MICROBIOTA ASSOCIATED WITH OYSTER MUSHROOM SUBSTRATES USING CULTURE DEPENDENT AND TARGETED-METAGENOMICS METHODS

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MICROBIOTA ASSOCIATED WITH OYSTER MUSHROOM SUBSTRATES USING CULTURE DEPENDENT AND TARGETED-METAGENOMICS METHODS

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ABSTRACT

The globally mushroom cultivation industry is challenged with frequent green mold contamination that is often thought to be caused by the green mold - *Trichoderma* spp. However, the farmers and researchers do not have much insight on the possible factors that lead to the frequent but random green mold contamination in the mushroom cultivation industry. Therefore, this study aims to investigate the possible role of the natural microbiota present in the mushroom substrate has on green mold contamination. To do so, both culture-dependent and culture independent (16 srRNA targeted-metagenomic) approaches were used in this study to identify and compare the diversity of microbiota found in both healthy and contaminated mushroom substrates collected from a local mushroom farm at different stages of harvest. The study found that the green mold contaminated mushroom substrates had the highest total heterophilic bacteria count, 9.5×10^7cfu/g. In general, the total heterophilic bacteria count in mushroom substrates increased from after sterilization stage (<10cfu/g) to reach the maximum count in contaminated stage (9.5×10^7cfu/g) of harvest. The findings revealed that bacterial community from the Proteobacteria phylum was the most abundant in both final harvest (healthy) and green mold contaminated substrates. However, there was a shift in the bacterial diversity in the cultured bacterial populations with the increase of Gammaproteobacteria and the decrease of Bacilli after sterilisation of substrate along the cultivation stages. Gammaproteobacteria, the highest occurring class of bacteria in contaminated substrate with Enterobacteriaceae being the largest community indicates possible unhygienic practices during cultivation process. High abundance of *Pseudomonas, Enterobacter* and *Stenotrophomonas* in contaminated substrate from the cultured population and metagenomic data may also be contributing to *Trichoderma*

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colonisation. The results from 18S sequencing targeting the universal ITS region identified *Trichoderma pleurotum* from the contaminated substrate. However, *Graphium penicillioies* recorded highest relative abundance in metagenomic reads from contaminated substrate and occurred persistently throughout harvest stages. Thus, this study not only reveals the microbial diversity in oyster mushroom substrate which may influence the health of the mushroom, but also prompts further research to be done to validate correlation between *Graphium* and *Trichoderma* contamination in local mushroom farms.

Keywords:

Mushroom cultivation, green mold contamination, microbial diversity, *Trichoderma, Graphium*

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MENDEDAHKAN KEPELBAGAIAN MICROBIOTA YANG TERDAPAT DALAM SUBSTRAT CENDAWAN MENGGUNAKAN KAEDAH KULTUR DAN KAEDAH NYAH KULTUR

ABSTRAK

Industri penanaman cendawan global mengalami pencemaran kulat hijau yang sering dilaporkan disebabkan oleh - *Trichoderma spp*. Walau bagaimanapun, para petani dan penyelidik tidak mempunyai banyak pandangan mengenai faktor-faktor yang mungkin menyebabkan pencemaran kulat hijau yang kerap tetapi rawak dalam industri penanaman cendawan. Oleh itu, kajian ini bertujuan untuk mengkaji kemungkinan peranan mikrobiota semula jadi yang terdapat pada substrat cendawan sebagai penyeybab terhadap pencemaran kulat hijau. Untuk melakukannya, kedua-dua pendekatan yang bergantung pada kaedah kultur dan kaedah nyah-kultur (16 srRNA-metagenomic) digunakan dalam kajian ini untuk mengenal pasti dan membandingkan kepelbagaian mikrobiota yang terdapat pada substrat cendawan yang sihat dan tercemar yang dikumpulkan dari ladang cendawan tempatan pada peringkat penuaian yang berbeza. Kajian mendapati bahawa substrat cendawan yang tercemar kulat hijau mempunyai jumlah bakteria heterofilik tertinggi, 9.5×10^7cfu / g. Secara amnya, jumlah bakteria heterofilik dalam substrat cendawan meningkat dari peringkat selepas pensterilan (<10 cfu/g) hingga mencapai jumlah maksimum pada peringkat substrat tercemar (9.5×10^7cfu / g). Hasil kajian menunjukkan bahawa komuniti bakteria dari Proteobacteria filum adalah yang paling banyak terdapat pada substrat penuaian akhir (sihat) dan substrat tercemar dengan kulat hijau. Walau bagaimanapun, terdapat perubahan kepelbagaian bakteria dalam populasi bakteria kultur dengan peningkatan Gammaproteobacteria dan penurunan Bacilli setelah pensterilan substrat sepanjang tahap penanaman. Gammaproteobacteria, kelas bakteria tertinggi dalam substrat yang tercemar dengan Enterobacteriaceae menjadi komuniti terbesar menunjukkan

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kemungkinan amalan pengendalian tidak bersih semasa proses penanaman. *Pseudomonas, Enterobacter* dan *Stenotrophomonas* dikesan paling tinggi dalam substrat yang tercemar dari populasi dikultur dan data metagenomik juga dapat menyumbang kepada kolonisasi *Trichoderma*. Hasil dari penjujukan 18S yang mensasarkan kawasan ITS sejagat mengenal pasti *Trichoderma* pleurotum dari substrat yang tercemar. Walau bagaimanapun, *Graphium penicillioies* mencatatkan kelimpahan relatif tertinggi dalam bacaan metagenomik dari substrat yang tercemar dan berlaku secara berterusan sepanjang peringkat penuaian. Oleh itu, kajian ini tidak hanya mengungkapkan kepelbagaian mikrobiota dalam substrat cendawan tiram yang dapat mempengaruhi kesihatan cendawan, tetapi juga mendorong penyelidikan lebih lanjut yang harus dilakukan untuk mengesahkan korelasi antara pencemaran *Graphium* dan *Trichoderma* di ladang cendawan tempatan.

Kata kunci:

Penanaman cendawan, pencemaran green mold, kepelbagaian mikrob, *Trichoderma, Graphium*

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	:	celcius
µL or µl	:	microliter
µM	:	micromolar
µm	:	micrometer
>	:	more than
%	:	percentage
Bp	:	base pair
CO	:	chromogenic agar
CFU	:	colony forming unit
dNTP	:	Deoxynucleoside triphosphate
DNA	:	deoxyribonucleic acid
g	:	gram
ITS	:	internal transcribed spacer
MEA	:	malt extract agar
$MgCl_2$	:	magnesium chloride
Min	:	minute
ml	:	milliliter
mm	:	millimeter
mM	:	millimolar
NA	:	nutrient agar
NA*	:	no significant value
PCR	:	polymerase chain reaction
PDA	:	potato dextrose agar
rpm	:	revolutions per minute
rDNA	:	ribosomal deoxyribonucleic acid

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SE	:	standard error
sec	:	seconds
spp	:	several species
sp	:	species
SFU	:	spore forming unit
16S rRNA	:	16 Svedberg ribosomal ribonucleic acid
US$	:	United States dollar
[v/v]	:	[volume/volume]

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CHAPTER 1: INTRODUCTION

Mushroom cultivation presents an economically important biotechnological industry that expanded all over the world. Mushrooms serve as delicacies for human consumption and as nutraceuticals (Kües and Liu, 2000). In Malaysia, cultivated mushrooms are one of the highest-valued crops in Malaysia where grey oyster (*Pleurotus pulmonarius*) is said to be the popular choice for local cultivation (Haimid et al., 2013) comprising of 91% from the total mushroom cultivated in 2014 (Rosmiza et al., 2016). The high demand for *P. pulmonarius* in Malaysia offers high income to cultivators due to the ability to grow and sporulate in tropical region (Islam et al., 2016).

Bioconversion of lignocellulosic residues through cultivation of oyster mushroom encourages utilization of renewable resources in the production of edible yet protein-rich food in developing countries (Díaz-Godínez and Sánchez, 2002). Many different lignocellulosic wastes are used as substrates for the cultivation of oyster mushroom and the usage depends on the availability of local agricultural wastes (Cohen et al., 2002). However, sawdust is commonly used in most Southeast Asian countries (Mandeel et al., 2005). Sawdust is said to be the best substrate for oyster mushroom cultivation as it has proved to produce high yield and better biologically efficiency (Shah et al., 2004).

During the last decades, several species of *Trichoderma* have been reported to be causing the green-mould contamination in mushroom farms resulted in significant loss (Kredics et al., 2010). In the case of the causal agents of the green-mould contamination of *Pleurotus ostreatus*, two new *Trichoderma* species in South Korea, *Trichoderma pleurotum* and *T. pleuroticola* were reported (Kredics et al., 2009, Komón-Zelazowska et al., 2007; Park et al., 2006). In a recent study, the green-mould contamination is still

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listed as one of the challenges in development of mushroom industry in Malaysia (Rosmiza et al., 2016).

In the past, studies to reveal interaction and relationship of microorganisms with mushroom were extremely limited. Most of the studies focused on compost as substrate and how microbial population and microbial activity in compost changes with inoculation of mushroom spawn (Silva et al., 2009, Adams and Frostick, 2008). Siyoum et al. (2016) reported the microbial population dynamics in white button mushroom supply chain which used compost as substrate. The study suggested that the microbial population may affecting the healthy growth of mushroom. Nonetheless, these publications did not study further to reveal the succession of microbial population in the mushroom substrate during the cultivation process. A research gap in the past has opened way for the current study to focus on the microbiota in sawdust substrate used for oyster mushroom cultivation. Therefore, this study aimed to determine the progression of microbial population in the mushroom substrate along the cultivation process, as well as associate the microbial population to disease development in mushroom. This information may be used in improving cultivation processes in local mushroom farm (Figure 1.1) by constructing a more sustainable agricultural practice.

Figure 1.1: Oyster mushroom cultivation in local mushroom farm

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1.1 Objectives

The objectives of the study were:

1. to determine the abundance and diversity of microbial population in the mushroom substrate collected at different stages of mushroom cultivation using culture dependent and targeted-metagenomic methods.
2. to compare the diversity between microbial populations associated with green-mould contaminated and healthy mushroom substrate.

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CHAPTER 2: LITERATURE REVIEW

2.1 Mushroom cultivation industry

Mushrooms are in kingdom of Fungi which separated from plants, animals, and bacteria. The living frame of the fungus is mycelium made of hyphae and under precise conditions, these sexually compatible structures fuse to sporulate. The larger spore producing structures (bigger than about 1 mm) are called mushrooms (Oei and Nieuwenhuijzen, 2005). Mushrooms can be classified into three groups: saprophytes, parasites, and mycorrhiza, however most of the cultivated mushrooms are saprophytic fungi (Cheung (Ed.). 2008).

According to Oei and Nieuwenhuijzen (2005), mushrooms are categorized as crops with high monetary value as they are easy to grow with short span of time (between spawning and harvesting), rich in protein, vitamins, and minerals. Mushroom cultivation adheres to sustainable farming using agricultural waste and the spent substrate can be used for soil conditioning. Edible, medicinal, and wild mushrooms are the three major produce in global mushroom industry and edible mushrooms production has increased more than 30-fold since 1978 worldwide. Due to the health benefits of mushroom and consumer awareness, the consumption per capita is increasing each year (Royse et al., 2017).

According to Kumla et. al. (2020), more than 50 species of edible mushrooms have been commercially cultivated globally (Figure 2.1). The top four cultivated edible mushrooms worldwide are genus *Lentinula* (shiitake), *Pleurotus* (oyster mushroom), *Auricularia* (wood ear mushroom) and *Agaricus* (button mushroom) (Rodríguez et al., 2008; Ma et al., 2018). *Pleurotus* mushroom cultivation is said to be most suitable and profitable in tropical, subtropical, and temperate regions. Mushroom farming as overall

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is a labor-intensive activity that can sustain revenue generation and provide livelihood, especially in progressing countries. (Raman et al., 2021)

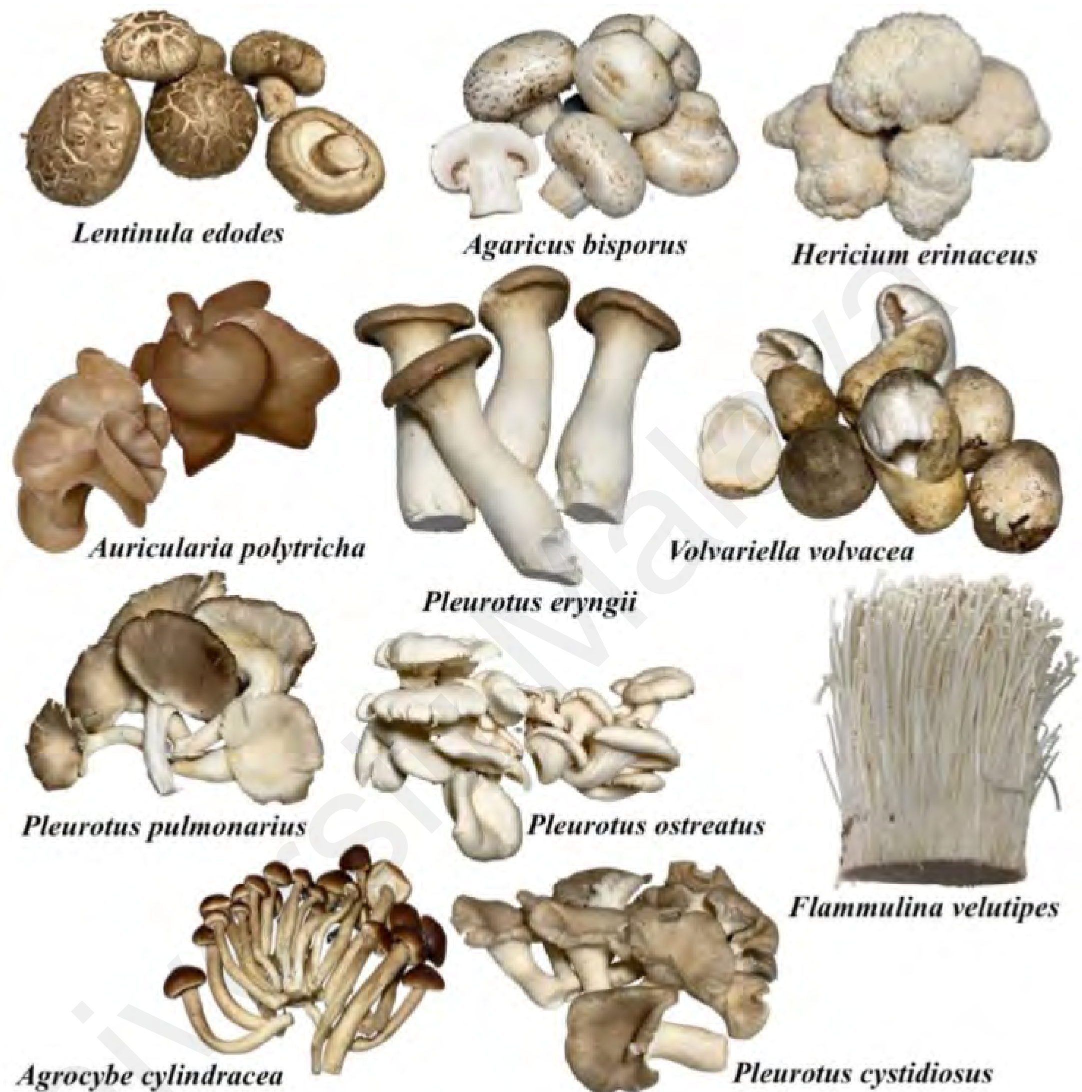

Figure 2.1: Examples of commercially important cultivated mushrooms. (Source: Kumla et al., 2020)

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In 2017, a report by the Transparency market research revealed that the global mushroom market is estimated to be worth US$69,267.9 million by 2024. Consumers′ willingness to change beyond the dependency on meat-based food is an important driving force for this transition. In Malaysia, mushrooms have been identified as one of the high-value crops under National Agro-Food Policy (2011-2020) (Zakil et al., 2020) as one of the sources of food and wealth for local farmers (Haimid 2013). The climatic

condition in Malaysia is found to be suitable for mushroom cultivation all year long. Hence, Malaysia has the potential to be one of the large mushroom producers in the market (Zakil et al., 2020).

2.2 Substrate used in mushroom cultivation

The medium used for the mushroom mycelium proliferates is called substrate (Oei and Nieuwenhuijzen, 2005). They could be branching out through soil, compost, wood log, or other lignocellulosic (Cheung (Ed.). 2008). Mushroom cultivation basically requires carbon, nitrogen, and inorganic compounds as nutritional sources. Oyster mushrooms require less nitrogen and more carbon source. Thus, most organic matters containing cellulose, hemicellulose and lignin can be used as the mushroom substrate (Besufekad 2020). Therefore, the type of mushrooms and the microbiota formed depends very much on the properties of a substrate. Generally, a combination of fine and coarser sawdust or wood chippings provides the best starting substrate and substrates are usually given a heat treatment before spawning (Oei and Nieuwenhuijzen, 2005)

Cultivation of mushrooms on lignocellulosic wastes denotes one of the most economically and cost-efficient organic recycling processes. Various agricultural by-products are being used as substrates for the cultivation of the oyster mushroom. Rice straw, cotton waste, banana leaves, Chrysalidocarpus lutescens leaves, wood chippings has been used as substrates to cultivate *P. pulmonaris* (Raman et al., 2021).

Besufekad (2020) studied oyster mushroom growth on different substrates namely cotton seed, enset waste, sawdust, and teff straw with contrasting composition. The highest bioconversion efficiency and yield were obtained from the combination of sawdust and teff straw and lowest from the combination of teff straw and enset waste.

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Zakil (2020) reported that sugarcane bagasse as oyster mushroom substrate gave higher biological efficiency than using rubber tree sawdust.

2.3 Common contamination associated with mushroom cultivation

Pests and diseases have always been a major problem in agriculture. Different organisms of bacteria and fungi may cause disease and threat to cultivated mushroom (Obire 2013). Fungi including *Aspergillus* spp., *Trichoderma* spp., *Cladobotryum* spp., *Penicillum* spp., *Gliodadium* spp. and *Verticiluium* spp. have been reported to be associated with contamination in *Pleurotus* mushroom (Sobowale 2018). According to Agbagwa (2020), larvae of *Megaselia* spp., *Scaria fenestralis, Lycriella* spp. and *Lepidocyrtus* spp. feed on the mushroom mycelium causing pest contamination. Mushroom production of *Agaricus bisporus* have been dealing with bacterial blotch disease attributed to *Pseudomonas tolaasii*. However, there are also other *Pseudomonas* species which induce similar diseases on edible mushrooms (Osdaghi, 2019).

In Malaysia, Munirah et al., (2013) developed a system called Expert System for Diagnosing Oyster Mushroom Diseases in local farms. The diseases to be identified are caused by green-mould, *Neurospora* (kulat jingga), *Penicillium* (kulat kuning kehijauan), *Rhizopus* spp. (kulat hitam), *Coprinus* spp. (kulat dakwat hitam). The diseases and pests are synonym with the quality and production of mushroom. Hence, by using the system may resulted in improvement of mushroom quality for the mushroom farm.

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2.4 Green-mould contamination in mushroom substrates

2.4.1 *Trichoderma*

2.4.1.1 General characteristics

Trichoderma spp. are ubiquitous soil dwellers and are found to be prominent component of soil fungal populations. They are well-known to produce degradative enzymes including chitinases involved in lysis of fungal mycelia and cellulases. Due to to rapid growth rate and having minimal nutritional requirements, *Trichoderma* spp. are regarded as adept colonizers and have been used as biocontrol agents and plant growth stimulators (Klein and Eveleigh 2002).

2.4.1.2 Origin, prevalence and virulence

Green-mould contamination in *Pleurotus ostreatus* was reported to be caused by *Trichoderma* species resulted in crop losses worldwide (Kredics et al., 2010). *Trichoderma* was first proposed by Persoon (1794). A large number of *Trichoderma* species have been linked to *Hypocrea* teleomorphs, such as *T. harzianum*, the anamorph of *H. lixii*, or *T. virens*, the anamorph of *H. virens*. However, some common and important species such as *T. asperellum* are not connected to a teleomorph and are seemingly clonal. Around 1995, DNA sequence analysis became the new standard in fungal systematics aid in developing taxonomy of *Trichoderma* and *Hypocrea* (Samuels 2006).

According to Samuels et al. (2002) *Trichoderma aggressivum.* and *T. aggressivum f. europaeum* were identified as the cause of the green-mould epidemic for commercially grown *Agaricus bisporus* in North America and Europe. These two species are described to be distinct from morphologically similar *T. harzianum* and *T. atroviride*. Park et al. (2006) described *T. pleurotum* (Figure 2.2) which is said to

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resemble *T. virens* was the most predominant and virulence species than other *Trichoderma* species found in oyster mushroom cultivation (Park et al., 2005a).

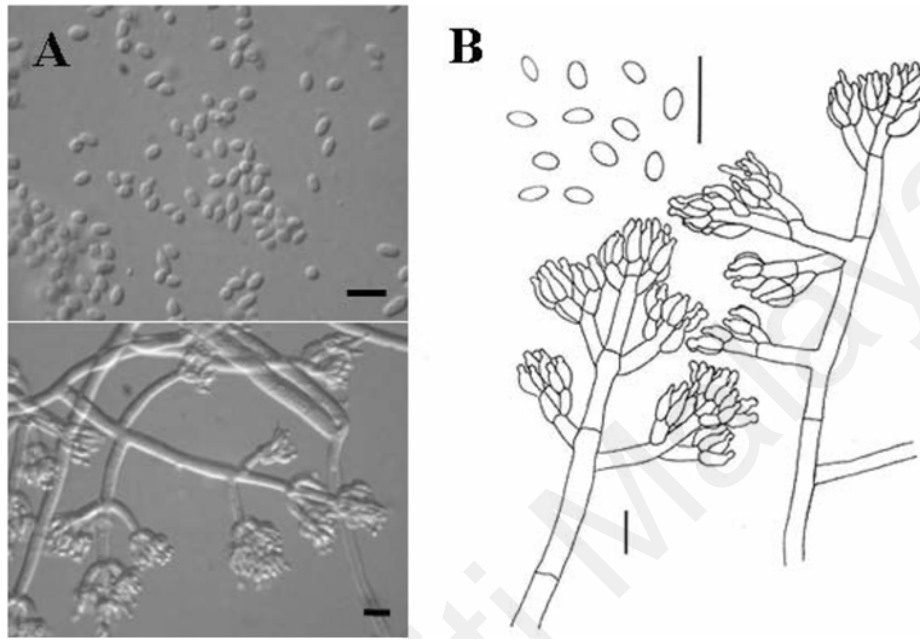

Figure 2.2: Micrographs (A) and drawings (B) of conidiophores and conidia of *Trichoderma pleurotum* S. H. Yu & M. S. Park. Scale bar = 10μm (Source: Park et al., 2006)

Luković et al. (2020) described twenty-two strains of *Trichoderma* spp. causing green-mould disease on edible mushrooms (button mushroom, shiitake and oyster mushroom), collected during 2004–2018 from Serbia, North Macedonia, Croatia, and Hungary. Based on the study, *Trichoderma* strains from shiitake mushroom in Serbia were members in the complex group of *T. harzianum*, while the *Trichoderma* strains from Serbian and North-Macedonian obtained from oyster mushroom farms were in the complex group of *T. harzianium*, *T. pleuroti* and *T. pleuroticola*.

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2.4.2 *Graphium*

2.4.2.1 General characteristics

The anamorph genus *Graphium* Corda (1837) has traditionally included species with darkly pigmented, determinate synnemata, percurrently proliferating conidiogenous cells and slimy, aseptate, hyaline to pale brown conidia (Ellis, 1971; Crane and Schoknecht, 1973). *Graphium* strains isolated in a past study were from wood or forest soil and had a consistent association with wood infested by bark insects (Lackner & de Hoog, 2011).

2.4.2.2 Origin, prevalence and virulence

The original description by Corda was based on a specimen collected in Prague on *Populus nigra* cv. *italica*. Ellis listed the fungus as occurring on *Populus* wood in Europe and North America. According to Okada et. al. 2000, the literatures Sutton and Laut (1970) and Sutton (1973) identified *G. penicillioides (*Figure 2.3) as a common secondary colonizer of bark beetle tunnels in *Ulmus* trees (Dutch elm disease), and this fungus is still common in Canada.

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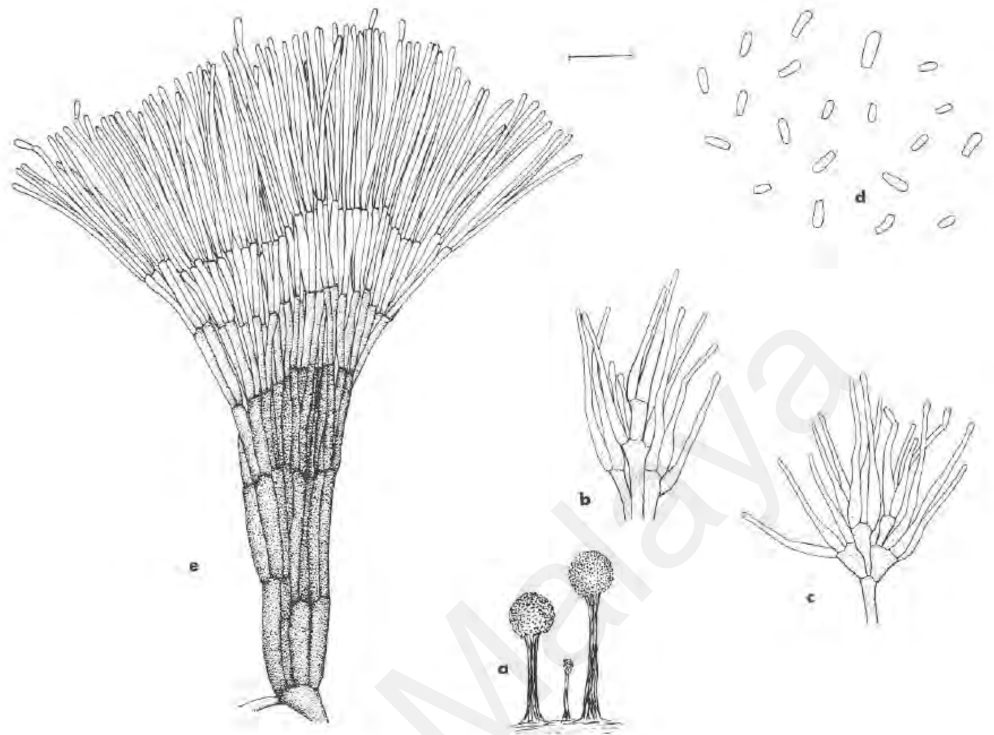

Figure 2.3: ***Graphium penicillioides*** **on** ***Populus*** **twigs. a. Habit of synnemata (not to scale). b, c. branching of conidiophores from synnemata. d. Conidia. e. reduced synnemata on MEA. (Source: Okada et al.,2000)**

Graphium pseudormiticum was isolated from a pine-infesting bark beetle on non-native pine trees in South Africa and most likely a fungus native to Europe (Mouton *et* al. 1994). Insects are said to be vectors of *Graphium* species (Jacobs et al. 2003, Geldenhuis et al. 2004, Alamouti et al. 2007, Hulcr et al. 2007) and the insects in Africa and Madagascar may be of the same genus (Cruywagen et al., 2010). *G. penicillioides* was isolated from a wood core of *Populus nigra* in the Czech Republic and *G. basitruncatum* was isolated from soil (Solomon Islands and Japan) and a diseased person (Kumar et al. 2007, Lackner and Hoog 2011).

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2.5 Microbiota of mushroom substrate

2.5.1 Definition

The words “micro” and “biota” are of Ancient Greek origin. It is a combination of “Micro” (μικρος, small), with the term “biota” (βιοτα), which means the living organisms of an ecosystem or a particular area. The microbiota consists of the assembly of microorganisms belonging to different kingdoms (Prokaryotes [Bacteria, Archaea], Eukaryotes [Protozoa, Fungi, and Algae]) (Berg et al., 2020). Proteobacteria is an evolutionarily, geologically, and environmentally chief group of microorganisms. All proteobacteria are Gram-negative bacteria and species of this phylum exhibit extreme metabolic diversity, including chemoautotrophic, chemoorganotrophic, and phototrophic microorganisms representing many medical, industrial, and agricultural significance bacteria (Marín 2014).

2.5.2 Bacterial community in mushroom substrate

Danon et al. (2008) showed significant shifts in bacterial community structure during an extended maturation period of compost curing. *Bacteroidetes* and *Gammaproteobacteria* were ubiquitous, but their comparative dominances were inversely related; as at the beginning, *Bacteroidetes* were dominant but after a long curing period, *Gammaproteobacteria* were more abundant.

Siyoum et al. (2016) reported a significantly higher total bacterial counts and total fungal colony for compost samples than for casing and fresh mushroom samples which showed significant rise in total bacterial counts from casing to pinning. Bacteria in casing samples were more diverse than those in mushroom samples. The phylum *Proteobacteria* was most prevalent across all compost, casing, and mushroom samples. Low diversity of culturable compost bacteria in the sample could be due to the difference in the type of the compost materials (vegetable, fruit, and garden waste) used.

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According to Johri (2011), a diverse population consisting mainly of *Bacillus* sp., followed by *Ochrobactrum* sp., *Arthrobacter arilaiti, Stenotrophomonas maltophilia* are responsible for the degradation of the physical, chemical, and fibrous characteristics of the compost used in the cultivation of button mushroom.

Carrasco et al. (2019) showed that *Pseudomonas* and *Flavobacterium* are abundant in fresh *Agaricus bisporus* suggested that the bacterial diversity and population in basidiomes is highly conditioned by the casing microbiome. The increased abundance of bacteria belonging to the genus *Pseudomonas* detected in the casing over the duration of the trial could be partially explained by their well-known ability to colonize substrates while disturbing the native microbial community (Thomas and Sekhar, 2016). The presence and proliferation of Pseudomonads in button mushroom cultivation could decreasing yield of mushroom or could help to suppress bacterial diseases and post-harvest spoilage (Carrasco et al., 2019). Silva et al., (2009) reported that *Bacillus* and *Paenibacillus* spp. were the principal microorganisms present throughout the composting process (bagasse-based compost) used to cultivate *Agaricus brasiliensis*.

2.6 Culture dependent & targeted-metagnomic method

2.6.1 Culture dependent isolation & detection

Conventionally, diversity was measured using the selective plate method and viable count (Boulter et al., 2002). Johnsen et al. (2001) reported that direct counting by fluorescent microscopy gave multiple fold more the number of cells obtained by plate counting. These approaches offer data on the active, heterotrophic component of the population (Trevors 1998a, b). According to Sutton (2011), microbiological data are inherently variable. The plate count method is best to interpret of an approximation of the number of cells present.

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Besides, culturing and plant count method are vital in ecological microbial population identification. Siyoum et al., (2016) used a combination of methods (cultural and non-cultural) to better understand the microbial dynamics of mushrooms from seeding through growth, postharvest up to ready-to-eat at the consumer end of the supply chain. Fungi and yeasts which dominated by the genus *Penicillium* were able to be detected on plates even in the low diversity. In another study by Vela´zquez-Ceden˜o et al. (2008), plate counting method was used to isolate bacteria inhibiting *T. harzianum* growth in cultivation substrate of *P.ostreatus.*

The added benefit of plate culture is that the isolates can be used for biotechnological potentials. A simple and rapid method of microbial characterization accelerate the study on complete or partial characterization of microorganisms in the selection of potential microbial isolates for further work. Matrix assisted laser desorption/ionization time-of-flight mass-spectrometry (MALDI-TOF MS) is said to be one of the techniques in microbial culturomics (Lagier et al., 2012, 2015; Stafsnes et al., 2013; Dubourg et al.,2014). MALDI-TOF MS is a rapid and dependable technique for microbial identification. This technique is based on fingerprinting analyses of primarily ribosomal proteins, which are synthesized under all growth conditions and are the most abundant cellular proteins (Ryzhov and Fenselau, 2001). Therefore, Rahi et al. (2016) is confident that MALDI-TOF MS based biotyping play a key role in bringing a revolution in microbial ecology and diversity studies.

2.6.2 Targeted-metagenomic method

Polymerase chain reaction (PCR)-based molecular methods provide a rapid and sensitive alternative as the molecular methods are based on the analysis of single cells, opening an opportunity to analyze the microbial community in its full diversity (Fakruddin et al., 2013). Currently, the study of microbial communities has grown

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vastly using both phylogenetic and metagenomic approaches (Hugenholtz and Tyson, 2008). The 16S rRNA gene sequences have been used most extensively to classify the bacteria biodiversity (Swofford et al., 1996; Agrawal, 2015). Illumina sequencing is a technology generating multimillion partial 16S rRNA gene sequence reads and is capable to meet the throughput demands of soil microbial ecology studies besides being cost efficient (Bartram et al., 2011; Wu et al., 2010; Vasileiadis et al., 2012).

Siyoum et al. (2016) revealed species that were not retrieved via plate culture method previously and could possibly be novel using denaturing gradient gel electrophoresis (DGGE). Kredics et al. (2010) used specifically designed multiplex PCR assay to target for *T. pleurotum* and *T. pleuroticola.* However, the assay does not differentiate whether the substrate contains only *T. pleurotum* or both species. Metagenomics conducted through NGS on the other hand can work with crude environmental samples without any enrichment step detect and quantify non-culturable microbes. In a paper by Singh et. al. (2018) on *T. polysporum* inhibition against fungal pathogen in soil sample, they found that the combined culture-based and metagenomics approaches allowed them to follow the fate of the biocontrol agent and its target in the treated soil.

Metagenomics provides a more holistic approach with a more powerful workflow when compared with methods such as DGGE and Terminal restriction fragment length polymorphism (T-RFLP) (Massart et al., 2015). Several next generation sequencing techniques have been developed and introduced to microbial ecology as sequencing of specific short (~100 to 210 bp) rRNA or functional gene tag-sequences from marine biosphere (Sogin et al., 2006; Huber et al., 2007) and soil (Leininger et al., 2006; Roesch et al., 2007). The amount of data gained with this approach is huge as the number of sequences was from 26,000 to 900,000.

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Metagenomics approaches can be categorized into two groups, one is being the whole metagenomics and the other is the targeted metagenomics. Whole metagenomics is based on random sequencing strategy revealing microbial domains such as the bacteria, archaea, viruses, and their respective characteristic profiles (Suenaga, 2015). According to Suenaga, (2012), targeted metagenomics provide wide coverage for targeted genes and reveal specific genome areas even at low abundances within a metagenome. Targeted metagenomics via PCR-based approach were utilized widely to retrieve specific genes from a pool of DNA. As an alternative to cloning all the extracted DNA, primers are designed exclusively against an identified target gene (Suenaga, 2015).

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CHAPTER 3: MATERIALS AND METHODS

3.1 Research flow

This study applied both culture dependent and targeted-metagenomic method to reveal the microbial community of oyster mushroom substrate. Figure 3.1 shows the overall research methodology of this study.

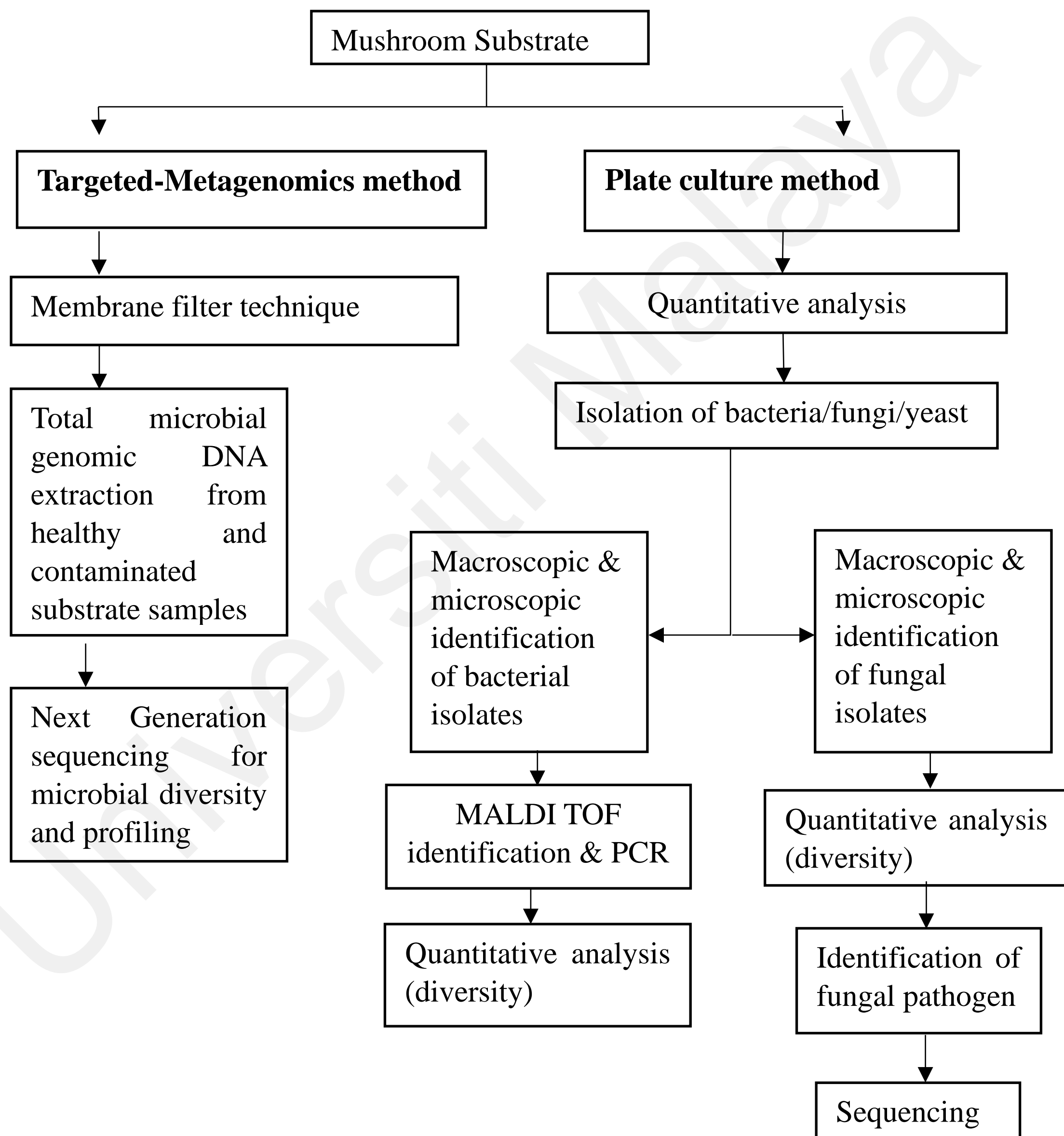

Figure 3.1: Overall research methodology

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3.2 Collection of mushroom substrates

Mushroom sawdust bags were sampled from a local mushroom, JW Mushroom Cultivation located at Kuala Pilah, Malaysia. The farm is a medium scale mushroom cultivation farm producing around 50-500kg fresh grey oyster mushroom daily for local market. Figure 3.2 shows the arrangement of mushroom substrate bags on the shelves used for cultivation and harvest. Figure 3.3 shows comparison between healthy and green-mould contaminated sawdust substrate bags.

Figure 3.2 Mushroom substrate bags on cultivation shelves at JW Mushroom Cultivation

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Figure 3.3: Substrate bags collected from the farm. (A) Healthy sawdust substrate bag – before sterilisation stage, (B) green-mould contaminated sawdust substrate bag – contaminated stage

A total of 14 mushroom sawdust samples were randomly collected at different stages of cultivation on 23/2/2016. The samples collected were sealed in biohazard bags and labeled respectively before transported to the laboratory for further analysis within 2 hours at room temperature. Table 3.1 shows the stages of samples collected.

Table 3.1: Samples collected at different stages of cultivation

Stages	Number of substrate bags (Sample)
Before Sterilization	2
After Sterilization	2
Spawn Running	2
First Harvest	2
Second Harvest	2
Final Harvest	2
Contaminated	2

3.3 Microbiological analysis

3.3.1 Sample preparation

Serial dilution was carried out by diluting 25g of each substrate sample in 225ml of sterile saline solution (0.85% NaCl) and homogenised for 5 min using the

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STOMACHER Lab Blender. The homogenised samples were further diluted up to 6th fold serial dilution (10^{-1} to 10^{-6}) for plating procedure.

3.3.2 Total bacterial count

A total of 100µL of each 4th to 6th fold diluted sample were plated out in duplicates on full strength nutrient agar (NA) and 1/10 strength nutrient agar (NA 1/10) respectively to retrieve culturable bacteria using the spread plate technique. The urine streak technique was used to streak 1µL of each 4th to 6th fold dilutions of each sample on CHROMagar™ Orientation (CO) to check for the presence or absence of possible enteric bacteria. The inoculated NA, NA 1/10 and CO plates were incubated at 37°C for 24 hours. The bacterial isolation plates were incubated at the mentioned temperature as it is the optimum temperature for the growth of foodborne pathogens this study aims to retrieve (Bryan, 2004). The cultured colonies from all the plates were enumerated and the total mean colony forming unit (CFU/ml) for bacteria were determined using the bacterial count from the 4th fold diluted sample as the counts were within enumerable range of 25-250 colonies.

3.3.3 Total fungal count

A total of 100µL of each 4th to 6th fold diluted sample were plated out in duplicates on potato dextrose agar (PDA) was used to retrieve culturable fungi using the spread plate technique. The inoculated PDA plates were incubated at 25°C for 5 days. The cultured colonies from PDA plates were enumerated and the total mean spore forming unit (SFU/ml) per plate for fungi were determined using the fungal count from the 4th fold diluted sample.

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3.3.4 Isolation and preservation of microbial isolates

Bacterial and fungal colonies of different colony morphology were purified on their respective culture agar as shown in Figure 3.4. The bacterial isolates were subjected to Gram staining (Smith & Hussey, 2005) and the protocol is as follows. Purified single colony of cells was smeared on a glass slide with a drop if sterile distilled water to form a thin layer. It was then heat-fixed. Crystal violet staining reagent was used to flood the smear for 1 minute. The slide was then washed for 2 seconds in and indirect stream of tap water. The slide was flood with Gram's iodine for a minute. The wash step was repeated. The slide was flood with decolorizing agent for 15 seconds and then let to run down the slide by tilting it to a side. Safranin was used as the counterstain by flooding for another minute. The wash step was repeated until no colour on the slide and then blot dried with absorbent paper. The glass slide was then observed under oil immersion using a Brightfield microscope for the gram stain results. Fungal isolates on the other hand, were identified based on phenotypic and microscopic characteristics. Agar stabs of purified isolates were prepared for long term preservation.

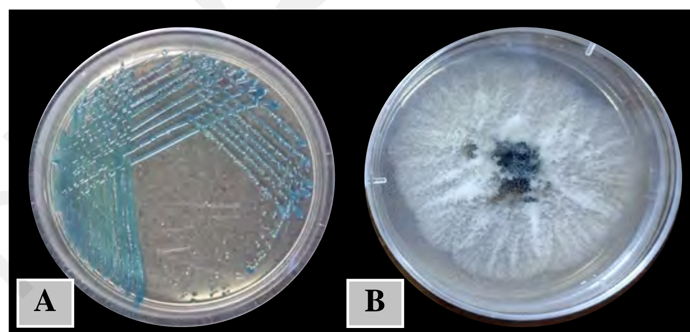

Figure 3.4 Bacterial and fungal colonies isolated from the sample. (A)Enteric bacteria isolation on CO plate, (B) fungal isolation PDA plate

3.4 Bacterial identification by Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS)

The matrix solubilisation procedure was carried out as described in a manual (APPENDIX A) by Bruker Daltonik in section 8.1 and 8.2 (Bruker Daltonik GmbH)

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using (50:50 [v/v] acetonitrile: 0.1% TFA in water) ratio to prepare the solvent containing α-Cyano-4-hydroxycinnamic acid(HCCA) matrix. One to two colonies of each purified bacterial isolates were picked using a sterile toothpick and smeared onto one target position each on the MSP 96 target polished steel BC. Then 1µL of matrix solvent was deposited onto each smeared target position and let to air dry. The MSP 96 target polished steel BC plate was then placed carefully into the Microflex MALDI-TOF instrument to allow the MALDI Biotyper system acquire bacterial ID. MALDI-TOF MS had been used in many past studies and is said to offer the possibility of accurate, rapid, and inexpensive identification of microorganisms (Murray, 2012).

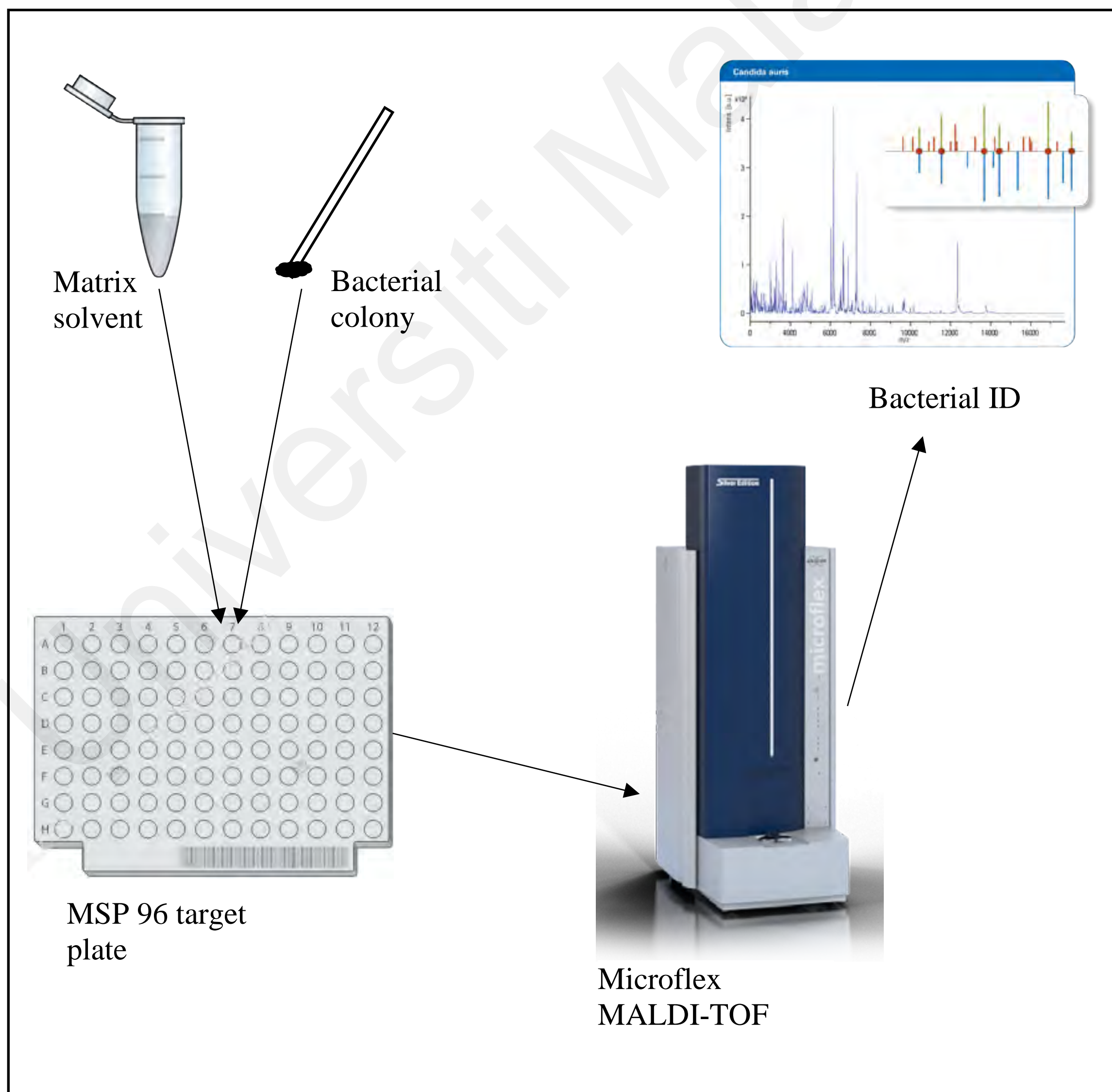

Figure 3.5 Flow of MALDI-TOF MS Bacterial Identification

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3.5 Fungal microscopic and macroscopic identification

Fungal colonies from the culture plates were purified on PDA and grown at 25°C for 3 to 5 days. Visual observation of phenotypic and microscopic characteristics using simple staining method for the fungal fruiting body were carried out for genus level identification by using book references (Roberts et al., 1981, Jennings, 1984). The colony morphology observed were the shape, size, elevations/surface, pigmentation, and edges. As for the microscopic observation hyphal branching and sporulation were noted.

3.6 Bacterial DNA extraction

Gram positive isolates which were not identifiable using the MALDI Biotyper system were prepared for 16S rRNA gene sequencing. Bacterial DNA were extracted using the boiling method. One colony of purified bacterial isolate was dispersed in a 1.5 ml micro centrifuge tube containing 100 µL of sterile ultrapure water and vortexed. The suspension is then incubated at 100°C for 5 min using a heat block, chilled at -20°C for 5 min and centrifuged at 14000×rpm for 1 min. The supernatant was transferred into a new sterile 1.5 ml micro centrifuge tube and stored at -20°C

3.7 Fungal DNA extraction

Trichoderma isolates were extracted using E.Z.N.A.®Forensic DNA Kit (OMEGA bio-tek) with modification. A total of 100 mg mycelia was grounded in 200 µl STL buffer and incubated at 55°C, 15 min. 25 µl of OB protease solution was added in it, vortexed and incubated at 60°C, 45 min with occasional mixing. 225 µl BL buffer was added, vortexed and incubated at 60°C, 10 min. 225 µl of absolute ethanol was then added to the mixture and vortexed. The entire sample mixture was transferred into a Hibind DNA column with 2ml collection tube which was centrifuged earlier with 100 µl equilibrium buffer. The column holding the sample was centrifuged at 8000 g, 1 min

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and the flow through is discarded. The column was washed with 500 µl HB buffer with a new collection tube and centrifuged at 8000 g, 1 min the flow through is discarded. The washing and centrifugation step are repeated twice with 750 µl wash buffer both times. The column is then dried by centrifugation at maximum speed at 13000 g, 2 min and placed in a 2 ml microcentrifuge tube to sit at room temperature, 3 min with 50 µl preheated elution buffer. The column was then centrifuged at 8000 g, 1 min to elute and the freshly prepared DNA solution was kept at -20°C until further use.

3.8 Identity confirmation of microbial isolates

3.8.1 16S rRNA gene-amplification

PCR reactions to detect 16S universal markers (V1-V9 region) in bacteria were performed by preparing a final volume of 25 µL containing 1 × Colourless GoTaq® Flexi Buffer (Promega, Madison, WI USA), 1.5 mM of $MgCl_2$, 0.2 mM of dNTP Mix, 1.0 unit of GoTaq® Flexi DNA Polymerase, primers 27F: 5′-AGA GTT TGA TCC TGG CTC AG-3′ (Lane, 1991) and 1492R: 5′-TAC GGY TAC CTT GTT ACG ACT T-3′ (Turner et al.,1999) at 0.2 µM each, and 2.0 µL of DNA template. The PCR amplification was run using Swift™ Maxi PCR thermal cycler (Esco, Barnsley, UK) with the conditions: 2 min at 95 °C; followed by 30 cycles of 95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 1 min; and 7 min at 72 °C. For each PCR run, a negative control of sterile ultrapure water was included. The amplification was verified via agarose gel electrophoresis where 2 µL of PCR product was mixed with 1 µL of 1 × Green GoTaq® Flexi Buffer (Promega, Madison, WI USA) and loaded into the gel wells to run at 75 volts for 30 min in a horizontal electrophoresis using MS Major Science. The visualization of PCR product was done using UVP DigiDoc-It 130. The amplified PCR products of bacteria were sent for sequencing to Genomics Bioscience & Technology Co., Ltd., (Taiwan).

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3.8.2 ITS-amplification

PCR reactions to detect internal transcribed spacer (ITS) region markers in *Trichoderma sp.* were performed with preparing a final volume of 50 µL containing 1 × i-Taq^{TM} Plus PCR Buffer (INtRON Biotechnology), 0.4 mM of i-Taq^{TM} Plus dNTP Mixture, 1.0 unit of i-Taq^{TM} Plus DNA Polymerase, primers ITS1-F: CTTGGTCATTTAGAGGAAGTAA (Gardes and Bruns, 1993) and ITS4: TCCTCCGCTTATTGATATGC (White et al.,1990) at 0.5 µM each, and 2.0 µL of DNA template. The PCR amplification was run using MyCycler^{TM} thermal cycler (BioRad) and the conditions adhered to were: 4 min at 95 °C; followed by 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 1 min; 10 min at 72 °C. For each PCR run, a negative control of sterile ultrapure water was included. The amplification was verified via agarose gel electrophoresis where 5 µL of PCR product was mixed with 1 µL of Gel Loading Dye, Blue (6X) and loaded into the gel wells to run at 100 volts for 40 min in a horizontal electrophoresis using MS Major Science. The visualization of PCR product was done using AlphaDigiDoc RT 2 digital imaging system. The amplified PCR products of fungi were sent for sequencing to Genomics Bioscience & Technology Co., Ltd., (Taiwan).

3.8.3 BLAST analysis

All data tabulation and analysis were conducted using Excel 2016®. ChromasPro 2.0.1 was used to assemble and edit the raw sequence of bacterial and fungal sequencing data to produce a consensus sequence for BLAST analysis. Fungal DNA sequences were identified using BLASTn(https://blast.ncbi.nlm.nih.gov/) and confirmed using TrichOKEY2.0((http://www.isth.info) (Druzhinina et al., 2005; Druzhinina and Kopchinski, 2006).

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3.9 Targeted- Metagenomic Method

Samples collected as mentioned in 3.2 were subjected to serial dilution by diluting 25 g of each substrate sample in 225 ml of sterile saline solution (0.85% NaCl) and homogenised for 5 min using the STOMACHER Lab Blender. Selected diluted samples (healthy mushroom substrate and green-mould contaminated substrate) for metagenomic analysis were subjected to membrane filtration technique (MFT). The diluted samples were first subjected to filtration using sterilized cheese cloth where 100 ml of the homogenized diluted sample was poured into a sterilized beaker through the cloth. The cloth filtered sample was then filtered using MFT to retrieve the total microbial content present on 0.2 µm polycarbonate filter membrane. This was done by filtering the sample by 50 ml volumes in 2 parts. The 0.2 µm polycarbonate filter membrane which contains the filtered microbial cells were then stored in sterilized airtight falcon tube in 4 ℃ to be subjected to total genomic DNA extraction using kit.

PowerSoil® DNA Isolation Kit from MO BIO Laboratories, Inc. was used to extract the total genomic DNA according to manufacturer′s instructions (Appendix B) with a few modifications. In step 1, instead of 0.25 g of soil sample, ¼ portion of the filtered 0.2 µm polycarbonate filter membrane was cut into small pieces and added into the PowerBead Tube. In step 3, Solution C1 was heated to 70℃ until dissolved before use. After step 4, the PowerBead Tube was incubated at 70℃ for 10 minutes before proceeding to step 5.

The DNA extract for each sample were concentrated according the PowerSoil® DNA Isolation Kit from MO BIO Laboratories, Inc. instructions (Appendix C) with modification. Each ¼ portion of the filtered 0.2 µm polycarbonate filter membrane extracted and concentrated were resuspended with 25 µl of sdH_2O and combined to pool

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a total volume of 100 µl DNA concentrate. The concentrated DNA extract (30 ng/uL) for each sample is subjected to PCR amplification targeting 16S(V1-V9) and ITS gene region universal markers (same procedure as in 3.8.1 and 3.8.2 respectively). Once the presence of bacterial and fungal DNA is confirmed, the DNA extracts of selected samples were sent to Majorbio Shanghai, China through MyTACG Bioscience Enterprise, Malaysia for sequencing via MiSeq Next Generation Sequencing (NGS) to screen for microbial diversity and profiling. According to the report from Majorbio, PCR amplicons were extracted and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's instructions and quantified using QuantiFluor™ -ST (Promega, U.S.). Library was constructed and pooled in equimolar and paired end sequenced (2 × 250/300 bp) on an Illumina MiSeq platform (or other sequencer when necessary) according to the standard protocols.

3.10 Calculation of Menhinick's Index and Shannon's Diversity Index

Species richness and diversity for our cultured bacterial isolates were calculated across the harvest stages as shown in Table 4.4. Species were measured using Menhinick's index, D.

$$D = \frac{s}{\sqrt{N}}$$

where s equals the number of different species in the sample, and N equals the total number of isolates cultured from the sample.

As for species diversity, the Shannon index, H was calculated.

$$H = \sum(p_l)|ln\, p_l|$$

where (p_l) is the ratio of the total number of isolates retrieved in the population that were in species "l".

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Both these indexes are classified under Species Richness indices. According to Mirzaie et al., (2013), these indexes can be used in a study if it is possible to identify all the species in the sample successfully. In this study were able to identify all culturable bacteria using the nutrient agar culture medium of 2 different strength across harvest stages, therefore these indices are suitable to be used to estimate richness of cultured bacterial population from the samples.

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CHAPTER 4: RESULTS

4.1 Microbial analysis of mushroom substrates

Mean bacterial count on both NA and 1/10 strength NA plates were highest from the contaminated substrate with the count of 9.5×10^7 CFU/g, respectively. Bacterial colonies were observed on CO plate for second harvest substrate and contaminated substrate each with the mean count of 1.5×10^6 CFU/g and 5.0×10^5 CFU/g, respectively (Table 4.1). Total bacterial mean count increased from first harvest to final harvest but were highest at contaminated substrate. To conclude, the contaminated substrate is the most abundant, diverse, and rich in terms of microbiota compared to other stages of harvest. With regards to the mean fungal count on PDA, the data did not show a significant trend as they were retrieved abundantly across harvest stages

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Table 4.1: Culturable bacterial and fungal count from sawdust substrates of oyster mushroom before sterilization process, during spawn run, after first harvest, second harvest and final harvest as well as green-mould contaminated substrate.

HARVEST STAGES	FUNGAL COUNT ON PDA		BACTERIAL COUNT ON NA		BACTERIAL COUNT ON NA (1/10 STRENGTH)		BACTERIAL COUNT ON CO	
	Mean(sfu/g)	**SE**	**Mean(cfu/g)**	**SE**	**Mean(cfu/g)**	**SE**	**Mean(cfu/g)**	**SE**
BEFORE STERILIZATION	4.6×10^{7}	1.4×10^{7}	1.0×10^{6}	1.0×10^{6}	1.4×10^{7}	3.8×10^{6}	< 10	ND
AFTER STERILIZATION	< 10	ND	< 10	ND	<10	ND	< 10	ND
SPAWN	< 10	ND	1.0×10^{6}	5.0×10^{5}	<10	ND	< 10	ND
FIRST HARVEST	1.3×10^{6}	2.5×10^{5}	1.1×10^{7}	1.3×10^{6}	4.0×10^{6}	4.0×10^{6}	< 10	ND
SECOND HARVEST	2.5×10^{5}	2.5×10^{5}	4.5×10^{7}	5.0×10^{6}	2.8×10^{7}	ND	1.5×10^{6}	ND
FINAL HARVEST	1.3×10^{6}	2.5×10^{5}	5.0×10^{7}	7.5×10^{6}	2.3×10^{7}	4.0×10^{6}	< 10	ND
CONTAMINATED	1.0×10^{6}	ND	9.5×10^{7}	1.4×10^{7}	9.5×10^{7}	ND	5.0×10^{5}	ND

PDA, potato dextrose agar; sfu, spore-forming unit; NA, nutrient agar; cfu, colony-forming unit; CO, ChromAgar Orientation; ND, not detected; SE, standard error

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4.2 Diversity of cultivated bacterial population in mushroom substrates for all harvest stages based on culture-dependent approach

In total, 116 cultured bacteria were obtained consisting phylum *Proteobacteria* (76 isolates), *Firmicutes* (18 isolates), *Bacteroidetes* (21 isolates) and Actinobacteria (1 isolate) (Table 4.3). Bacteria cultured from substrate before sterilisation consist mostly of *Bacilli* class bacteria from the *Firmicutes* phylum. Majority of bacteria cultured from substrates after first harvest, were mostly from phylum *Proteobacteria* and *Pseudomonas* was the highest cultured genus consisting of 21 isolates out of 76 *Proteobacteria* isolates. The highest number of isolates were from contaminated substrate and majority were in the class *Gammaproteobacteria*. Bacteria of genus *Raoultella*, *Enterobacter, Pseudomonas* and *Stenotrophomonas* were the most notable genus of bacteria retrieved.

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Table 4.2 Culturable bacterial population from sawdust substrates of oyster mushroom before sterilization process, during spawn run, after first harvest, second harvest and final harvest as well as green-mould contaminated substrate

Phylum	Class	Order	Family	Genus	Species	Before Sterilisation	After Sterilisation	Spawn	First Harvest	Second Harvest	Final Harvest	Contaminated	Grand Total	Identification
Actinobacteria	*Actinobacteria*	*Micrococcales*	*Micrococcaceae*	*Citricoccus*	*Citricoccus parietis*				1				1	16s
Actinobacteria Total									1				1	
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Chryseobacterium*	*Chryseobacterium gleum*				1	5		1	7	MALDI
					Chryseobacterium indologenes			1	1			2	4	16s, MALDI
					Chryseobacterium moechotypicola				2				2	16s
					Chryseobacterium sp.				1		1	1	3	16s, MALDI
	Sphingobacteriia	*Sphingobacteriales*	*Sphingobacteriaceae*	*Sphingobacterium*	*Sphingobacterium multivorum*					1	1	2	4	16s, MALDI
					Sphingobacterium thalpophilum							1	1	MALDI
Bacteroidetes Total								1	5	6	2	7	21	
Firmicutes	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	*Bacillus cereus*						2		2	16s, MALDI
					Bacillus circulans	1							1	16s
					Bacillus megaterium							1	1	16s
					Bacillus pumilus	1							1	16s
					Bacillus sp.	1		1					2	16s
					Bacillus subtilis	5		1		1			7	16s, MALDI
			Planococcaceae	*Rummeliibacillus*	*Rummeliibacillus stabekisii*	1							1	16s
					Rummeliibacillus suwonensis	1							1	16s
			Staphylococcaceae	*Staphyloccocus*	*Staphylococcus hominis*	1							1	MALDI
		Lactobacillales	*Lactobacillaceae*	*Lactobacillus*	*Lactobacillus pentosus*	1							1	MALDI
Firmicutes						12		2		1	2	1	18	

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Phylum	Class	Order	Family	Genus	Species	Before Sterilisation	After Sterilisation	Spawn	First Harvest	Second Harvest	Final Harvest	Contaminated	Grand Total	Identification
Total														
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Brucellaceae*	*Ochrobactrum*	*Ochrobactrum intermedium*				1			1	2	MALDI
	Betaproteobacteria	*Burkholderiales*	*Alcaligenaceae*	*Achromobacter*	*Achromobacter denitrificans*					1			1	MALDI
					Achromobacter insolitus							1	1	MALDI
					Achromobacter xylosoxidans				1		1		2	MALDI
			Comamonadaceae	*Corticibacter*	*Corticibacter populi*	3							3	16s
				Delftia	*Delftia* sp.						2		2	16s
				Diaphorobacter	*Diaphorobacter* sp.					1			1	16s
	Gammaproteobacteria	*Enterobacteriales*	*Enterobacteriaceae*	*Cedecea*	*Cedecea lapagei*							1	1	MALDI
					Cedecea neteri							2	2	MALDI
				Enterobacter	*Enterobacter asburiae*					1		3	4	MALDI
					Enterobacter cloacae				1	2		2	5	MALDI
					Enterobacter cowanii							1	1	MALDI
					Enterobacter kobei					1			1	MALDI
				Klebsiella	*Klebsiella variicola*	1			1				2	MALDI
				Pantoea	*Pantoea dispersa*							1	1	MALDI
				Raoultella	*Raoultella ornithinolytica*				1	2	5	6	14	16s
				Serratia	*Serratia marcescens*					2	1		3	MALDI
		Pseudomonadales	*Moraxellaceae*	*Acinetobacter*	*Acinetobacter baumannii*						1		1	MALDI
			Pseudomonadaceae	*Pseudomonas*	*Pseudomonas aeruginosa*					2	2	2	6	MALDI
					Pseudomonas citronellolis	1							1	MALDI
					Pseudomonas mendocina					2			2	16s, MALDI

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Phylum	Class	Order	Family	Genus	Species	Before Sterilisation	After Sterilisation	Spawn	First Harvest	Second Harvest	Final Harvest	Contaminated	Grand Total	Identification
					Pseudomonas monteilii				3			2	5	MALDI
					Pseudomonas nitroreducens					1		1	2	MALDI
					Pseudomonas putida							4	4	16s, MALDI
					Pseudomonas taiwanensis				2				2	16s, MALDI
		Xanthomonadales	*Xanthomonadaceae*	*Stenotrophomonas*	*Stenotrophomonas acidaminiphila*					1			1	MALDI
					Stenotrophomonas maltophilia				1	1		2	4	MALDI
					Stenotrophomonas sp.	2							2	16s
Proteobacteria Total						7			11	17	12	29	76	
Grand Total						19		3	17	24	16	37	116	
Species Richness(D)						2.75	NA*	1.73	3.15	3.06	2.25	3.29		
Shannon(H)						2.27	NA*	1.10	2.48	2.55	2.01	2.84		

NA*, no significant va

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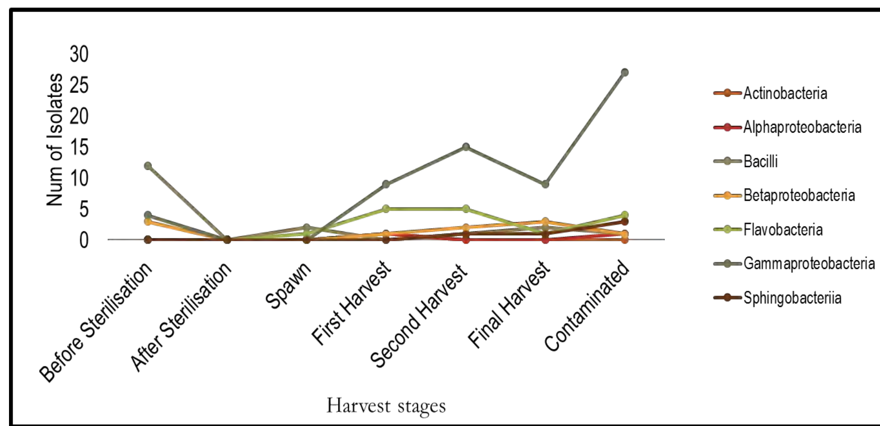

Figure 4.1: Diversity shift in cultured bacterial community

However, a diversity shift was observed in the cultured bacterial community across harvest stages as shown in Figure 4.1. Bacteria retrieved from the class *Bacilli* significantly dropped after sterilization of substrate and it is notable that this class of bacteria dominated the cultured bacterial community in substrate before sterilisation. Large number of isolates of *Bacillus* sp. from substrates before sterilisation indicates the predomination in the substrates which may occur naturally in the sawdust. However, an increase in the number of *Gammaproteobacteria* class bacterial community was observed along the harvest stages.

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4.3 Diversity of cultivated fungal population in mushroom substrates for all harvest stages based on culture-dependent approach

The culturable fungi population consists of 28 fungal isolates in total, 18 isolates were *Trichoderma* spp.*,* while the others were identified as genus *Graphium, Fusarium,* and *Penicillium*. No fungi were isolated from after sterilisation, spawn run and second harvest stage (Table 4.3). All the 15 *Trichoderma* isolates from the contaminated substrate were identified as *Trichoderma pleurotum* and 3 out of 4 *Graphium* isolates were identified as G*raphium penicillioides* (Table 4.4). Figure 4.2 shows the plate morphology and microscopic morphology of *Graphium penicilloides* and *Trichoderma pleurotum* isolated from the contaminated mushroom substrate bags.

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Table 4.3 Culturable fungal population from sawdust substrates of oyster mushroom before sterilization process, after first harvest and final harvest as well as green-mould contaminated substrate.

Genus and Species of Fungal Isolate	Before Sterilisation	First Harvest	Final Harvest	Contaminated	Grand Total
Fusarium sp.			1		1
Unidentified			1		1
Graphium sp.	1	2		1	4
G. penicilloides		2		1	3
Unidentified	1				1
Penicillium sp.		3	2		5
P. cinnapurpureum			1		1
P. citrinum		2			2
Unidentified		1	1		2
Trichoderma sp.			3	15	18
T. pleurotum				15	15
Unidentified			3		3
Grand Total	**1**	**5**	**6**	**16**	**28**

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Table 4.4: Identification of *Trichoderma* and *Graphium* species isolated from green-mould contaminated substrate

Isolate code	Species	Query cover (%)	E value	Identity (%)	Accession
CD1(2)-1F	*Trichoderma pleurotum*	100	0.0	100	KP263552
CD1(2)-2F					
CD1(2)-3F					
CD1(2)-4F					
CD2(2)-1F					
CD2(2)-2F					
CD2(2)-5F					
CD2(2)-A2F					
CD2(2)-B1F					
CD2(2)-B2F					
CD2(2)-F1F					
CD2(2)-F2F					
CD2(2)-G2F					
CD2(2)-G5F					
CD2(2)-H5F					
HIDI(2)-2F	*Graphium penicillioides*	100	0.0	97	KM433841
H1D2(2)-2F		100	0.0	97	AB038431
CD2(2)-3F		98	0.0	97	

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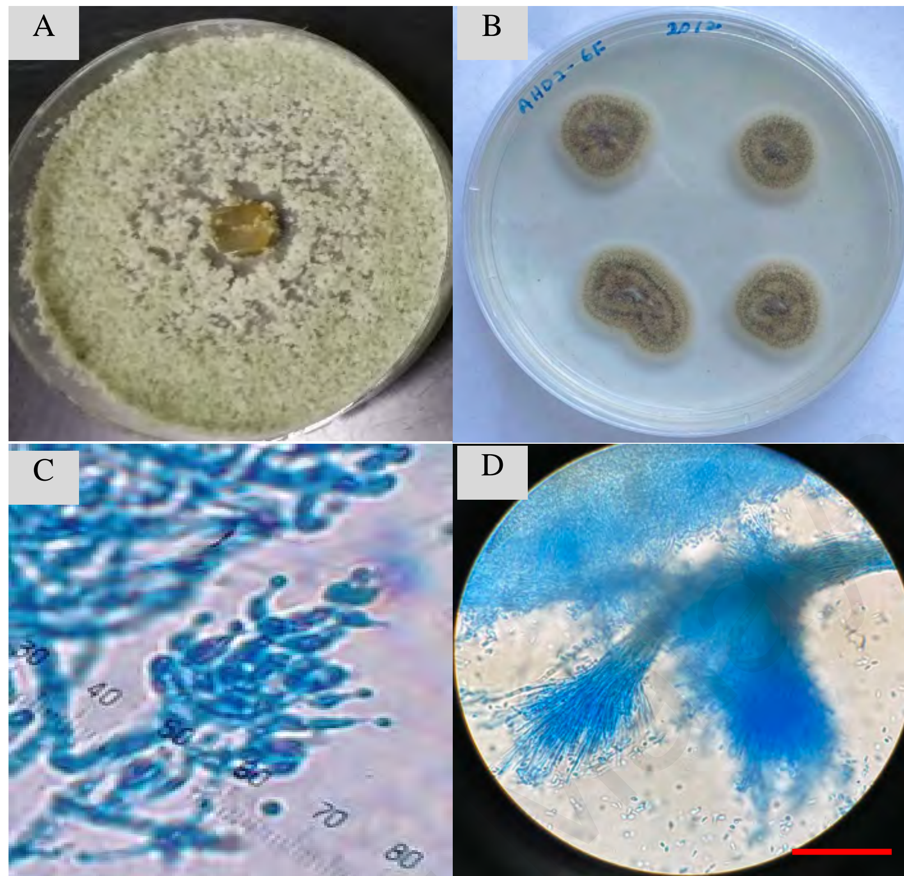

Figure 4.2: ***Graphium penicilloides*** **and** ***Trichoderma pleurotum*** **isolated from the contaminated mushroom substrate bag. A. Colony of** ***T. pleurotum*** **on PDA culture. B)** ***G. penicilloides*** **colony on PDA culture. C) Conidiophores of** ***T. pleurotum*****, magnification 1000x; D) Conidiiophores of** ***G. penicilloides*****, maginification 1000x. Scale bars: A-B= 10 mm, C-D= 10 µm**

4.4 Metagenomic analysis of bacterial and fungal composition in healthy and contaminated substrates

Shanghai Majorbio reported back the metagenomic data of the DNA extracts from both healthy and contaminated substrate bags sent for NGS and the statistics of samples are as shown in Table 4.5.

Table 4.5: Statistics of DNA extracts from healthy and green-mould contaminated substrate

Sample	DNA extract	Sample code	Sequences	Bases(bp)	Average length (Bp)
Final harvest (Healthy)	Bacteria	A_B	55033	24329160	442.08
	Fungi	A_F	59875	19596727	327.29
Contaminated (Green-mould)	Bacteria	C_B	72859	32425282	445.04
	Fungi	C_F	60382	15908061	263.46

The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier against the Silva (SSU123) 16S rRNA gene database using confidence threshold of 0.7 and Fungus ITS database via Unite by Majorbio.

The metagenomic analysis results from contaminated substrate revealed that *Gammaproteobacteria* made up to 58% of the total quality reads consisting largely of 73.5% *Enterobacteriaceae*, 13.7% *Pseudomonadaceae* and 10.7% *Xanthomonadaceae* within total Gammaproteobacteria quality reads as shown in Figure 4.3. *Enterobacteriaceae* could be assigned to 10.53% *Raoultella*, and 17.51% *Cedecea*; *Pseudomonadaceae* could be assigned to 7.96% *Pseudomonas*; and *Xanthomonadaceae* could be assigned to 5.36% *Stenotrophomonas* of the total quality reads obtained from the contaminated substrate. The relative abundance in terms of metagenomic reads of *Proteobacteria* community shows similar trend when compared with cultured bacterial community from final harvest and contaminated substrate. The species richness and diversity calculated for the samples using Mehenick's Index measure and Shannon's

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Diversity Index as shown in Table 4.2 respectively indicated that contaminated substrate had the highest value (D=3.29) and (H=2.84).

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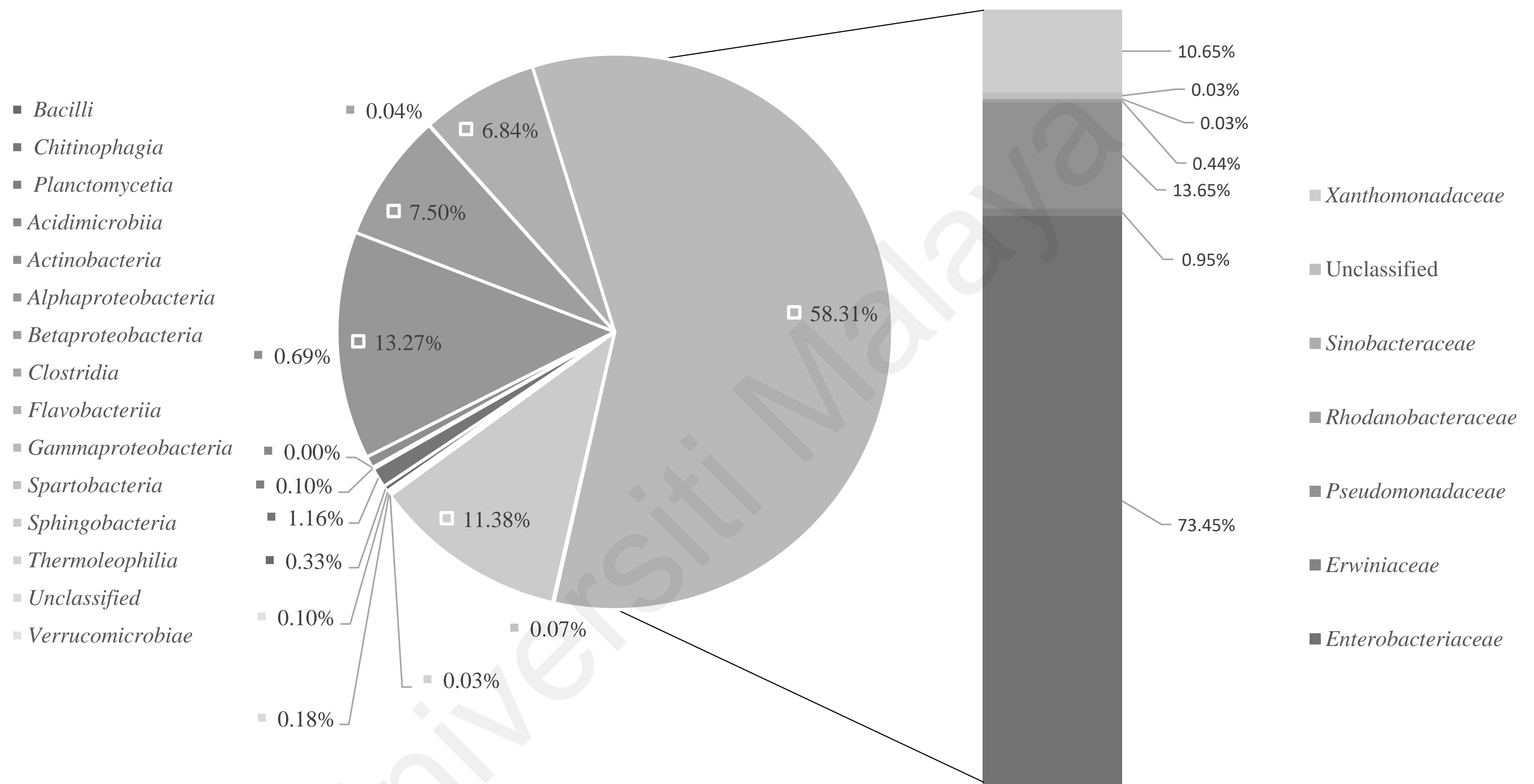

Figure 4.3: Metagenomic composition of bacterial community in green-mould contaminated substrate

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In terms of bacterial diversity, Figure 4.4 shows both culture dependent and targeted-metagenomic method revealed that bacteria from the phylum *Proteobacteria* dominated the bacterial community in both final harvest substrate and contaminated substrate.

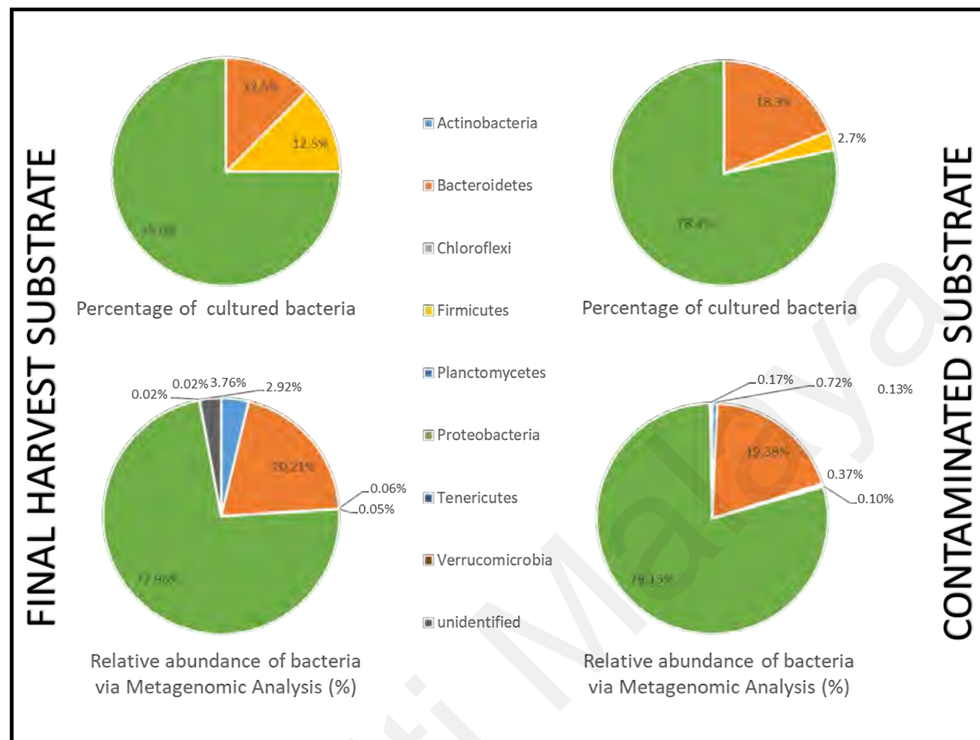

Figure 4.4: Bacterial community retrieved from culture dependent and independent method

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(a) Bacterial Taxon	Healthy mushroom bag	Green-mould contaminated mushroom bag
480-2_norank	0.074	0.004
*Acetobacteraceae*_unclassified	0.028	0
Achromobacter	1.668	0.969
*Acidimicrobiaceae*_uncultured	0.008	0.002
Acinetobacter	0.019	0
Advenella	0	0.044
Agaricicola	0.076	0.002
Agromyces	0.011	0.002
*Alcaligenaceae*_unclassified	0.444	0.229
*Alphaproteobacteria*_unclassified	0.068	0
Altererythrobacter	0.751	0.423
Asticcacaulis	0.095	0
*Bacteria*_unclassified	0.017	0
Bordetella	8.376	2.45
Bosea	0.571	0.578
*Bradyrhizobiaceae*_unclassified	0.322	0.174
Brevundimonas	5.36	0.91
*Brucellaceae*_unclassified	0.722	1.282
Camelimonas	0.006	0.925
Candidatus_Odyssella	0.013	0
Candidatus_Xiphinematobacter	0.004	0.072
Caulobacter	1.839	0.087
*Caulobacteraceae*_unclassified	0.068	0.104
Cedecea	0.349	17.519
Cellulosimicrobium	0.072	0.114
Cellvibrio	0.19	0
Chitinophaga	0.514	0
*Chitinophagaceae*_unclassified	0.034	0.03
*Chitinophagaceae*_uncultured	0.002	0.053
Chryseobacterium	0.497	4.876
CL500-29_marine_group	0.015	0
*Clostridium*_sensu_stricto_3	0	0.013
Cohnella	0.049	0.292
*Comamonadaceae*_unclassified	0.415	1.354
DB1-14_norank	0.004	0
Devosia	1.069	0.169
Dokdonella	0	0.254
Dongia	0.032	0
Elizabethkingia	0	0.639
Enterobacter	0.023	0.214
*Erythrobacteraceae*_unclassified	0.14	0.169
Filimonas	0.019	0
Flavobacterium	0.719	0.59
Fluviicola	0	0.398
*Gracilibacteria*_norank	0.131	0
*Hydrogenedentes*_norank	0	0.047
Hydrogenophaga	0.036	0
Hyphomicrobium	0.559	0.004
Isosphaera	0	0.008
JG30-KF-CM45_norank	0.059	0
Kaistia	0.032	0.241

(b) Fungal Taxon	Healthy mushroom bag	Green-mould contaminated mushroom bag
Alternaria	0	0.003
Bjerkandera	0	0.007
*Chaetothyriales*_unclassified	0	0.015
Cryptococcus	0.002	0.043
*Eurotiomycetes*_unclassified	0	0.012
Fonsecaea	0.026	0.128
*Fungi*_unclassified	0.012	2.352
Graphium	0.003	56.296
*Hypocreales*_unclassified	0.079	0.145
Kurtzmanomyces	0	0.003
*Microascaceae*_unclassified	0	0.007
*Nectriaceae*_unclassified	0.015	0.073
Penicillium	0.01	0.174
Pleurotus	98.783	0.246
*Saccharomycetales*_unidentified	0.007	0
Schizophyllum	0.031	0.003
Schizothecium	0.005	0.002
*Sordariomycetes*_unclassified	0.792	6.38
Trichoderma	0.236	34.11

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(a) Bacterial Taxon	Healthy mushroom bag	Green-mould contaminated mushroom bag
Lachnoclostridium	0	0.023
LD29_norank	0	0.049
Leucobacter	0.148	0.068
Listeria	0	0.008
Luteimonas	0.036	0.002
Luteolibacter	0.019	0.097
Lysinimonas	1.515	0.021
Marmoricola	0.108	0.004
Mesorhizobium	0.472	0.07
Methylobacillus	3.085	0.148
Methylobacterium	0.017	0.074
Methylophilus	6.243	0.768
*Microbacteriaceae*_unclassified	0.842	0.006
Microbacterium	0.815	0.343
*Micrococcales*_unclassified	0.002	0.03
Moheibacter	0.017	0.104
*Mollicutes*_RF9_norank	0.017	0
Mumia	0.025	0
Mycobacterium	0.004	0.061
Myroides	0	0.233
Niabella	0.317	0.038
Nocardioides	0.023	0
NS11-12_marine_group_norank	0.015	0
Nubsella	0.03	0.104
*Oligoflexales*_norank	0.034	0
Olivibacter	1.888	3.299
Oxalicibacterium	22.732	0.944
Paenalcaligenes	0	0.038
Paenibacillus	0	0.034
Pantoea	0.004	0.554
Parapedobacter	9.639	1.177
Patulibacter	0.011	0.025
Pedobacter	0.303	0.032
Pedomicrobium	0.002	0.032
Peredibacter	0.019	0
Persicitalea	0.008	0
Phenylobacterium	0.696	0.053
Pigmentiphaga	0.438	0.072
Pleomorphomonas	0.032	0.025
*Propionibacteriaceae*_uncultured	0.023	0
*Proteobacteria*_unclassified	0.286	0.049
Providencia	0.008	14.563
*Pseudomonadales*_unclassified	0.021	0.485
Pseudomonas	0.008	7.961
Pseudospirillum	0.284	0
Pseudoxanthobacter	0	0.023
Pseudoxanthomonas	2.994	0.849
Ralstonia	0.038	0.053
Ramlibacter	0.066	0.254
Raoultella	0.343	10.534
Reyranella	0.004	0.144
*Rhizobiales*_unclassified	0.07	0.07

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(a) Bacterial Taxon	Healthy mushroom bag	Green-mould contaminated mushroom bag
Rhizobium	3.295	3.587
Rhizomicrobium	0.44	0.059
*Rhodobacteraceae*_unclassified	0.063	0
Rhodoligotrophos	0	0.03
*Rhodospirillaceae*_uncultured	0.095	0
Roseococcus	0	0.176
Roseomonas	0.119	0.028
*Saccharibacteria*_norank	2.755	0
SHA-109_norank	0	0.036
Shinella	0.218	3.214
Singulisphaera	0	0.093
SM2D12_norank	0.161	0
Solimonas	0.072	0
*Sphingobacteriaceae*_unclassified	0.586	0.176
*Sphingobacteriaceae*_uncultured	0.48	0.573
Sphingobacterium	1.206	5.244
Sphingobium	1.445	0.722
*Sphingomonadaceae*_unclassified	1.06	0.023
*Sphingomonadales*_norank	0.586	0.021
Sphingomonas	0.411	0.209
Sphingopyxis	0.178	0.008
Starkeya	0.057	0.303
Stenotrophomonas	0.195	5.356
Steroidobacter	0.032	0.017
Streptomyces	0.068	0.036
Taibaiella	0.681	1.039
Thermomonas	0.074	0
Xanthobacter	0.049	0.106
*Xanthomonadaceae*_unclassified	6.317	0.002
Xenophilus	0.157	0.18

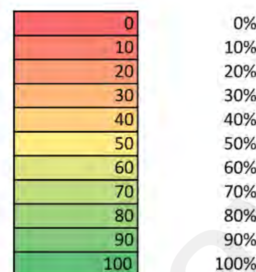

Figure 4.5 Comparison of the (a) bacterial and (b) fungal population in the mushroom substrates of the healthy substrate bag and green-mould contaminated substrate based on targeted-metagenomic method. Numbers in coloured cells are relative abundance (%) of each taxon

Metagenomic analysis (Figure 4.5) revealed that the relative abundance of *Graphium* was the highest in the contaminated substrate, making up to 56.3% as compared to *Trichoderma,* which makes up only 34.1% of the total quality reads, 58587. However, the relative abundance of *Graphium* in the healthy bag (final harvest substrate) was close to nil (0.003%) and the relative abundance of *Trichoderma* was higher (0.24%). The relative abundance of *Pleurotus* in the healthy bag (final harvest substrate) made up most of the fungal population of about 98.8% whereas it decreased drastically in the contaminated bag making up to only 0.25%.

CHAPTER 5: DISCUSSION

5.1 High bacterial count in contaminated substrate

Colavolpe et al. (2014) suggested that the presence of total microflora may increase the production of phenoloxidases by *Pleurotus ostreatus* which acts as a defence mechanism on itself. However, our study (Table 4.1) showed that the contaminated substrate which had the most abundant microbiota did not deter the contamination and colonization by *Trichoderma.* The same paper by Colavolpe et al. (2014) also mentioned that reduction of the natural microbial flora of the substrate by the sterilization may increase the prospect of *Trichoderma* contamination due to a lower presence of competitive microflora which reduces the possibility of mycelial growth. This statement also contradicts with our study as after sterilization, even though the bacterial mean count was zero *Trichoderma* did not contaminate the substrates until after second harvest. This opens a questionable discussion on the source of contamination which could be from the hands or tools of the harvesters′ during harvest routines.

On a different note, the high mean bacterial count in contaminated substrate could also be due to rapid breakdown of lignocellulose in sawdust into simpler sugar by cellulase produced by *Trichoderma* sp., making it easier for the majority of microbiota to proliferate and colonize. The relative abundance in terms of metagenomic reads of Proteobacteria community shows similar trend when compared with cultured bacterial community from final harvest and contaminated substrate. The species richness and diversity calculated for the samples using Mehenick′s Index measure and Shannon′s Diversity Index as shown in Table 4.2 respectively indicated that contaminated substrate had the highest value (D=3.29) and (H=2.84).

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5.2 Diversity Shift in Culturable Bacterial Community

Bacillus spp. a genus commonly recognised as plant growth promoting bacteria (Weller, D. M., 2007) and specifically, *Paenibacillus polymyxa* from cultivation substrates were reported to have implicated in their selectivity by both inhibiting the growth of *T. harzianum* and stimulating defences of the mushroom *P. ostreatus* through the induction of laccases (Velázquez-Cedeño et al., 2007). According to this study, the sterilisation process seems to have killed most of the naturally occurring *Bacilli* class bacteria resulting in lesser isolates retrieved after sterilisation (Figure 4.1). Besides that, the targeted-metagenomic analysis also did not trace any reads for *Bacili* from both healthy and contaminated bags. This may weaken the defence mechanism of *P. ostreatus* and triggers the colonization of *Trichoderma*. Bacteria from the families *Enterobacteriaceae* and *Pseudomonadaceae* were cultured across harvest stages no specific trend (Table 4.2). However, metagenomic data from the healthy and contaminated substrate shows that the members of both the above-mentioned families were retrieved higher in contaminated substrate than in healthy substrate (Figure 4.5) and is discussed in the following subchapter.

5.3 Bacterial population in healthy and contaminated substrate

The targeted-metagenomic analysis results from the healthy bag (final harvest substrate) showed a different group of bacteria communities with a higher abundance such as 22.7% *Oxalicibacterium*, 6.3 % *Xanthomonadaceae*, 3.1% *Methylobacillus,* and 6.2% *Methylophilus,* which were totally absent in the contaminated bag. The total quality reads of the contaminated bag revealed a bacterial community largely made up of *Gammaproteobacteria* with 17.5% *Cedecea*, 14.6% *Providencia*, 10.5% *Raoultella,* and 8.0% *Pseudomonas,* which were in turn absent in the healthy bag (Figure 4.5).

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An interesting phenomenon is observed in terms of the dominating bacterial population groups found in the healthy and contaminated bags from the metagenomic analysis. A group of bacteria which are known for their ability to produce secondary metabolites and has antifungal properties which are commonly used as plant biocontrol agents are largely present in the healthy bags but absent in the contaminated bag. They are *Oxalibacterium* (known to produce oxalic acid), *Xanthomonadaceae* (used in disease suppressive soils (Gómez Expósito et al., 2017)), *Methylobacillus* and *Methylophilus* (methylotrophs known to be antagonistic towards plant pathogens (Kumar et al., 2016). This could be the reason why the healthy bags were immune against *Graphium* and *Trichoderma.* Their absence in the contaminated bag may have induced undeterred proliferation of both the above-mentioned fungi.

Enterobacteriaceae on the other hand was the largest group of bacteria retrieved in the contaminated bag (58.31%) as shown in Figure 4.3. *Raoultella ornithinolytica* from the *Enterobacteriaceae* family, were isolated across the whole cultivation process indicate that it could be part of the natural flora in the sawdust (Drancourt et al., 2001) which remained dormant through sterilisation process. The genus *Raoultella* held high percentage of quality reads from contaminated substrate via metagenomic analysis. Being a cellulose degrader (Drancourt et al., 2001), it could have also contributed to the increase in abundance of microflora in the contaminated substrate. *Pseudomonas* also held a significant percentage of reads and it is not surprised to be present in the contaminated bags as they are cellulose degrading bacteria known for causing spoilage in crop. The soft flesh of mushroom is easily bruised which could hasten the colonisation *Pseudomonas* spp.. *Cedecea* held the highest percentage of total quality reads from contaminated substrate, and it is human pathogen (Chan et al., 2014). Since harvesting is done manually, human pathogens may be transferred to the fruiting bodies. Therefore, the possible source of enterobacteria occurring in the substrates could be due

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to unhygienic handling practice during the harvesting process or the water source used for washing and spraying the mushroom house.

5.4 High diversity in fungi population of Green-mould contaminated substrate

The targeted-metagenomic analysis showed that the relative abundance of *Pleurotus* is highest in the healthy bag (98.8%) which decreased drastically to 0.25% of the total fungal population in the contaminated bag, indicating inhibition of *Pleurotus* growth by green-mould. In relation to that, the fungal community in the contaminated bag was more diverse compared to the healthy bag with an increase in the population of filamentous fungi such as *Hypocreales* (0.15%), *Penicillium* (0.17%), *Sordariomycetes* (6.38%), besides *Graphium* and *Trichoderma* (Figure 4.5). An increase in population of other filamentous fungi in the contaminated bag may have suppressed the growth of *Pleurotus* in the contaminated mushroom bag. Besides the fact that certain species of *Trichoderma* are strong growth inhibitors to *Pleurotus* sp. (Parani & Eyini, 2016), it was found that many fungi isolated from the *Pleurotus* beds (Velazquez-Cedeno et al., 2007) were reported to be nutritional competitors, or antagonists to the *Pleurotus* being cultivated. Therefore, there could be a correlation between an increase in fungal population diversity in the contaminated bag and the stunted growth of *Pleurotus.* The dynamics interactions among the fungal microbiome could have played a role in promoting the rapid and widespread growth of *Graphium* and *Trichoderma* but suppressing the growth of *Pleurotus*. Further study is required to investigate how the fungal networks function and interact to suppress or promote growth of oyster mushroom during commercial cultivation.

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5.5 Co-existence of *Graphium* and *Trichoderma* in the green-mould contaminated mushroom substrate

Concerning green-mould contamination, phenotypic and microscopic observation of the fungal isolates from contaminated bags were presumed to be *Trichoderma* sp. On the same note, *Graphium* a genus with similar macroscopic morphology to *Trichoderma,* was also cultured from the contaminated bags (Figure 4.2). The *Trichoderma* isolates were identified as *Trichoderma pleurotum* based on micro-morphology and molecular analysis (Table 4.4). Colony of *T. pleurotum* showed a single green concentric ring with a cluster of green and white conidia scattered throughout the plate. The spores turn from greenish white to dark green, powdery, and dense after maturation (Figure 4.2). Under microscope, the conidiphores are branched and the branches are packed closely with philiades. Each philiades are terminated with conidia. The colony and microscopic characteristics of of *T. pleurotum* matched the description by Park et al., (2006).

The slow growth *Graphium penicillioides* took at least 7 to 14 days to grow till 1 cm diameter in culture plate (Figure 4.2). The colonies of *G. penicillioides* appeared to be solid radial and white when young and turn brownish green as mature. Spares aerial mycelium are presented with hyphae immersed in the media. Under microscope, the conidiophores of *G. penicillioides* are heavily branched in a single synnemata where one of two synnemata branching from a hypha. Besides that, the conidiophores are surrounded by numerous free conidia which are cylindrical to obovoid in shape. The cultural and microscopic characteristics observed resembles the *Graphium penicillioides* characteristics described by Okada et al., (2000).

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Therefore, this study may unfold a new perspective on this issue of green-mould contamination in terms of the co-existence of *Graphium* and *Trichoderma* in the contaminated mushroom bags. Both *Graphium* and *Trichoderma* were found to predominate the green-mould contaminated bag according to metagenomic data. According to Figure 4.5, *Graphium* shows a higher relative abundance in terms of metagenomic reads in contaminated substrate compared to *Trichoderma*. This contradicts the culture dependent method as *Graphium* isolates were retrieved in low numbers from substrate before sterilization, first harvest and contaminated substrate compared to 15 *Trichoderma* isolates all confirmed to be *T. pleurotum* (Table 4.4) isolated from contaminated substrates bags (Table 4.3). This opens many questions on why such contradiction is observed between the culture dependent and independent methods. What are the dynamics of the co-existing genus which caused the contamination? Which one of the genera could be the true cause of contamination?

Graphium, being a plant pathogen for some species, could be part of the natural flora in the saw dust. Besides, *Graphium* was reported to be slow grower (Okada et al., 2000) as colonies on MEA after 8 days at 25°C measured only 1.2 cm in diameter. This indicates that the spores remained dormant throughout sterilization process and was retrieved again through the culture method from first harvest substrate (45-60 days after sterilization). The slow and persistent growth of *Graphium* may have caused the random contamination of substrate by *Trichoderma*. Therefore, the study hypothesize that *Graphium* may be the first colonizer degrading the saw dust particles as they are known to cause soft-rot decay in hardwoods (Singh, 2006). This carves the way for the fast growing second colonizer *Trichoderma,* which relies on simple sugar as its carbon source (Papavizas, 1985) to proliferate rapidly and eventually cause the green-mould contamination. *Trichoderma pleurotum* and *T. pleuroticola* were reported being found on cultivated *Pleurotus* and its substrate in Europe, Iran, and South Korea (Komón-

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Zelazowska et al., 2007). *Trichoderma* are said to produce several enzymes involved in degradation of the fungal cell walls namely chitinases and glucanases (Colavolpe et al., 2014, Ait-Lahsen et al., 2001, Geremia et al., 1993, Sivan & Chet 1989). This could be why once *Trichoderma* starts colonising the substrate, growth of the naturally occurring *Graphium* fungi is masked and is not able to be cultured out.

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CHAPTER 6: CONCLUSION

This study presents an overview of bacterial succession in oyster mushroom cultivation using sawdust as substrate with the application of both culture dependent and targeted-metagenomic methods, thus contributes to the main body of knowledge of microbial population succession in oyster mushroom cultivation. This study also contributes to the mushroom industry with revealing potential beneficial bacteria to be used in disease management. The first objective of this study is achieved as it has shown microbial abundance and bacterial population shift along the cultivation stages. Decrease in *Bacillus* sp. count in the cultured bacterial population shift, may be associated with the spread of *Trichoderma* contamination. The second objective of this study is achieved as it revealed the dominating bacterial population in contaminated and healthy substrate which may have contributed to disease development. The occurrence of *Enterobacteriaceae*, even after the sterilization stage, indicates the possibility of re-contamination during farming process and may have contributed to disease development in the substrate. In this study, the green-mould contamination in the farm was found to be *T. pleurotum*. However, the persisting revival of *Graphium* and its high metagenomic reads in contaminated substrate suggests a possible correlation with *Trichoderma* contamination. As a suggestion, further study could be conducted specifically focusing on *Graphium* and its correlation with *Trichoderma* contamination. As this could be an important factor to consider in the prevention and eradication of green-mould contamination in the mushroom cultivation industry.

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