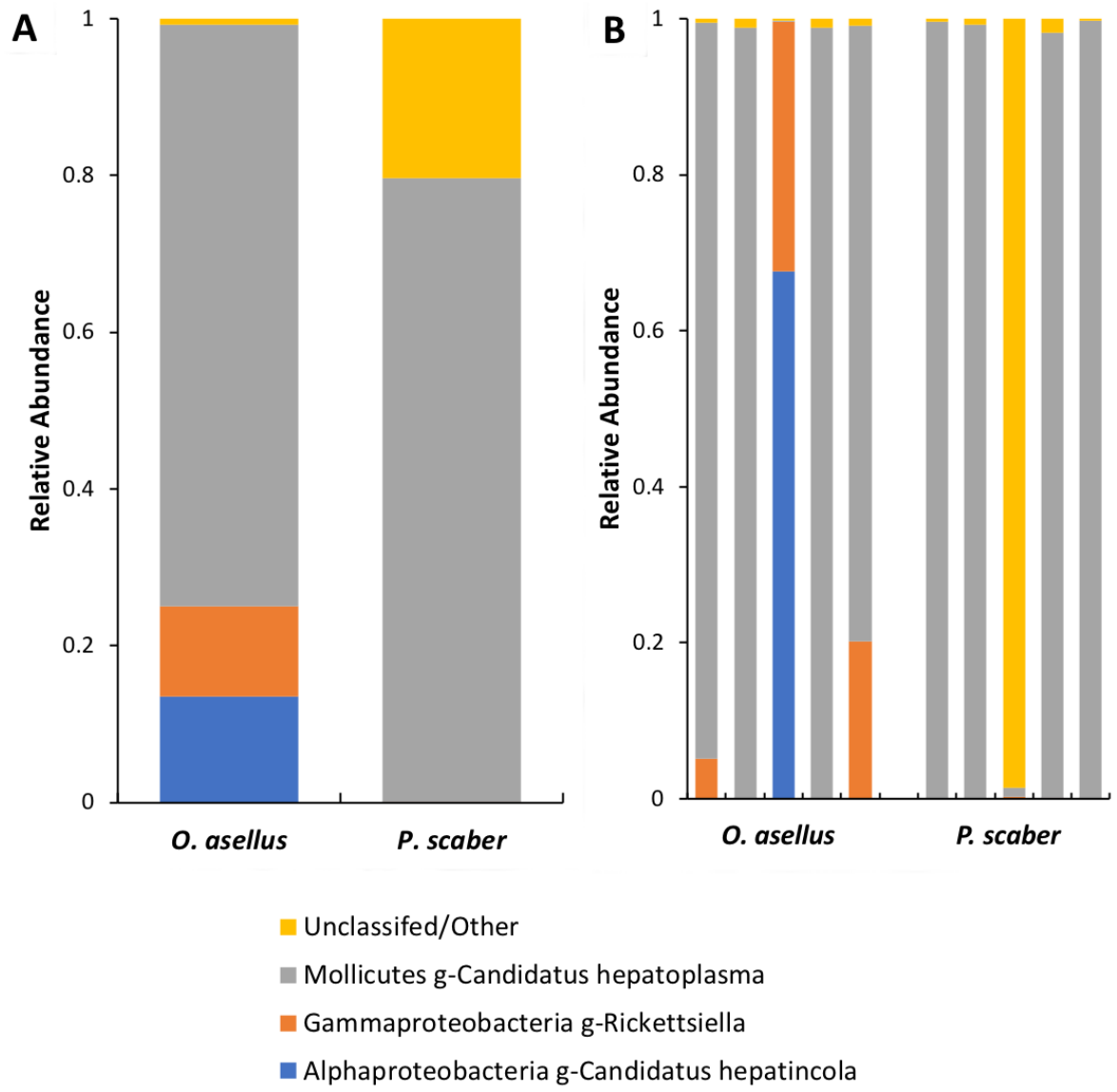

Figure 4:5: Taxonomic composition of the bacterial microbiome in the woodlouse hepatopancreatic lobes. Results are reported at class and genus (g). (A) Average relative abundance and distribution of the most abundant bacterial sequences (>1%) found in at least one woodlouse. (B) Relative abundance and distribution

of bacteria of individual woodlice. Results are from 16S rRNA gene Illumina sequencing from the hepatopancreas of *O. asellus* (n=5) and *P. scaber* (n=5). Sequences were assigned to OTUs with ≥97% sequence similarity.

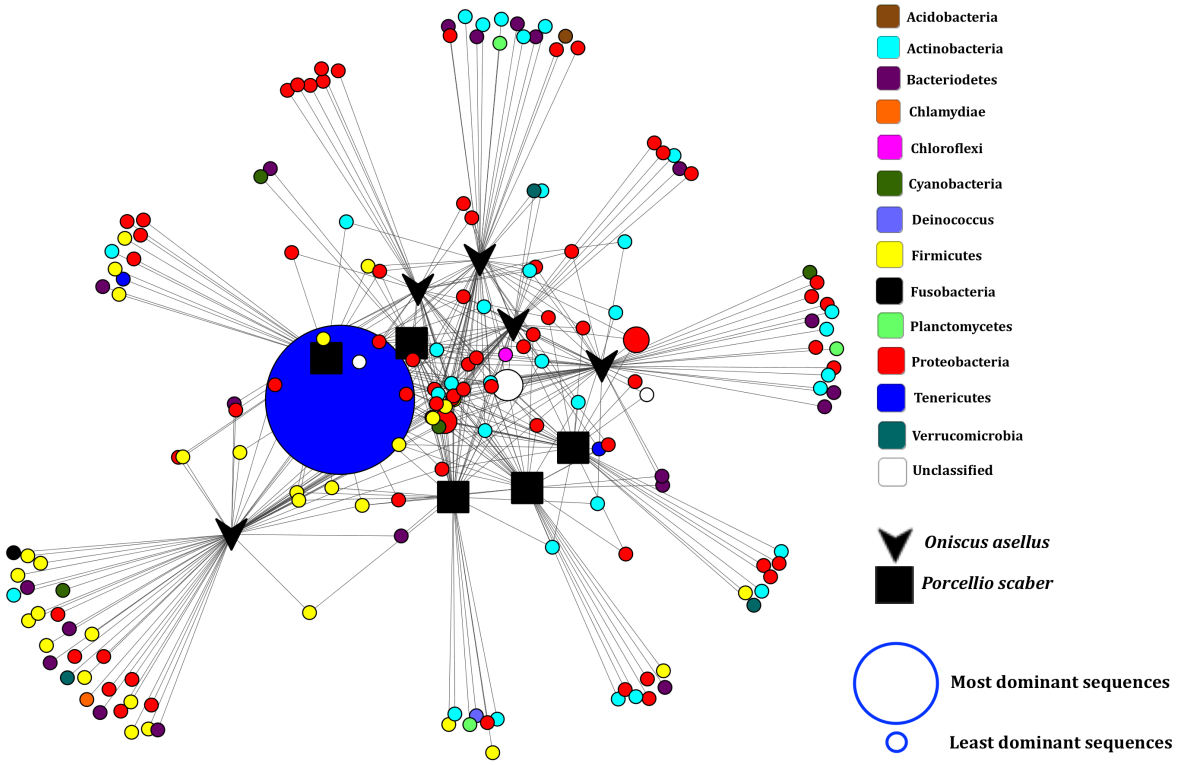

Figure 4:6: OTU network showing distribution of all bacterial OTUs within the hepatopancreas of woodlice. OTUs were identified to phylum detected via sequencing the 16S rRNA gene from the bacterial community present in the hepatopancreatic lobes of *O. asellus* and *P. scaber.* Node size indicates the dominance of reads assigned to an OTU and node colour indicates consensus taxonomy.

4.5 Analysis of Fungal ITS rDNA Sequences Detected in the Hepatopancreas of *O. asellus* and *P. scaber*

Fungal community alpha diversities within this tissue were compared after OTUs were binned into taxonomic groupings. After subsequent analysis using different parameters to the initial analysis carried out by LGC, sequences were assigned to 23 OTUs (*O. asellus*) and 20 OTUs (*P. scaber*) with Simpson's diversity very similar (Table 4.3).

Table 4:3: Comparison of ITS2 rDNA OTU species richness. Non-parametric estimates $Chao_1$ and Simpson's diversity indices, were used to estimate species diversity in the hepatopancreas of *O. asellus* and *P. scaber*. For species richness estimations, OTUs were binned to genera.

Woodlice species	Total observed OTUs	$Chao_1$ estimation	Simpson's diversity index - D
O. asellus	23	23	0.78
P. scaber	20	21	0.76

4.5.1 Alpha Diversity Analysis in the Hepatopancreas

To better compare fungal diversity in the hepatopancreas, the data was normalised to the sample containing the lowest number of sequences, 1,250 sequences (Schloss et al., 2011). The *O. asellus* sequence dataset showed more fungal OTUs at 1,250 sequences than in the *P. scaber* dataset (Fig. 4.7A). A t-test revealed there was no significant difference ($P = 1$) in the number of fungal OTUs observed between the hepatopancreas of both species of woodlice. To estimate the need for further sampling to discover more OTUs, $Chao_1$ estimations of diversity were applied to OTU distributions. These estimations (Fig. 4.7B) indicate the number of OTUs would not have increased with additional sampling, with no significant difference in the number of OTUs between the woodlice species ($P = 0.995$).

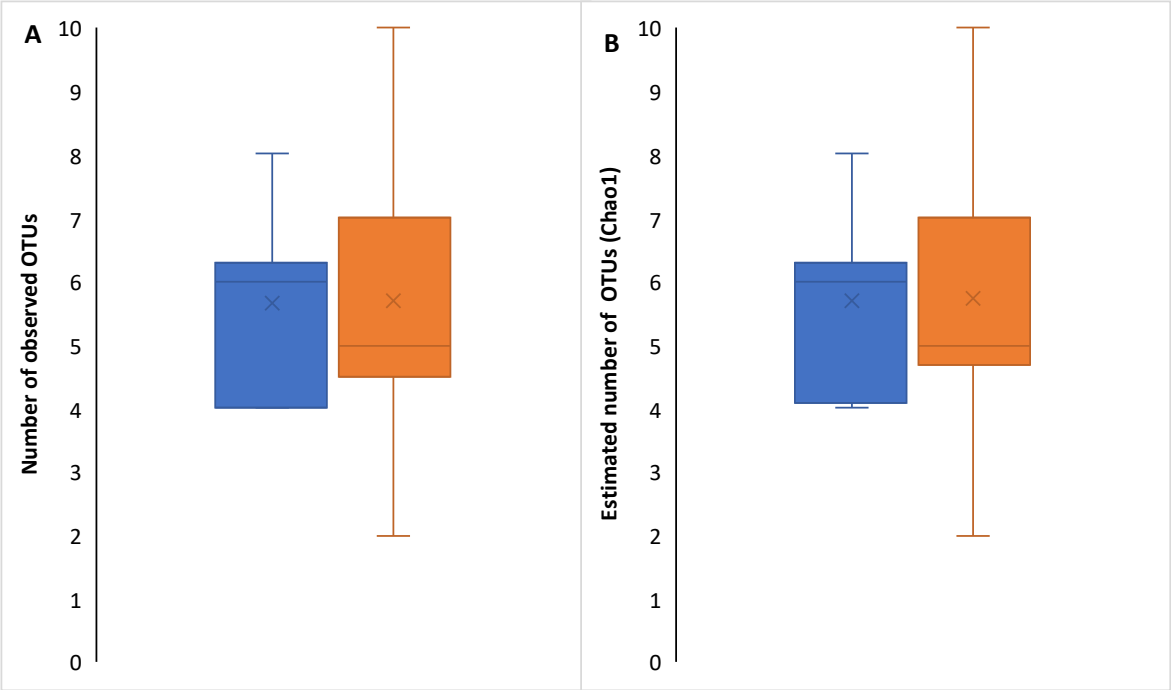

Figure 4:7: Alpha diversity boxplots showing diversity of fungal OTUs within the woodlice hepatopancreas. The mean (x), median (-) and range of fungal OTUs within the samples of *O. asellus* (blue) and *P. scaber* (orange) hepatopancreas at 1,250 sequences per sample. (A) Observed species (B) $Chao_1$ estimations.

Rarefaction analysis supported these findings (Fig. 4.8), which demonstrated *O. asellus* and *P. scaber* had similar average detectable species richness, 5.66 OTUs and 5.7 OTUs respectively. In both species of woodlice, ITS rDNA diversity had reached asymptote at 800 sequences and differences between S_{obs} and S_{chao1} (Fig. 4.8) suggests additional sampling would not allow more novel OTUs to be detected from both woodlice species.

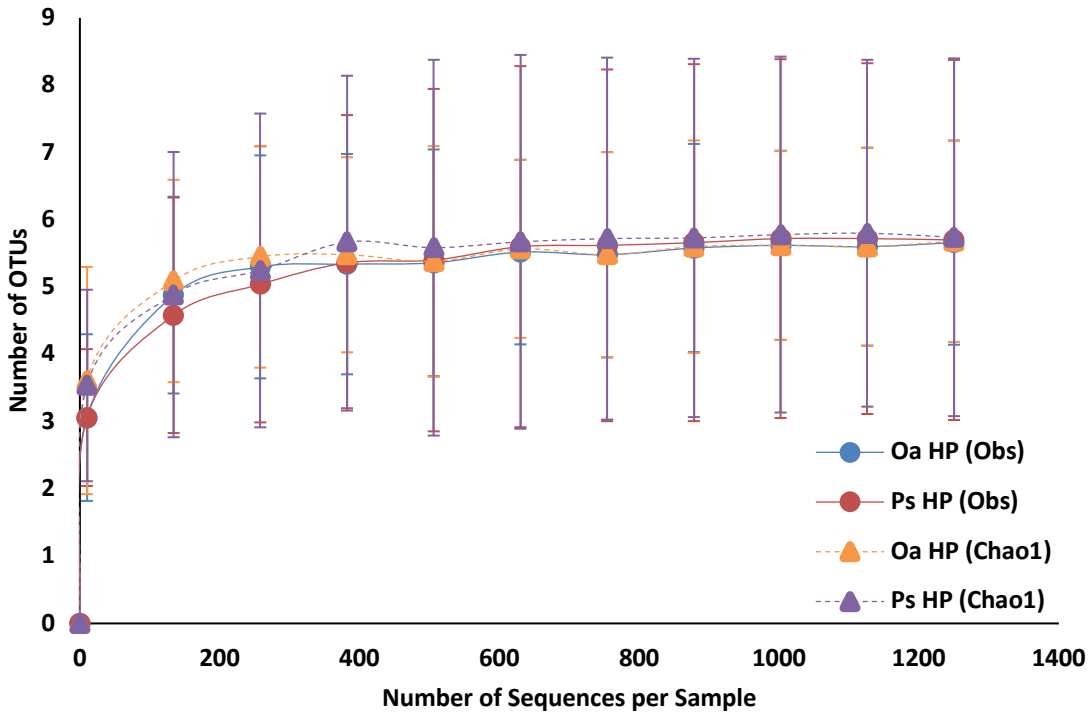

Figure 4:8: Rarefaction graphs with OTUs derived from sequencing the ITS2 rDNA region, binned to genera. The data was normalised on the sample containing the lowest number of sequences, 1,250 sequences were subsampled from the hepatopancreas of *O. asellus* (Oa) (n=5) and *P. scaber* (Ps) (n=5) and reported for observed species (Obs) and $Chao_1$ estimations.

4.5.2 Beta Diversity Analysis of the Hepatopancreas of *O. asellus* and *P. scaber*

Distinct fungal communities were observed in the lobes, between each sample of *O. asellus* and *P. scaber* (Fig. 4.9). One sample of *O. asellus* and one sample of *P. scaber* appear to be different from the other respective samples.

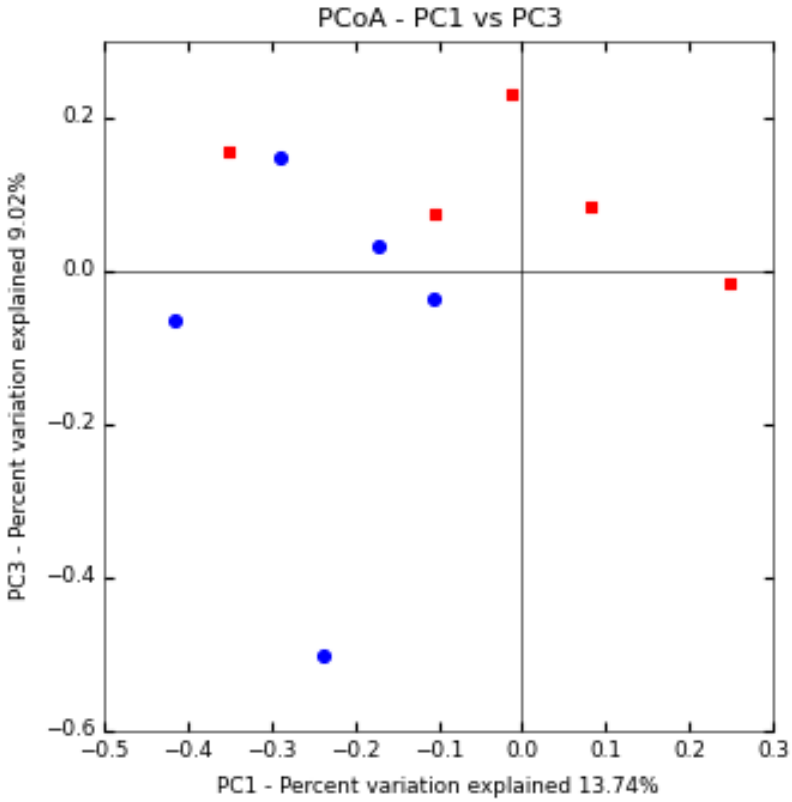

Figure 4:9: Similarities of fungal communities in the hepatopancreatic lobes of woodlice; *O. asellus* (red square) (n=5) and *P. scaber* (blue circle) (n=5). PCoAs shown were generated from unweighted UniFrac distances. Axes represent the two synthetic variables explaining the greatest proportion of variation in the data set. Figure supplied by LGC Genomics.

4.5.3 NGS Analysis of the Fungal Community Diversity in the Hepatopancreas

The majority of sequences were assigned to two phyla (Fig. 4.10), with the majority of OTU reads greater than 1% abundance assigned to sequences similar to Ascomycota (95.4%), followed by Basidiomycota (4.1%). There was a clear difference in diversity between the two species of woodlice with *O. asellus* being dominated by unclassified Sordariomycetes and Davidiellaceae, and *P. scaber* being dominated by Davidiellaceae and Sclerotiniaceae.

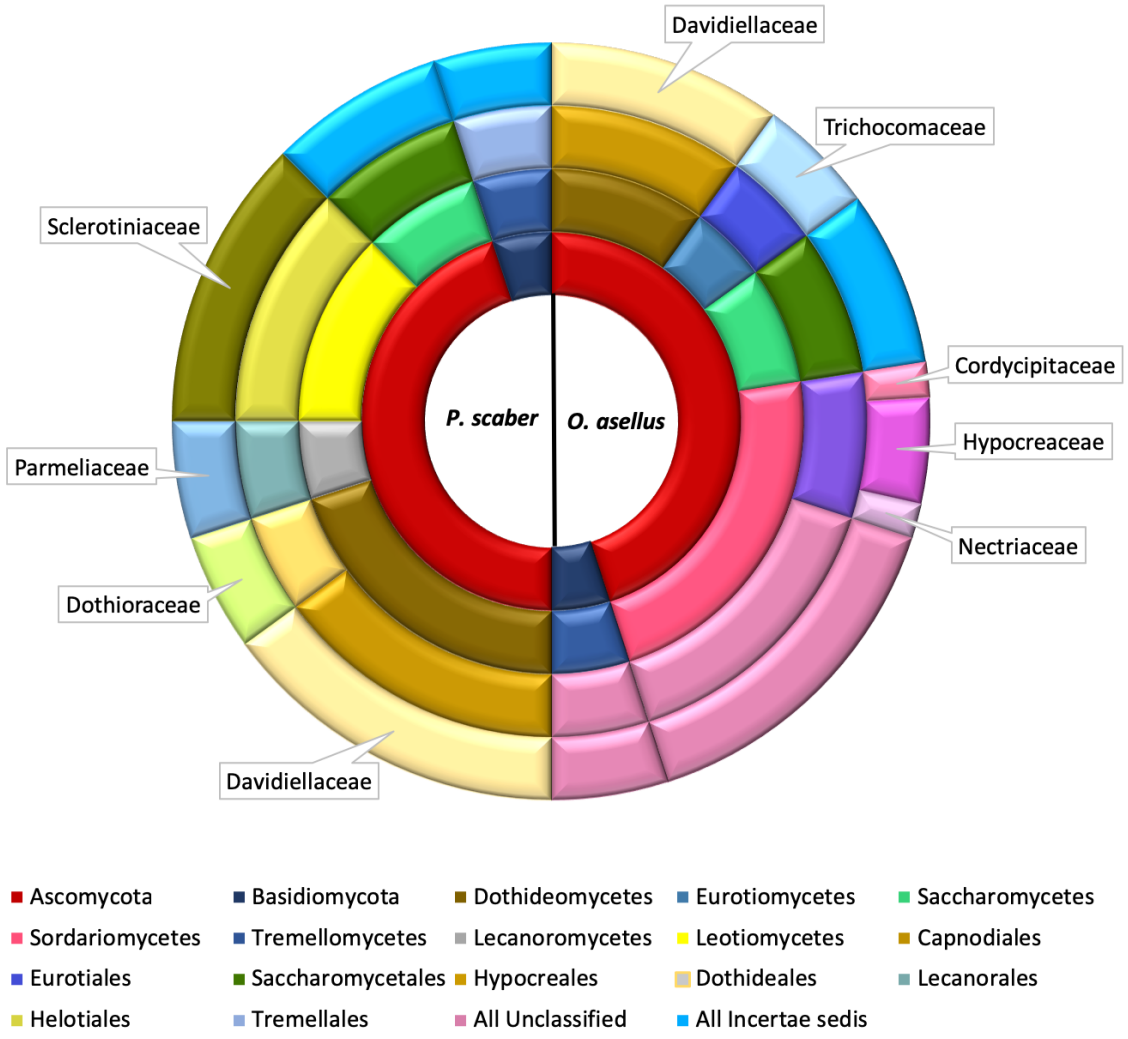

Figure 4:10: Relative abundance of fungal taxa from *O. asellus* and *P. scaber* hepatopancreas from amplifying the ITS2 rDNA region. Sequences greater than 1% abundance are shown.

The taxonomic composition of the fungal community (>1%) was diverse and different between *O. asellus* and *P. scaber*. Taxa were grouped and reported at class with lower classifications reported after if detected greater than 1% abundance, sequences found fewer than 1% abundance, unclassified fungi, Protista and Plantae sequences were grouped in unclassified/other (Fig. 4.11). Both woodlice species were dominated by sequences similar to Dothideomycetes; family Davidiellaceae (*O. asellus* 25.4% and *P. scaber* 38.7%). Sequences similar to unclassified Sordariomycetes (35.9%) and genus *Trichoderma* (11.4%) and Saccharomycetes; genus *Debaryomyces* (6.7%) prevailed in *O. asellus.* However, in addition to Davidiellaceae, *P. scaber* hepatopancreas detected Leotiomycetes; genus *Botrytis* (27.5%), Saccharomycetes; genus *Candida* (9.1%), Dothideomycetes; *Aureobasidium* (4.7%) and Saccharomycetes; genus *Debaryomyces* (3.4%) to be abundant (Fig. 4.11A).

The community diversity varied between individual samples (Fig. 4.11B, Table 4.4 and Fig. 4.12). Sequences similar to Saccharomycetes and Dothideomycetes classes were found in greater than 1% abundance in 60% of samples in both *O. asellus* and *P. scaber,* with the percentage of abundance being similar. From analysis of lower taxonomic levels, no clear fungal community dominated. Some OTUs were shared between two or more samples with many OTUs being unique to one sample (Fig. 4.12). Although sequences similar to Dothideomycetes; family Davidiellaceae dominated (Fig. 4.11A), they were only found in one sample of *O. asellus* and four samples of *P. scaber* (Fig. 4.11B & Table 4.4). However, when divided into genera, *Candida* was only present in two samples of *O. asellus* and three samples of *P. scaber*. Sequences similar to *Cyberlindnera* were present in no samples of *O. asellus* and only one sample of *P. scaber*. *Debaryomyces* was present in one sample of *O. asellus* and *P. scaber* and *Wickerhamomyces* present in one *O. asellus* but absent from *P. scaber* samples. *Trichoderma* was present in one sample of *O. asellus* and no sample of *P. scaber* (Table 4.4). Therefore, in respect of a core mycobiota, none could be established at 80% of samples, sequences similar to Sordariomycetes unclassified, Saccharomycetes; genus *Candida* and Dothideomycetes; family Davidiellaceae were OTUs in only 50% of samples and only Sordariomycetes; unclassified OTUs were found in 70% of samples. Compared to the bacterial community, the taxonomic composition of the fungal community was diverse and varied in individual samples of *O. asellus* and *P. scaber*.

Table 4:4: Variability of fungal classes (>1%) found in individual samples of *O. asellus* and *P. scaber* hepatopancreas and percentage of OTU reads.

Taxonomy	No. of woodlouse samples fungi were recovered from		% of OTU reads	
	O. asellus	***P. scaber***	***O. asellus***	***P. scaber***
Dothideomycetes	3	4	18	38.6
Eurotiomycetes	3	2	4	1.3
Lecanoromycetes	0	2	0	3
Leotiomycetes	0	1	0	16.6
Saccharomycetes	4	4	19.9	19.4
Sordariomycetes	4	2	37.8	0.6
Agaricomycetes	3	2	1.8	2.7
Tremellomycetes	1	1	7.6	7.9

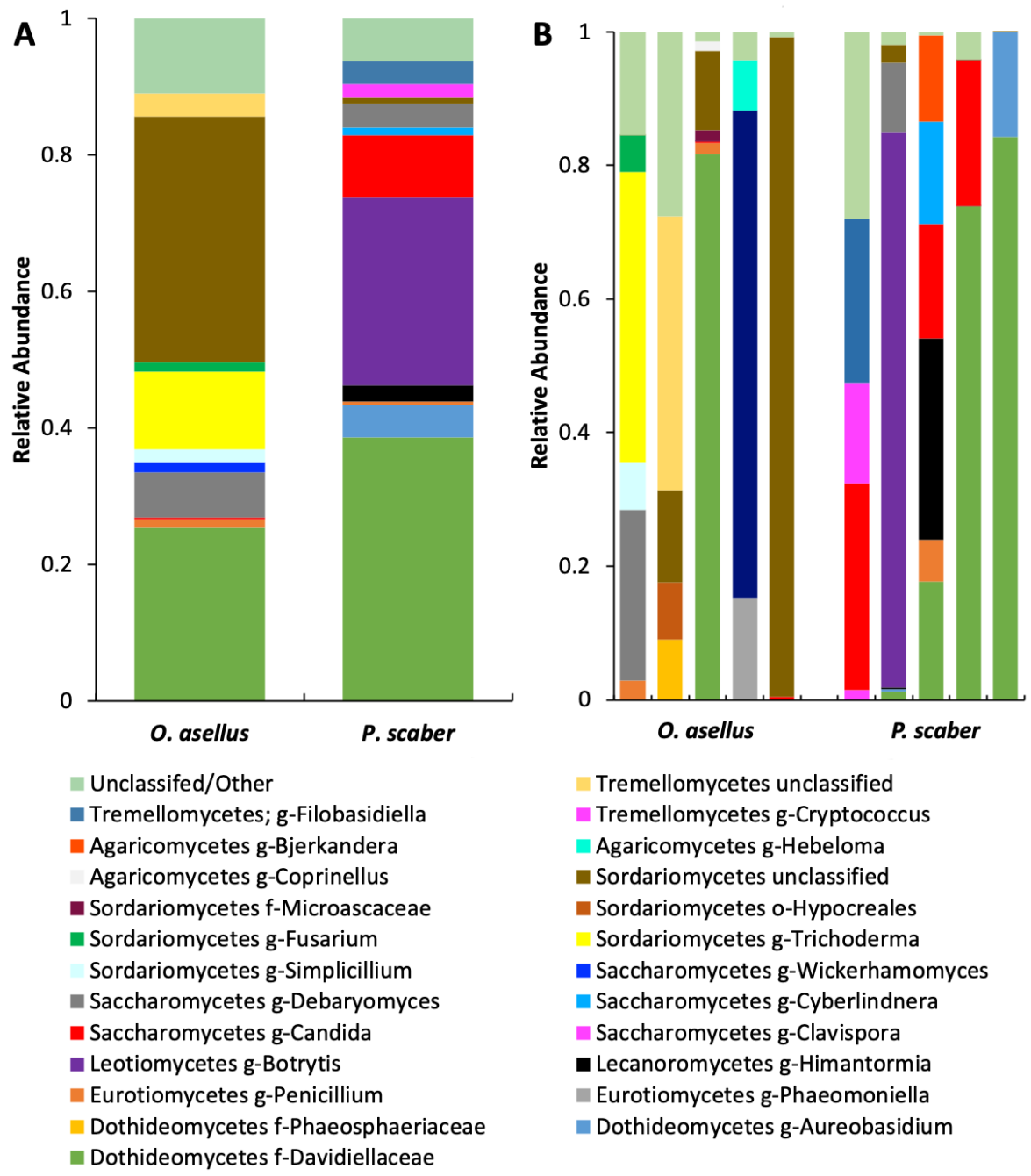

Figure 4:11: Taxonomic composition of the mycobiome in the hepatopancreatic lobes from individual woodlice. Taxa were grouped and reported at class and lower level taxonomy as indicated by o-order, f-family, g-genus. (A) Average relative abundance and distribution of the most abundant fungal sequences (>1%) found in at least one woodlouse. (B) Relative abundance of the mycobiome of individual woodlice. Results are from ITS2 rDNA Illumina sequencing from the hepatopancreas of *O. asellus* (n=5) and *P. scaber* (n=5). Sequences were assigned to OTUs with ≥97% sequence similarity.

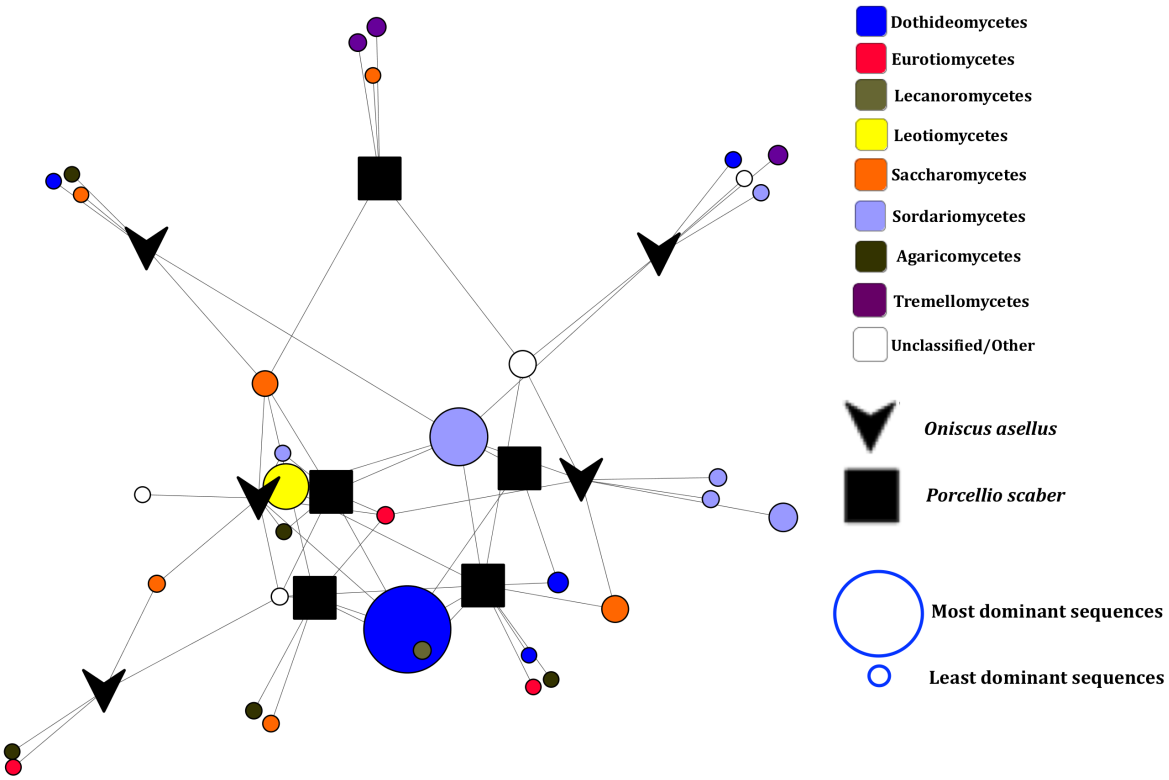

Figure 4:12: OTU network showing distribution of all OTUs identified to class detected via sequencing the ITS2 rDNA region from the hepatopancreatic lobes of *O. asellus* and *P. scaber.* Node size indicates the dominance of reads assigned to an OTU and node colour indicates consensus taxonomy.

4.6 Discussion

This study used the V3-V4 region of the 16S rRNA gene to sequence and study the bacterial diversity and the ITS2 rDNA region to study the fungal diversity in the hepatopancreas of *O. asellus* and *P. scaber*. Previous studies have investigated the bacterial microbiota in either *O. asellus*, *P. scaber* or other species of woodlouse (Table 1.1). This is the first study to put two species of woodlice under the same conditions allowing for a more reliable comparison of hepatopancreatic microbiota. This is the first study to investigate the mycobiota within the hepatopancreas of any woodlouse.

There was difficulty in amplifying fungal DNA from the total gut DNA using the ITS rDNA region using universal fungal primers. This is reported to be a more reliable region to amplify than using 18S rRNA primers (Halwachs *et al.*, 2017), however, preliminary experiments on isolates showed two bands at 650 bp and 300 bp and mixed environmental samples resulted in no bands. To optimise,

re-amplification of PCR product (1:10) proved to be the most effective method on previous environmental samples.

Multiple attempts were made to amplify the fungal DNA from the 10 hepatopancreatic samples, including re-amplification of the PCR product, but was unsuccessful in all samples. Samples were plated onto Malt agar and SDA and the presence of fungi was confirmed, therefore, samples were sent for NGS.

More OTUs were found by sequencing the 16S rRNA, than the ITS rDNA region. However, many of these bacterial OTUs were found in less than 1% abundance in samples. To allow working analysis to occur, abundant OTUs detected in greater than 1% abundance were reported. In comparison to bacteria, fungal diversity, greater then 1% abundance, was higher, being different between *O. asellus* and *P. scaber* and within individual samples.

A diverse range of fungi were found in the hepatopancreas despite previous studies indicating a filter between the foregut and the hepatopancreas, that prevents particles greater than 40 nm (Hames and Hopkin, 1989) or 1.7 μm (Wood and Griffiths, 1988) from entering and colonising, including cellulosic particles (Zimmer and Topp, 1998b). It has been suggested this filter is the cause of low bacterial diversity, however, fungi were able to enter and colonise. These results indicate otherwise and there may be other factors involved.

The mode of transmission of microbes into the hepatopancreas is unclear, mother isopods that screened positive for *Hepatoplasma* did not have embryos with this symbiont under sterile conditions (Wang *et al.*, 2007). However, under non-sterile conditions bacteria were detected in the hepatopancreas one week after hatching. The eggs were not surface sterilised in the pouch; therefore, maternal smearing of eggs cannot be excluded (Wang *et al.*, 2007). It has been suggested that *Hepatoplasma* could be transmitted horizontally among species of woodlice in the same habitat or through the environment, through coprophagy or cannibalism, however, as yet, *Hepatoplasma* has not been found in soil or leaf litter samples (Wang *et al.*, 2007; Bouchon *et al.*, 2016).

The most dominant bacterial genera in both *O. asellus* and *P. scaber* from this study were sequences similar to Mollicutes; *Hepatoplasma* and consistent between samples, revealing a core microbiome in 100% of samples in this study. Only one sample of *O. asellus* differed and was dominated by sequences similar to Alphaproteobacteria; *Hepatincola* and one sample of *P. scaber* was dominated

by an unclassified bacterial clone that had sequence similar to one found in the accessory nidamental gland of the Cuttlefish; *Sepiella maindroni,* found on the NCBI database.

A study by Wang *et al*. (2007) looked at the distribution of bacterial symbionts in the hepatopancreas of different species of isopod from different geographic locations. Using 16S rRNA gene sequencing and FISH, bacteria were detected in all species hepatopancreas' tested except marine species; *Idotea balthica* which had no bacterial cells in the 30 samples tested using FISH and PCR with bacterial primers. Sequences similar to *Hepatoplasma* were found in all terrestrial isopods studied; *O. asellus*, *P. scaber*, *Philoscia muscorum*, *Armadillidium vulgare*, *Trachelipus rathkil* and *Alloniscus perconvexus*. The only isopods to harbour sequences similar to *Hepatincola* were *O. asellus*, *P. scaber* and *T. rathkil*. Semi-terrestrial isopod *Ligia oceanica* had sequences similar to *Pseudomonas* whilst freshwater isopod *Asellus aquaticus,* from two sites, harboured sequences similar to Rhodobacter and Aeromonas. *Hepatoplasma* has been found in the hepatopancreas of all Oniscidean species studied to date, but *Hepatincola* has only been found in Crinocheta species (Wang *et al.*, 2004a; Fraune and Zimmer, 2008). These results support the hypothesis put forward by Zimmer (2002), that these symbionts are one of the adaptations acquired allowing colonisation to land, by contributing the necessary enzymes for digestion of terrestrial food sources and suggests *Hepatincola* only evolved in true terrestrial isopods (Fraune & Zimmer, 2008). *Hepatoplasma* is not exclusive to isopods, uncultured clones of *Hepatoplasma* have also been discovered in the hepatopancreas of Norway lobster *Nephrops norvegicus* (Meziti *et al.*, 2012).

Unlike Wang *et al*. (2007), Fraune and Zimmer (2008) found sequences similar to *Hepatoplasma* in *L. oceanica*. They detected *Hepatoplasma* in the hepatopancreas of eight out of nine species tested from different sites indicating isopod host specificity. The only species that did not have this symbiont was *Ligidium hypnorum*. They reported the relationship between isopod and symbiont to be highly specific and thought it improved host survival under cellulose-based, low nutrient conditions. This study found that isopods that harboured *Hepatoplasma* survived longer than those with other bacteria, including *Hepatincola*. Rickettsia-like *Hepatincola* had a negative effect on the host, with isopods harbouring this pathogenic symbiont, having a lower life expectancy than individuals without it.

Another pathogenic bacterium found in this study was opportunistic pathogen; *Rickettsiella*. The sample that had the greatest abundance of *Rickettsiella*, was also dominated by pathogenic *Hepatincola*. It was found in less than 1% abundance in 7 samples, in these samples *Hepatoplasma* dominated, this may indicate *Hepatoplasma* protects the woodlouse from this opportunistic

pathogen. It has been reported in numerous terrestrial isopod species, including *A. vulgare, A. nasatum, A. granulatum, Eluma purpurascens, O. asellus, P. laevis and P. gallicus* (Bouchon *et al.*, 2016), *Helleria brevicornis* and *Philoscia muscorum* (Cordaux *et al.*, 2007), however, it is unknown which tissue it inhabited. *Candidatus Rickettsiella isopodorum* has been discovered in the hepatopancreas of *P. scaber* and *A. vulgare* via light microscopy and TEM (Kleespies *et al.*, 2014). *Rickettsiella* in *O. asellus* and *P. scaber* was found by DNA extraction and microscopy from sectioning the whole organism (Abd El-Aal and Holdich, 1987; Drobne *et al.*, 1999; Dittmer *et al.*, 2016). *Rickettsiella* has also been described using cloning methods in the hepatopancreas of freshwater isopod *A. aquaticus* (Wang et al. 2007). Rickettsiaceae harbouring vacuoles in mature cells of the hepatopancreas has also been observed in *Palaemon elegans* (Crustacea; Decapoda) (Vogt and Štrus, 1998).

Sequences amplified from the fungal ITS2 region found all samples from this study were dominated by sequences similar to phyla Ascomycota. *O. asellus* was dominated by Sordariomycetes; unclassified and genus; *Trichoderma*, Dothideomycetes; family Davidiellaceae and Saccharomycetes; genus *Debaryomyces* and *P. scaber* was dominated by Dothideomycetes; family Davidiellaceae, genus *Aureobasidium*, Leotiomycetes; genus *Botrytis* and Saccharomycetes; genus *Candida*. The fungal diversity between samples was varied with no core microbiome and many fungal OTUs were found in only one or two hepatopancreatic samples. The mycobiome detected was different, however, it is plausible that their function for lignocellulose degradation pathways may be similar. Several cellulase-producing fungi found in the present study, including Dothideomycetes (Schoch *et al.*, 2006; Schneider *et al.*, 2012), Sordariomycetes (Zhang *et al.*, 2006; Schneider *et al.*, 2012), *Botrytis* (van Kan, 2006; Fernández-Acero *et al.*, 2009), *Trichoderma* (Beldman *et al.*, 1985; Sarsaiya *et al.*, 2018), *Bjerkandera* (Fukasawa *et al.*, 2011b) and yeasts *Aureobasidium* (Kudanga and Mwenje, 2005; Thongekkaew *et al.*, 2012), *Debaryomyces* (Giese *et al.*, 2017) and *Candida* (Thongekkaew *et al.*, 2012; Urbina *et al.*, 2013). This is not surprising as fungi are thought to be the main players in leaf litter decomposition due to their ability to degrade recalcitrant compounds (de Boer *et al.*, 2005; Meidute *et al.*, 2008) and being the main producers of leaf degrading enzymes (Schneider *et al.*, 2012).

Unclassified Sordariomycetes was found to dominate one *O. asellus* individual, interestingly, some species have been previously reported to be pathogenic to arthropods. Several Sordariomycetes genera (*Cordyceps, Elaphocordyceps, Hypocrella, Metacordyceps, Torrubiella and Ophiocordyceps*) from three families (Clavicipitaceae, Cordycipitaceae and Ophiocordycipitaceae) (Sung *et al.*, 2007; Johnson *et al.*, 2009) have been shown to directly parasitise a range of arthropods. To date, most

research has been carried out on spiders, beetles, scale insects and white flies (Sung *et al.*, 2007, 2008; Johnson *et al.*, 2009), however, as fungal research carried out on isopods is lacking, it is possible they could be parasitic to isopods. Sordariomycetes comprises of a diverse number of species and as well as arthropod-pathogens, they are also saprobes involved in decomposition and nutrient cycling (Zhang *et al.*, 2006) and as sequences could not be shown to have similarity to lower classifications, it is unknown whether these symbionts are mutualistic or antagonistic.

Woodlice used in this study, indicated bacteria had a lower diversity than fungi in the hepatopancreas, suggesting bacterial symbionts in these samples are obligate. The fungi detected had higher diversity and lacked a core microbiome and may be involved in a role irrespective of the host. However, by examining other terrestrial isopods, the association with *Hepatoplasma* was found to be not obligate, with only 44-80% of isopods harbouring this symbiont (Fraune and Zimmer, 2008). In terrestrial isopod *A. vulgare*, only 36% harboured *Hepatoplasma* (Dittmer *et al.*, 2014), whilst semi-terrestrial isopods *Ligia pallasii* from 20 locations, *Hepatoplasma* was detected in 96% of samples and *L. occidentalis* from 10 locations, 52% of samples had this symbiont (Eberl, 2010). Therefore, the benefit of bacterial symbionts to the host is unclear. It has been postulated that *Hepatoplasma* was beneficial to its host under low nutrient conditions (Fraune and Zimmer, 2008), enabling colonisation of new habitats, extending their geographic distribution (Wang *et al.*, 2004a), or to protect the isopod from harmful parasitic or pathogenic bacteria. It was also previously thought that *Hepatoplasma* and *Hepatincola* were mutually exclusive (Wang *et al.*, 2007), supporting this theory of protection, but a recent study on *A. vulgare* found that 46/119 samples tested, harboured either *Hepatoplasma* (42/46) or *Hepatincola* (5/46) with one sample harbouring both, at an average ratio of 46/16%, respectively (Dittmer *et al.*, 2014). A study on feminising manipulator *Wolbachia* and pathogenic *Rickettsiella* were found in the hepatopancreas with *Hepatoplasma*, however, results were represented as averages for replicate tissue and it is unclear if they inhabited the same individual (Dittmer *et al.*, 2016). Our study found three samples contained both symbionts, but *Hepatincola* was found at less than 0.05%, suggesting *Hepatoplasma* has a stronger protective role. This study also found *Hepatoplasma* and pathogen *Rickettsiella* in two individual samples at a ratio of 94/5% and 79/20%, respectively.

Terrestrial isopods imbibe a diet rich in cellulose and to exist on this recalcitrant, low nutrient diet, cellulases would be needed to facilitate digestion. It is well documented that fungi play a vital role in lignocellulose degradation, due to their production of enzymes that accelerate this process (Aneja *et al.*, 2006; Schneider *et al.*, 2012; Purahong *et al.*, 2014), working in a three-way symbiosis together with bacteria and the host, however, the fungal role has never been investigated in the

hepatopancreas of isopods. Mixed communities of bacteria have shown greater decomposition rates in wood (Schmidt and Liese, 1994), so it is interesting that the diversity within the hepatopancreas is so low, this may explain the higher diversity of fungi. An early study indicated it was highly probable that cellulase originated from the hepatopancreatic tissue of the *O. asellus* (Hartenstein 1964), but later studies concluded that cellulase activity originates from the unculturable endosymbiotic bacteria in the hepatopancreas and isopods may have developed a symbiotic relationship with microbes to degrade this resource (Zimmer and Topp, 1998a). It has now been shown that the hepatopancreas does represent the major site of transcription of endogenous cellulases, whilst very few were found in the microbes (Bredon *et al.*, 2018). The published genome of *Hepatoplasma* from *A. vulgare* (Leclercq *et al.*, 2014) has enabled genes to be examined for their function, with five coding sequences assigned to enzyme classes characterised by the CAZy database (Lombard *et al.*, 2014). These include glyosidic hydrolase family GH13, and two carbohydrate esterases and two glycosyltranferases (Bouchon *et al.*, 2016). The hepatopancreas, hindgut and legs of *P. scaber* were examined for cellulase activity and an endogenous GH9 cellulase gene was found exclusively in the hepatopancreas (Kostanjšek *et al.*, 2010). Other isopods such as *Limnoria quadripunctata*, have also been found to possess endogenous cellulases aiding the digestion of wood (King *et al.*, 2010; Kern *et al.*, 2013; Bredon *et al.*, 2018). A recent paper by Bredon *et al*. (2018), investigated lignocellulose degradation using transcriptomics and shot gun metagenomics in *A. vulgare*. Pooling the hepatopancreas with the nerve cord, gonads and haemolymph identified a high diversity of lignocellulose-degrading enzymes, including cellulases, hemicellulases and lignocellulose-modifying enzymes, prevalent in both *A. vulgare* and its microbiome including the pooled tissues samples. All this evidence suggests a symbiotic relationship between the host and the microbiota in a two-stage process for cellulose digestion. Bredon *et al*. (2018) concluded that *A. vulgare* hydrolyses cellulose with endoglucanases from the hepatopancreas and the microbiota completes the process with other endoglucanases and β-glucosidases from the hindgut. In our study, lignocellulose degrading bacteria and fungi were found colonising the hepatopancreas and it is plausible these microbes are involved in a symbiotic relationship with the woodlice to enable them to eat the terrestrial diet, helping their colonisation to land from sea.

These endogenous genes discovered in *A. vulgare* (Bredon *et al.*, 2018) and *P. scaber* (Kostanjšek *et al.*, 2010), together with reports that *Hepatoplasma* not being an obligate symbiont, suggests it being less relevant or partly redundant in its role in cellulose degradation (Bouchon *et al.*, 2016). This redundancy could suggest other factors involved, it has been postulated that GH5 and GH45

family in Nematodes has been acquired from bacteria and fungi, respectively, via ancient HGT (Smant *et al.*, 1998).

Although two species of terrestrial woodlice were used, they were collected from the same area and if other terrestrial isopods were used or were sampled from a different site, the diversity may be different from the replicates used in this study.

These results give first insights into the bacterial and fungal microbiota colonising the hepatopancreas of two species of terrestrial woodlice in the same study. It was previously hypothesised a filter between the foregut and hepatopancreas prevented particles the size of fungi entering, therefore, this has not been previously investigated. This is the first to study the show the presence of a resident fungal community within the hepatopancreas of *O. asellus* and *P. scaber*. In this study, bacterial symbiont *Hepatoplasma* appears to be obligate, densely colonising the hepatopancreas and forming a symbiotic relationship with the host, this is plausible but other studies featuring *A. vulgare* have shown this symbiont not to be obligate. It is intriguing why other bacteria did not abundantly colonise the hepatopancreas, given the filter did not prevent colonisation of fungi.

5 Characterisation of the Resident Microbial Communities in the Hindgut of Woodlice

5.1 Introduction

The microbial communities within the hindgut of woodlice are poorly understood. This may be due to the reported transient nature of the food due to the structure of the short, straight, tube-like cuticular lined hindgut (Hames and Hopkin, 1989; Kostanjšek *et al.*, 2002). It is hypothesised the cuticular lining of the hindgut sheds at ecdysis (Lichtwardt, 2001, 2008; Strus and Blejec, 2001; Kostanjšek *et al.*, 2007). Prior to 2000, studies into this region used culture techniques and biochemical tests to investigate the presence and identity of bacteria (Table 1.2). Studies since 2000 have focused on either the effect of heavy metal pollution on the bacterial communities (Lapanje *et al.*, 2010) or examined the presence of one species of bacterium, such as, *Wolbachia* or *Candidatus Bacilloplasma* (Kostanjšek *et al.*, 2010; Dittmer *et al.*, 2016). Only one study focused on the bacterial diversity within the hindgut of one species of woodlouse; *P scaber*, via cloning, PCR and sequencing, but many sequences found did not meet the ≥97% similarity threshold (Kostanjšek *et al.*, 2002). The hindgut was not previously thought to be the site of endogenous lignocellulases, however, a recent study has shown the hindgut of *A. vulgare*, does express these enzymes (Bredon *et al.*, 2018). This suggests the hindgut and its resident microbiota may have a role in explaining how woodlice have evolved to eat a diet rich in lignocellulose.

Therefore, this chapter will investigate the bacterial and fungal communities in the hindgut of two species of woodlice, using non-culture methods, after the hindguts have been emptied to ensure the microbes present are resident and not transient. Moving on from research carried out by Kostanjšek (2002), using new advanced NGS technologies will allow for better microbial species identification. This is the first study to characterise the resident fungal communities within the hindgut of any woodlouse.

5.2 Aims

The major aims of this study include:

- Characterisation of the presence of resident bacterial communities in the hindgut of two species of woodlouse; *O. asellus* and *P. scaber*
- Investigation into the resident fungal communities in the hindgut of woodlice.

5.3 Characterising the Bacteria and Fungal Microbiota in the Hindgut of Two Species of Woodlice

DNA samples from the hindgut were subjected to PCR to ensure the presence of bacteria and fungi in all replicates. All DNA samples were sent to LGC Genomics for NGS sequencing as described in the material and methods (2.3.7 & 4.3). This enabled the analysis of bacterial and fungal sequences detected in the hindgut of *O. asellus* (n=5) and *P. scaber* (n=5).

A total of 5,296,040 reads were generated by sequencing the bacterial 16S rRNA gene and 5,505,732 reads from sequencing the eukaryotic ITS2 rDNA region in the hindgut of *O. asellus* and *P. scaber* (Table 5.1). After processing and subsampling the abundance of unique bacterial OTUs was higher than fungal OTUs (Table 5.1). The 16S rRNA gene data was subsampled at 50,000 reads (see 2.4.1.1) and 70% of samples generated 50,000 reads and the ITS2 rDNA data was subsampled to 20,000 reads and 90% of samples generated 20,000 reads. OTU instability was present in the initial analysis by LGC, therefore, subsequent analysis of OTU abundance took place on own data using different parameters (Table 5.1).

Table 5:1: NGS results from sequencing microbial communities in the hindgut of woodlice. Number of raw reads, total clusters, unique OTUs and own analysis of OTUs from sequencing the 16S rRNA gene and ITS2 rDNA region of *O. asellus* and *P. scaber* hindgut. Sequences were assigned to OTUs with ≥97% sequence identity.

Woodlice species	**No. of samples**	**Total raw reads**		**Total clusters (≥97%)**		**Unique OTUs (≥97%) LGC analysis**		**Unique OTUs (≥97%) Own analysis**	
		16S	**ITS2**	**16S**	**ITS2**	**16S**	**ITS2**	**16S**	**ITS2**
O. asellus	5	3,575,296	4,682,394	173,205	81,081	1,452	232	255	81
P. scaber	5	1,720,744	823,338	237,147	99,906	1,350	124	217	41
All		5,296,040	5,505,732	410,352	180,987	2,802	356	472	122

5.4 Analysis of 16S rRNA Gene Sequences Detected in the *O. asellus* and *P. scaber* Hindgut

Bacterial sequences corresponding to the V3-V4 region of the 16S rRNA gene were amplified via PCR from the hindgut of *O. asellus* and *P. scaber.* OTUs were binned into taxonomic groupings to allow for comparison of bacterial communities within the hindgut. After clustering sequences

assigned to the same consensus lineage, sequences were assigned to 255 OTUs (*O. asellus*) and 217 OTUs (*P. scaber*) with Simpson's diversity indices indicating higher diversity in *P. scaber* (Table 5.2).

Table 5:2: Comparison of V3-V4 16S rRNA gene OTU species richness within the hindgut of woodlice. Non-parametric estimates $Chao_1$ and Simpson's diversity indices were used to estimate species diversity in the hindgut of *O. asellus* and *P. scaber*. For species richness estimations, OTUs were binned to genera.

Woodlice species	Total Observed OTUs	$Chao_1$ estimations	Simpson's diversity index - D
***O. asellus* (n=5)**	255	388	0.74
***P. scaber* (n=5)**	217	272.7	0.89

5.4.1 Analysis of Alpha Diversity within the Hindgut of the *O. asellus* and *P. scaber*

These data were normalised to the sample containing the lowest number of sequences; 12,170, to better compare the microbial community within the other samples used in the study (Schloss *et al*., 2011). A t-test revealed there was no significant difference (P = 0.5) in the number of observed bacterial OTUs between the hindgut of both woodlouse species, however, *P. scaber* contained more diversity (Fig. 5.1A). To estimate the need for further sampling and to ensure all bacterial diversity was captured, $Chao_1$ estimations of diversity were applied (Fig. 5.1B). These estimations indicate more OTUs would be found with additional sampling, suggesting more unique and rare bacterial species were found, particularly in *O. asellus*. No significant difference (P = 0.092) in OTU distribution and diversity was detected using $Chao_1$ and predicted less diversity in *P. scaber* than *O. asellus* (Fig. 5.1B).

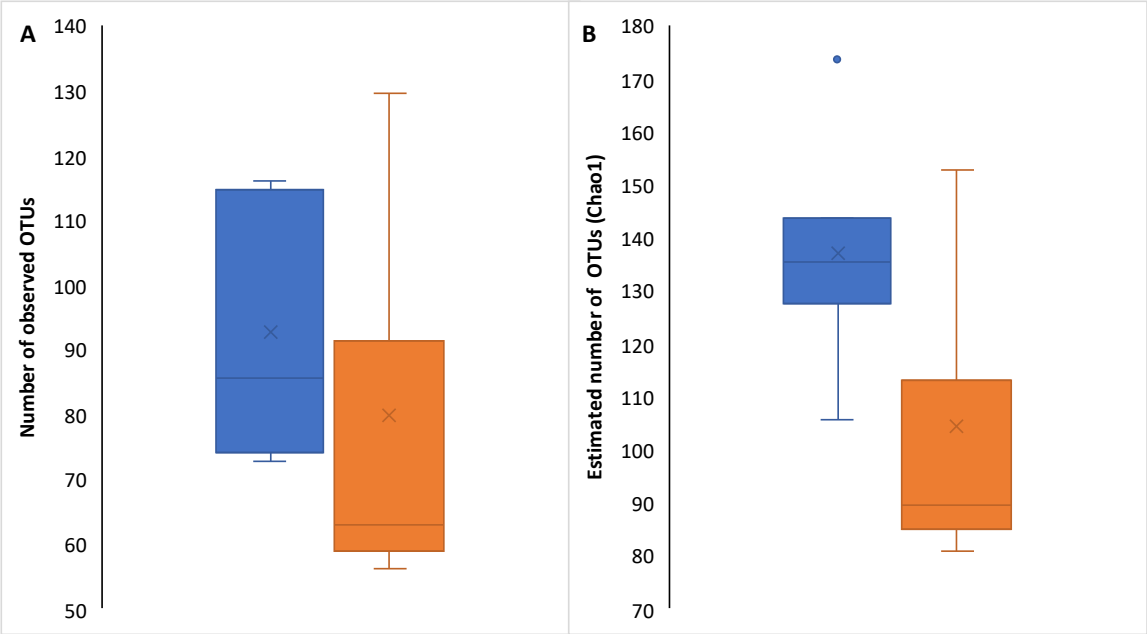

Figure 5:1: Alpha diversity boxplots showing bacterial OTUs within the hindgut of woodlice. The mean (x), median (-) and range of bacterial OTUs within the samples of *O. asellus* (blue) and *P. scaber* (orange) hindgut at 12,170 sequences per sample. (A) Observed species (B) $Chao_1$ estimations.

The alpha rarefaction analysis supported these findings, which demonstrated *O. asellus* had higher average detectable species richness than *P. scaber*, 92.6 OTUs and 80 OTUs respectively (Fig. 5.2). In both woodlouse species, the majority of bacterial diversity had been sampled as diversity of observed species was reaching asymptote. However, differences between S_{obs} and S_{chao1} (Table 5.2 and Fig. 5.2) suggests additional sampling would allow more novel OTUs to be detected in both woodlouse species, but more so in *O. asellus*.

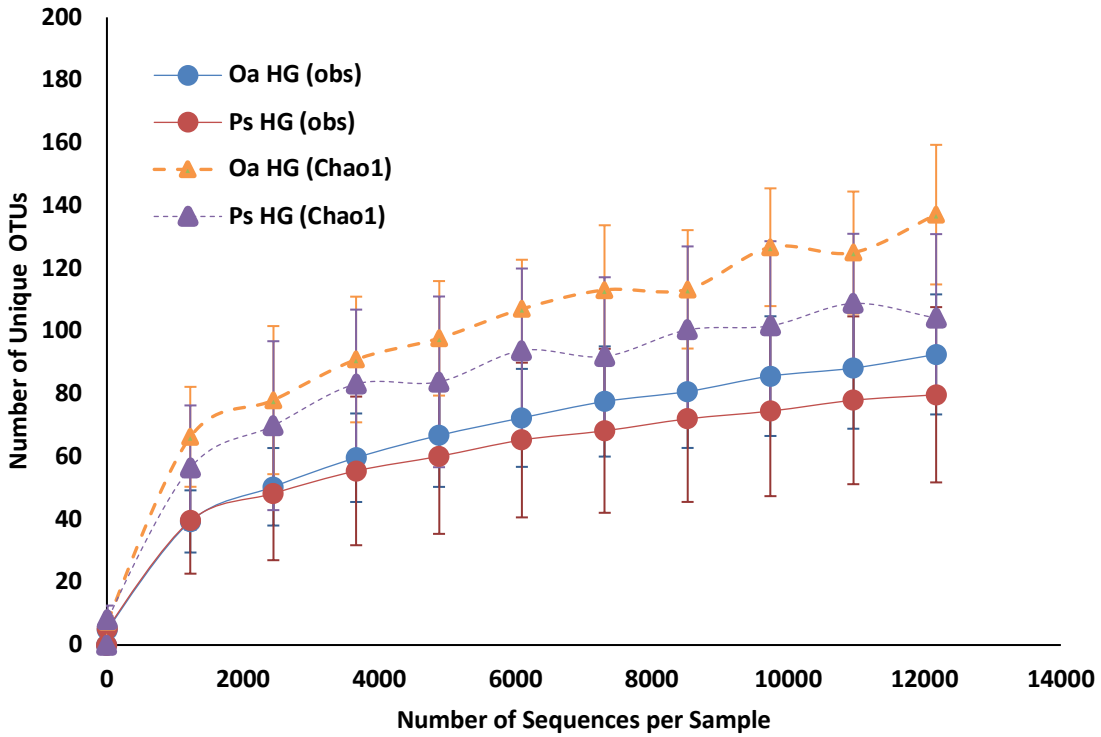

Figure 5:2: Rarefaction graphs with OTUs derived from sequencing the 16S rRNA gene, binned to genera. The data was normalised on the sample containing the lowest number of sequences, 12,170 sequences were subsampled from the hindgut of *O. asellus* (Oa) and *P. scaber* (Ps) and reported for observed species (Obs) and $Chao_1$ estimations.

5.4.2 Analysis of Beta Diversity

Beta diversity enabled a better understanding of the bacterial diversity present. Taxonomic analysis showed two distinct clusters corresponding to bacterial OTUs, each cluster representing communities found in the two species of woodlice (Fig. 5.3). The bacterial community of one sample of *P. scaber* suggest communities were different from the other four samples, which are clustered in two pairs.

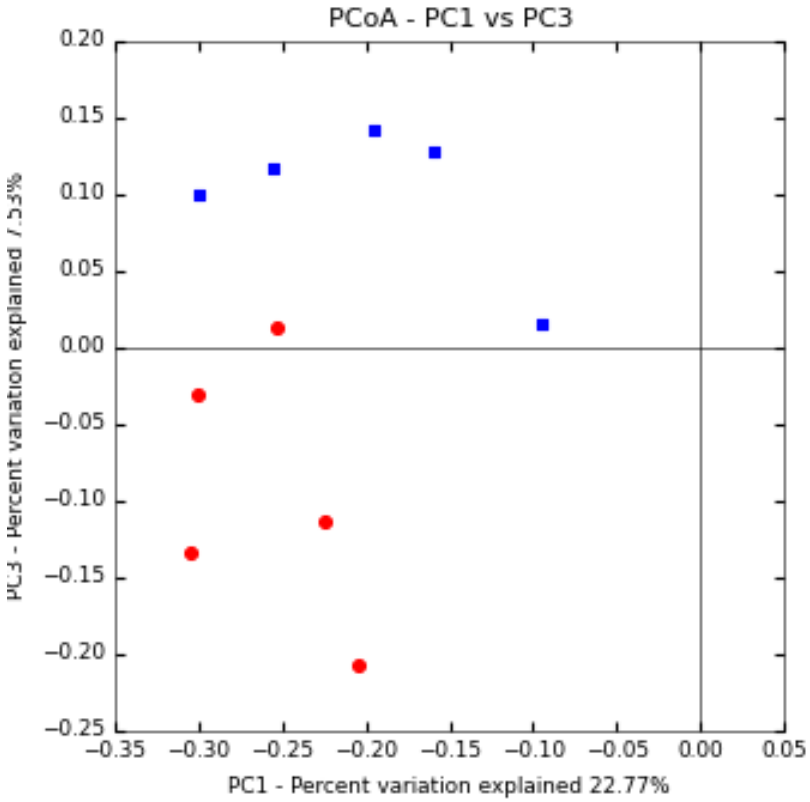

Figure 5:3: Similarities of bacterial communities in the hindgut samples of woodlice; *O. asellus* (red circle) (n=5) and *P. scaber* (blue square) (n=5). PCoAs shown were generated from unweighted UniFrac distances. Axes represent the two synthetic variables explaining the greatest proportion of variation in the data set. Figure supplied by LGC Genomics.

5.4.3 Bacterial Community Diversity Detected by NGS in the Hindgut

The majority of sequences detected were assigned to twenty phyla, however, only sequences detected in greater than 1% abundance were examined. From this analysis the hindgut appears to contain a diverse bacterial community with the majority of OTUs assigned to sequences similar to phyla; Proteobacteria, Actinobacteria, Tenericutes and Bacteroidetes (Fig. 5.4). Both woodlouse species were dominated by bacterial OTUs most closely related to class; Gammaproteobacteria with families; Enterobacteriaceae, Pseudomonadaceae and Vibrionaceae found in similar diversity, whilst family; Coxiellaceae was more dominant in *P. scaber*. The less abundant OTU diversity was similar between woodlice, having similar classes of bacteria, but detecting some different orders and families.

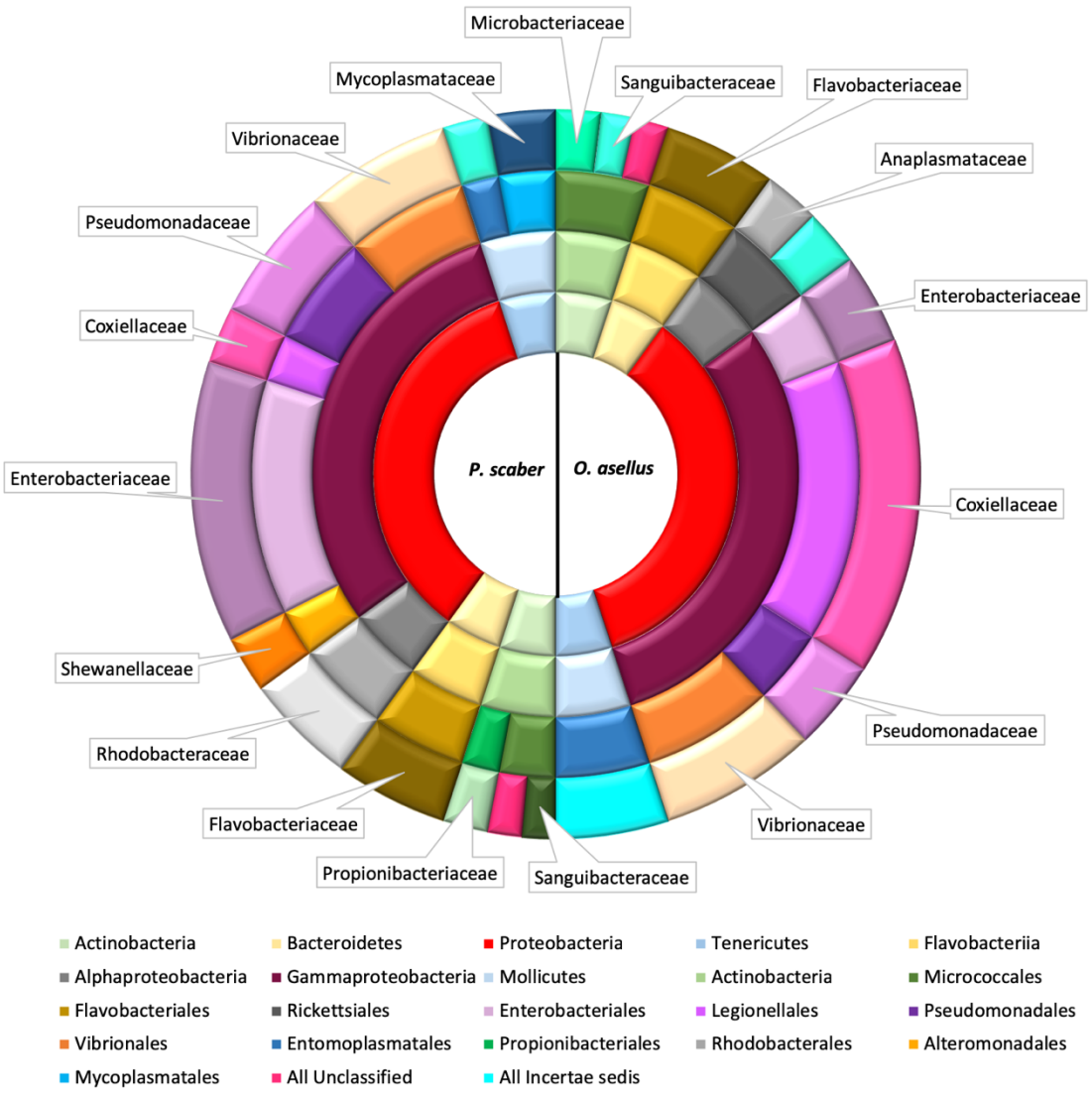

Figure 5:4: The relative abundance of bacterial taxa from *O. asellus* and *P. scaber* hindgut detected by amplifying the 16S rRNA gene. Sequences detected greater than 1% abundance are shown.

The bacterial communities in the hindgut of *O. asellus* and *P. scaber* was varied between woodlouse species (Fig. 5.5A), and also varied between individual samples (Fig 5.5B). Taxa were grouped and reported at class level with lower taxonomic classifications subsequently reported, if sequences were detected at greater than 1% abundance. Any sequences detected in less than 1% abundance and unclassified bacterial sequences were grouped into unclassified/other (Fig. 5.5). In order of abundance, sequences similar to *Rickettsiella*, *Vibrio*, *Hepatoplasma*, *Pseudomonas*, *Kluyvera* and *Microbacterium* were most abundant in *O. asellus*. In contrast sequences similar to *Kluyvera*, *Vibrio*, *Pseudomonas*, Enterobacteriaceae, *Bacilloplasma*, *Sanguibacter* and *Hepatoplasma* were most abundantly detected in *P. scaber* (Fig. 5.5A). Sequences similar to *Rickettsiella* dominated *O. asellus* in three samples (Fig 5.5B) (47% average), but was only found in 1% abundance in *P. scaber*. In

contrast, the most dominant sequences in *P. scaber* were sequences similar to *Kluyvera*, *Vibrio* and *Pseudomonas*. Sequences similar to Vibrio was found in similar abundance in *O. asellus* (14%) and *P. scaber* (17%).

The core microbiome in both woodlice is an OTU appearing in 80% of samples. Fifty-two sequences representing OTUs were found in at least 80% of samples with the majority being found in less than 1% abundance. OTUs that were detected in greater than 1% abundance were found in all samples are listed in Table 5.3, with *Rickettsiella*, *Vibrio*, *Kluyvera, Pseudomonas* and *Hepatoplasma* being the most abundant.

Individual replicates had unique microbial communities (Fig. 5.5B & 5.6), the OTUs most predominantly found in all samples were sequences similar to phyla Proteobacteria, OTUs with sequences similar to Actinobacteria detected in less abundance.

Table 5:3: The core bacterial microbiome in the hindgut of *O. asellus* and *P. scaber* in 100% of samples from Illumina sequencing the 16S rRNA gene. The average percentage of OTU reads detected between the two species of woodlice.

Taxonomy	Average % of OTU reads
Gammaproteobacteria; *Rickettsiella*	17.4
Gammaproteobacteria; *Vibrio*	14.8
Gammaproteobacteria; *Kluyvera*	12.2
Gammaproteobacteria; *Pseudomonas*	9.9
Mollicutes; *Candidatus Hepatoplasma*	8.7
Gammaproteobacteria; Enterobacteriaceae	4.9
Actinobacteria; *Sanguibacter*	4.3
Mollicutes; *Candidatus Bacilloplasma*	3.6
Actinobacteria; Micrococcales	3.4
Actinobacteria; *Microbacterium*	2
Flavobacteriia; *Chryseobacterium*	1.5
Flavobacteriia; *Flavobacterium*	1.4
Alphaproteobacteria; Rhodobacteraceae	1.2
Gammaproteobacteria; *Shewanella*	1

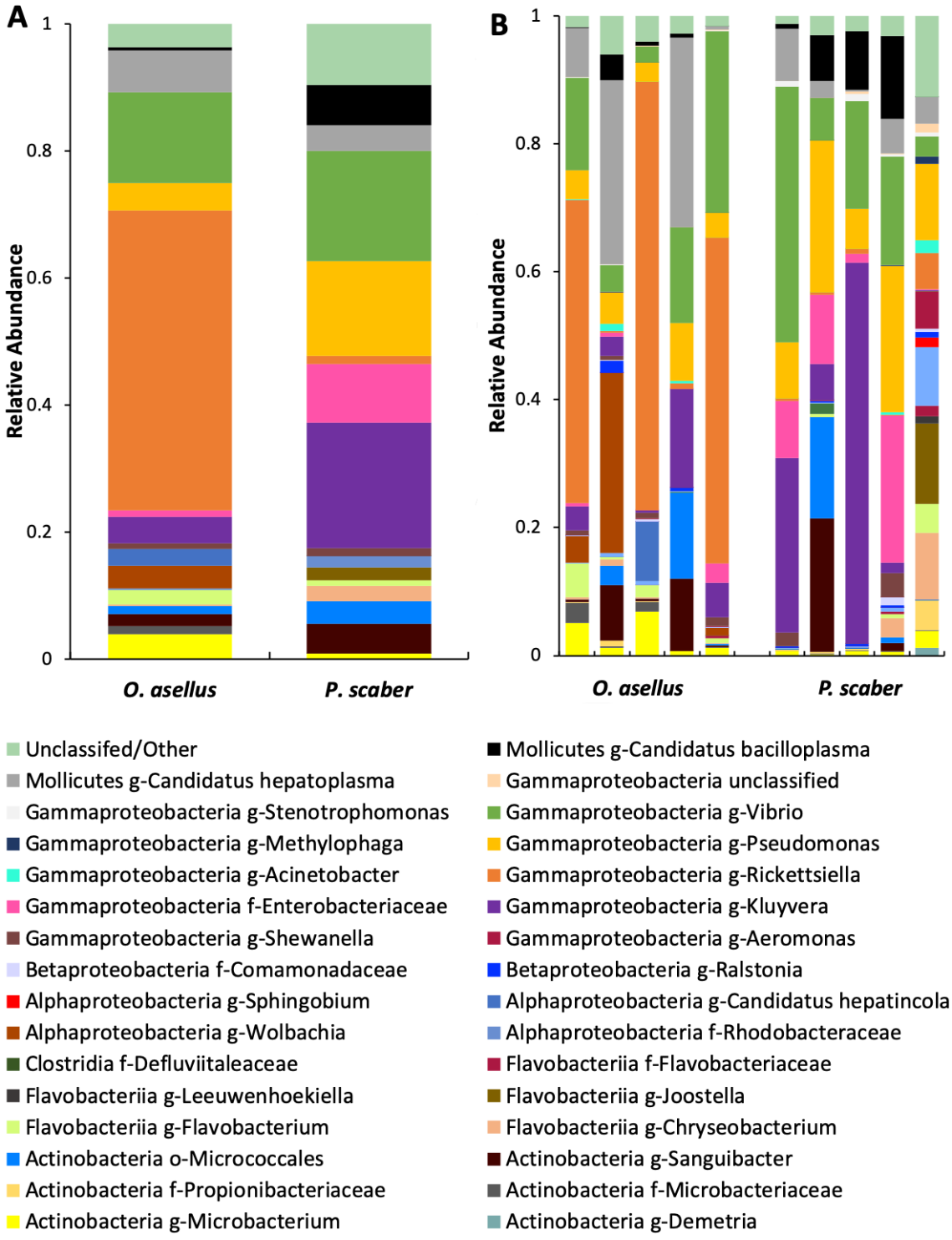

Figure 5:5: Taxonomic composition of the bacterial microbiome in the woodlouse hindgut. Results are reported at class and lower level taxonomy as indicated by o-order, f-family, g-genus. (A) Average relative abundance and distribution of the most abundant bacterial sequences (>1%) found in at least one woodlouse. (B) Individual woodlouse relative abundance and distribution. Results are from 16S rRNA gene Illumina sequencing from the hindgut of *O. asellus* (n=5) and *P. scaber* (n=5). Sequences were assigned to OTUs with ≥97% sequence similarity.

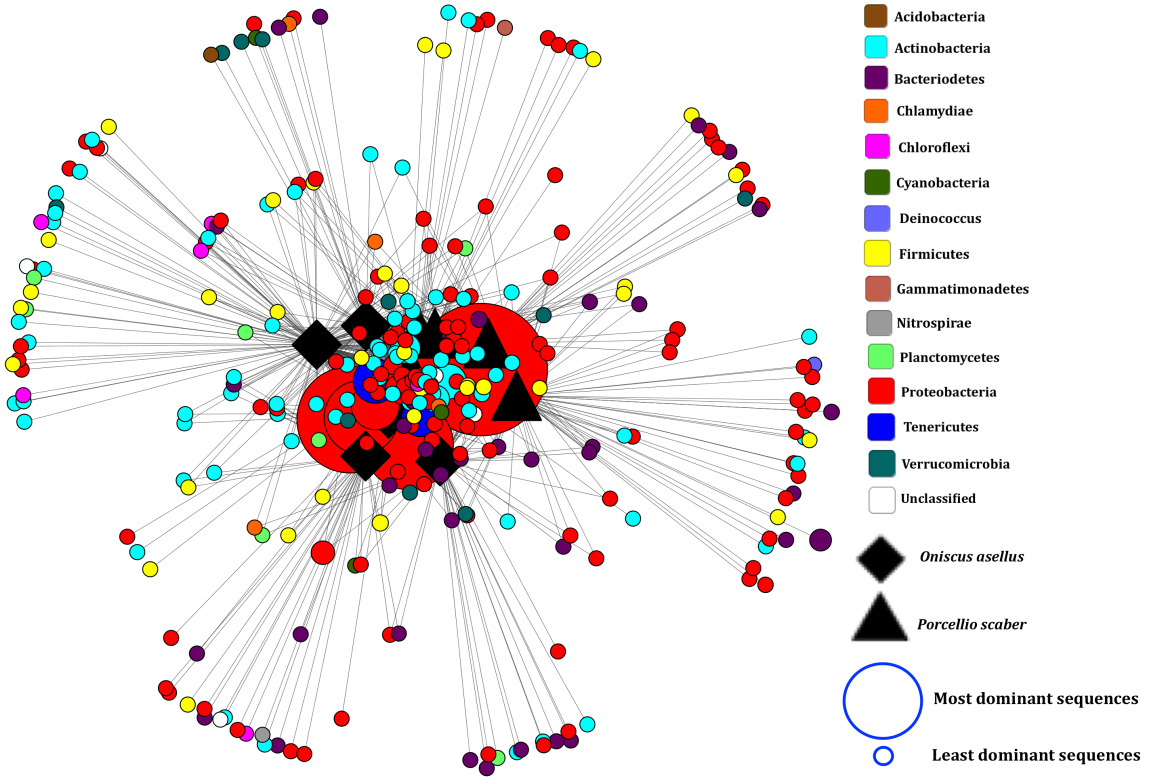

Figure 5:6: OTU network showing distribution of all OTUs identified to phylum detected via sequencing the 16S rRNA gene from the bacterial community present in the hindgut of *O. asellus* and *P. scaber.* Node size indicates the dominance of reads assigned to an OTU and node colour indicates consensus taxonomy.

5.5 Analysis of Fungal ITS rDNA Sequences Detected in the Hindgut of *O. asellus* and *P. scaber*

Fungal sequences corresponding to the ITS2 rDNA region were PCR amplified from the hindgut of *O. asellus* and *P. scaber*. Fungal communities of the hindgut tissue were compared after OTUs were binned to taxonomic groupings. After analysis sequences were assigned to 81 OTUs (*O. asellus*) and 41 OTUs (*P. scaber*) (Table 5.4).

Table 5:4: Comparison of ITS2 rDNA OTU species richness within the hindgut of woodlice. Non-parametric estimate $Chao_1$ and Simpson's diversity indices, were used to estimate species diversity in the hindgut of *O. asellus* and *P. scaber*. For species richness estimations, OTUs were binned to genera.

Woodlice species	Total observed OTUs	$Chao_1$ estimations	Simpson's diversity index - D
***O. asellus* (n=5)**	81	81	0.87
***P. scaber* (n=5)**	41	54	0.82

5.5.1 Analysis of Alpha Diversity within the Hindgut

To compare diversity of fungal communities in the hindgut, the data was normalised to the sample containing the lowest number of sequences; 1,130 (Schloss et al. 2011). A t-test revealed there was no significant difference (*P* = 0.42) in the number of fungal OTUs observed between the hindgut of both woodlouse species, however, sequencing detected more fungal OTUs in *O. asellus* hindgut compared to *P. scaber* (Fig. 5.7A). $Chao_1$ estimations indicate the number of OTUs would not have increased with additional sampling with no significant difference in number of OTUs between the woodlouse species (*P* = 0.36) (Fig. 5.7B).

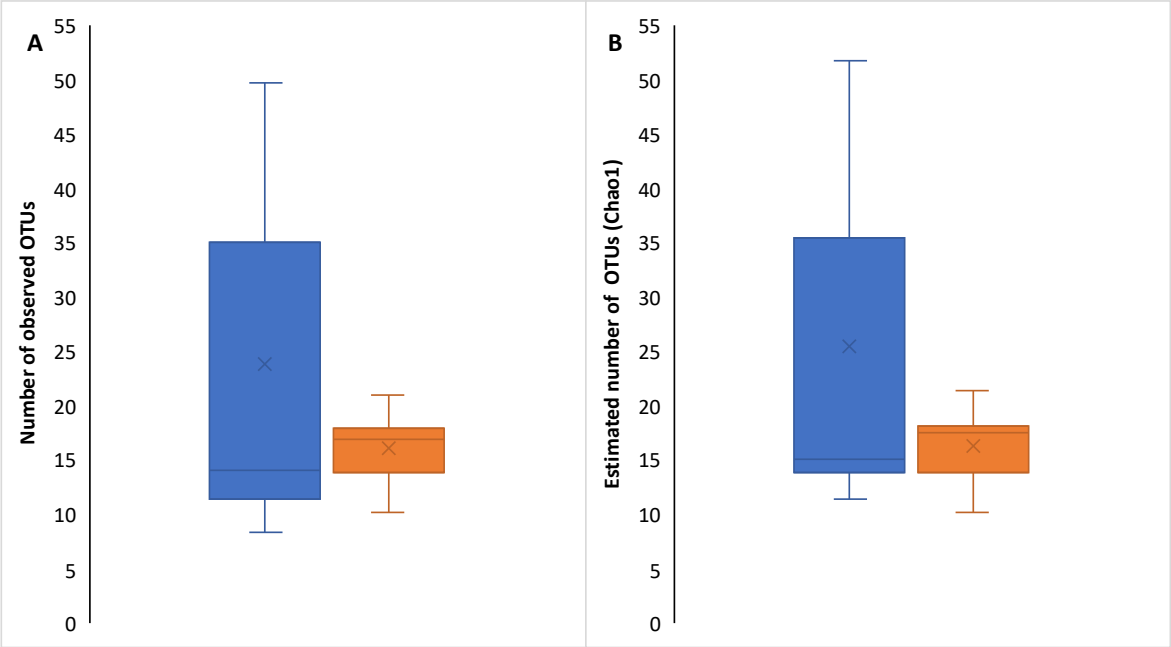

Figure 5:7: Alpha diversity boxplots showing diversity of fungal OTUs within the hindgut of woodlice. The mean (x), median (-) and range of fungal OTUs within the samples of *O. asellus* (blue) and *P. scaber* (orange) hindgut at 1,130 sequences per sample. (A) Observed species (B) $Chao_1$ estimations.

These findings were supported by rarefaction analysis (Fig. 5.8) of the hindgut which demonstrated *O. asellus* had higher average detectable species richness than *P. scaber*, 23.7 OTUs and 15.9 OTUs respectively. In both woodlouse species, the majority of diversity had been sampled as observed species had reached asymptote at 900 sequences. The differences between S_{obs} and S_{chao1} (Fig. 5.8) from both species of woodlice suggests additional sampling would not allow more novel OTUs to be detected.

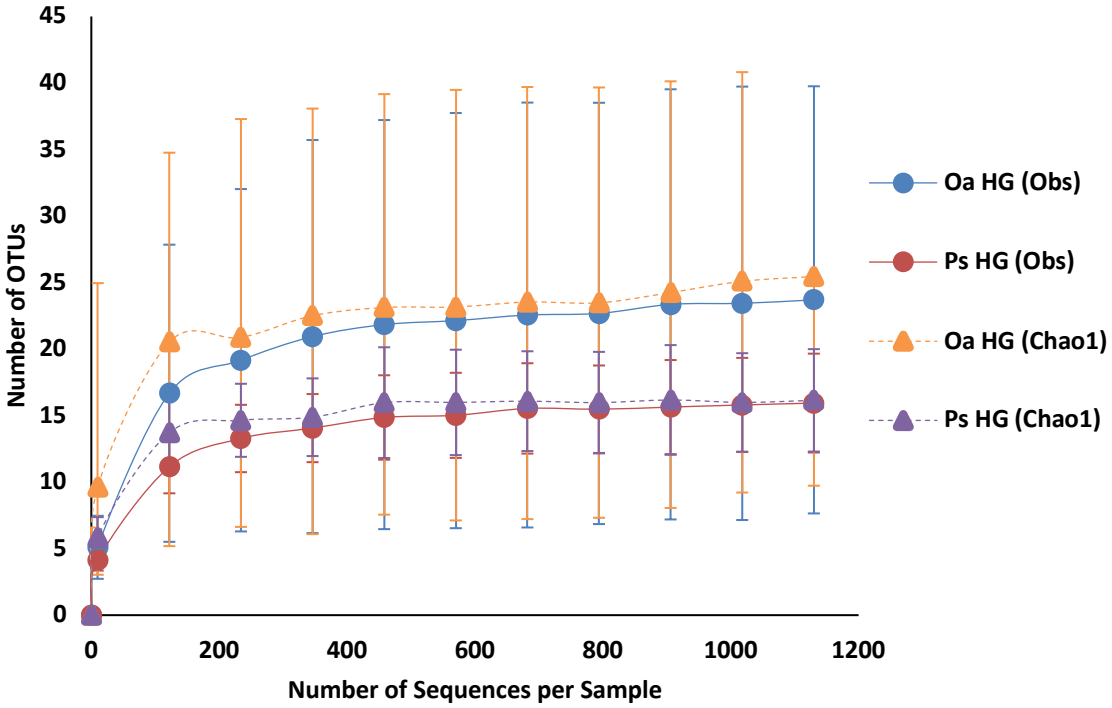

Figure 5:8: Rarefaction graphs with OTUs derived from sequencing the ITS2 rDNA region, binned to genera. The data was normalised on the sample containing the lowest number of sequences, 1,130 sequences were subsampled from the hindgut of *O. asellus* (Oa) and *P. scaber* (Ps) and reported for observed species (Obs) and $Chao_1$ estimations.

5.5.2 Analysis of Beta Diversity within the Hindgut

To enable a better understanding of fungal diversity present in the hindgut, taxonomic profiling was applied. This showed two clusters corresponding to three individual samples, one corresponding to *O. asellus* and the other *P. scaber*, however, both woodlouse species had two samples that indicated different communities when compared to the other three samples (Fig. 5.9).

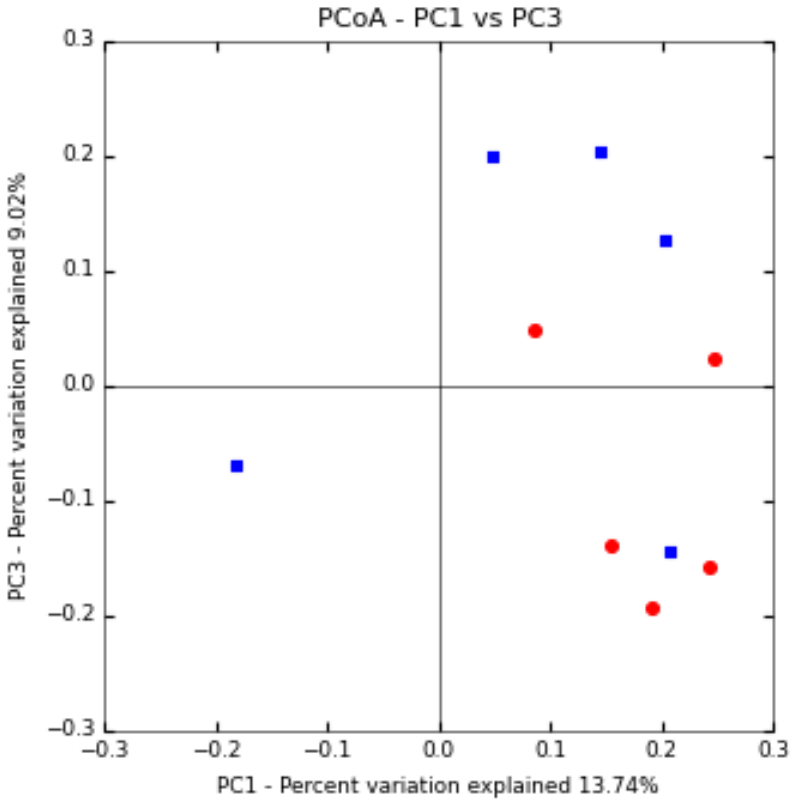

Figure 5:9: Similarities of fungal communities in the hindgut of woodlice; *O. asellus* (red circles) (n=5) and *P. scaber* (blue squares) (n=5). PCoAs shown were generated from unweighted UniFrac distances. Axes represent the two synthetic variables explaining the greatest proportion of variation in the data set. Figure supplied by LGC Genomics.

5.5.3 NGS Analysis of the Fungal Community Diversity in the Hindgut of *O. asellus* and *P. scaber*

The majority of sequences were assigned to sequences similar to three phyla, with the majority of OTU reads detected in greater than 1% abundance were assigned to Ascomycota (88.9%), followed by Basidiomycota (5.4%). The remaining phyla were assigned to Zygomycota in *P. scaber* and Rozellomycota in *O. asellus* (Fig. 5.10). The difference in diversity between the two species of woodlouse is clear, at family level, the only sequences that were present in both species of woodlice were sequences similar to Davidiellaceae, Trichocomaceae and Nectriaceae (Fig. 5.10).

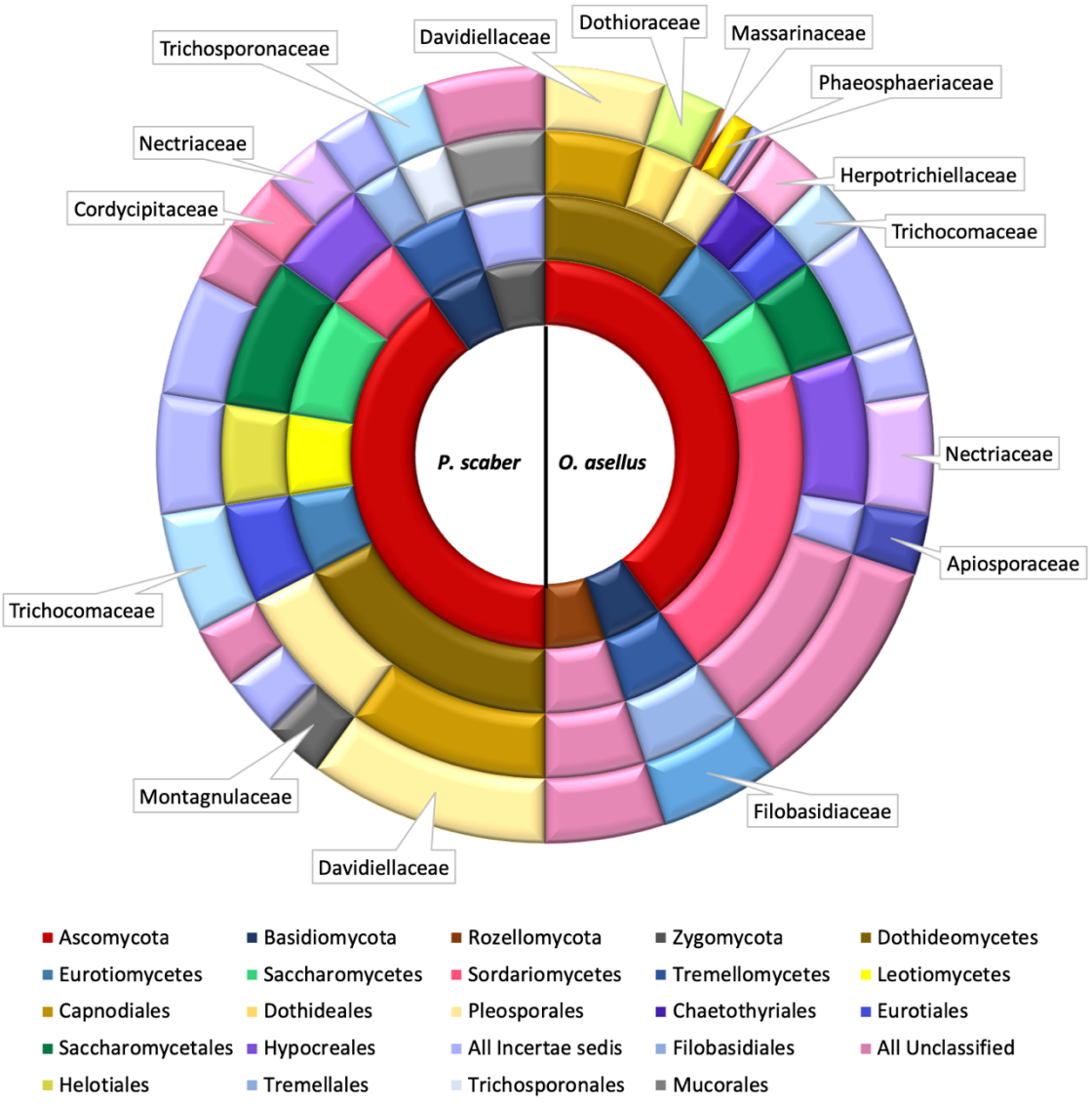

Figure 5:10: Relative abundance of fungal taxa from *O. asellus* and *P. scaber* hindgut from amplifying the ITS2 rDNA region. Sequences greater than 1% abundance are shown.

The composition of fungal communities greater than 1% abundant in the hindgut of *O. asellus* and *P. scaber* were different (Fig. 5.11). Taxa were grouped and reported at class with lower classifications subsequently reported if greater than 1% abundant, any sequences found lower than 1%, unclassified fungi, Protista and Plantae sequences were grouped in unclassified/other. The only sequences present in all hindgut samples were sequences similar to Davidiellaceae. Only one sample of *O. asellus* was very different and dominated by an unclassified Sordariomycetes but Davidiellaceae was still detected at 0.04%. In addition to Davidiellaceae, *O. asellus* had dominant sequences similar to unclassified Sordariomycetes and Nectriaceae. *P. scaber* had a different community sequence profile and was also dominated by sequences similar to unclassified Montagnulaceae, Pleosporales and genera; *Candida* (Fig. 5.11A).

The community diversity varied between individual samples (Fig. 5.11B and Fig. 5.12). Sequences similar to Dothideomycetes and Sordariomycetes were found greater than 1% abundance in 90% of samples and Tremellomycetes and Saccharomycetes were greater than 1% in 70% of samples (Fig. 5.11B). One sample of *O. asellus* differed from the other samples and the majority of sequences found were similar to unclassified Sordariomycetes. In respect to a core microbiota, sequences similar Davidiellaceae were detected in 100% of samples and greater than 1% abundant in 90% of samples. Sequences similar to *Penicillium* were present in 80% of samples but only 30% of samples had greater than 1% abundance. Some OTUs were found in two or more samples with many OTUs being unique to one sample (Fig. 5.12).

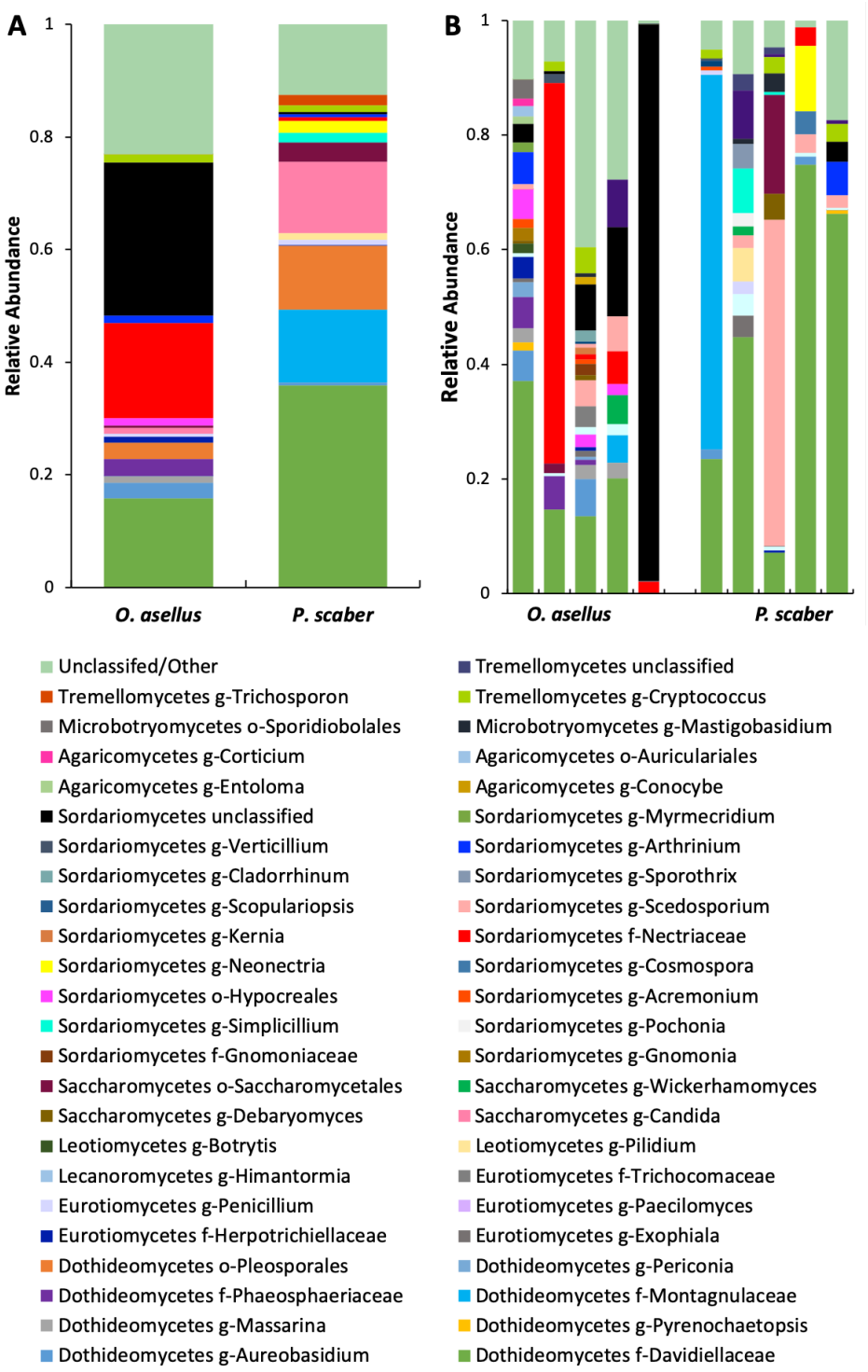

Figure 5:11: Taxonomic composition of the mycobiome in the woodlouse hindgut. Taxa was grouped and reported at class and lower level taxonomy as indicated by o-order, f-family, g-genus. (A) Average relative abundance and distribution of the most abundant fungal sequences (>1%) found in at least one woodlouse. (B) Relative abundance of individual woodlice. Results are from ITS2 rDNA Illumina sequencing from the hindgut of *O. asellus* (n=5) and *P. scaber* (n=5). Sequences were assigned to OTUs with ≥97% sequence similarity.

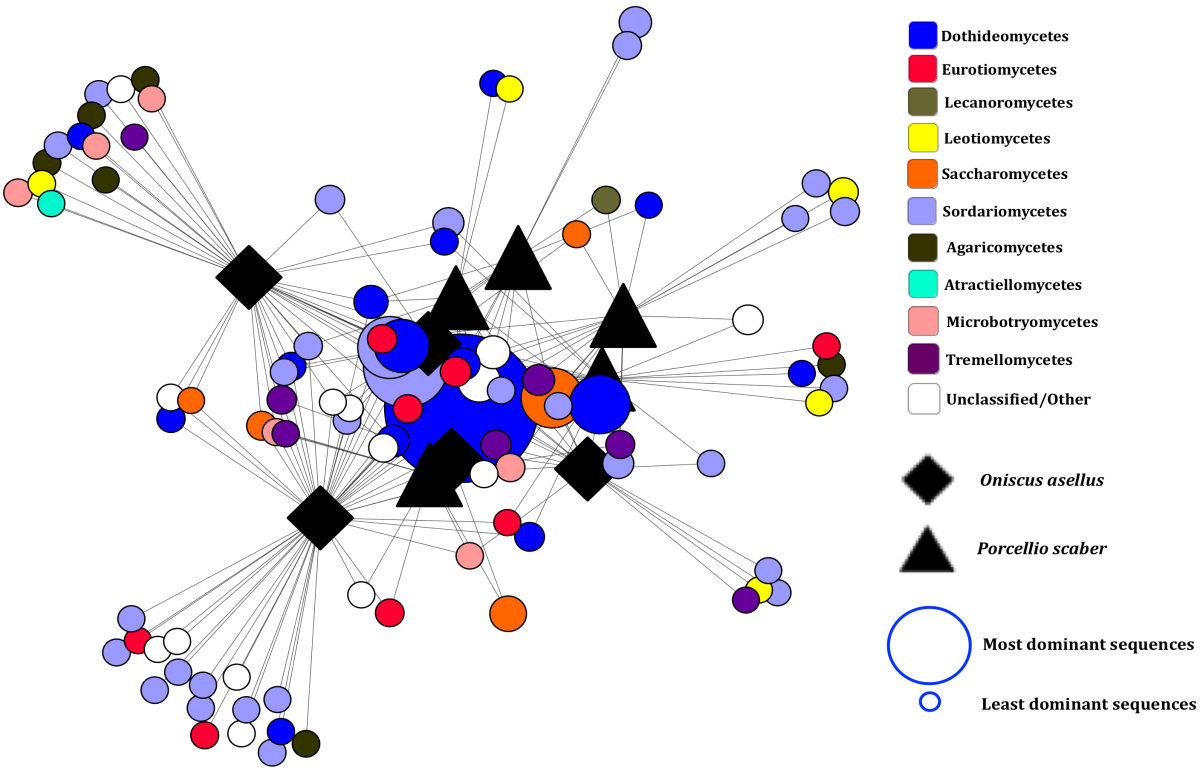

Figure 5:12: OTU network showing distribution of all OTUs identified to class detected via sequencing the ITS2 rDNA region from the hindgut of *O. asellus* and *P. scaber.* Node size indicates the dominance of reads assigned to an OTU and node colour indicates consensus taxonomy.

5.6 Discussion

This study used the V3-V4 region of the 16S rRNA amplification and subsequent NGS gene sequencing to study the bacterial diversity and the ITS2 rDNA region to study the fungal diversity in the hindgut of *O. asellus* and *P. scaber*. This is the first study to put two species of woodlice under the same conditions allowing for a more reliable comparison of hindgut microbiota. This is also the first study to use NGS to examine the bacterial microbiota found in the hindgut. Additionally, this is the first study to report the mycobiota within the hindgut in any species of woodlouse.

Analysis of the hindgut showed a similar number of sequences obtained from the 16S rRNA gene sequences and ITS rDNA sequences. It was hypothesised there would be more bacterial diversity than fungal, although the number of sequences were similar, fungal diversity was lower. This may be due to the number of sequences that could not be identified to lower taxonomic levels. In comparing the two species of woodlouse the microbial community of *O. asellus* and *P. scaber* hindgut found no significant difference in bacterial or fungal OTU abundance (see 5.4.1 & 5.5.1).

Samples of hindgut were subsampled to 50,000 bacterial sequences and 20,000 fungal sequences. To enable comparison of microbial communities in each individual hindgut, the data was normalised to the sample containing the least number of sequences; 12,170 bacterial sequences and 1,130 fungal sequences. Bacterial sequences obtained from seven hindgut samples had greater than 48,000 sequences and fungal sequences obtained from nine hindgut samples were greater than 19,000 sequences. This could have distorted OTU abundance, however, the analysis shows further sampling would not prove to be beneficial in finding more fungal OTUs. A disadvantage to normalising to the lowest number of sequences found in a sample is the sequences are randomly subsampled and some OTU abundance may be omitted in the process.

The hindgut of terrestrial isopods is known to be colonised by a diverse bacterial microbiota, with the majority represented by ubiquitous microorganisms (Kostanjšek *et al.*, 2002, 2006; Zimmer, 2002; Bouchon *et al.*, 2016). There is debate whether the microbiota in the hindgut are transient, gained from the wider environment, such as ingested with food, or if they are resident. The structure of the hindgut is a short, straight tube and terrestrial isopods have a short retention time of food, with several studies reporting the hindgut cuticle regularly sheds at ecdysis, therefore, the environment may not be not suitable for a resident microbial community (Lichtwardt, 2001, 2008; Strus and Blejec, 2001). Hindgut shedding is common amongst crustaceans with the newly molted hindgut surface of desert millipedes (*Orthoporus ornatus*) shown to be virtually devoid of microbes, thought to be replaced from the anterior gut regions, ingested exuviae and ingested soil (Crawford *et al.*, 1983). A study into the microbial communities within the hindgut of *P. scaber* has cast doubt on this, with attachment of a bacterium, *Candidatus Bacilloplasma*, to the surface of the host cell of the hindgut (Kostanjšek *et al*. 2007). This questions how the infection is maintained or re-established after disturbance, as *Bacilloplasma* has not, as yet, been found in soil, associated with leaf litter or in isopod faeces (Bouchon *et al.*, 2016). This is not unsurprising as *Bacilloplasma* has no cell wall and would not likely survive outside its host. A study on shrimp hypothesised the bacteria attached to the posterior midgut could function as a bacterial inoculum (Soonthornchai *et al.*, 2015). Reinfection could be achieved by horizontal transmission infecting individuals via cannibalism and re-ingestion of the shed cuticle, which is reported to be common in terrestrial isopods and presumed to restore minerals lost during the molt (Kostanjšek *et al*. 2007). However, in this current study, many individual woodlice were isolated and ecdysis was observed and the exuvia not re-ingested. A study on cannibalism and predation, in relation to infection route of *Wolbachia*, reported that starved *A. vulgare* and *P. dilatatus* resorted to predation and cannibalism (Le Clec'h *et al.*, 2013). Therefore, re-ingesting the cuticle may only occur when there is no other

food source available. After examining the literature, it was concluded more studies need to be carried out to substantiate whether the hindgut cuticle does shed in terrestrial isopods.

Previous studies (Table 1.2) have examined the bacterial populations in the hindgut of *O. asellus*, *P. scaber* and other terrestrial isopods, however, comparing two woodlouse species using new sequencing technologies, will build upon this previous research. Similar to previous research, our current study also found sequences similar to *Achromobacter, Arthrobacter, Bacillus, Enterococcus, Hepatincola, Halomonas, Listeria*, Micrococcaceae, *Ochrobactrum*, Pseudomonadaceae and *Shewanella* but all were found in less than 0.1% abundance. More abundant sequences found similar to previous studies, were sequences similar to *Rickettsiella* (25%), *Pseudomonas* (9.5%), *Hepatoplasma* (5%), *Bacilloplasma* (3.3%), and *Wolbachia* (1.5%). Abundant bacteria in the hindgut found in this study, were sequences similar to *Rickettsiella*, *Kluyvera*, *Vibrio*, *Pseudomonas*, *Hepatoplasma*, *Bacilloplasma* and *Sanguibacter*. Only one study has attempted to look at the diversity of the bacterial community within the hindgut of *P. scaber* as a whole, via cloning, PCR and sequencing with most resulting sequences not meeting the ≥97% identity threshold (Kostanjšek *et al.*, 2002).

To survive on a diet rich in lignocellulose, a consortium of enzymes, from the organism itself, the microbiota or both, is required to degrade such a recalcitrant material. A recent study by Bredon *et al.* (2018) examined lignocellulose degradation within the holobiont of *A. vulgare* obtained from both laboratory lineages and a natural population. Bredon *et al*. (2018) found microbial communities in the hindgut were different between laboratory reared and natural conditions using NGS. Results showed sequences similar to Enterobacteriaceae and Vibrionaceae from the laboratory lineages, and Coxiellaceae, Microbacteriaceae and Anaplasmataceae from natural conditions and had the highest contribution of lignocellulose-degrading genes. At genus level, *Vibrio*, *Kluyvera* and *Enterobacter* contributed to the most cellulases and hemicellulases and *Vibrio*, *Buttiauxella* and *Halomonas* contributed most lignin modifying enzymes from laboratory linages. The woodlice from the natural environment had sequences similar to *Rickettsiella*, *Wolbachia* and *Microbacterium* contributing the most cellulases, *Rickettsiella*, *Microbacterium* and *Cellulosimicrobium* were most frequently associated with hemicellulases, and *Streptomyces*, *Microbacterium, Arthrobacter* and *Leucobacter* were associated with genes encoding lignin modifying enzymes (Bredon *et al.*, 2018). Our current study found all these families at greater than 1% abundance in the hindgut both *O. asellus* and *P. scaber*. At genus level, similar results were found with the exception of *Cellulosimicrobium*, indicating many of the dominant genera present in this study are involved in lignocellulose degradation. In our study, in order of dominance,

Rickettsiella, *Vibrio*, *Enterobacte*r, *Kluyvera*, *Wolbachia* and *Microbacterium* were found at greater than 1% abundance within *O. asellus* and/or *P. scaber*. *Halomonas*, *Buttiauxella*, *Streptomyces*, *Arthrobacter* and *Leucobacte*r were detected, but with less than 1% abundance. Our study also found other sequences that have been reported to have lignocellulase activity, sequences similar to *Pseudomonas*, *Hepatoplasma* and *Sanguibacter* were also detected (McMahon *et al.*, 2007; Behera *et al.*, 2014; Leclercq *et al.*, 2014; Prabhakaran *et al.*, 2015; Bouchon *et al.*, 2016; de Lima Brossi *et al.*, 2016). It has been suggested the abundant consortia of microbes found in the population contains enzymes working symbiotically to allow lignocellulosic degradation (Jiménez *et al.*, 2016).

Bredon *et al*. (2018) carried out the first study to demonstrate endogenous enzymes in the hindgut of isopods with all regions of the *A. vulgare* gut expressing enzymes to assist with lignocellulose degradation. A high diversity of cellulases, hemicellulases, and lignocellulose-modifying enzymes were expressed in *A. vulgare* hindgut tissue. These findings casts doubt on an earlier study which compared cellulase activity in the different regions of the *P. scaber* gut and concluded that cellulases were produced by bacteria in the hepatopancreas and drawn into the hindgut with digestive fluids, but could not assign this activity to the microbes, the isopod or both (Hassall and Jennings, 1975; Zimmer and Topp, 1998a). These endogenous enzymes, along with those expressed from the hepatopancreas and the gut microbiota, suggest symbiosis between the host and its microbiota, allowing terrestrial isopods to eat the terrestrial diet, helping their colonisation from sea to land.

As discussed above *Wolbachia* and *Rickettsiella* contributed the most cellulases and hemicellulases in isopods from the natural conditions (Bredon *et al*., 2018). However, they may have another role in the isopod gut, *Rickettsiella* can also be an arthropod pathogen and has been discussed in Chapter 4.6. *Wolbachia* has previously been found to be prevalent in terrestrial isopods, expressing cellulases and hemicellulases (Bouchon *et al.*, 2011; Le Clec'h *et al.*, 2013; Bredon *et al.*, 2018). It is diverse in its interactions with the host, illustrated by a variety of strategies exhibited in order to secure vertical transmission, which is its main mode of transmission (Braquart-Varnier *et al.*, 2008). This would suggest some *Wolbachia* have evolved mutualistic relationships with their hosts, with vertical transmission offering favourable mutualism (Werren *et al*., 2008). *Wolbachia* was found in our study, although not prevalent, it can be hypothesised that both *Rickettsiella* and *Wolbachia* are contributing the breakdown of cellulose and hemicellulose allowing woodlice to each a diet rich in cellulose.

An interesting species *Candidatus Bacilloplasma* was found in our study. It is thought to exhibit specific adaptations to the host gut environment and indicates a long-term association and coevolution to the host (Kostanjšek *et al*., 2007). *Bacilloplasma* was discovered using microscopy and as it was attached to the hindgut wall, this indicates there may be a resident bacterial community as it has not yet been found in soil, plant litter or isopod faeces (Kostanjšek *et al.*, 2007; Zhang *et al.*, 2014). Few studies have focused on the relationship between *Bacilloplasma* and diseases in the host, however it is thought to be commensal in isopods, as hosts do not display any disease symptoms (Kostanjšek *et al*., 2007). It could be inferred that *Bacilloplasma* has part of the adaptation that enabled terrestrial isopods transition to land as all studies, to date, have found sequences similar to *Bacilloplasma* as a gut symbiont from animals from the aquatic environment (Durand *et al.*, 2010; Dhanasiri *et al.*, 2011; Zhang *et al.*, 2014; Chen *et al.*, 2015; Li *et al.*, 2016; Zhang *et al.*, 2016; Hou *et al.*, 2018; Wang *et al.*, 2018; Gao *et al.*, 2019).

The hindgut microbiota of some other wood-eating arthropods differs from those found in this study. The hindgut of *P. scaber* and *O. asellus* in this study was different from termites and cockroaches and was colonised by Proteobacteria, Actinobacteria, Bacteroidetes and Tenericutes. Lower termites have been found to be dominated by Spirochaetes, Actinobacteria and Firmicutes and higher termites dominated by Spirochaetes, Fibrobacteres, Firmicutes and TG3 with Spirochaetes being major agents of hemicellulose degradation (Brune, 2014; Rossmassler *et al.*, 2015; Mikaelyan *et al.*, 2016; Tokuda *et al.*, 2018). The microbiota of wood feeding cockroaches was dominated by Firmicutes and Bacteroidetes (Bauer *et al.*, 2014). Our results show a more similar microbiota to xylophagous beetles, which were dominated by Proteobacteria Actinobacteria, Bacteroidetes and Firmicutes (Rizzi *et al.*, 2013; Mohammed *et al.*, 2018; Ziganshina *et al.*, 2018).

Fungal mycobiota within the guts of isopods have not been previously studied. An early study observed fungi in *O. asellus* faecal pellets using plate counts, however it has been hypothesised the fungi observed is transient, ingested with food (Gunnarsson and Tunlid, 1986; Zimmer, 1999). Another study examined the effect of isopods (*Burmoniscus*) grazing on microbial dynamics, using identification tests and found fungal communities observed in the hindgut to be *Penicillium, Trichoderma, Fusarium, Cladosporium, Aspergillus* and *Mucor* (Kayang *et al.*, 1996). This current study found sequences similar to *Penicillium* to be the only genera found greater than 1% abundance, *Mucor* and *Cladosporium* had less than 1% abundance, and *Fusarium* and *Trichoderma* were not present.

Saccharomycete and Basidiomycete yeasts are common in the hindgut of beetles carrying out fermentation of cellobiose and xylose (Suh *et al.*, 2005; Urbina *et al.*, 2013). Recently, the whole mycobiota of wood-eating beetles has been examined. Similar to our current study, findings showed variability of fungi between individuals for all beetles examined (Ziganshina *et al*., 2018; Mohammed *et al*., 2018). For example, in beetle *Cucujus cinnaberinus,* one larva had members of Ascomycota; Microascaceae and Hypocreaceae, whilst Zygomycota; Mortierellaceae dominated in the second larva, whereas the third individual had Ascomycota; Chaetosphaeriaceae and Davidiellaceae and Zygomycota; Mortierellaceae, (Ziganshina *et al*., 2018). Our current study found Davidiellaceae the be the most abundant family of fungi in *O. asellus* samples and was present across all samples of *O. asellus* and *P. scaber*, whilst other fungi varied in samples. Unclassified Sordariomycetes was most dominant in *P. scaber* hindgut and can be mutualistic or pathogenic to arthropods and discussed in Chapter 4.6.

From our current study, many abundant fungal sequences could only be identified to higher taxonomic levels, such as unclassified classes, orders or families, within these would be many different genera that have different roles and functions. Therefore, it is hard to predict which of these fungi have lignocellulose-degrading abilities. Sequences similar to specific genera were *Candida* found in *P. scaber* at 12% abundance and all other individual genera were found less than 2% abundance. In a study by Bredon *et al*. (2018), eleven fungal genes encoding lignocellulose-degrading CAZymes were detected in *A. vulgare* and were all affiliated with *Aureobasidium pullulans.* Genera; *Aureobasidium* was found in this study at 1% total abundance. Bredon concluded the low number of fungal lignocellulose-degrading CAZymes identified could be due to the prokaryotic enrichment process used when constructing the metagenomic libraries and further research is needed enriching for eukaryotes (Bredon *et al*., 2018).

This is the first study to characterise the presence of fungi in the hindgut of any woodlouse. We found distinct and different fungal communities present within each individual woodlouse. Individual differences were identified which show the communities are not completely uniform. This is also the first study to investigate the bacterial microbiota via NGS and to compare two species of woodlice with many other bacterial genera found than had previously been reported. This evidence of resident bacterial and fungal communities in the hindgut, indicates the microbiota can persist in the hindgut and more research is needed into the shedding of the hindgut cuticle.

6 Comparison of Hepatopancreas and Hindgut Microbiota of Two Species of Woodlice

6.1 Introduction

In terrestrial ecosystems invertebrates feed on substrates rich in cellulose, and are therefore, important vehicles of wood decomposition (Byzov, 2005). Woodlice degrade lignocellulose substrates, both physically and enzymatically, masticating and compressing food to reduce its size and decrease the crystallinity of cellulose (Kostanjšek *et al*., 2002). The ability to digest lignocellulose is primarily due to the symbiotic relationship between woodlice and their gut microbiota (Bredon *et al*., 2018). A plethora of lignocellulose degrading enzymes have been recently detected in the tissues of *A. vulgare* and also their associated gut microbiota (Kostanjšek *et al*., 2010; Bredon *et al*., 2018).

When dissimilar organisms live together, they enter a broad range of symbiotic interactions, which are a continuum and can change over time, during evolution or under the changing conditions between the individual organisms (de Bary, 1879; Werren *et al.*, 2008). Previous studies have found the hepatopancreas to be densely colonised but harbouring a low diversity of bacteria, dominated by *Hepatoplasma* or *Hepatincola* (Wang *et al.*, 2004a; Wang *et al.*, 2004b). This suggests a mutualistic symbiotic relationship between the bacteria and host, contributing to host health and working together with the endogenous enzymes to enable woodlice to survive on such a recalcitrant diet (Kostanjšek *et al*., 2010; Bredon *et al*., 2018). Fungi within the hepatopancreas have never been investigated, this may be due to the reported presence of a filter preventing particles from entering and colonising (Storch, 1987; Wood and Griffiths, 1988; Hames and Hopkin, 1989). The presence of fungi colonising the hepatopancreas and hindgut, would suggest a symbiotic relationship between these two different organisms in the breakdown of lignocellulose. Bacteria have an important endosymbiotic relationship with host development and health, therefore it is plausible to suggest the presence of fungi may also have a similar role (Mueller *et al.*, 2012; Llewellyn *et al.*, 2014; Bouchon *et al.*, 2016).

6.2 Aims

The major aims of this chapter are to:

- Compare the microbial communities in the hepatopancreas and hindgut of two species of woodlouse
- Compare the microbial communities detected from culture and non-culture experiments

6.3 Investigation into the Microbial Communities within the Hepatopancreas and Hindgut of *O. asellus* and *P. scaber*

DNA samples from the hepatopancreas and hindgut of *O. asellus* and *P. scaber* were subjected to PCR to amplify the bacterial 16S rRNA gene and fungal ITS2 rDNA region (see section 2.3). Samples were sent to LGC Genomics for NGS and analysis (see section 2.3.7). After bioinformatic analysis (see section 2.4), sequences were assigned to unique OTUs. The total unique bacterial and fungal OTUs were more abundant in the hindgut than the hepatopancreas (Table 6.1). There were also more bacterial OTUs in the hepatopancreas and hindgut compared to fungal OTUs colonising the same region.

Table 6:1: Number of unique OTUs from sequencing the bacterial 16S rRNA gene and fungal ITS2 rDNA region from the hepatopancreas and hindgut of *O. asellus* and *P. scaber.* Sequences were assigned to OTUs with ≥97% similarity.

Woodlice species	**No. of samples**	**Unique bacterial OTUs (≥97%)**		**Unique fungal OTUs (≥97%)**	
		Hepatopancreas	**Hindgut**	**Hepatopancreas**	**Hindgut**
O. asellus	5	137	255	23	81
P. scaber	5	92	217	20	41
All		229	473	46	122

6.3.1 Alpha Diversity Analysis

To enable comparison between each individual woodlouse sample, DNA sequences were normalised to the sample containing the lowest number of sequences; 12,170 bacterial sequences and 1,132 fungal sequences. A t-test revealed there was no significant difference between the bacterial and fungal communities within *O. asellus* or *P. scaber,* irrespective of digestive tract region (Table 6.2 *P*= <0.05). A t-test indicated there was a significant difference in bacterial communities

between the hepatopancreas and hindgut of *O. asellus* and *P. scaber*, in contrast, there was no significant difference in fungal communities between the hepatopancreas and hindgut of *O. asellus* or *P. scaber* (Table 6.2 *P*= >0.05).

Table 6:2: T-test showing p-value results indicating differences in bacterial (16S) and fungal (ITS2) communities in the GI tract of *O. asellus* and *P. scaber* from NGS analysis. Significant differences *P*= <0.05 (bold text).

	P. scaber		***O. asellus* hindgut**		***P. scaber* hepatopancreas**		***P. scaber* hindgut**	
	16S	ITS2	16S	ITS2	16S	ITS2	16S	ITS2
O. asellus	0.57	0.51						
***O. asellus* hepatopancreas**			**0.036**	0.09	0.79	1.0	**0.006**	**0.02**
***O. asellus* hindgut**					0.06	0.16	1.0	1.0
***P. scaber* hepatopancreas**							**0.024**	0.08

The hepatopancreas and hindgut were extracted from the same individual woodlouse, therefore, a comparison of bacterial and fungal communities could be examined between the two tissues (Fig. 6.1). The number of bacterial OTUs were higher than fungal OTUs in both regions on the GI tract, with the most bacterial OTUs present in one *P. scaber* hindgut sample was 130, compared to 48 fungal OTUs in one *O. asellus* hindgut sample. The diversity of bacterial OTUs in the hepatopancreas was significantly lower compared to the hindgut (Fig. 6.1A & Table 6.1). In comparison, similar to bacterial OTUs, the number of fungal OTUs in the hepatopancreas was lower than the hindgut, however, the number of fungal OTUs in the hindgut did not differ significantly from hepatopancreas (Fig. 6.1B & Table 6.2).

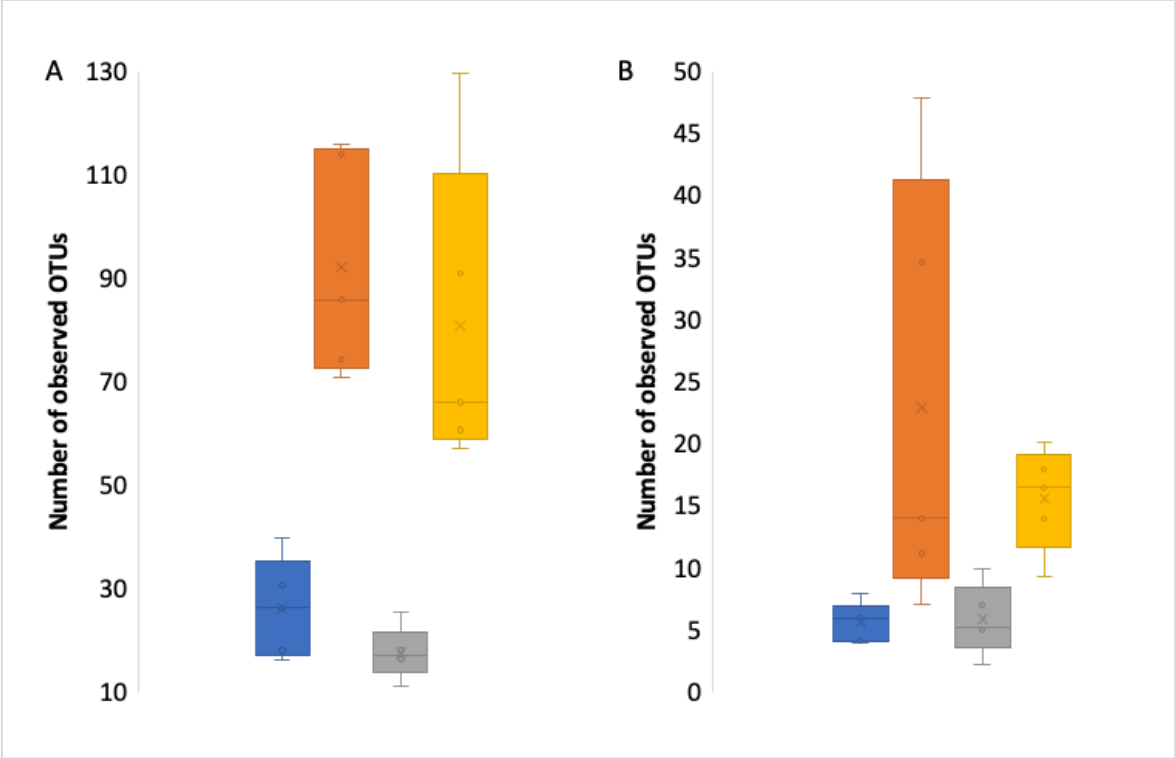

Figure 6:1: Alpha diversity boxplots showing microbial diversity within the GI tract of woodlice. The mean (x), median (-) and range of the average number of observed bacterial (A) OTUs and fungal (B) OTUs within the samples of *O. asellus* hepatopancreas (blue) and hindgut (orange) and *P. scaber* hepatopancreas (grey) and hindgut (yellow) at 12,170 bacterial and 1,132 fungal sequences per sample.

6.3.2 Beta Diversity Analysis

Beta diversity enabled better understanding of bacterial and fungal diversity present. Taxonomic profiling indicated two different bacterial communities separating the hepatopancreas and hindgut, that appeared to be similar in each GI tract region between woodlouse species (Fig. 6.2A). The fungal communities indicate differences between the hepatopancreas and hindgut (Fig. 6.2B), however, not as stark as the differences in the bacterial communities.

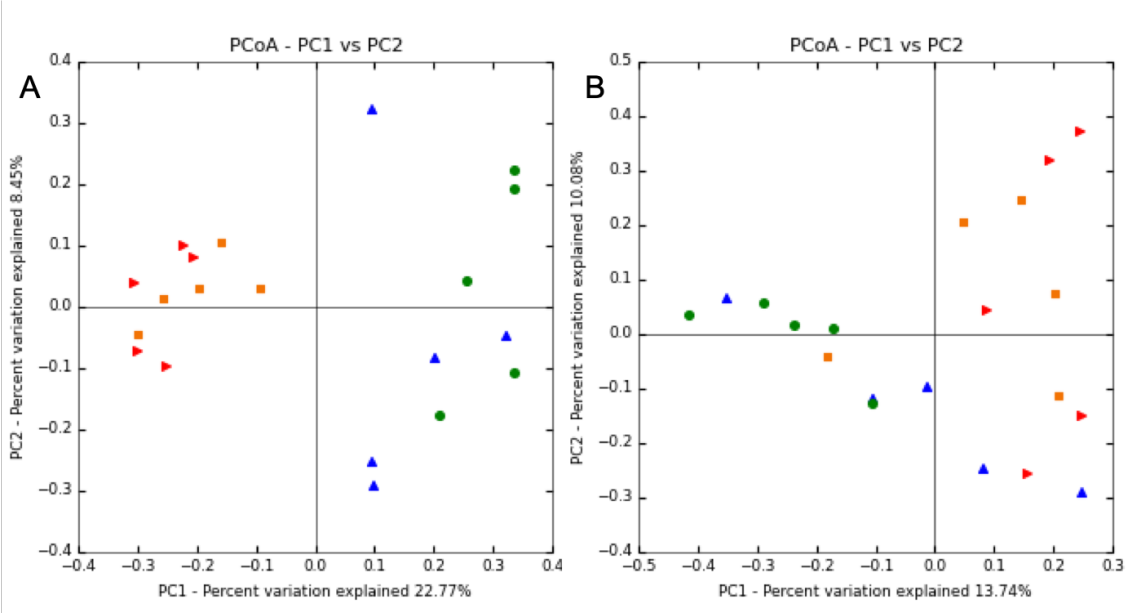

Figure 6:2: Similarities of microbial communities in woodlice GI tract. (A) Bacterial communities and (B) fungal communities in the hepatopancreas (HP) and hindgut (HG) samples of *O. asellus* (Oa) and *P. scaber* (Ps). (OaHP blue triangle, OaHG red triangle, PsHP green circle, PsHG orange square). PCoAs were generated from unweighted UniFrac distances. Axes represent the two synthetic variables explaining the greatest proportion of variation in the data set. PCoA charts supplied by LGC Genomics.

6.3.3 Comparison of Bacterial Community Diversity from the Hepatopancreas and Hindgut of *O. asellus* and *P. scaber*

Examining the microbial community composition in both the hepatopancreas and hindgut (Fig. 6.3) allows comparison of microbial diversity, found greater than 1% abundance, between the hepatopancreas and hindgut of both species of woodlice. The hepatopancreas has significantly less diversity than the hindgut, with one bacterium; sequence similar to *Hepatoplasma*, in both species of woodlice colonising abundantly. As seen in Fig. 4.5B and 5.5B, showing bacterial communities in individuals, it is observed that *O. asellus* hepatopancreas, sample 3, is numerically dominated by sequences similar to pathogenic bacteria *Rickettsiella* and *Hepatincola* and the hindgut of sample 3 is colonised by *Rickettsiella*. *Rickettsiella* is prevalent in both the hepatopancreas and hindgut of samples 1, 3 and 5 of *O. asellus*, but not in the tissues of *P. scaber*. Apart from colonisation by pathogenic bacteria, there are no similarities between the hepatopancreas and hindgut.

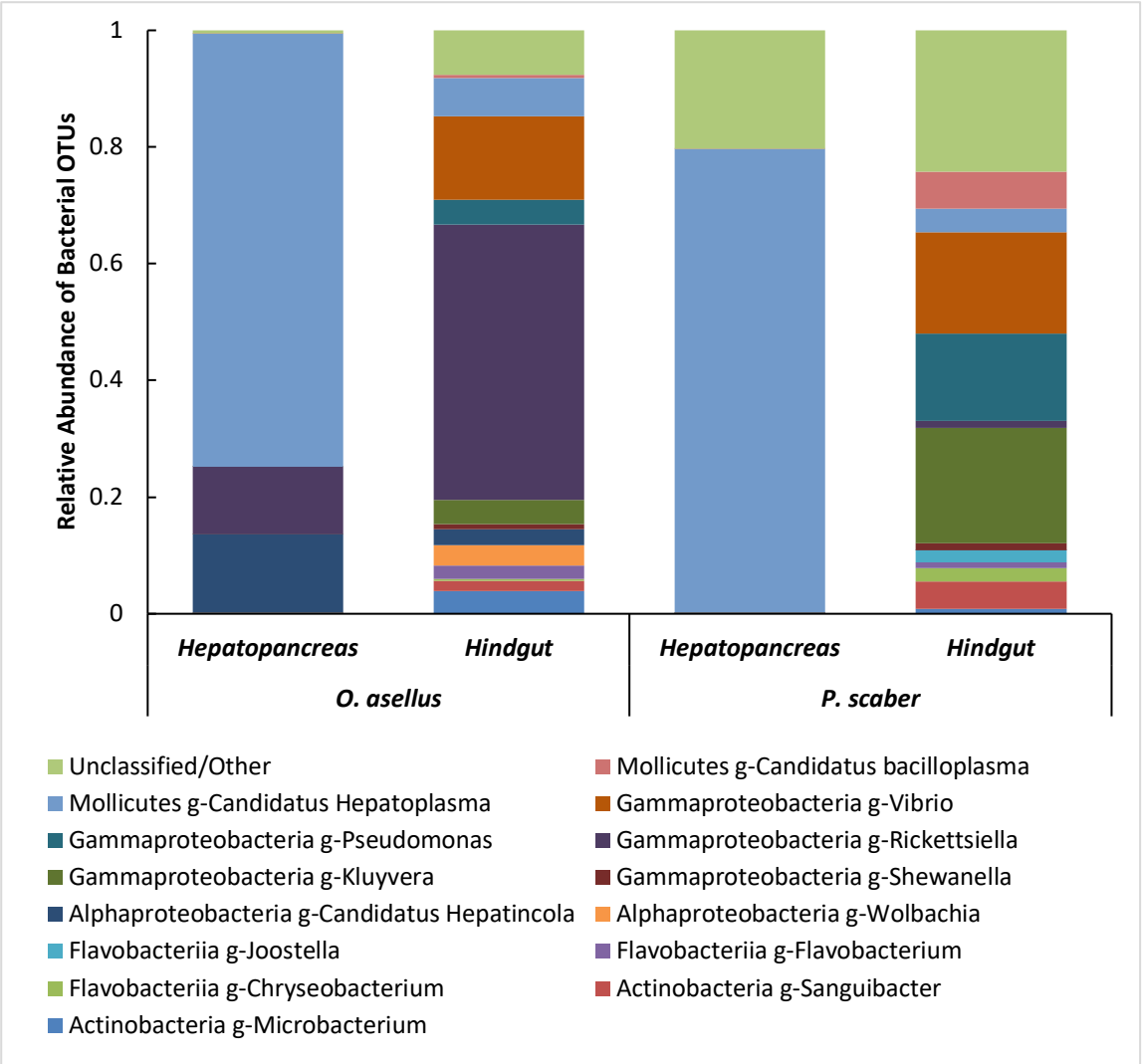

Figure 6:3: Taxonomic composition of the 16S rRNA sequences from the hepatopancreas and hindgut of both species of woodlice (all samples n=5). Results are reported at class followed by genera (g). Average relative abundance and distribution of the most abundant bacterial sequences found in greater than 1% abundance in at least one woodlouse. Sequences were assigned to OTUs with ≥97% sequence similarity.

6.3.4 Comparison of Fungal Community Diversity from the Hepatopancreas and Hindgut of both Species of Woodlice

The fungal communities within the woodlice were varied (Fig. 6.4). There was less diversity in the hepatopancreas than the hindgut in both woodlouse species, the only similarity between all tissue regions was the presence of sequences similar to Davidiellaceae (Fig. 6.4). The hepatopancreas and hindgut of *O. asellus* was dominated by unclassified Sordariomycetes, however, making up 97% of the total fungal abundance in the hepatopancreas and 99% total abundance in the hindgut in the same individual woodlouse, therefore not representative of all samples. In *P. scaber,* in addition to

Davidiellaceae, sequences similar to *Candida* were present in 3 out of 5 hepatopancreas and 4 out of 5 hindgut samples. When comparing fungal diversity and bacterial diversity (Fig. 6.3), the number of fungal OTUs were lower than bacterial OTUs (Fig. 6.1), however, the fungal communities in the hepatopancreas were more diverse than bacterial communities (Fig. 6.4).

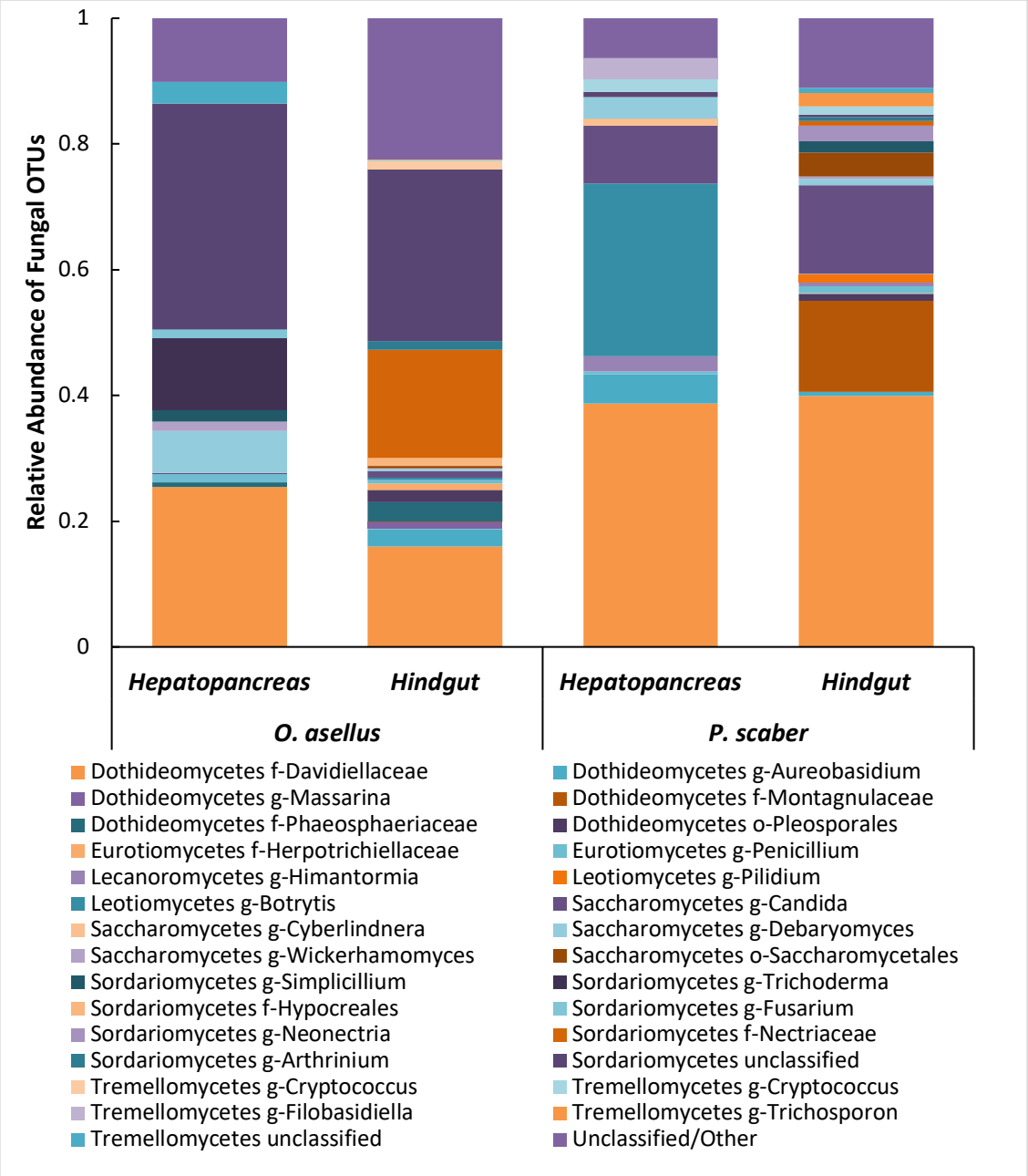

Figure 6:4: Taxonomic composition of ITS rDNA sequences from the hepatopancreas and hindgut of both species of woodlice (all samples n=5). Results are reported at class followed by lower taxonomic classifications; order (o), family (f) and genus (g). Average relative abundance and distribution of the most abundant fungal sequences found in greater than 1% abundance in at least one woodlouse. OTUs were assigned to closest species by sequence with ≥97% similarity.

6.4 FISH

To examine the location of the bacteria and fungi in the different tissues, FISH was attempted (see section 2.2.2.2). Initially this was carried out on whole organisms, however, it was concluded the tissues were too substantial for the reagents to penetrate. Subsequently tissues were sliced using the cryostat and mounted onto slides, a different slide was withdrawn after each step of the method and visualised via microscopy to try to identify which step the experiment needed optimisation. After troubleshooting and attempting various washing techniques, sample adhesion was the major issue and therefore, there were no results from this experiment.

6.5 Culturable Bacteria from the Hepatopancreas of *O. asellus*

The hepatopancreas of *O. asellus* and *P. scaber* samples used in this study contained little diversity and was dominated by *Hepatoplasma*, with one individual of *O. asellus* dominated by *Hepatincola*, which both, as yet, have not been cultured (Wang *et al.*, 2004a; Wang *et al.*, 2004b). The hepatopancreas extracted from *O. asellus* was inoculated onto agars using different environmental conditions (See section 2.2.2.3). Isolates were sent for 16S rRNA gene sequencing (Table 6.3). Results showed that some isolates had sequences similar to those found from NGS analysis, however, the abundant sequences found via NGS were not cultured. Isolates with sequences similar to six genera (*Raoultella, Erwinia, Psychrobacillus, Buttiauxella, Ornithinibacillus* and *Nocardia*) were not found in either *O. asellus* or *P. scaber* NGS community profiles.

Table 6:3: Bacterial isolates cultured from the hepatopancreas of *O. asellus* using different environmental conditions. The 16S rRNA gene sequence isolate results were compared against the BLAST database. Isolate results were compared against the possible NGS detection results.

Sequence similarity to (≥97%)	Accession number	Similarity score	% detected from NGS analysis of *O. asellus*	% detected from NGS analysis of *P. scaber*
***Rahnella*_sp**	NR_025337.1	99%	<0.0005	0
***Raoultella*_sp**	NR_114502.1	100%	0	0
***Erwinia*_sp**	NR_118431.1	98%	0	0
***Pseudomonas*_sp**	NR_113651.1	100%	<0.0005	<0.0005
***Psychrobacillus*_sp**	NR_025409.1	99%	0	0
***Buttiauxella*_sp**	NR_036919.1	99%	0	0
***Staphylococcus*_sp**	NR_025922.1	100%	<0.0005	<0.0005
***Stenotrophomonas*_sp**	NR_112030.1	99%	<0.0005	<0.0005
***Acinetobacter*_sp**	NR_117625.1	99%	<0.0005	<0.0005
***Micrococcus*_sp**	NR_134088.1	99%	0	<0.0005
***Ornithinibacillus*_sp**	NR_117927.1	97%	0	0
***Rhodococcus*_sp**	NR_116275.1	98%	<0.0005	0
***Mycobacterium*_sp**	NR_158073.1	99%	<0.0005	0
***Nocardia*_sp**	NR_104776.1	99%	0	0

6.6 Culturable Wood Degrading Bacteria and Fungi from the Hindgut of *O. asellus* and *P. scaber*

The hindgut tissue of *O. asellus* and *P. scaber* were inoculated onto a minimal agar with differing long chain polymers, such as, lignin, cellulose and sawdust. Plates were incubated at different temperatures to compare culturable isolates that were able to degrade cellulose or lignin, to sequences obtained from non-culture method. Several bacteria and fungi were isolated by culturing, but not present in the NGS community profile (Tables 6.4 & 6.5). Some culturable bacterial and fungal isolates had sequences similar to those detected in the NGS profile, *Pseudomonas* was detected in culture-dependant and independent methods, in greater than 1% abundance in both species of woodlice. Bacterial isolates with sequences similar to *Raoultella*, *Rouxiella* and *Pusillimonas* were isolated, but were not detected in either *O. asellus* or *P. scaber* hindgut NGS community profile (Table 6.4). Fungal isolates with sequences similar to *Trichoderma*, *Phoma*, *Plectosphaerella* and *Paraphaeosphaeria* were isolated, but not found when amplified

using molecular analysis, but sequences similar to *Candida* and *Penicillium* were found in NGS analysis in *P. scaber* (Table 6.4).

Table 6:4: Bacterial isolates cultured from the hindgut of *O. asellus* and *P. scaber* using agar infused with carbon and lignin sources. The 16S rRNA gene sequence isolate results were compared against the BLAST database. Isolate results were compared against possible NGS detection results.

Culturable bacterial sequence similarity to (≥97%)	**Accession number**	**Similarity score**	**% detected from NGS analysis of *O. asellus***	**% detected from NGS analysis of *P. scaber***
***Raoultella*_sp**	NR_037085.1	98%	0	0
***Pseudomonas*_sp**	NR_025103.1	99%	4	15
***Stenotrophomonas*_sp**	NR_112030.1	99%	0.05	0.6
***Rouxiella*_sp**	NR_156932.1	99%	0	0
***Pusillimonas*_sp**	NR_116103.1	97%	0	0

Table 6:5: Fungi isolated from the hindgut of *O. asellus* and *P. scaber* using minimal agar infused with carbon and lignin sources. The ITS rDNA region sequence isolate results were compared against the BLAST database. Isolate results were compared against the possible NGS detection results.

Culturable fungal sequence similarity to (≥97%)	**Accession number**	**Similarity score**	**% detected from NGS analysis of *O. asellus***	**% detected from NGS analysis of *P. scaber***
***Trichoderma*_sp**	KT323198.2	100%	0	0
***Cladosporium*_sp**	MG975638.1	98%	0	0.08
***Candida*_sp**	MF616363.1	99%	1	13
***Phoma*_sp**	JF817319.1	99%	0	0
***Verticillium*_sp**	HG935575.1	100%	0.4	0.06
***Penicillium*_sp**	KX664360.1	100%	0.05	1
***Plectosphaerella*_sp**	MK120895.1	100%	0	0
***Paraphaeosphaeria*_sp**	JX49609.1	99%	0	0

6.7 Discussion

Woodlice have adapted to diverse terrestrial environments and survive on a low nutrient diet, rich in lignocellulose (Wieser, 1966; Sfenthourakis and Taiti, 2015). To survive on such a diet, detritivores must either rely on endogenous lignocellulose degrading enzymes or endosymbiotic microorganisms. It is well accepted there is a symbiotic relationship between the gut microbiota and the host, playing a vital role in host development, such as, immunology, physiology and health and digestion of complex carbohydrates, such as lignocellulose (Bouchon *et al*., 2016; Mueller *et al*., 2012). Woodlice have been shown to exhibit a consortium of endogenous enzymes in their tissues capable to degrading lignocellulose (Kostanjšek *et al*., 2010; King *et al*., 2010; Bredon *et al*., 2018). Recently, it has also been confirmed that these genes are produced by the gut microbiota too (Bredon *et al*., 2018).

In agreement with other studies (Wang *et al.*, 2004a; Wang *et al.*, 2004b; Wang *et al.*, 2007; Fraune and Zimmer, 2008; Leclercq *et al.*, 2014), our study found the hepatopancreas of individual woodlice to be colonised by either *Hepatoplasma* or *Hepatincola*. Having a low, stable and specialised resident bacterial community, that appears to be repeatable and core is strongly evident of a symbiotic relationship between the bacteria and the host. It has been suggested that bacteria in the hepatopancreas are involved in hydrolysis of cellulose and breakdown of lignin and tannins, therefore, these symbionts may enable woodlice to digest leaf litter and directly contribute to litter decomposition (Zimmer and Topp, 1998c; Zimmer, 1999; Zimmer *et al.*, 2002). It has been reported that having *Hepatoplasma* symbionts colonising the hepatopancreas affects the isopod host and survivorship on a low-quality diet, compared to those that had other bacterial symbionts (Fraune and Zimmer, 2008). In contrast, colonisation in the hepatopancreas of *Hepatincola*, negatively correlated with host life longevity, even when fed high-quality food (Fraune and Zimmer, 2008).

The bacterial community within the hindgut was more diverse than the hepatopancreas, indicating more roles are being utilised. To date, compared to the hepatopancreas, little is known about the function of the hindgut. The hindgut is the passage for food, from the mouth, travelling through a short tube and expelled through the anus and reported to have absorptive properties for water and ions (Hassall and Jennings, 1975; Hames and Hopkin, 1989; Storch and Strus, 1989; Kostanjšek *et al.*, 2002). Therefore, more diverse communities would be expected to carry out other roles, such as, fermentation, nitrogen fixation, sulphate reduction, lignocellulose degradation (Kostanjšek *et al*., 2004). This is also suggested in studies examining the bacterial communities within the hindgut of *P. scaber* from high heavy metal polluted environments and non-polluted environments. Results

have shown the bacterial communities with mercury resistant genes proliferate and are transferred through HGT, enabling adaptation to the environment, enabling better host survival lowering the concentration of mercury in the woodlouse gut (Lapanje *et al*., 2008; Lapanje *et al*., 2010). A previous study showed bacterial sequences from the hindgut of *P. scaber*, are generally unable to exist outside their host, indicating a specific bacterial microflora exists and that a symbiotic interaction between these and the host has developed (Kostanjšek *et al*., 2002).

This is the first to study the fungal communities in the hepatopancreas and hindgut of any woodlouse. Despite reports of a filter preventing fungi from entering the hepatopancreas (Storch, 1987; Wood and Griffiths, 1988; Hames and Hopkin, 1989), it was hypothesised that fungi would colonise both the hepatopancreas and hindgut, due to woodlice having a diet rich in lignocellulose. Fungi are known to be the main degraders of wood and in environments with a high proportion of dead wood, until recently fungal communities have been the main focus of attention, in preference to the bacterial communities (Johnston *et al.*, 2016). It is accepted that interactions between bacteria and fungi work synergistically in the breakdown of lignocellulose in the environment (Folman *et al*., 2008; Hoppe *et al*., 2014). Therefore, it is plausible to hypothesise the fungal communities in the digestive tract of wood-eating animals, including woodlice, would have a symbiotic relationship with the bacterial community in the breakdown of lignocellulose and may have the same relationship with the host that the bacteria do. Our study found many species of fungi know to have lignocellulosic properties (see Chapter 4 & 5), however, the results in individual woodlice are not repeatable and core, which indicates their presence may primarily assist the other microbes in lignocellulose degradation rather than any beneficial interaction with the host.

When examining the microbial communities in the GI tract of *P. nigrolineatus, O. asellus and P. scaber,* our results suggest there are resident bacterial and fungal communities in both systems. These two systems are very different, one being aquatic and the other terrestrial. The structures of the GI tract are also very different, *P. nigrolineatus* does not have defined fore, mid and hindgut (German, 2009). In contrast, the woodlouse has very defined short foregut, four hepatopancreatic lobes (midgut), that do not connect to the straight, tubular hindgut (Hames and Hopkin, 1989). The microbial communities in the foregut of the woodlice were not investigated in this study, but the resident bacterial and fungal communities detected in the midgut and hindgut in each organism were different. *P. nigrolineatus* and the two species of woodlice were abundant in Ascomycota (Marden *et al.*, 2017) and at class level, the wood-fed diet fish and *P. scaber* were abundant in Dothideomycetes, whilst the mixed-fed diet fish and *O. asellus* were more abundant in Sordariomycetes. However, at lower taxonomic levels there was minimal similarity. Order;

Pleosporales was abundant in the mid and hindgut of *P. nigrolineatus*, but was detected in 5% abundance in the hindgut but not abundant in the hepatopancreas of the woodlice. In the mixed-fed diet fish, order; Microascales and family; Microascaceae was abundant, but was not abundant in the woodlice. Sordariomycetes; *Fusarium* was abundant in *P. nigrolineatus*, whilst, Dothideomycetes; Davidiellaceae was abundant in the woodlice. Neither of these were abundant in the other organism. Similarly, examination into the bacterial communities in the GI tract of *P. nigrolineatus* indicated different communities present to those detected in the woodlouse GI tract. The hepatopancreas of *O. asellus* and *P. scaber* were dominated by phyla Tenericutes, in contrast, *P. nigrolineatus* was abundantly colonised by Proteobacteria (McDonald *et al.*, 2012, 2015; Watts *et al.*, 2013). The hindgut of *O. asellus* and *P. scaber* was dominated by Proteobacteria and *P. nigrolineatus* by Bacteroidetes (McDonald *et al.*, 2012, 2015; Watts *et al.*, 2013). In fact, many of the dominant sequences at lower taxonomic levels in *P. nigrolineatus*, were either not detected or in less than 1% abundance in the woodlice. Therefore, although they both have a diet rich in cellulose and cellulose degrading microorganisms have been detected within their GI tracts, these differing results suggest they have adapted and evolved to suit their specific environment, regardless of their diet.

To detect the location of these symbionts in the woodlice, FISH was performed (see 2.2.2.2). These attempts were unsuccessful due to attachment of bacteria and fungi on the slides during washing procedures. Therefore, future work would focus on optimising this technique to contain more gentle reagents.

The advent of culture-independent analysis, such as sequencing, has enabled a more comprehensive snapshot into the microbial communities in a given environment, as results are independent of any physiological state the microbe is in or any specific requirements needed for growth on a culture medium. However, whilst the results from these techniques give a snapshot into the microbial communities colonising the lumen of these tissues, these techniques may not give the entire picture.

To try and increase bacterial isolates capable of growing under different conditions, woodlouse gut samples were inoculated onto agar using different environmental conditions (see section 2.2.2.3 and 2.2.2.4) and isolates obtained were sequenced. Only three microbes; a sequence similar to bacterium; *Pseudomonas* and fungi; *Candida* and *Penicillium* from the hindgut were found in greater than 1% abundance in the NGS community profile in greater than 1% abundance. Many isolates did not appear in the NGS community profile or were found in less than 0.005% abundance.

An isolate is one organism and if results are inconclusive, other conditions may be adopted, such as the use of different primers. However, by using NGS, a sample of thousands or millions of sequences are produced and there may be biases for certain groups of microbes involved in each step of the process (Fonseca *et al.*, 2012; Ihrmark *et al.*, 2012; Wang *et al.*, 2015). Culture-dependent methods only detect easily culturable organisms, whilst members that need enrichments or are in a particular physiological condition are lost (Harwani, 2012). The diversity found from NGS sequencing did not correlate to the culture experiment. The term 'the great plate count anomaly' was introduced to examine the discrepancy between the viable plate count grown on agar medium and total direct microscopic counts of bacteria in a given environment (Staley and Konopka, 1985). Some microbes require specific conditions for successful culture, such as pH conditions, incubation temperatures or levels of oxygen in the atmosphere or there may be competition for nutrients among a mixed environmental sample being cultured together (Harwani, 2012). Species may be in a specific stage of growth, such as dormancy, which would prevent growth in a nutrient rich media, these cells are viable but not in the right conditions for growth (Harwani, 2012).

The bacterial communities in the hepatopancreas from our study and other studies appear to be stable, this is the first time fungal communities have been characterised in this region. The fungal communities were resident and did not appear to have a core element, indicating their presence is primarily involved in lignocellulose degradation. This is the first time the bacterial communities have been investigated in the hindgut using more advanced molecular techniques and the first time fungal communities have been characterised. Our results indicate more diverse communities, probably involved in other processes as this is the passage of ingested food.

7 Investigation in Antimicrobial Resistant Bacteria in the Digestive Tract of *O. asellus*

7.1 Introduction

The spread and persistence of AMR poses an ongoing threat to human health (WHO, 2014), however, although there is still a lack of knowledge surrounding the interplay between the environment and AMR, awareness is increasing (Woolhouse *et al.*, 2015; Bengtsson-Palme *et al.*, 2017). AMR factors can happen at any time, through mutation, rearrangement or HGT, however, resistance factors are associated with a fitness cost and only selected for if there is a strong pressure to maintain it (Bengtsson-Palme *et al*., 2017). Environmental factors, such as, untreated sewage and effluent containing resistance genes from wastewater treatment plants are released into bodies of water. Water contaminated by effluents is often used for farmland irrigation and drunk as surface water by domestic animals (Bengtsson-Palme *et al*., 2017). Sewage sludge and manure from animals treated with antibiotics are used as fertiliser on farmlands, with humans consuming these foods grown on the farms (Woolhouse *et al*., 2015). Effluent and run-off from farms and fields will follow rivers into the sea, polluting estuaries, coastal waters and beaches with faecal matter (Arnold *et al*., 2016). Physical forces, such as wind and water movement, can also move bacteria with AMR genes over large distances (Allen *et al.*, 2010).

Relatively little is known about the flow and fate of AMR in highly mobile species acting as AMR dispersers (Arnold *et al*., 2016). However, another physical force of dissemination could be highly mobile terrestrial woodlice, which colonise a diverse number of environments, including environmentally polluted soils in fields and farmlands, as well as heavy metal polluted environments (Lapanje *et al*., 2010). It is estimated the soil microbes, which have a very abundant and diverse microbiome, have been producing antibiotics for 2 billion years and is a reservoir for resistance gene pools (D'Costa *et al.*, 2011; Gaze *et al.*, 2013). The transfer of genetic material between bacteria is induced by stressors such as antibiotics and heavy metals (Bengtsson-Palme *et al*., 2017). Woodlice have been shown to persist in heavy metal polluted environments, developing heavy metal resistance genes that share similar structural and functional resistance systems as AMR (Lapanje *et al.*, 2010; Singer *et al.*, 2016). Therefore, it is plausible that terrestrial isopods assist in the spread of antimicrobial genes.

To date, no studies have looked at antimicrobial resistant bacteria in the GI tract of terrestrial isopods and there is limited research into AMR in other arthropods.

7.2 Aim

The major aim of this chapter is to examine AMR bacteria isolated from the GI tract of *O. asellus.*

7.3 Culture-Dependant Analysis to Examine AMR in the Hepatopancreas and Hindgut of *O. asellus*

7.3.1 Obtaining and Enumeration of Antibiotic-Resistant Bacteria by Culture-dependant Method

O. asellus (n=13) were euthanised as stated in 2.2.2.1 and the hepatopancreas and hindgut was extracted. AMR screening was carried out (see section 2.2.2.5) using agar infused with cycloheximide, acting as an anti-fungal compound and a combination of bacterial inhibitors; ampicillin, cycloheximide, gentamicin and tetracycline. Colony forming units (CFUs) were recorded and data showing the CFU/g of resistant bacteria in the hepatopancreas and hindgut of *O. asellus* (Table 7.1). The hindgut had the most CFU/g prior to the addition of bacterial inhibitors compared to the hepatopancreas. Results indicate the number of colonies resistant to bacterial inhibitors decrease in the hepatopancreas with the increasing number of antibiotics infused into the media. No bacterial growth was observed on agar infused with AG, GC, GT, ACG, CGT and ACGT. The number of resistant colonies from the hindgut decreased with counts remaining at similar levels with two or more antibiotics added. There was no growth on agar infused with ACG or AGCT (Fig. 7.1).

Table 7:1: Number of CFU/g isolated from the GI tract of woodlice on agar infused with antibiotics. Nutrient agar was infused with fungal inhibitor cycloheximide (50 mg/L) and a combination of bacterial inhibitors (Ampicillin 100mg/L (A), chloramphenicol 100 mg/L (C), gentamicin 50 mg/L (G), tetracycline 20 mg/L (T)).

	Hepatopancreas	**Hindgut**
Agar infused with Antibiotics	**CFU/g**	**CFU/g**
None	$3.90*10^{12}$	$1.34*10^{14}$
A	$1.04*10^{10}$	$2.06*10^{9}$
C	$3.84*10^{7}$	$6.70*10^{6}$
G	$1.62*10^{9}$	$2.72*10^{8}$
T	$4.96*10^{8}$	$7.30*10^{6}$
AC	$2*10^{6}$	$1.76*10^{7}$
AG	0	$1.31*10^{8}$
AT	$9*10^{3}$	$1.04*10^{7}$
CG	0	$3*10^{6}$
CT	$2.70*10^{4}$	$1.66*10^{6}$
GT	0	$1.08*10^{7}$
ACG	0	0
ACT	$3.70*10^{4}$	$5*10^{4}$
AGT	$2*10^{3}$	$2.96*10^{6}$
CGT	0	$6.20*10^{5}$
ACGT	0	0

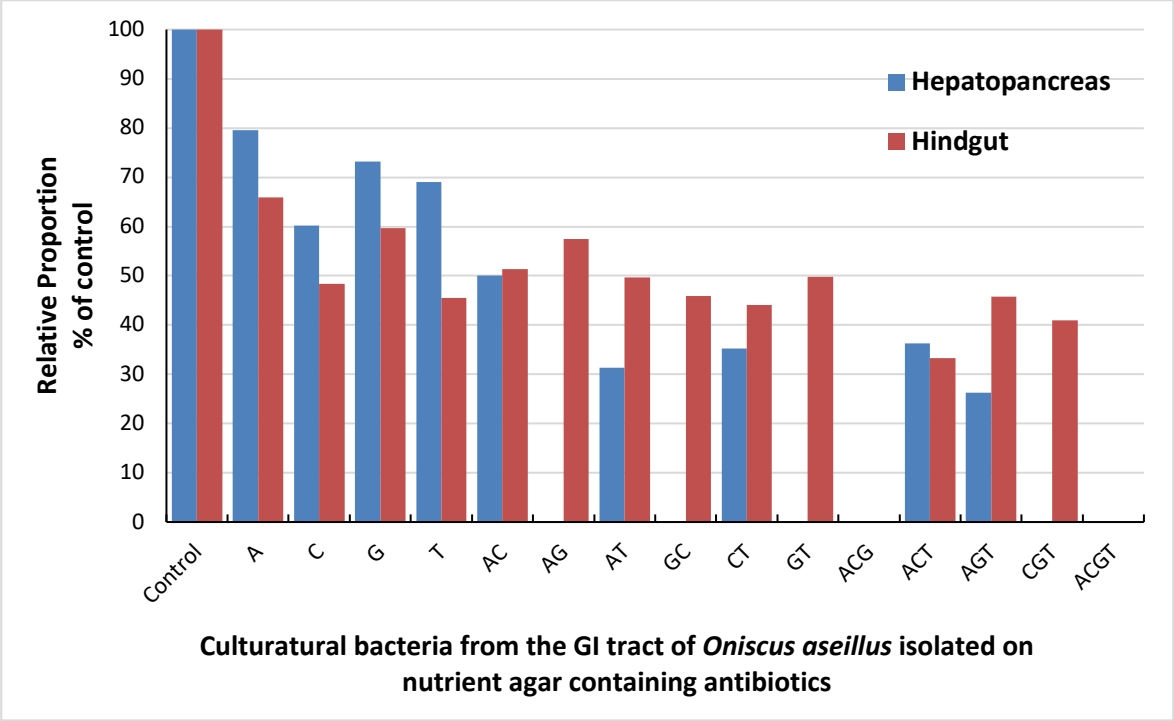

Figure 7:1: Percentage relative proportion against control samples of culturable bacteria from the GI tract of *O. asellus* recorded grown on media infused with one or more antibiotics. Hepatopancreatic and hindgut tissue were extracted from *O. asellus*. Ampicillin (A) (100 mg/L), Chloramphenicol (C) (100 mg/L), Gentamicin (G) (50 mg/L), Tetracycline (T) (20 mg/L).

7.3.2 Identification of AMR Isolates

Unique colonies were isolated onto nutrient agar and incubated at 20°C. DNA was extracted (see section 2.3.1) and the 16S rRNA gene was amplified by PCR (see 2.3.3). Results were sent for analysis by Sanger sequencing technology (see 2.3.6) and results recorded (Table 7.2). Sequences similar to the same genera, but with a distinct morphological profile on the agar plate were given a reference number after the possible identification.

Table 7:2: Isolates taken from antibiotic infused agar by sequencing the 16S rRNA gene. Isolates were grown from the hepatopancreas (HP) and hindgut (HG) of *O. asellus*. Numbers after genera refer to isolates with distinct features on the agar plate.

Isolate	Region of GI tract	Sequences similar to genera (≥97%)	Species (≥97%)	Accession number	% identity
AMR-2	HP	*Pseudomonas (1)*	*helmanticensis*	NR_126220.1	97%
AMR-4	HP	*Pseudomonas (2)*	*koreensis*	NR_025228.1	99%
AMR-5	HP	*Pseudomonas (3)*	*marginalis*	NR_112072.1	99%
AMR-9	HP	*Stenotrophomonas (1)*	*rhizophila*	NR_121739.1	99%
AMR-10	HP	*Stenotrophomonas (2)*	*maltophila*	NR_112030.1	99%
AMR-C	HG	*Sphingobacterium (1)*	*faecium*	NR_113744.1	99%
AMR-D	HG	*Pseudomonas (4)*	*fragi*	NR_113578.1	98%
AMR-E	HG	*Stenotrophomonas (3)*	*rhizophila*	NR_121739.1	99%
AMR-G	HG	*Delftia*	*acidovorans*	NR_113708.1	99%
AMR-J	HG	*Sphingobacterium (2)*	*kitahiroshimense*	NR_041636.1	99%
AMR-L	HG	*Pseudomonas (5)*	*fluorescens*	NR_113647.1	97%
AMR-M	HG	*Pseudomonas (6)*	*helmanticensis*	NR_126220.1	99%
AMR-N	HG	*Stenotrophomonas (4)*	*pavanii*	NR_116793.1	100%

Samples AMR-8, AMR-A and AMR-F could not be identified by sequencing the 16S rRNA gene. A variety of techniques were used, including reamplification of the PCR product, gel extraction and DNA extraction using phenol chloroform. Due to the consistency of AMR-A, prior to DNA extraction the sample was also frozen then thawed and extending the initial denaturing step in PCR to 5 minutes. Attempts were also made to sequence these samples using other universal primers. However, these samples had unsuccessful results when sequencing the 16S rRNA gene, but morphologies of these colonies were recorded (Table 7.3).

Table 7:3: Morphology of AMR isolates that were unable to be sequenced.

Morphology	Unsuccessful isolates from sequencing the 16S rRNA gene		
	AMR-8	**AMR-A**	**AMR-F**
Gram stain	Positive	Positive	Positive
Shape	Cocci	Cocci	Rod
Size	2 mm	1 mm	5 mm
Colour	Yellow, luminescence	White opaque	Yellow
Texture	Soft, sticky, mucoid	Dry	Sticky, soft, mucoid
Elevation	Flat	Raised	Raised
Edges	Irregular	Filamentous edge	Circular

7.3.3 Zones of Inhibition from the Isolates Obtained from the Hepatopancreas and Hindgut of *O. asellus*

A total of 16 different isolates from the hepatopancreas and hindgut were screened for AMR using 6 different antibiotic disks (Fig. 7.2 & 7.3). Isolates were plated onto nutrient agar and incubated at 20°C, to look at antimicrobial resistance that has been acquired under environment conditions and Mueller Hinton agar (Mueller & Hinton 1941) following the Kirby Bauer disk diffusion susceptibility test, for clinical resistance (Bauer et al. 1966). ZOIs defined are routinely measured and results from the Kirby Bauer method are compared to CLSI susceptibility guidelines. Antibiotic discs were applied to triplicate plates. There was more resistance when isolates were grown on Mueller Hinton agar at 35°C as opposed to nutrient agar at 20°C (Fig. 7.2 & and 7.3).

All isolates cultured on both agars were resistant to ampicillin and penicillin with the exception of AMR-G (presumptive *Delftia*) which was susceptible to ampicillin on Mueller Hinton (Fig. 7.2 & 7.3). All isolates, with the exception of AMR-4, indicated different resistance profiles when comparing the result between nutrient agar incubated at 20°C and Mueller Hinton incubated at 35°C. AMR-4 had similar ZOIs for gentamycin, streptomycin and tetracycline on both agars. Several isolates cultured on Mueller Hinton had a ZOI around an antibiotic, but under the CSLI guidelines, the size of the ZOI produced resulted in intermediately or completely resistant (Fig. 7.2 & 7.3).

Isolates AMR-2, AMR-4, AMR-5, AMR-D, AMR-L and AMR-M indicated they were sequences similar to *Pseudomonas* species (Table 7.2). With the exception of AMR-D, all had similar susceptibility profiles, when grown on nutrient agar at 20°C. AMR-D (presumptive *Pseudomonas*) had a different profile and was the only isolate grown on nutrient agar in this study to be resistant to tetracycline

and not resistant to erythromycin, as the other isolates similar to *Pseudomonas* were (Fig. 7.2 & Fig. 7.3). Incubating these isolates at on Mueller Hinton agar indicated that AMR-2, AMR-5, AMR-D and AMR-L were resistant to all tested antibiotics. Isolate AMR-4 was susceptible to gentamicin and tetracycline but was resistant to other antibiotics used (Fig 7.2). AMR-M showed a large enough ZOI around tetracycline to make it intermediately susceptible (Fig. 7.3). AMR-5 and AMR-L (Fig. 7.2 & Fig. 7.3) had similar resistance when grown on both agars but AMR-5 was hypothesised to be a different species as it produced a pink hue when grown on both types of agars.

Isolates AMR-9, AMR-10, AMR-E and AMR-N indicated sequences similar to *Stenotrophomonas*. AMR-10 and AMR-N showed resistance to all antibiotics, however, the ZOIs were not reproducible (Fig. 7.2 & 7.3). Different resistance profiles were observed for all presumptive *Stenotrophomonas* isolates, however, AMR-9 and AMR-E were both susceptible to gentamycin and tetracycline but resistant to the remaining antibiotics used here.

Sequences similar to *Sphingobacterium* were only isolated from the hindgut. AMR-C and AMR-J had different resistance profiles when grown on nutrient agar at 20°C (Fig. 7.3). AMR-C was resistant to all antibiotics and AMR-J was resistant to all, except intermediately resistant to tetracycline (Fig. 7.3).

Isolate AMR-G had a sequence similar to *Delftia* and indicated the lowest resistance of all isolates, being resistant to only penicillin and gentamycin when grown on Mueller Hinton agar. It was intermediately susceptible to erythromycin and susceptible to penicillin and tetracycline. This was the only isolate found in the hepatopancreas and hindgut that had any ZOIs for ampicillin (Fig 7.3).

AMR-8 had the lowest resistance profile to the antibiotics used, when grown on nutrient agar, but when grown on Mueller Hinton agar only had intermediate susceptibility to tetracycline and resistant to the other antibiotics used.

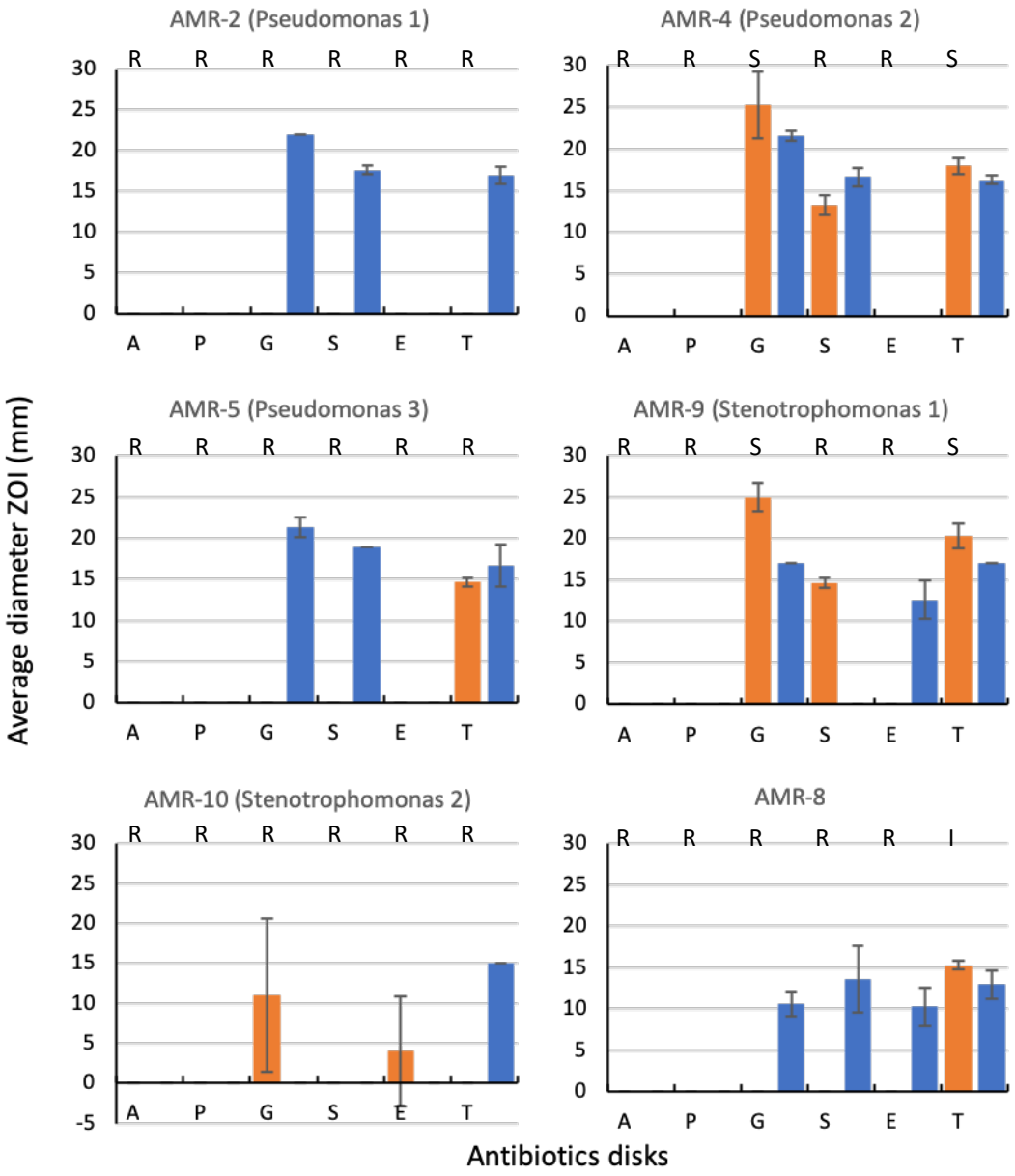

Figure 7:2: Bacterial isolates showing AMR from the hepatopancreas of *O. asellus* isolated on different agars. Nutrient agar (n=3) (blue) and Mueller Hinton agar (n=3) (orange) were used showing the susceptibility to antibiotics as indicated by the measurement of each zone of clearing (mm). Putative identification in brackets. Results of the Kirby Bauer indicate resistance (R), intermediate (I) and susceptible (S) to the different antibiotics; Ampicillin (A) (10 μg), Penicillin (P) (10 units), Gentamicin (G) (10 μg), Streptomycin (S) (10 μg) and Erythromycin (E) (15 μg), Tetracycline (T) (30 μg).

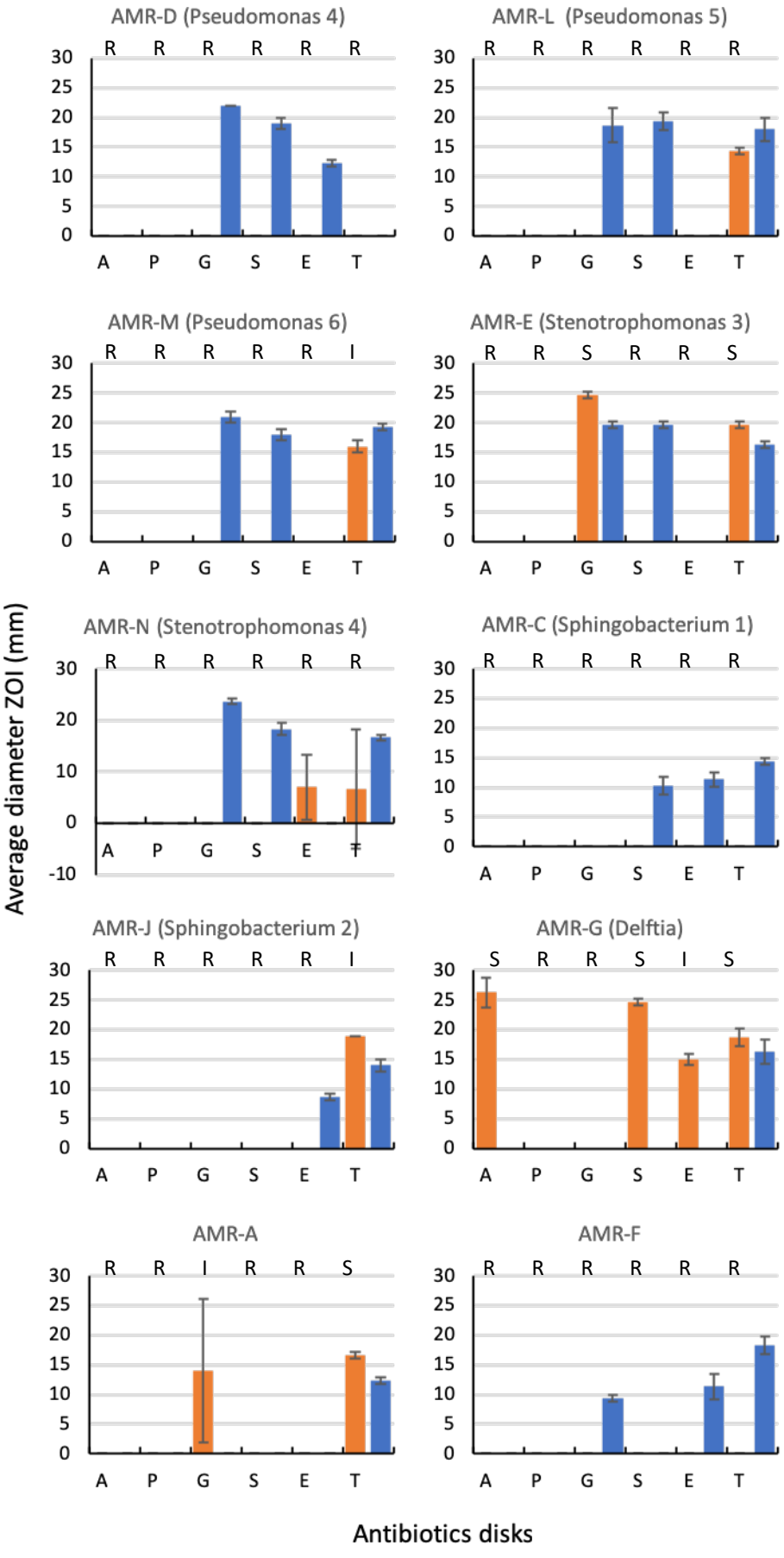
AMR-D (Pseudomonas 4)
R R R R R R
AMR-L (Pseudomonas 5)
R R R R R R
AMR-M (Pseudomonas 6)
R R R R R I
AMR-E (Stenotrophomonas 3)
R R S R R S
AMR-N (Stenotrophomonas 4)
R R R R R R
AMR-C (Sphingobacterium 1)
R R R R R R
AMR-J (Sphingobacterium 2)
R R R R R I
AMR-G (Delftia)
S R R S I S
AMR-A
R R I R R S
AMR-F
R R R R R R
A P G S E T
Average diameter ZOI (mm)
Antibiotics disks

Figure 7:3: Bacterial isolates showing AMR from the hindgut of *O. asellus*. Nutrient agar (n=3) (blue) and Mueller Hinton agar (n=3) (orange) was used to show susceptibility to antibiotics as indicated by the measurement of each zone of clearing (mm). Putative identification in brackets. Results of the Kirby Bauer indicate resistance (R), intermediate (I) and susceptible (S) to the different antibiotics; Ampicillin (A) (10 μg), Penicillin (P) (10 units), Gentamicin (G) (10 μg), Streptomycin (S) (10 μg) and Erythromycin (E) (15 μg), Tetracycline (T) (30 μg).

Half the isolates grown following the Kirby Bauer disk diffusion susceptibility test, showed resistance to all antibiotics used in this study (Fig. 7.4). AMR-2, AMR-5, AMR-D and AMR-L (presumptive *Pseudomonas*), AMR-10 and AMR-N (presumptive *Stenotrophomonas*), AMR-C (presumptive *Sphingobacterium*) and AMR-F (unidentified via sequencing) showed resistance to all antibiotics used. Some results did have a ZOI around the disk, however, when compared to the CLSI guidelines, they were still considered resistant.

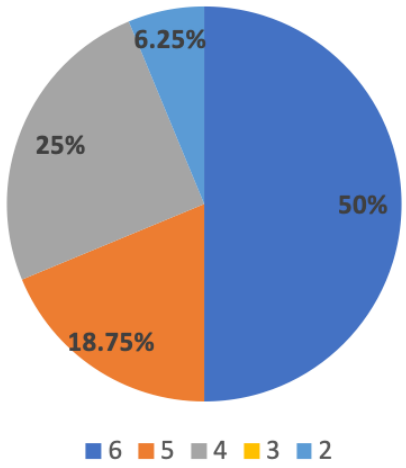

Figure 7:4: The percentage number of isolates from the GI tract of *O. asellus* indicating resistance to the number of antibiotics used.

7.4 Discussion

A culture experiment was carried out to investigate AMR in the hepatopancreas and hindgut of *O. asellus*. There is a worldwide AMR crisis and it is essential to understand the link between the environment and the clinic (WHO, 2014; Arnold *et al.*, 2016). It has been demonstrated that AMR genes and associated genetic elements disperse between environmental and human associated bacteria, supporting the hypothesis that AMR in the environment is linked to AMR in the medical setting via the mobilisation of novel resistance genes (Wellington *et al.*, 2013; Amos *et al.*, 2018).

Antimicrobial resistant bacteria colonise the hepatopancreas and hindgut of *O. asellus*, this was determined by an initial experiment using solid agar infused with single or multiple bacterial inhibitors. It was not possible to test for differences in colony counts as replicates could not be obtained due to the small weight of the woodlouse tissues, therefore tissues were pooled. Despite this, it was concluded there were more culturable bacterial colonies in the hindgut than the hepatopancreas, with the hindgut maintaining abundance more effectively with multiple antibiotics, than the hepatopancreas. Morphologically different colonies were isolated and tested for multi-drug resistance using the disk diffusion method against 6 different antibiotics. To provide a more environmentally relevant assay, nutrient agar was used. This is a general-purpose medium which supports cultivation of a wide range of non-fastidious bacteria and was incubated at 20°C in dark conditions to emulate environmental conditions. Mueller Hinton agar is a non-selective, non-differential growth medium, that contains starch to absorb toxins released from bacteria and is commonly used for antibiotic susceptibility testing (Mueller and Hinton, 1941). This is the most commonly used method in medical testing for determining antibiotic susceptibility, following the Kirby-Bauer method (Bauer *et al.*, 1966) and is low in sulfonamide, trimethoprim and tetracycline inhibitors and reproducible due to the conditions being strictly controlled (See 2.2.2.5) (Hudzicki, 2009). Unlike the assay using the strict conditions as determined by the Kirby-Bauer method, there is no guide to indicate susceptibility when resistant isolates were grown on nutrient agar. Many isolates gave ZOIs but may have been deemed resistant if an equivalent susceptibility test was applied.

Results prior to the susceptibility test being applied, showed more resistance when isolates were grown on Mueller Hinton agar at 35°C as opposed to nutrient agar at 20°C. These isolates were from woodlice that inhabited an environment where temperatures were often less than 20°C. Isolates also showed more variability in the measurement of zones of inhibition on Mueller Hinton agar at 35°C. This may have relevance to researchers examining AMR in environmental systems as the tool being used may not be useful.

Results from the disk diffusion assay showed all resistant isolates obtained from the antibiotic infused agar grew on both nutrient agar and Mueller Hinton agar. This study found the least effective form of antibiotics were β-lactams; ampicillin and penicillin which inhibit the formation of the peptidoglycan cross-link cell wall (Tomasz, 1979). Both aminoglycosides (gentamicin and streptomycin) and an inhibitor of protein synthesis; tetracycline being the most effective. Only one isolate, AMR-8 (unidentified through sequencing), grown on nutrient agar, indicated all tested pathways of protein synthesis were inhibited and the cells were lysed, inhibiting any growth. This

was in contrast to results from Mueller Hinton which indicated AMR-8 was resistant to the β-Lactams and aminoglycosides used, inhibiting cell wall synthesis and the aminoglycoside pathway of protein synthesis. When grown on nutrient agar, no species of bacteria was completely resistant to all 6 antibiotics, however AMR-A (unidentified via sequencing), AMR-G (presumptive *Delftia*) and AMR-10 (presumptive *Stenotrophomonas*) showed resistance to all antibiotics used except tetracycline. There is no official guide to susceptibility when measuring ZOIs on nutrient agar, if a similar guide of susceptibility was used, these and other isolates tested may be deemed resistant or not to all antibiotics used. When tested using the Kirby-Bauer method, AMR-2, AMR-5, AMR-D and AMR-L (presumptive *Pseudomonas*), AMR-10 and AMR-N (presumptive *Stenotrophomonas*), AMR-C (presumptive *Sphingobacterium*) and AMR-F (unidentified via sequencing) showed resistance to all antibiotic used in this assay. This resistance seems to be multifaceted with the cell able to withstand the antibiotics used in this study, inhibiting protein synthesis and cell wall synthesis.

Arthropods serve as vectors for a number of pathogens that cause human and animal diseases (Abraham *et al.*, 2017). Amongst other Arthropoda, the gills of edible snow crabs (Crustacea) demonstrated AMR from *Pseudomonas* and *Stenotrophomonas* (Kim *et al.*, 2013). Resistance amongst members of Enterobacteriaceae and other Gammaproteobacteria, including *Sphingobacterium* and *Pseudomonas*, have been found in the midguts of the gypsy moth (Insecta), also showing variation among isolates of closely related species (Allen *et al*., 2009). Collembolans (known as springtails) are microarthropods found in soil throughout the world, living alongside woodlice, culturable isolates from the GI tract were screened for inhibitory activity against pathogens. Results suggested a large proportion of microbes associated with Collembola have potential for antimicrobial production, including three *Pseudomonas* and *Stenotrophomonas* (Agamennone *et al.*, 2018).

In our study, variation in resistance profiles from bacteria from closely related species, including presumptive *Pseudomonas*, *Stenotrophomonas* and *Sphingobacterium* were observed. Some bacteria may be naturally antibiotic resistant whilst others may have differing profiles due to transference of mobile genetic elements or mutation, which are examples of how bacteria are evolving to resist the drugs that have been used to combat them. Bacteria can develop resistance either from mutations or by acquisition of antibiotic resistance genes from other bacteria in the environment through HGT (as reviewed in Martínez, 2012; Hiltunen *et al.*, 2017 and references therein). For resistance genes to be transferred from environmental to pathogenic bacteria, they need to share the same habitat, if only temporarily (Wiedenbeck and Cohan, 2011). There are many

links between human and environmental areas, allowing the movement of bacteria, mobile genetic elements and antibiotics themselves (Woolhouse *et al.*, 2015). Across all major classes of antibiotics, multiple examples of resistance genes in the soil was found to have 100% similar to ones found in clinical isolates, providing evidence for HGT between soil and pathogens (Forsberg *et al.*, 2012).

Species of *Pseudomonas*, *Stenotrophomonas*, *Sphingobacterium*, *Delftia* and three unidentified genera indicated multi-drug resistance in the GI tract of *O. asellus*. Finding high levels of AMR in the environment indicates a role for these compounds. Resistant bacteria are a natural occurrence, predating modern selective pressures of medical and agricultural use of antibiotics (D'Costa *et al.*, 2011). In fact, several studies have demonstrated resistance factors found in areas not impacted by anthropogenic antibiotics, such as bacteria in faeces of tribes untouched by 'modern civilisation' and a cave that has been isolated for over 4 million years (Gardner *et al.*, 1969; Bhullar *et al.*, 2012). The spread of resistance genes is contributed to many factors such as physical forces, animals and humans, with human activity probably increasing the prevalence of multi-drug resistant bacteria in air, soil, freshwater and marine ecosystems (Allen *et al.*, 2010; Davies and Davies, 2010; D'Costa *et al.*, 2011).

Woodlice have successfully colonised land and adapted to many different terrestrial environments. This has led to studies investigating how their adaptation has enabled them to thrive in many environments, in particular in heavy metal polluted areas (Zimmer, 2002; Lapanje *et al.*, 2007, 2008, 2010). Chemical agents, such as detergents, biocides and heavy metals, discharged into the environment can hasten the movement of resistance genes across bacterial populations by increasing selection for maintenance of the mobilome or increasing the rate of HGT (Cambray *et al.*, 2011; Gaze *et al.*, 2011). It is unsurprising that woodlice harbour AMR bacteria, as genes often co-select for resistance to heavy metals and antibiotics (Baker-Austin *et al.*, 2006). All presumptive genera found in this study; *Stenotrophomonas*, *Pseudomonas*, *Sphingobacterium* and *Delftia* show resistance to heavy metals from different environments polluted with multiple toxic metals (Malik and Jaiswal, 2000; Pages *et al.*, 2008; Karelová *et al.*, 2011; Ubalde *et al.*, 2012; Teixeira *et al.*, 2016). The most studied is *S. maltophilia* and exhibits resistance to a broad range of antibiotics, including the ones used in this study, with the mechanisms of AMR acquired via HGT through plasmids, transposons and integrons (Brooke, 2012). In addition to AMR, strains of *S. maltophilia* have genes encoding multiple metal resistance (Pages *et al.*, 2008; Holmes *et al.*, 2009). *S. maltophilia* reportedly grew to a high density in the presence of high concentrations of heavy metals and was resistant to a wide range of antibiotics, indicating overproduction of some multidrug efflux pumps

extruding antibiotics and metals (Sanchez *et al.*, 2005; Pages *et al.*, 2008). There is evidence of a physical link between antibiotic and metal resistance genes on the same plasmid. Some are efflux pumps which confer multiple resistances, whilst others are plasmids containing distinct regions of AMR and metal tolerance (Sanchez *et al.*, 2005; Teixeira *et al.*, 2016). These observations of metal resistance in environmental isolates suggest that similar to the acquisition of antimicrobial drug resistance, the acquisition of metal resistance also occurs in the natural environment.

Our study shows AMR bacteria residing in GI tract of woodlice. As these are highly mobile invertebrates, colonising many environments that are known to be colonised with bacteria with AMR genes, it is plausible to suggest they are involved in the spread of these genes. This dispersal may contribute to the spread of AMR genes from the environment to human bacterial flora.

8 General Discussion

The central aim of this thesis was to characterise the microbial communities within the GI tract of organisms that have a diet rich in lignocellulose. This study was the first to establish the mycobiota of the different areas of the GI tract of *P. nigrolineatus.* It was also the first to investigate the gut mycobiota and bacterial microbiota of two species of woodlice, in our study *O. asellus* and *P. scaber* were investigated. Additionally, no other study has compared the microbiome of the hepatopancreas and hindgut of the same individual woodlouse. As well as culture-independent methods, culture-dependent methods were used to compare the results gained from both methods. Woodlice inhabit diverse environments and it has been reported that the bacterial communities within the gut adapt to an environment with heavy metal pollution (Lapanje *et al.*, 2008, 2010). With this in mind and the co-selection of heavy metals resistance genes and AMR genes, the digestive tract of *O. asellus* was investigated for AMR (Huijbers *et al.*, 2015).

8.1 Methodological Considerations

The study of gut microbiota continues to be a challenge for microbiologists. The limitations of culture methods have restricted scientists to investigate only a very small fraction of the many microbial species present. The results gained from NGS technology provides a snapshot of what microbial sequences are in a sample, which can then be assigned a species from public databases. Many factors can affect the results, including the DNA extraction method (Krsek and Wellington, 1999; McOrist *et al.*, 2002; Fredricks *et al.*, 2005), the chosen primer sets (Anderson *et al.*, 2003; Baker *et al.*, 2003; Wang and Qian, 2009; Ihrmark *et al.*, 2012), the PCR itself (Fonseca *et al.*, 2012; Sergeant *et al.*, 2012; Wang *et al.*, 2015) and the quality of databases (Nilsson *et al.*, 2006; Edgar *et al.*, 2011; Wang *et al.*, 2015; Meyer *et al.*, 2016). Previous studies into DNA extraction methods for bacteria and fungi concluded no single extraction method was optimal for all organisms as each produces markedly different results, thus affecting downstream PCR success. Recovery of DNA from fungi can be particularly difficult as cell lysis can be impeded due to their cell walls (Fredricks *et al.*, 2005). Biases can be introduced at all steps in the process affecting the outcome of the community analysis.

To examine community structure and diversity in this study, the bacterial 16S rRNA gene and the fungal ITS rDNA region were used. Primers used to amplify this gene and region were chosen based on the literature. NGS technologies enable deep coverage of samples, however one limitation is the length of sequence produced. The entire 16S rRNA gene is not sequenced, therefore, a short region

within the gene is amplified, this is reported to an unreliable representation of bacterial communities (Mizrahi-Man *et al.*, 2013). With the current read length of twice 300 bp, the V3-V4 region of the rRNA gene, used in this study, presents the optimal target for sequencing, providing a sufficient overlap of forward and reverse paired-end reads (Mizrahi-Man *et al.*, 2013). Assembling these paired-end reads increases the quality and confidence in the overlapping region (Gloor *et al.*, 2010).

Amplifying the entire fungal ITS region (including the 5.8S region) may be more phylogenetically informative, but may also artificially reduce microbial richness and bias community structure (Huber *et al.*, 2009). In addition, the incidence of chimeric sequences also increases due to the conservation of the 5.8S region and less likely to occur when amplifying one region (Fonseca *et al.*, 2012). Amplifying either the ITS1 or ITS2 region rather than the whole region, can lead to higher PCR efficiencies with diversity and community structure better preserved (Ihrmark *et al.*, 2012). To study the fungal communities in *P. nigrolineatus*, both the ITS1 and ITS2 rDNA regions were amplified separately to compare results. It has been debated whether ITS1 or ITS2 provides better taxonomic resolution with results from other studies being varied. Some studies have reported that although each region has advantages and disadvantages, producing similar results, with any differences being caused by primer biases (Nilsson *et al.*, 2008; Blaalid *et al.*, 2013). Other studies concluded one region was more effective than the other, however, most studies used different sets of primers than the ones used in this study (Lindahl *et al.*, 2013; Li *et al.*, 2014; Halwachs *et al.*, 2017). Results from *P. nigrolineatus* concluded that the ITS2 region was a more suitable barcode marker on this occasion and gave better results at lower taxonomic levels. Based on the literature and results from the previous study, the ITS2 region was used to study fungal diversity in *O. asellus* and *P. scaber*, however, using the additional ITS1 region may have revealed more sequences, not revealed by amplifying the ITS2 region.

The studies into *P. nigrolineatus, O. asellus and P. scaber* used the same primer set to amplify the ITS2 rDNA region. The same primer set was used to amplify the V3-V4 region of the 16S rRNA gene from *O. asellus* and *P. scaber*. The poor results obtained from the fungal ITS1 region could be due to the sample type or biases and limitations of the primer sets (Fonseca *et al.*, 2012; Ihrmark *et al.*, 2012; Wang *et al.*, 2015). To limit PCR bias, it has been suggested, when using high-throughput sequencing of environmental samples, either different parts of the ITS region should be analysed or using additional sets of primer combinations (Bellemain *et al.*, 2010; Ihrmark *et al.*, 2012). In fact, both sets of primers used for amplifying ITS1 and ITS2 are biased from certain groups, primers used for amplifying ITS2 are reported to be biased for Dikarya, as non-Dikarya lineages lack information

on their diversity (Lindahl *et al.*, 2013). This primer bias is also applicable to bacterial 16S rRNA gene-based analysis (Bellemain *et al.*, 2010). The primer fITS7 used for amplifying the ITS2 region has been found to exclude most *Penicillium* species, as well as some *Orbiliales* and *Mucorales* species (Ihrmark *et al.*, 2012). More recently, newly designed primers have been developed targeting the ITS1 region *in silico*, showing an average taxonomic coverage of 80% compared to 45% using previous published primers, recovering an average of 22,000 reads from fungal isolate culture samples, compared to previously published primers averaging at 3,300 (Usyk *et al.*, 2017). Research into more effective fungal primers is ongoing.

As new sequencing technologies become more advanced and cost effective, these technologies have been applied to numerous environments to investigate microbial diversity, often revealing dynamics of rare taxa (Sinclair *et al.*, 2015). This study used the MiSeq Illumina platform. However, all methods used are not without issues, such as sequencing errors and base miscalling together with PCR errors, chimera formation and pseudogenes introducing noise, therefore, leading to biased estimates of diversity and abundance (Sinclair *et al.*, 2015).

Data analysis from NGS technologies typically assigns sequences to OTUs. The workflow of amplicon data has four main steps; pre-processing, OTU picking, taxonomic classification and visualisation and statistical analysis. Microbial community data analysis can produce different results, dependant on the effectiveness of the programs used. Microbiota analysis tools have been developed mainly for targeting bacteria, such as the 16S rRNA gene and can often be inadequate if applied to fungal investigations, such as ITS regions (Halwachs *et al.*, 2017). The QIIME package is a popular diversity analysis program, however, if used for every step of the analysis, it has been shown to greatly exaggerate bacterial species richness and between samples diversity, with mock communities from Illumina reads giving 56% to 88% of predicted genus names being false positives (Edgar, 2017). The workflow used here was not limited to one program but used several, with Mothur being used for data pre-processing and the recommendation to switch tools to QIIME for further diversity analysis was employed (Halwachs *et al.*, 2017).

OTUs are clustered together based on their similarity to other sequences in the community (Schloss and Westcott, 2011). OTUs have been defined with a ≥97% nucleotide identity cut off, with the remaining 3% scattered among the hypervariable regions (Stackebrandt and Goebel, 1994). Whilst ≥97% is considered the accepted level for defining an OTU, there are various caveats to consider. Some species have rRNA/rDNA genes that share a ≥97% identity resulting in OTUs representing multiple species. Artefacts from chimeras or read errors can result in false identification of OTUs,

and single species can have multiple copies of the 16S rRNA gene/ITS rDNA region that are below the threshold, causing more than one representative OTU (Schloss and Westcott, 2011; Větrovský and Baldrian, 2013). This can lead to over inflation of diversity, although some errors can be corrected by quality filtering tools (Edgar *et al.*, 2011; Edgar, 2013).

Analysing fungal community roles and identities in a novel environment is challenging due to the paucity of literature and lack of available sequences. Compared to bacteria sequences on databases, the quality of fungal sequence databases are underdeveloped and highly variable, often lacking proper annotation and lineage designations (Nilsson *et al.*, 2006; Chen *et al.*, 2015; Tang *et al.*, 2015; Meyer *et al.*, 2016). Species identification relies on comparison of DNA sequences to those known in a database. Bacterial databases are more established than fungal and the quality of datasets of fungal sequences is highly variable, with comparisons limited. With the advance in technology giving higher quality and better assembly of genome sequences, automated annotation, which is dependent on existing annotated genomes as a reference, gives rise to poor annotation and propagation of errors as fungi lack well-supported and verified annotations, with up to 20% of fungal DNA sequences having inaccurate lineage designations (Nilsson *et al.*, 2006; Meyer *et al.*, 2016). The genome of a single serotype may not reflect the diversity of the species as over time one strain may have lost its natural characteristics (Meyer *et al.*, 2016). The International Nucleotide Sequence Databases Collaboration (INSDC) has been the most comprehensive nucleotide data resource, having free and unrestricted access to its data (Nakamura *et al.*, 2016), which can be problematic as the quality of sequences are unknown from public databases (Wang *et al.*, 2015). There is a vast difference between genomes recorded for bacteria and fungi; the NCBI database holds 181,538 shotgun sequences for bacterial genomes and 4,070 fungal (NCBI 08/01/2019). This difference shows the lack of research conducted on fungi making identification more difficult and this has been demonstrated in this project with many sequences being similar to those of higher taxonomic levels.

Samples were sent to a third party for sequencing and bioinformatics analysis. A major issue in this study became apparent with the diversity analysis carried out using QIIME and preliminary results showed OTU instability (He *et al.*, 2015). OTU clustering can cause OTU instability, where sequences are assigned to different OTUs based on their differing similarity, if unnoticed this can inflate diversity and the identification of represented OTUs (He *et al.*, 2015). This instability can vary depending on the clustering technique used. Closed-reference clustering produces completely stable OTUs, however sequences that do not match a pre-existing reference sequence collection are excluded, making novel OTUs impossible to analyse (He *et al.*, 2015). In our study, over inflation

of diversity was more pronounced with bacterial sequences than fungal sequences. This could be due to the higher volume of sequences generated from amplifying the 16S rRNA gene than the ITS rDNA region, resulting in more unique OTUs being generated but being assigned the same consensus lineage. From the initial analysis carried out by LGC, bacterial diversity in the hepatopancreas and hindgut were clustered into 1277 OTUs and 2802 OTUs respectively. After our own analysis, diversity was reduced to 229 OTUs and 356 OTUs respectively. Therefore, sequences with the same consensus lineage were clustered together into one and re-labelled and diversity analysis was carried out on the re-clustered OTU data.

A limitation of 16S rRNA gene sequencing is the difficulty to assign OTU taxonomy further than genus level, therefore, the functions of observed OTUs could be difficult to assign and predict. If the observed OTUs are phenotypically and genotypically similar to a closest bacterial relative, it could be assumed have similar traits and functions. Fungal OTUs can be classified to sequences similar to species from databases, however, as seen in *O. asellus* and *P. scaber*, between 80% and 92% in the hepatopancreas and in the hindgut, between 50% and 95% of OTUs were unclassified due to the low levels of fungal classification.

8.2 Suggestions for Future Work

Although all initial aims in this study were completed, in many cases this research raised additional questions. The structure and function of the filters between the foregut and hepatopancreas, needs further investigation as it was reported they filter out particles, including large bacteria and fungi, however, our research indicated a resident fungal community in the hepatopancreas.

The hindgut and the presence of bacteria and fungi within it, is another area of interest and needs to be further investigated. It has long been reported the hindgut cuticle sheds with the posterior exoskeleton, making the microbial communities in the hindgut transient. The discovery of *Bacilloplasma* attachment to the hindgut wall, put doubt onto this theory, although the paper did make some plausible conclusions for this resident bacterium, such as re-ingestion of the cuticle (Kostanjšek *et al.*, 2007). Additional molecular research could be carried out on the hindgut tissues immediately after the shedding of the posterior exoskeleton together with the hypothesised hindgut cuticle. This may give an insight into the number of sequences obtained and any microbial communities present, pre-shedding and post-shedding. Theoretically, the number of sequences would be greatly reduced if the hindgut cuticle did shed.

The bacterial communities within the hepatopancreas and hindgut were very different, indicating the bacteria was located within the lumen of the tissues and not on the outside of the digestive tract, which could lead to contamination of samples. An attempt to clarify this was made using FISH, however, due to time constraints, this part of the experiment ceased. Continuation of this experiment would answer to some of the questions that did arise. It would confirm the location of the microbial communities, the hindgut is divided into two areas, the anterior region and the papillate region, which is reported to be more densely colonised with bacteria, than the anterior region. Additionally, any attachment of microbes to the hindgut cuticle could be confirmed. This could also give insight into the positioning of *Hepatoplasma* and other bacteria inhabiting the hepatopancreas and may indicate any inhibition from *Hepatoplasma*. As this study is looking at wood degradation, the location of bacteria and fungi could be observed and relationships between them may be inferred.

The advent of NGS has given major advances in our understanding of microbial ecology. NGS studies use universal marker genes and cannot directly identify metabolic or other functional capabilities of the communities present (Langille *et al.*, 2013). Having an insight into the microbial communities present in an environment gives valuable information, however, predicting their functional composition would add more value to these results. Colleagues working with *P. nigrolineatus* have already carried out this analysis on the bacterial community's metagenome from its 16S profile using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Langille et al. 2013; McDonald et al. manuscript in preparation). However, the same analysis carried out from the 16S results obtained from NGS in our study into *O. asellus* and *P. scaber* would give an indication of what metabolic or other functional capabilities the bacterial community was having, particularly in respect of wood degradation. Unfortunately, to date, there is not a similar analysis tool for examining fungal communities.

Many of the fungal sequences within the different areas of the GI tract of *P. nigrolineatus, O. asellus* and *P. scaber* were classified as sequences similar to higher taxonomic levels. Phylogenetic trees could be utilised using known fungal sequences of higher taxonomic levels to predict the unknown sequences may be compared to the other known sequences to predict their close relatives. This could allow for prediction of unknown sequences, for example, many sequences in the woodlouse data set were unclassified Sordariomycetes, with some Sordariomycetes species known to infect arthropods (Sung *et al.*, 2007; Johnson *et al.*, 2009) or other species involved in decomposition and nutrient cycling (Zhang *et al.*, 2006). Analysing these sequences, may give insights into their inferred function based on the hypothesised function of their closest relative.

The purpose of our studies was to characterise the microbial communities within the GI tract of wood-eating organisms, after it was hypothesised that both bacteria and fungi have a symbiotic role in the breakdown of lignocellulose substrates. To further investigate this hypothesis, a microcosm symbiosis experiment could be set up using wood blocks and sawdust to investigate wood degradation when the microcosm was inoculated with bacteria or fungi or bacteria and fungi isolated from the GI tract of woodlice.

The preliminary results in our study confirmed the presence of bacteria having AMR to one or multiple antibiotics. Culture-independent methods, such as NGS, could provide more comprehensive information about the extent of AMR bacteria in the digestive tract of woodlice using primers targeting ARGs.

8.3 Conclusions

As discussed, there are limitations to the methodologies used, however, there is no conclusive way, as of yet, to identify all microbial sequences within an environment, therefore, this method gives a snapshot of what species are there. Despite these limitations, the advanced technologies of NGS enables researchers to examine the microbial communities in any environment, irrespective of the environmental conditions needed for successful culturing. Studies into organisms that imbibe a diet rich in cellulose, to date, including the ones used in our study, have examined the bacterial microbiota within the GI tract and the symbiotic relationship with each other and the host. As fungi are major wood degraders (Boddy and Watkinson, 1995; de Boer *et al.*, 2005; Kavanagh, 2011; Voříškova and Baldrian, 2013; Haitjema *et al.*, 2014; Chandra *et al.*, 2015; Johnston *et al.*, 2016), it needs to be questioned whether fungi detected in the GI tract of animals contribute in the breakdown of lignocellulose. In fact, when studying dead wood, the fungal communities have received more attention than bacteria in the same habitat (Johnston *et al.*, 2016). Therefore, examining the fungal communities in the GI tract of wood-eating organisms gives new insights into investigating how organisms are able to survive on a diet rich in lignocellulose.

The first in-depth study into wood-feeding cockroaches gut bacterial microbiota was found to be diverse and composed of obligate anaerobic bacteria dominated by Bacteroidetes and Firmicutes, with the addition of Proteobacteria in the midgut (Bauer *et al.*, 2014). There was presence of bacteria from other host groups, such as termites, mammals and fish suggesting bacteria have adapted to intestinal habitats (Bauer *et al.*, 2014). Also, a study on the herbivorous giant panda that has a diet typical rich in cellulose but the gut bacterial microbiota has been found to resemble that

of a carnivore, with Firmicutes and Proteobacteria dominating the gut and lacked cellulolytic bacteria, questioning the effectiveness of cellulose utilisation (Xue *et al.*, 2015). This study concluded 'the giant panda appears not to have evolved a gut microbiota compatible with its newly adopted diet, which may adversely influence the coevolutionary fitness of this herbivore', however, if the fungal communities were investigated, this may give more rounded information about their evolution to eat their diet rich in cellulose.

This is the first study into the fungal communities within the GI tract of wood-eating *P. nigrolineatus*. It has been previously reported there is a resident bacterial community in the GI tract of *P. nigrolineatus*, with phylotypes similar to cellulose degrading and nitrogen fixing bacteria found (McDonald *et al.*, 2012, 2015; Di Maiuta *et al.*, 2013; Watts *et al.*, 2013; Marden *et al.*, 2017). Our results are the first to demonstrate a resident fungal community in the GI tract of *P. nigrolineatus* with the most dominant fungi; *F. oxysporum* previously been shown to excrete enodocellulases, exocellulases and β-glucosidase (Alconada and Martinez, 1996; Marden *et al.*, 2017). It was unfortunate, in this environment, many ITS rDNA sequences could not be identified to lower taxonomic levels, hindering conclusions into any roles they may play in wood degradation. It is accepted that the gut microbiota has a tight, coordinated connection with a variety of functions of its host, such as metabolism, immunity, energy utilisation and health maintenance (Stevens and Hume, 1998; Nicholson *et al.*, 2005; Velagapudi *et al.*, 2010; Bäckhed, 2011; Xue *et al.*, 2015). These results pave the way for further research into the relationship of bacteria and fungi in wood degradation and whether the fungal communities present in the GI tract also have an effect on the various functions and health of its host, in a similar way to the role of the bacterial community.

Our research is the first to subject two species of woodlice to the same conditions to make a comparison in bacterial communities present. It is also the first study to sequence the microbial metagenome from the hepatopancreas and hindgut from the same individual. Prior to 2004, researchers were aware that the hepatopancreas was densely colonised by bacteria, as visualised by microscopy work, however, attempts to culture them were unsuccessful (Wood and Griffiths, 1988; Hames and Hopkin, 1989; Zimmer and Topp, 1998b; Zimmer, 1999). A recent study examined the microbiota in the tissues of another woodlouse species, *A. vulgare,* using 454 amplicon sequencing and found sequences similar to *Hepatoplasma*, *Shewanella*, *Rickettsiella*, *Halomonas*, *Wolbachia* and *Hepatincola* dominating (Dittmer *et al.*, 2016). In our study the hepatopancreas was dominated by sequences similar to *Hepatoplasma*, *Rickettsiella* and *Hepatincola*, sequences similar to *Halomonas* were found in *P. scaber* in less than 1% abundance and sequences similar to *Shewanella* was absent. Other research into the bacteria in the hepatopancreas have been very

focused, investigating the phylogenetic affiliation, diversity, location and comparison of the, to date, unculturable symbionts via cloning and sequencing the 16S rRNA genes in *P. scaber* (Wang *et al.*, 2004a; Wang *et al.*, 2004b; Fraune and Zimmer, 2008). Our research indicates sequences similar to *Hepatoplasma* to be the most abundant symbiont, dominating 4 out of 5 individuals, with the other individual being dominated by *Hepatincola*. Although, as previously mentioned, the bacterial metagenome of *A. vulgare* hepatopancreas has been investigated (Dittmer *et al.*, 2016), no study has sequenced the bacterial metagenome in the hepatopancreas of two species of woodlice in the same study. This study gives valuable insights into what bacteria colonise the hepatopancreas. Further research is needed to investigate the role of *Hepatoplasma* and the function of its dense colonisation, appearing to outcompete many other species of bacteria.

Other studies examining the hepatopancreas and hindgut have focused on one bacterium *Rickettsiella*, *Wolbachia*, *Candidatus Rhabdochlamydia porcellionis* or *Candidatus Rickettsiella isopodorum*, rather than the entire bacterial community (Abd El-Aal and Holdich, 1987; Kostanjšek *et al.*, 2004; Sixt *et al.*, 2013; Dittmer *et al.*, 2014; Kleespies *et al.*, 2014; Kostanjšek and Marolt, 2015). Our study found sequences similar to *Rickettsiella* in both *O. asellus* and *P. scaber*, dominating 3 out of 5 individual *O. asellus* but was not so prevalent in *P. scaber*. Sequences similar to *Wolbachia* was present in 3 out of 5 *O. asellus* individuals but was not *P. scaber*. It has been postulated that the most dominant bacterium found in the hepatopancreas, *Hepatoplasma*, is unable to colonise the same environment as pathogenic *Hepatincola* (Wang *et al.*, 2007). However, our study found *Hepatoplasma* dominating 8/10 of samples and one sample dominated by *Hepatincola* (68% abundance), this sample had sequences similar to *Rickettsiella* (32%) and *Hepatoplasma* (0.1%), therefore indicating they are not mutually exclusive, but they could outcompete each other depending on the health of the individual woodlouse. Whilst investigating an individual pathogenic bacterium gives some valuable information into the bacterium itself and its effect on the individual woodlouse, by examining the bacterial metagenome of each part of the digestive tract, it can be observed which other species are able to reside alongside these pathogenic bacteria and which are not.

The hindgut of the woodlouse has not been the focus of attention, due to other research reporting the frequent removal of the cuticular lining along with residual material during the ecdysis (Hartenstein, 1964; Hassall and Jennings, 1975; Hames and Hopkin, 1989; Kostanjšek *et al.*, 2007; Mrak *et al.*, 2015). This study is the first to sequence the bacterial community in the hindgut of two species of woodlice. Despite the reported frequent removal of the cuticular lining, our study, suggests a resident bacterial community in the hindgut of *O. asellus* and *P. scaber* and concluded

more research needs to be done to substantiate the shedding of the cuticular lining. Furthermore, a study into *Bacilloplasma* attachment to the hindgut in *P. scaber*, has previously indicated the possibility of a bacterium to persist in the digestive tract for longer periods, therefore, it is plausible that other species of bacteria could persist (Kostanjšek *et al.*, 2007). Previously, the bacterial communities within the hindgut of *P. scaber* have been investigated via PCR and cloning, however, many sequences in that study did not meet the ≥97% sequence identity threshold, sequences that did, were similar to *Enterococcus* and *Neisseria* (Kostanjšek *et al.*, 2002). *Enterococcus* were found in our study but in less than 1% abundance. The microbiota in the hindgut of another woodlouse species, *A. vulgare* was examined via 454 amplicon sequencing, which found sequences similar to *Wolbachia*, *Halomonas*, *Rickettsiella*, *Pseudomonas*, *Hepatoplasma*, *Shewanella*, *Bacilloplasma* and *Hepatincola* dominating (Dittmer *et al.*, 2016). Our study detected all these sequences at greater than 1% abundance, except *Halomonas* which was found in less than 1% abundance and *Shewanella* which was not detected. The similar results of Dittmer *et al.* (2016) and our study indicates a resident microbial community within the hindgut of woodlice. Previous studies have been more focused on one bacterium or environment (Kostanjšek *et al.*, 2007; Lapanje *et al.*, 2007, 2008, 2010). As aforementioned, *Bacilloplasma* has been associated with the gut lining of the hindgut in *P. scaber* (Kostanjšek *et al.*, 2007). Our study found sequences similar to this bacterium in the hindgut of both *O. asellus* and *P. scaber*.

This is the first study to investigate the fungal communities in the hepatopancreas and hindgut of any woodlouse species. Whilst the bacterial community had limited diversity the hepatopancreas was found to harbour a more diverse fungal community. Our study indicated a resident fungal community, although unlike the bacterial communities, the fungal diversity within each individual varied. Our results highlight the need for further investigation into the presence of the filter, that reportedly prevented particles, such as, fungi from entering and colonising the hepatopancreas (Storch, 1987; Wood and Griffiths, 1988; Hames and Hopkin, 1989). Our study indicates other factors involved that prevent the colonisation of a diverse microbial community and further research is needed to substantiate the role of the filter.

Our results into the fungal communities in the hindgut of woodlice, indicates a resident fungal community that was more diverse than those found in the hepatopancreas. Similar to the results of the hepatopancreas, diversity was varied between samples. Further research into the shedding of the cuticular lining of the hindgut and investigating the attachment of fungi to the gut wall may give an insight into whether fungi can persist, similar to the bacterium, *Bacilloplasma*.

These results highlight the need for fungal communities to be investigated in other organisms to get more complete and balanced information about the absence and presence of microorganisms. This may give insights into whether the bacterial and fungal communities affect themselves, each other or the host and whether any interaction is mutual, commensal or pathogenic. The relationship between bacteria and fungi in decaying wood is well documented. Fungi in decomposing wood are hypothesised to play a role in shaping the bacterial communities in wood. Competitive and antagonistic interactions between bacteria and Basidiomycetes have already been described, however, not in such a niche environment as the ones investigated in our study (de Boer *et al.*, 2005; de Boer and van der Wal, 2008). A recent commentary has highlighted the hidden fungal diversity in many environments, with fungi reported to be important for their symbiotic interactions with all groups of organisms (Blackwell and Vega, 2018).

Bacterial communities were detected in the guts of woodlice adapted to environments polluted with heavy metals. As heavy metal resistance genes and AMR genes often co-select (Baker-Austin *et al.*, 2006), preliminary AMR investigations were carried out as part of our study. As woodlice are highly mobile, inhabiting many diverse environments, including areas that are often contaminated with antibiotics from human and animal sources, it was unsurprising that AMR bacteria were found to inhabit the digestive tract of *O. asellus*. This part of the research used culture-dependant methods for isolation which as discussed previously, only allows identification for isolates that are easily culturable under the conditions used. Due to the interplay between environmental bacteria and human pathogens through various anthropogenic practices, studying AMR in the environment is important in examining its transmission to humans.

9 Appendices

TOWSON UNIVERSITY ANIMAL CARE AND USE FORM

1. Dates of Study: ***08/01/2009-7/31/2012***

2. Title: ***Microbial Processes Facilitating Xylophagy in a Loricariid***

3. Principal Investigator: [redacted]

3a Briefly summarize qualifications and experience of the PI and personnel working on the protocol.

All animal procedures will be performed by co-PI [redacted]. [redacted] has 28 years experience working with fish and their anesthetization. He has also performed research on the effects of anesthesia on fish. Nelson has twelve years of surgical experience with fish and has researched loricariid catfish biology for about 6 years.

4. Animal Use:

   1. Species/Strain/Age/Sex: ***Royal Plecostomus: Panaque nigrolineatus/unkown/both sexes***

   2. Source: ***Aquarium wholesalers and stores***

   3. Total Number required: ***100***

   4. Average stay in animal facilities: ***6 months***

   5. Animal use for***:*** ***X*** Research _____ Training/Teaching

5. On an attached sheet, provide a description of the project. In addition, briefly describe the experimental manipulations that will be performed with the animals.

   See attached

6. On an attached sheet, describe the scientific rationale for use of animals, electing the animal model, and for the number of animals required. Provide written assurance that the proposed activities do not unnecessarily duplicate previous experiments.

See attached

7. Would the procedures described above cause pain or distress to the animals if anesthetics or analgesics were not used? ***Yes***

If yes, check the appropriate statement (s):

___X___ Anesthetics or analgesics will be used in the course of the experiment. List drugs to be used, dosages, routes and frequency of administration.

-3-aminobenzoic acid, ethyl ester (MS-222), trans cutaneous; water concentration of 200 mg/L before sampling. All handlers will wear gloves.

8. List euthanasia techniques (drugs, dose, route of administration).

-3-aminobenzoic acid, ethyl ester (MS-222), trans cutaneous; water concentration of 200 mg/L before sampling. All handlers will wear gloves.

9. Please provide a written summary of the literature search regarding alternatives to animal use and painful procedures, consulting at least two data bases (ex. Animal Welfare Information Center, Medline). ***This applies to all species covered by the Animal Welfare Act (excludes laboratory mice, laboratory rats, and birds).***

I certify that I am aware that the principal investigator is responsible for all aspects of her/his research or teaching protocol, including the humane treatment of animals. I understand that compliance with the policies for the humane treatment of animals is a prerequisite for purchasing and housing animals, and for the use of animals at Towson University. I assure that the proposed use of animals does not unnecessarily duplicate previous research or teaching.

_____ _____

Date **Principal Investigator**

IACUC approval page 2.

5.) PROJECT DESCRIPTION

Cellulose, the major constituent of wood is the most abundant biological compound on Earth, however, xylophagy (wood-eating) is a relatively rare behavior in animals. Preliminary studies have determined that Panaque nigrolineatus can survive on a wood only diet and that the microbial community is providing enzymes that facilitate this xylophagus lifestyle. The main goals of this proposal are to identify and characterize the microbial consortia responsible for digestion of recalcitrant carbon polymers and nitrogen fixation allowing P. nigrolineatus to flourish in the environment with a xylophagus lifestyle.

To investigate the microbial community present in the fish total community DNA will be extracted from the GI tract and subjected to a number of molecular tools. DGGE will provide a rapid microbial community screening for the different regions of the digestive tract. Universal 16S rRNA clone libraries will also be constructed from different regions of the digestive tract providing a detailed phylogeny of the phylotypes present in the different regions. To determine functional activity in the digestive tract a metagenomic library will be created from the digestive tract and screened for specific genes for cellulose degradation and nitrogen metabolism. Microbial enrichment cultures will also be created using ^{13}C-cellulose and DNA-SIP-DGGE will be performed to link individuals with cellulose degradation.

All P. nigrolineatus will be sacrificed by an overdose of anesthetic before sampling their gastrointestinal tracts for microbial flora. Some P. nigrolineatus will be held in Smith 363 for a variable period of time, individually housed in 10-gallon aquaria at 28ºC and fed varying diets.

6) SCIENTIFIC RATIONALE

The Neotropical catfish family Loricariidae is a vertebrate success story about which we know little. With over 600 described species (and many additional undescribed) this family evolved entirely after the Cretaceous separation of South America from Africa and accounts for between 1 and 2% of all current vertebrate diversity. Unlike other speciose fish families that usually exploit multiple trophic niches, virtually all loricariids are herbivores and eat near the bottom of the food chain. Field collections and laboratory experiments suggest that some loricariid catfishes are even utilizing wood in their diet. This is the first and only documentation of wood-eating (xylophagy) in the more than 25,000 species of bony fish. These observations alone justify undertaking more detailed studies of the biology of loricariids. However, all cellulolytic organisms thus far cultured from loricariids are either aerobic or facultative anaerobes, and all loricariids that have been tested can breathe air as well as water. Thus it is possible that loricariids are the only vertebrates to be digesting cellulose under aerobic conditions that would entail novel biochemical pathways. Unfortunately, little is currently known about the gut microbiology of loricariids, especially in situ.

The experiments do not duplicate any previous work; there is some previous work on microbiology in loricariids (e.g. Nelson et al. 1999) and there is work on digestive processing in loricariids (e.g. Arua Lima et al. 1986 and Nelson et al. 1999), but there have been no molecular investigations of microbiology of loricariid guts.

Long-term dietary experiments to be performed the US with 50 fish obtained from aquarium dealers, twice. Three fish will be sacrificed before dietary treatment to establish a baseline. The remaining fish will be randomly split into dietary groups and anti-biotic treatments and kept in individual tanks with aeration and shelter. Feces collection, food consumption and growth will be charted over a period of 6 months, after which the animals will be sacrificed by an overdose of anesthetic. The requested 100 animals provides for repetition of the experiment, if needed.

Araujo-Lima C. A. R. M., Forsberg, B. R., Victoria, R., & Martinelli, L. 1986. Energy sources for Detritivorous fishes in the Amazon. Science 234: 1256-1258.

Nelson, J.A., M.E. Whitmer, E.A. Johnson, D. Wubah and D.J. Stewart. 1999. Wood-eating Catfishes of the genus Panaque (Eigenmann and Eigenmann): Gut microflora and Enzyme Activities. Journal of Fish Biology 54: 1069-1082.

9)ALTERNATIVES

Panaque nigrolineatus is the most accessible and available member of the only proven xylophagous genus of fish. There are no alternatives to sampling them or their congener Panaque maccus, also available from the aquarium trade, if we are to understand the microbial contribution to xylophagy in freshwater fishes.

FORM UPR16

Research Ethics Review Checklist

UNIVERSITY OF PORTSMOUTH

Please include this completed form as an appendix to your thesis (see the Research Degrees Operational Handbook for more information

Postgraduate Research Student (PGRS) Information			**Student ID:**	119279
PGRS Name:	Caroline Marden			
Department:	Biology	**First Supervisor:**	Joy Watts	
Start Date: (or progression date for Prof Doc students)		**February 2015**		
Study Mode and Route:	Part-time ☒ Full-time ☐	MPhil ☐ PhD ☒	MD ☐ Professional Doctorate ☐	

Title of Thesis:	Characterisation of the microbial communities within the gastrointestinal tract of wood-eating organisms
Thesis Word Count: (excluding ancillary data)	36,904

If you are unsure about any of the following, please contact the local representative on your Faculty Ethics Committee for advice. Please note that it is your responsibility to follow the University's Ethics Policy and any relevant University, academic or professional guidelines in the conduct of your study

Although the Ethics Committee may have given your study a favourable opinion, the final responsibility for the ethical conduct of this work lies with the researcher(s).

UKRIO Finished Research Checklist:
(If you would like to know more about the checklist, please see your Faculty or Departmental Ethics Committee rep or see the online version of the full checklist at: http://www.ukrio.org/what-we-do/code-of-practice-for-research/)

a) Have all of your research and findings been reported accurately, honestly and within a reasonable time frame?	YES ☒ NO ☐
b) Have all contributions to knowledge been acknowledged?	YES ☒ NO ☐
c) Have you complied with all agreements relating to intellectual property, publication and authorship?	YES ☒ NO ☐
d) Has your research data been retained in a secure and accessible form and will it remain so for the required duration?	YES ☒ NO ☐
e) Does your research comply with all legal, ethical, and contractual requirements?	YES ☒ NO ☐

Candidate Statement:

I have considered the ethical dimensions of the above named research project, and have successfully obtained the necessary ethical approval(s)

Ethical review number(s) from Faculty Ethics Committee (or from NRES/SCREC):	071509JW-01

If you have *not* submitted your work for ethical review, and/or you have answered 'No' to one or more of questions a) to e), please explain below why this is so:

Signed *(PGRS)*:	C. Marden	**Date:** 19/03/19

UPR16 – April 2018

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Research article

Investigation into the fungal diversity within different regions of the gastrointestinal tract of *Panaque nigrolineatus*, a wood-eating fish

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Abstract: The Amazonian catfish, *Panaque nigrolineatus* have several physiological adaptions enabling the scraping and consumption of wood (xylivory), facilitating a detritivorous dietary strategy. Composed of lignocellulose, wood is a difficult substrate to degrade and as yet, it is unclear whether the fish obtains any direct nutritional benefits from wood ingestion and degradation. However, there are numerous systems that rely on microbial symbioses to provide energy and other nutritional benefits for host organisms via lignocellulose decomposition. While previous studies on the microbial community of *P. nigrolineatus* have focused upon the bacterial population, the role of fungi in lignocellulose degradation in the fish has not yet been examined. This study describes the detection of fungi within the fish gastrointestinal tract. Using next generation sequencing, the effects of diet on enteric fungal populations were examined in each gastrointestinal tract region. Fungal species were found to vary in different regions of the gastrointestinal tract as a function of diet. This study is the first to examine the fungal community in a xylivorous fish and results support the hypothesis that diet influences fungal distribution and diversity within the gastrointestinal tract of *P. nigrolineatus*.

Keywords: fungi; microbiome; symbiosis; fish; *Panaque nigrolineatus*; gastrointestinal tract; wood; lignocellulose

1. Introduction

Fungi are a diverse and ubiquitous eukaryotic kingdom that inhabit the terrestrial and aquatic environment and play a key role in global biogeochemical cycling as primary decomposers of organic material [1]. Fungal decomposition of wood is dependent on a number of factors including host species, abiotic conditions, and community composition. Three types of fungi are the predominant decomposers in the environment: white rot, brown rot, and soft rot fungi [2]. These degrade wood according to their enzymatic arsenal, breaking down cell wall polymers, penetrating the cell wall, and altering its chemistry, with the resulting constituents being taken up by the hyphae for energy and anabolism [3,4]. Fungi and bacteria inhabit and compete for resources in ecological niches; these interactions can be powerful mutual drivers with positive and negative feedbacks [5,6]. These bacterial-fungal interactions can be highly specific with symbiotic associations developing between bacterial cells and fungal hyphae [7].

The interactions between fungal and bacterial populations can play a critical role in the breakdown of lignocellulose in the environment [8,9,10]. These synergistic interactions can alter microbial community structure, development, and composition [7,11]. Wood degradation by fungi can be inhibited or promoted by bacteria depending on the species present and the growth stage at which the association is initiated [12]. Bacteria can alter the structural integrity of wood, providing more favourable attack sites for fungi and increasing overall decomposition rates [13]. Furthermore, bacteria can also provide nutritional benefits to wood degrading fungi by supplying biologically fixed nitrogen that fungal hyphae transport to the wood degradation site [13,14,15].

Woody plant material is a challenging dietary resource for animals, as plants contain recalcitrant polymers including cellulose, hemicelluloses and lignins [16]. Employing a wood-based dietary strategy with its low nutritional quality and lack of nitrogenous compounds, xylivores must either rely on the activities of endosymbiotic microbes or produce the essential cellulose and lignin degrading enzymes. Some wood eating animals such as cockroaches and longicorn beetles have gut structures that imply cellulose digestion is primarily accomplished by endogenous cellulases rather than microbial cellulases [17]. In contrast, the termite gut requires a greater contribution from microbial cellulose digestion [17], which works synergistically with endogenous cellulases to enable them to degrade 74–99% of cellulose and 65–87% hemicellulose [17,18]. A complex resident microbiota inhabits the digestive system of the bovine rumen, which converts cellulose-rich plant mass into volatile fatty acids that are subsequently absorbed by the rumen epithelium. Additional compounds such as amino acids and vitamins B and C, are also supplied to the host [19,20].

Fish represent the greatest diversity of all vertebrates [21], however, understanding their gut microbiota and its significance is lacking compared to terrestrial vertebrates. Loricariidae is a speciose family of fish distributed in freshwater ecosystems of the Neotropics [22,23]. One member of the Loricariidae, *Panaque nigrolineatus*, has been the focus of study by several groups due to its ability to imbibe large amounts of wood (up to 70% of the GI contents) [24]. This fish uses spoon- shaped teeth and a suckermouth to allow for ingestion of woody material by rasping [24]. Stable isotope studies provide support for the consumption of large amounts of cellulose as part of their diet [24,25,26], which may offer selective advantage when river nutrients are limited during the dry season [27]. The *P. nigrolineatus* gastrointestinal (GI) tract is approximately 10× its body length,

providing a large surface area with many different microenvironments [24].

Previous studies have described the isolation of cellulolytic bacteria from the GI tracts and faeces of *Panaque* demonstrating the presence of a consortium of microorganisms performing cellulose breakdown [28,29]. Using 16S rRNA and culture-based analyses, the enteric bacterial community of *P. nigrolineatus* appears distinct and specialised in each region of the GI tract. The dominant bacteria have 16S rRNA gene sequences similar to *Proteobacteria, Firmicutes, Bacteriodetes* and *Actinobacteria* [30,31,32]. The midgut contains phylotypes with high sequence similarity to cellulose degrading bacteria *Clostridium, Cellulomonas, Bacteroides, Eubacterium* and *Aeromonas spp.* as well as nitrogen-fixing *Bradyrhizobium* and *Agrobacterium spp.* that are capable of in situ nitrogen fixation [32]. The hindgut is dominated by *Bacteroidetes* [31,32]. Bacterial species richness has been shown to decrease distally from foregut, through to the midgut and hindgut. While the bacterial microbiota within the GI tracts of other fish have been studied [33–42], comparatively little is known about the diversity, abundance, and role of the fungal microbiota in these systems [21]. It is likely that fungi play an important role in the fish microbiome, yeasts have been identified as part of the normal microbiota of fish GI tract [43–47] and have been studied with specific relevance for fish health and yields in aquaculture.

The GI tract of *P. nigrolineatus* is enriched with lignocellulose and provides a unique microenvironment. The fungal population within this fish warrants investigation to better understand their role in this process and possibly identify new lignocellulose degradation pathways and microbial interactions. The aim of the present study was to examine and compare the diversity of fungal communities in different GI tract regions as a function of diet. To our knowledge, this is the first description of fungal populations using molecular techniques in the GI tract of fish.

2. Materials and Methods

2.1. Fish rearing conditions and tissue preparation

P. nigrolineatus (L-190) were imported from South America by the fish wholesaler Aquascapesonline (Belleville, NJ). They were randomly assigned to individual, filtered, and aerated tanks kept at 29 ± 1 ℃. Fish (40 mm, standard length) were fed a mixed diet of hearts of palm (*Euterpe precatoria*), algae pellets (Hikari Tropical Sinking Algae Wafers, Hayward, CA), and date palm wood (*Phoenix dactylifera*) during an acclimation period of three weeks under conditions specified by IACUC 071509JW-01. For the duration of the experiment, the fish on a mixed diet were provided with palm hearts and algae every second day while wood was constantly available. Wood was thoroughly soaked in water and autoclaved three times prior to being provided to the fish. Fish were then converted to a palm wood-only diet or a mixed diet of palm hearts and palm wood. This feeding regimen was maintained for three weeks prior to termination.

After the feeding period, one fish from each treatment was sacrificed by anaesthetic overdose in 3-aminobenzoic acid ethyl ester (MS-222, 50 mg/L) as described previously [30]. After removing the ventral body plate, sterile ice-cold phosphate buffered saline (PBS) was added to the abdominal cavity. The intestine was separated immediately distal to the stomach, removed from the body cavity, uncoiled, and measured rapidly in cold PBS. The auxiliary lobe was separated from the intestine,

which was then divided into three parts of equal length, defining foregut, midgut, and hindgut regions. Tissue samples were processed using the Qiagen (Germantown, MD, USA) DNeasy Blood and Tissue Kit with pre-treatments for Gram-positive and Gram-negative bacteria according to the manufacturer's instructions. DNA extracted from three samples of each GI tract region was pooled and processed for PCR amplification.

2.2. Internal transcribed spacer (ITS) polymerase chain reaction (PCR) amplification and sequencing

Primers ITS1 and ITS2 were used to amplify the ITS1 region and primers fITS7 and ITS4 to amplify the ITS2 region (Table 1) using the following parameters: initial denaturation step of 2 min at 96 ℃ followed by 30 cycles of denaturation for 15 s at 96 ℃,annealing for 30 s at 50 ℃,elongation for 60 s at 72 ℃. Sequencing was performed on the Illumina MiSeq V3 platform (LGC Genomics GmbH, Berlin, Germany). Barcode sequences, adapters and primer dimer products were removed from the resulting sequence fragments using Illumina bcl2fastq 1.8.4 software and submitted to GenBank (Accession numbers SRR5808488–SRR5808499).

Table 1. Primers used for amplification of fungal ITS rDNA genes.

Primer	Sequence (5'–3')	Reference
ITS1	TCCGTAGGTGAACCTGCGG	[48]
ITS2	GCTGCGTTCTTCATCGATGC	[48]
ITS4	TCCTCCGCTTATTGATATGC	[48]
fITS7	GTGARTCATCGAATCTTTG	[49]

2.3. ITS fungal community analysis

ITS pre-processing and OTU picking was carried out with mothur 1.35.1 [50]. Sequences were subsampled in mothur to 60,000 reads per sample, with distances generated using USEARCH [51]. Chimeras were eliminated using the UCHIME algorithm [52]. The similarity threshold for ITS sequences belonging to the same operational taxonomic unit (OTU) was set to 97% and clustered by CD-HIT-EST [53] with cluster representative sequences selected based on abundance. Taxonomic classification of OTUs was performed against the UNITE v6 database [54] with species assigned at 97% identity threshold. Samples were normalised to 28,090, the lowest number of reads per sample for downstream analysis by Quantitative Insights into Microbial Ecology 1.9.0 (QIIME) [55]. Alpha diversity was measured using parallel_alpha_diversity.py script using observed_species and $Chao_1$ metrics. An OTU network was generated using the make_OTU_network.py script. The resulting network was visualized in Cytoscape (3.5.1) using a spring-embedded layout.

3. Results

3.1. Distribution and diversity of fungi in the GI tract

Fungal sequences corresponding to the ITS1 and ITS2 regions were PCR amplified from all GI tract regions of both diets. A total of 256,280 sequences, clustering into 207 OTUs, were obtained from the ITS2 amplification and analysed using USEARCH. The ITS1 sequence analysis was found to be considerably less sensitive and useful for this study and the results for this region are included in supplementary information (Table S1). For the ITS2 analysis, OTUs were binned into taxonomic groupings allowing comparison of fungal community alpha diversities across tissues and diet.

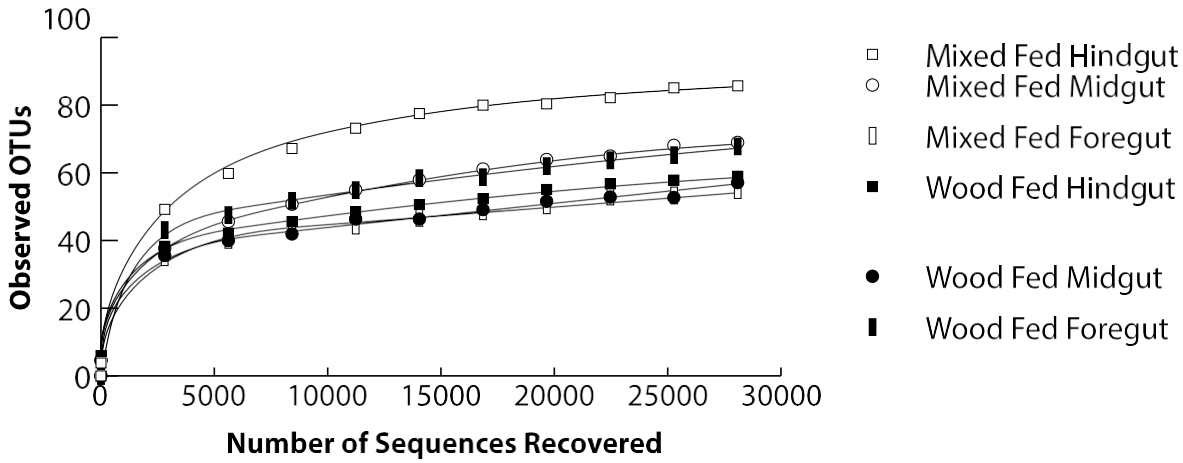

Figure 1. Rarefaction graphs with OTUs derived from sequencing of the ITS2 region, binned to species. The data was normalised on the sample containing the lowest number of sequences, 28,090 sequences were subsampled from the wood-fed fish and mixed-fed fish.

To detect changes in fungal alpha diversity, $Chao_1$ estimations of diversity were applied to OTU distributions (Table 2). Fungal diversity increased distally with the greatest diversity in the hindgut of the mixed-diet fed fish. The opposite trend was observed in the wood-fed fish with the foregut having the most fungal diversity. These findings were supported by the rarefaction analysis, which demonstrated that the hindgut had the highest detectable species richness, while the foregut of mixed-diet fed fish had the lowest. The rarefaction analysis suggests that additional sequencing would allow more novel OTUs to be detected but the majority of the diversity present had been sampled (Figure 1). T-tests revealed there was no significant difference ($P = 0.4$) in the average number of fungal OTUs observed between wood or mixed-diet fish. Differences in S_{obs} and S_{Chao1} suggest more unique or rare OTUs being present in the wood-fed foregut (Table 2) than any other tissue region examined.

Table 2. Comparison of ITS2 rDNA region OTU species richness. A nonparametric estimate $Chao_1$, was used to compare species diversity in different regions of the GI tract. For phylotype richness estimations, OTUs were binned to species.

	Wood Fed Fish			Mixed Fed Fish		
	Foregut	Midgut	Hindgut	Foregut	Midgut	Hindgut
Observed OTUs	67.8	57.1	59	54.9	69	85.7
$Chao_1$	95.2	79.6	70	76.9	82.3	96.1

The taxonomic composition of the microbial community varied across tissue type and diet (Figure 2, Figure 3 and Figure S1). Figure 3 provides visualisation of fungal OTUs unique to each fish GI tract region and those that were shared between two or more regions. Different OTUs were detected within each region of each fish, but the core microbiome of the fish is dominated by sequences with high similarity to Sordariomycetes and Dothideomycetes in all regions with the former more prevalent in the midgut and hindgut of the mixed-diet fed fish and the latter dominated in the midgut and hindgut of the wood-diet fed fish (Figure 3). Sequences with high sequence similarity to *Fusarium oxysporum* were the major OTUs detected in all tissue regions, except the wood-fed hindgut, which was dominated by sequences similar to *Cirrenalia macrocephala.* Other sequences present throughout the GI tracts of both dietary regimens included *Aureobasidium pullulans* and *Debaryomyces prosopidis* (both more abundant in wood-fed fish), and *Malassezia restricta*.

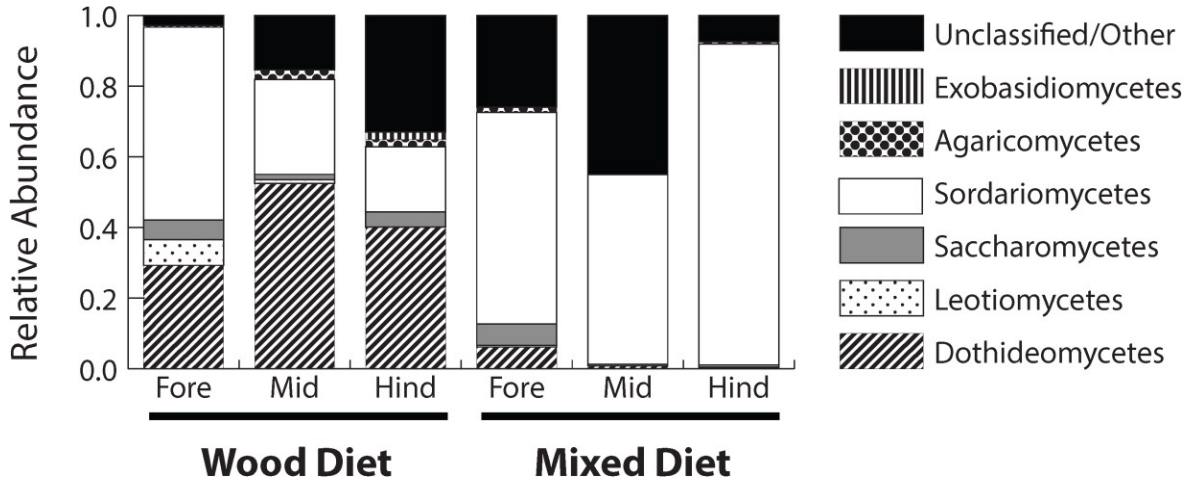

Figure 2. Relative abundance of dominant fungal classes (>1%) detected by sequencing the ITS2 region from the various GI tract regions of *P. nigrolineatus* fed either a wood diet or a mixed diet. Sequences were assigned to OTUs with over 97% sequence identity.

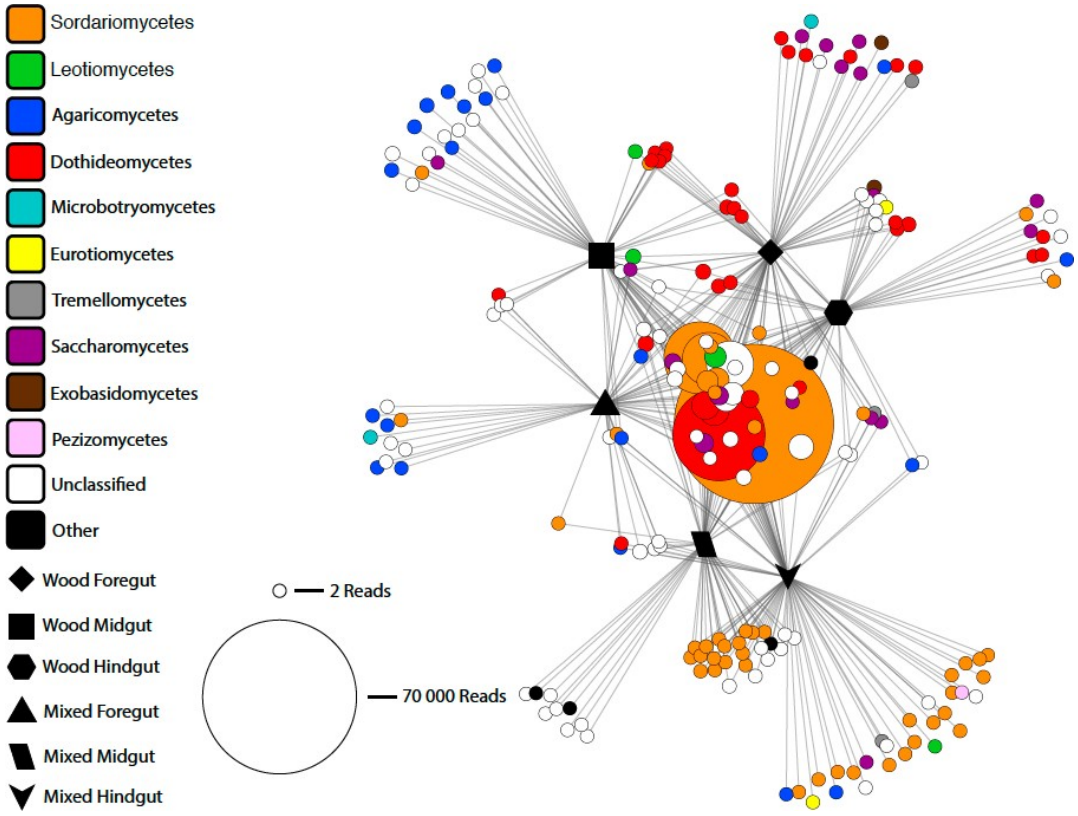

Figure 3. OTU network showing distribution of all OTUs identified to class detected via sequencing of the ITS2 region from different regions of the GI tract of *P. nigrolineatus* fed either a wood diet or a mixed diet. Node size indicates the number of reads assigned to an OTU while node colour indicates consensus taxonomy.

3.2. Relative distribution and diversity of fungi based on dietary regimen

OTUs were binned into taxonomic groupings allowing comparison of fungal diversity within the GI tract of wood-fed fish and mixed-fed fish, irrespective of GI tract region. The GI tract of the mixed-fed fish was found to have the most ITS2 region sequence diversity. $Chao_1$ analysis indicated that the wood-fed fish had a higher number of rare and unique species compared to mixed-diet fed fish (Table 2). The most prevalent classes in the wood-diet fed fish were sequences similar to Dothideomycetes (40%) and Sordariomycetes (36%) and the mixed-diet fed fish were sequences similar to Sordariomycetes (73%). Three OTUs detected from the Dothideomycetes class were found in the foregut, midgut and hindgut of the wood-diet fed fish but absent in the mixed-diet fed fish.

Each region of the GI tract was analysed for differences in community composition. Analysis did not reveal any tissue specific fungal communities, but sequences similar to the Saccharomycete genus *Metschnikowia* were found exclusively in the foregut of both fish while OTUs with sequences similar to Tremellomycetes and the Agaricomycete genus *Stereaceae* were found solely in the hindgut. These OTUs were not found in high abundance in either feeding regimen suggesting limited biological significance. However, analyses were based on the sequences of one foregut, midgut and hindgut from each diet and may not reflect actual variability amongst individuals [57].

4. Conclusions

In this study, the fungal communities of wood and mixed-diet fed *P. nigrolineatus* GI tracts were investigated via rDNA ITS sequencing. Due to complications associated with fish acquisition and rearing, we were only able to analyse one fish raised on each diet. While we recognise that a rigorous analysis of the GI tract communities requires the utilisation of several fish, we found that each GI tract region possessed a distinct fungal community and this is the first report of the presence of fungi throughout a fish GI tract.

P. nigrolineatus imbibes large quantities of wood in its diet and may have developed a symbiotic relationship with microbes to degrade this resource. Wood decomposition is dynamic and the rate of decomposition depends on many factors including priority effects [58] and successional changes in microbial communities [59]. Fungi have been shown to shape the composition of the bacterial communities [60] and are thought to be more abundant in the early stages of decay, with fungal mycelia doubling faster than bacterial cells [61], and bacterial-fungal interactions facilitating decomposition [7,11,60]. Although bacterial-fungal interactions are vital in plant cell wall digestion, aerobic and anaerobic fungal activity has been shown to be responsible for most cell wall degradation by penetrating into plant tissues not normally available to bacteria [62].

Our finding that *P. nigrolineatus* fed a wood-diet or a mixed-diet has different fungal communities is consistent with previous and current research showing similar variations for the bacterial communities in different regions of the GI tract [30, McDonald, Watts and Schreier, manuscript in preparation]. Of particular interest is the hindgut, which contains cellulolytic bacteria [29,30,31] as well as sequences similar to *C. macrocephala*, which has been associated with waterlogged wood [63,64]; any relationship between these bacteria and fungi and their role in the fish GI tract remains to be determined.

Combined with previous studies [29–32], our results indicate that bacteria and fungi co-inhabit the GI tract of *P. nigrolineatus*, with the potential of degrading dietary wood. Ongoing studies [30, McDonald, Watts and Schreier, manuscript in preparation] suggest that the enteric bacterial community may lack selected enzymatic activities essential for lignocellulolytic digestion, which may be provided by the fungal community and/or host. ITS1/ITS2 sequences for several cellulase-producing fungi have been found in the present study, including those having similarity with *F. oxysporum*, *A. pullulans, Botrytis caroliniana, Metschnikowia, Alternaria* and *Debaryomyces*.

F. oxysporum, which dominated foregut and midgut regions, excretes enodocellulases, exocellulases and β-glucosidase [65]. These cellulolytic activities might allow the bacteria to benefit from the primary stage of cellulose degradation as part of a synergistic relationship. While there are different fungi in different regions of each fish, many may be carrying out equivalent roles, acting on cellulose to enhance and augment bacterial activities. Future studies will examine whether genes utilised for wood degradation are differentially expressed.

While we have identified the fungal microbiota within the GI tract, the type of symbiotic relationship that this partnership takes with bacteria and host is unknown. It is conceivable that during the breakdown of lignocellulose, microbes produce volatile fatty acids and amino acids, which are absorbed by the fish and provide a source of energy. Bacteria facilitate fungal decomposition of lignin [5,7] by altering wood chemistry, structure, and permeability [13]. They

may also provide nutritional benefits to wood degrading fungi via nitrogen fixation, allowing fungi to decompose nitrogen-sparse wood [15]. An active nitrogen-fixing community has been identified in the GI tract of *P. nigrolioneatus* [32], suggesting that a mutualistic symbiosis between fungi and bacteria is possible within the fish GI tract. Confirmation of such a relationship will require further studies.

Fungi are commercially important and play a critical role in global environmental health. There is considerable interest in applied fungal research, which includes commercial production of important compounds, human health, food safety and security and crop protection [66]. Most research to date has focused on fungi that affect human health or have commercial applications. Comparatively little is known about many of the fungal species found in the environment. Analysing fungal community roles and identities in a novel environment is challenging due to the paucity of literature and lack of available sequences. The quality of fungal sequence databases is highly variable [67] and often lacks proper annotation and lineage designations [66,68]. Amplifying the entire ITS region (including the 5.8S region) may be more phylogenetically informative, but may also artificially reduce microbial richness and bias community structure [69]. In addition, the incidence of chimeric sequences also increases due to the conservation of the 5.8S region [70]. In this study, both ITS1 and ITS2 were used for community analysis. Although, both sets of primers used for their amplification are biased for certain groups [49,71]. In this study, the ITS2 region was found to be more sensitive in detecting novel OTUs, which could be due to the sample type or the biases and limitations of the primer sets [49,67,70].

Different fungal communities were detected across tissue regions and dietary regimens, indicating diet and tissue type affects fungal diversity in fish. Since *P. nigrolineatus* does not appear to gain energy directly from the digestion of wood [26,72] it is possible that enteric fungal communities are important for wood-only diets that lacks readily available carbon and nitrogen by supplying a digestible source of carbon or other micronutrients. This study is the first to examine the fungal community in a xylivorous fish and our results indicate the presence of a diverse fungal population that may play critical roles in cellulose degradation with potential nutritional benefits for the fish. Furthermore, these previously under-studied fungal species may have novel cellulolytic and lignin degrading capabilities that could have implications for biofuel generation. Understanding the role of the fungal communities in lignocellulose degradation and their interaction with GI tract bacteria in this process is the focus of future studies.

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Conflict of Interest

All authors declare no conflicts of interest in this paper.

Ethics Statement

Fish were maintained and sacrificed under strict accordance with the recommendations of the IACUC protocol, approved by the Committee on the Ethics of Animal Experiments at Towson University (071509JW-01).

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