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The microbiome of *Agaricus bisporus* (Lange) Imbach and the prevalence of foodborne pathogens and mycoparasitic fungal pathogens in white button mushroom production systems

by

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Submitted in partial fulfilment of the requirements for the degree

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SUMMARY

The microbiome of *Agaricus bisporus* (Lange) Imbach and the prevalence of foodborne pathogens and mycoparasitic fungal pathogens in white button mushroom production systems

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Keywords: *Agaricus bisporus*; microbial diversity; food safety; *Escherichia coli*; *Listeria monocytogenes*; *Staphylococcus aureus*; *Salmonella enterica*; coagulase-negative staphylococci; antibiotic resistance; mushroom pathogens; green mould disease, dry bubble disease, cobweb disease, wet bubble disease; disease monitoring; droplet digital PCR; farm health checks

Food safety related disease outbreaks and challenges could cause serious crop and market related losses in any industry. This is also true for the commercial mushroom sector in South Africa. Disease outbreaks adversely affect economic viability of the industry, on both the production and consumer margins. In order to secure the economic feasibility of the industry this study focussed on production practices and diseases that contribute towards losses. Included in this research are investigations into method development for foodborne and mushroom antagonistic organisms in production systems. An important research objective was to understand the dynamics of the microbiological significance in each major aspect of the mushroom production chain to determine the viability of early detection systems and ultimately prevention of disease outbreaks. Different molecular techniques were optimized and employed to confirm prevalence and persistence of the various microorganisms identified to be of importance. Successfully developed and implemented aspects of the work performed in this study will be directed to the South African mushroom industry for further development of a holistic mushroom quality and safety management programme. Not only will it provide a better understanding of the mushroom microbial ecology, prevalence of foodborne pathogens and accurate identification of mushroom pathogens, but also contribute to improved product quality and safety for consumers. These aspects have a level of novelty that has the potential to offer the South African industry with solutions to improve disease management and overall safety standards.

DECLARATION

I declare that this thesis, which I hereby submit for the degree Philosophiae Doctor in Plant Pathology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

ETHICS STATEMENT

The author, whose name appears on the title page of this thesis, has obtained, for the research described in this work, the applicable research ethics approval.

The author declares that he has observed the ethical standards required in terms of the University of Pretoria's Code of Ethics for Researchers and the policy guidelines for responsible research.

Werner Rossouw
DECEMBER 2019

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"Greatest Achievement"
Have an open mind in life,
And you will be okay.

- Shari Forman

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Finally, and most important, I wish to dedicate my work to our Heavenly Father, through Jesus Christ, for none of this would have been possible if it hadn't been for His grace and blessings. He gave me strength and wisdom, guiding me on the path which I believe He meant me to follow.

"The one who goes out weeping, carrying a bag of seeds, will surely return with a joyful song, bearing sheaves of grain from his harvest."

-Psalms 126:6

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LIST OF ABBREVIATIONS

C	Celsius (Centigrade)
cfu	colony forming unit(s)
DNA	Deoxyribonucleic Acid
e.g.	for example
EtBr	Ethidium Bromide
EtOH	Ethanol
FAO	Food and Agriculture Organisation
SAMFA	South African Mushroom Farmers' Association
g	gram(s)
h	hour(s)
kg	kilogram(s)
L	litre(s)
mg	milligram(s)
min	minute(s)
mL	millilitre(s)
rpm	revolutions per minute
UV	ultraviolet
µ	micron (0.001 mm); use micrometre (µm)(10^{-6} m)
µg	microgram(s) (10^{-6} g)
µL	microlitre(s)

LIST OF SYMBOLS

%	percent (parts per 100); percentage
>	more than; greater than; above; exceeds
<	less than; under; below
≤	not more than; not greater than; equal or less than
≥	not less than; equal to or greater than; equal to or more than
°	degree

CHAPTER 1

GENERAL INTRODUCTION

1.1. BACKGROUND AND OVERVIEW

For hundreds of years prior to mass production of mushrooms, people have harvested them from natural habitats for food and use as medicine (Boa, 2004). While harvesting of wild mushrooms continues today, most of the world's domestic supply comes from commercial mushroom growers (Carrera *et al*., 2005). White button mushrooms, *Agaricus bisporus* (J.E. Lange) Imbach 1946, were first "domesticated" for large scale production in France in 1650 where after the United States pioneered mushroom agriculture in the late 1880s (Barney, 1997). Commercial mushroom cultivation is a unique agronomic practice that combines industrial and automated production systems with practices and aspects of traditional agriculture (Kumar, 2013; Pandey *et al*., 2018). Mushroom farming is now commercially practiced in more than 100 countries (Das, 2016), including South Africa, and to this day Agaricus stays the most popular mushroom crop produced worldwide (Leiva, 2015).

Due to popularity and versatility of mushrooms as a culinary additive, production volumes for the past eight years exceeded 5 million metric tons per annum, whilst continuing to steadily increase and as predicted by Kumar *et al*. (2011) is currently starting to surpass 7 million metric tons per annum. White button mushrooms account for 38% of this production (ISMS Edible mushrooms, 2018). It was projected that the global mushroom market would have grown at a compound annual growth rate of 8% from 2016 to 2020 (Technavio, 2016). According to Transparency Market Research the growth rate of the industry was 8.2% by mid-2018.

There is no argument that the global mushroom market is growing and diversifying, which emphasises the worth of mushrooms with regards to culinary and economic demand, therefore the future viability of the commercial mushroom sector depends not only on monetary aspects or commercial popularity, but also on the sustainability of producing a biologically safe product (Grimm & Wösten, 2018). Fresh produce, including mushrooms, harbour transient as well as resident microbes (Simon *et al*., 2005). Unlike fresh produce such as vegetables or fruit, mushrooms themselves are fungi, making their surface characteristics and fruit body construct unique, and in theory more susceptible to contamination by exposure to environmental conditions and production practices. This means that quality of mushrooms could be directly related to the microbiological populations found around and on the surface of the product (Venturini *et al*., 2011). As an alternative, these microorganisms when present in a balanced population can also provide a buffering effect on the surface of the fruiting body against potential pathogens (Ippolito *et al*., 2000). It is well described in literature that the microbiome of a mushroom consists of bacteria, fungi, yeasts and viruses. Some of these microbes are naturally associated with mushrooms, whereas others are artificially introduced during watering, handling, harvesting, packaging or due to specific environmental conditions (polluted air, water or contaminated contact surfaces).

These microorganisms then persist and can ultimately affect consumer health if contaminated mushrooms are consumed (Lunden *et al.*, 2000).

Consumption of undercooked or raw food is one of the main reasons for foodborne pathogens associated with outbreaks around the world (Altekruse *et al*., 1996). Bacteria are of greatest concern when considering foodborne disease outbreaks. Some of the bacteria that may potentially be introduced include *Escherichia coli* O157:H7, various coliforms, *Staphylococcus aureus, Salmonella enterica* and *Listeria monocytogenes*. The presence of some of these organisms or groups of organisms i.e. coliforms are also used in food quality control systems as indicators to show possible hazards that may be associated with a product or production system (Busta *et al*., 2003). An example is highlighted by the recent foodborne outbreak of *Listeria monocytogenes* ST6 in South Africa, implicating a brand of processed meat products. A total of 1033 laboratory-confirmed cases have been reported since the beginning of 2017 (National Institute for Communicable Diseases, 2019), with 204 deaths as a result. This outbreak yet again illustrated the dynamism leading up to and during an outbreak within a ready-to-eat production-consumer system. No reported foodborne related outbreaks have been associated with fresh mushrooms in South Africa yet, but pathogenic microorganisms have been linked to recalls of mushrooms and mushroom products in the United Kingdom (Personal communication - Peter J McClure, Unilever, Research and Development, UK, 2014), Canada (Canadian Inspection Agency, 2011, 2012a, 2012b, 2014) and the United States (United States Food and Drug Administration, 2015). Part of this study therefor serves to establish a microbiological profile of the non-pathogenic microbiome of mushrooms and to isolate any potential human pathogens that may be associated with fresh mushrooms or the production environment. From a consumer perspective, it is important for farms and the industry to ensure that products produced are safe and will not harm consumers.

Still considering the microbiological associations of mushrooms, they not only act as a potential transport matrix for human pathogens but are also subject to fungal (mycoparasitic) pathogens, which can cause infection and disease of mushroom tissue. These pathogens are usually not harmful to humans and mostly affect only production yield and quality. A few mycoparasitic species that are of economic importance to the cultivation of white button mushrooms include: *Trichoderma aggressivum* Samuels & W. Gams 2002 (Green mould disease), *Lacanicillium fungicola* (Preuss) Zare & W. Gams 2008 (Dry Bubble disease), *Cladobotryum dendroides* (Bull.) W. Gams & Hooz. 1970 (Cobweb disease) and *Mycogone perniciosa* (Magnus) Delacr.1900 (Wet Bubble disease). These species can be introduced at any stage during production and usually establish during the pinning stages of mushroom growth causing the fruiting body to be deformed, spotted or not to develop causing significant losses on-farm. Detection of these pathogens are difficult as their presence is mostly only noticed once symptoms appear on the mushroom itself or in the growing beds. Early detection of these pathogens would be ideal,

allowing farmers the opportunity to introduce a more preventative approach rather than remedial actions.

The overall objective of this thesis is to assess the microbiological quality, safety and health status of white button mushrooms in production systems. From a producer's point of view, detection of pathogens within production systems, both crop and human related, are of great economic importance and contribute to industry feasibility and longevity. This study inclusively aims to consider two major research aspects relating to the improvement and assurance of safe agricultural production within the mushroom industry. These considerations are essential to achieve a sustainable development process that will contribute to reducing poverty and enhance food security and income alike (Goletti & Wolff, 1999). The themes identified for research are discussed according to their respective objectives, hypothesis statements, experimental strategies and significances below.

1.1.1. MICROBIOLOGICAL QUALITY AND FOOD SAFETY OF MUSHROOMS (CHAPTERS 3 to 5)

As with most foods that are consumed uncooked, mushrooms in their raw form, inherently possess a potential health risk due to the microorganisms that may be present on and within the product (Chapter 3). In order to better understand the safety status of fresh mushrooms and determine the relevant hazard associated with the potential presence of foodborne pathogens, a basic understanding of the microbial dynamics (Chapter 5) in the context of production and handling systems are required. These aspects of the study will focus on determining the potential presence of foodborne pathogens on mushrooms as well as quantify the populational load associated with different stages of on-farm production. A significant part of mushroom production requires physical handling of the fruiting bodies during picking and finally preparing the product for retail by packing staff. The potential of Staphylococcus being introduced onto mushrooms and the occurrence of this bacterium within growing rooms and production facilities will also be investigated (Chapter 4). In addition to determining which Staphylococcus species associate with mushroom production, the possible resistance of these organisms to commonly used antibiotics will similarly be evaluated.

1.1.2. MUSHROOM DISEASE DETECTION AND FARM BIOSECURITY (CHAPTERS 6 to 7)

Disease challenges and crop losses before harvest have a considerable impact on the yield and quality of mushrooms (Largeteau & Savoie, 2010). Four main mycoparasitic fungal diseases were considered which have the greatest economic significance in South Africa, these include green mould (*T. aggressivum*), dry bubble (*L. fungicola*), cobweb (*C. dendroides*) and wet bubble (*M. perniciosa*). Farm health checks will be developed to assist mushroom growers to devise more effective disease control

strategies. This novel concept for disease monitoring, intends to establish a health status for each farm as well as that of the industry by evaluating pathogen prevalence throughout the year. A series of existing and adapted test methods were used to detect and identify fungal pathogens prior to disease expression. Achieving success in this aspect will enable farmers to identify critical areas of contamination and infection early in production; enabling them to act in a preventative rather than a curative manner to more effectively manage outbreaks and losses.

1.2. HYPOTHESIS STATEMENTS AND OBJECTIVES

1.2.1. MICROBIOLOGICAL QUALITY AND FOOD SAFETY OF MUSHROOMS

Objective: Determining persistence of microorganisms on mushrooms, with the focus on food safety by means of pathogen detection, assessment of species diversity including microbial load during different stages of production. Lastly also to determine the dynamics of *Staphylococcus* spp., as a potential indicator organism known to be linked to hand-food transference during production and handling of food.

Hypothesis: An increase in microbial counts between mushrooms that are picked before harvest compared to those after harvesting up to and including packaging as handling and movement in transitional spaces from the beds to the packhouse could be conducive to increased microbial activity or potential microbial introduction. It is expected that total microbial counts will increase with each consecutive break or production cycle on mushrooms before to after packaging. Foodborne pathogens are expected to occur more readily after harvest on picked mushrooms. Similarly, it is likely that human associated Staphylococcus will also be introduced onto mushrooms as they are being handled during picking and packing.

Experimental strategy: To determine the average microbial load and species diversity of fresh, whole white button mushrooms, isolation and identification of the most abundant species of bacteria, fungi and yeasts will be performed using standardized plating and enumeration, selective media as well as molecular and spectrometric identification methods. Establishing a progression or trend for microbiological change between selected production stages on mushrooms will require consistent sampling of mushroom, hand and water samples in the same production batches. Experimental repeats will be performed on commercial farms with similar preparation, cultivation and production practices. Matrix-assisted laser desorption/ ionisation time-of-flight mass spectrophotometry (MALDI-TOF MS) will be used in a novel application to rapidly and accurately identify isolates obtained from mushroom samples. Verification of the reliability and accuracy of the identities assigned by the MALDI-TOF MS will be done via sequencing and genetic database comparisons for selected isolates. Handling (picking and packing) is considered an important point of possible contamination when the final product is

prepared for market and will be investigated specifically using swabs as collection method, together with selective plating and molecular identification techniques.

1.2.2. MUSHROOM DISEASE DETECTION AND FARM BIOSECURITY

Objective: 1) Determining prevalence of the four most important fungal pathogens affecting mushrooms within the South African mushroom industry, which include *T. aggressivum*, *L. fungicola*, *C. dendroides* and *M. perniciosa*. 2) Developing and optimising a detection and identification approach for these pathogens, which can be implemented easily and reliably, 3) Monitoring and surveying, on-farm presence of these pathogens.

Hypothesis: Genus and species-specific molecular primer design and development will enable detection and identification by means of organism-specific and generic genomic regions for the four mushroom pathogens identified. Upon establishment of a successful detection method, seasonal and persistent monitoring of farm health will be possible for both smallholder and commercial mushroom farms in South Africa. Trends in on-farm pathogen presence within different production phases will be established, including identification of areas which are prone and susceptible to harbouring pathogenic organisms.

Experimental strategy: The international mushroom culture collection will be used as reference in identifying representative cultures from each pathogen group. Using nucleic-acid-based strategies specifically targeting the internally transcribed spacer (ITS) regions, which are nested in the nuclear rDNA repeats, will be used to investigate the diversity within pathogenic species groups as well as distinguishing identities between selected pathogens. After establishment of reliable and repeatable molecular amplification through consideration and design of the necessary primer sets, establishment of the best and most robust technique for sampling and DNA extraction will be performed. Sample collection and preparation will be crucial to the successful detection of pathogens as it will be a challenge to allow for direct extraction of PCR-amplifiable genetic material from samples taken on various surfaces containing several environmental and non-specific contaminants. On completion of the molecular detection and sample process, disease prevalence will be monitored through systematic and regular sampling on different farms with varying production and farming strategies. This will enable assessment of the robustness and repeatability as well as sensitivity of the developed method.

Development of pathogen specific primer sets will mean that screening for collective pathogen presence can be combined into a single detection reaction. Multiplexing can be completed in one PCR reaction, together with quantification of pathogen concentration, in contrast to running a conventional first-generation PCR reaction for each pathogen and evaluating results on agarose gels. Once a reliable

detection and identification method has been designed and implemented, frequent monitoring of pathogens and disease incidence on individual farms will be done to determine occurrence and prevalence patterns.

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CHAPTER 2

A LITERATURE SURVEY ON WHITE BUTTON MUSHROOMS

2.1. INTRODUCTION

At least 14 000 species of mushroom are known, of which 2000 are edible (Chang, 1999a). From this group, the most consumed is *Agaricus bisporus* (J.E. Lange) Imbach 1946, also known as white button mushrooms or cultivated mushrooms (Kumar *et al*., 2018). Specific microorganisms can associate both internally and externally with fresh mushrooms. Some of these microbes are naturally associated, whereas others are artificially introduced through watering, handling and packaging. Mushroom cultivation practices and the nature of mushrooms as a fresh product allow for various microbial interactions and possible presence of microorganisms that could affect the quality of the final product (Zhang *et al*., 2018). As global trade in fresh food has increased, associated foodborne disease outbreaks linked with fresh and processed products have also increased (Kaczmarek *et al*., 2019). With extended trade systems the risk of introducing foodborne pathogens in the food chain became greater (Behzadi *et al*., 2017). Some of these risks are mitigated by reducing breaks in the cold chain and mechanizing or automating certain processes in the product processing chain, but mushroom harvesting and packaging processes are still reliant on extensive handling by workers.

Comparable to potential pathogen association on post-harvest mushrooms, disease challenges and crop losses before harvest have a considerable impact on the final yield of mushrooms (Largeteau & Savoie, 2010). Mushrooms are susceptible to various diseases (Fletcher *et al*., 1989) of which the causal agent may be bacterial, fungal or viral pathogens. Four main mycoparasitic fungal diseases are of economic importance in South Africa, these include *Trichoderma aggressivum* Samuels & W. Gams 2002 (Green mould disease), *Lacanicillium fungicola* (Preuss) Zare & W. Gams 2008 (Dry bubble disease), *Cladobotryum dendroides* (Bull.) W. Gams & Hooz. 1970 (Cobweb disease) and *Mycogone perniciosa* (Magnus) Delacr.1900 (Wet bubble disease). Growers and farm managers observe infection of crops once disease symptoms are visible, at which stage the crop is already infected and the pathogen spread is difficult to control. Through a holistic approach to increase production and curb product losses, development and implementation of standard microbiological methods, advanced identification techniques and molecular methods should serve as platform for accurate as well as reliable monitoring and diagnostics. This chapter aims to provide an overview of the economic importance of commercial white button mushroom production both globally and locally. Concepts underlining microbiological significances of mushroom production, food safety of mushrooms and diseases of mushrooms, that could influence sustainability of mushroom production will be reviewed.

2.2. WHITE BUTTON MUSHROOM PRODUCTION AND TRADE

2.2.1. GLOBAL MUSHROOM PRODUCTION

Mushrooms in general, may be described as ‘macrofungi’ with a distinctive fruiting body, usually large enough to be seen with the naked eye and which can be picked by hand (Chang & Miles, 1992). From a taxonomic point of view, mainly Basidiomycetes, but also some species of Ascomycetes belong to

the mushroom group (Lindequist *et al*., 2005). Mushrooms in turn belong to a larger taxon called Myceteae (Miles & Chang, 1997), which contains edible and non-edible species. From the edible mushrooms, the most well-known is *A. bisporus*, with species such as *Lentinus edodes* (Berk.) Singer 1941 (shiitake mushroom), *Pleurotus* spp. (oyster mushroom) and a few others also being very popular (Chang, 1999b; Singh *et al*., 1999; Boa, 2004). The production and consumption of edible mushrooms have grown continuously during the last fifteen years (Royse, 2001; Boa, 2004). According to FAOSTAT (2017), total commercial mushroom production worldwide has increased more than 12 times in the last 46 years, from 634 878 tons (1976) to 10 408 485 tons in 2014 (Figure 2.1). With more than 65% input into the global market annually, Asia is the largest contributor of mushrooms, with China producing almost 10 times more fresh mushrooms than its nearest rival, the United States of America.

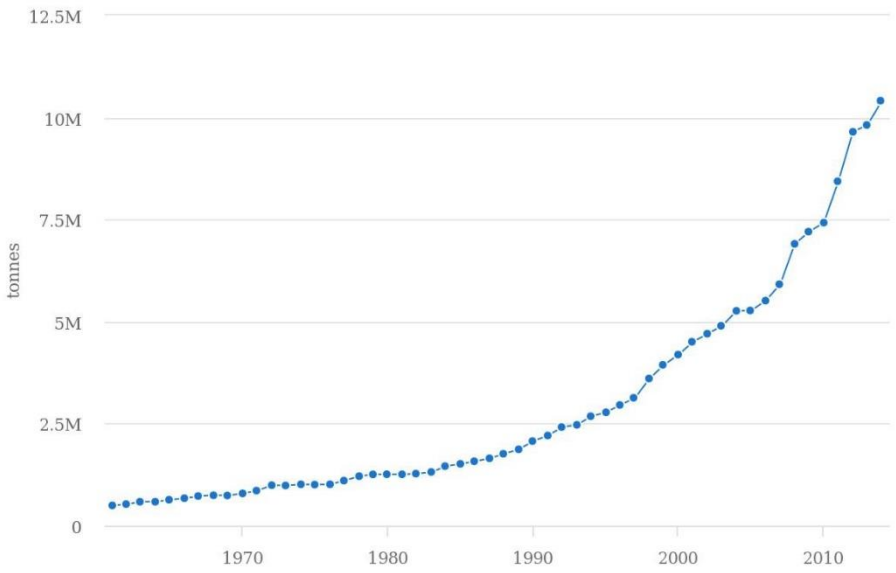

Figure 2.1 *Global mushroom production trend over time (FAOSTAT, 2017)*

2.2.2. MUSHROOM PRODUCTION IN SOUTH AFRICA

The most common mushroom species cultivated in South Africa, based on production volumes are the white button mushroom with a moving average of 18 500 t/an (SAMFA Production Statistics, 2018). Despite the popularity of white button mushrooms in South Africa, production of other edible mushrooms such as oyster, shitake and shemeji, amount to an average of 4000 t/an (Personal communication with Dr Marmari van Greuning, General Manager, Sylvan South Africa, Sept 2018). Comparable to global trends, mushroom production in South Africa has also displayed a gradual increase in volumes from 2001 (16 378 t/an) to 2017 (21 192 t/an) (Figure 2.2). According to FAOSTAT 2017, South Africa ranks as the 24th largest mushroom producer of fresh mushrooms in the world.

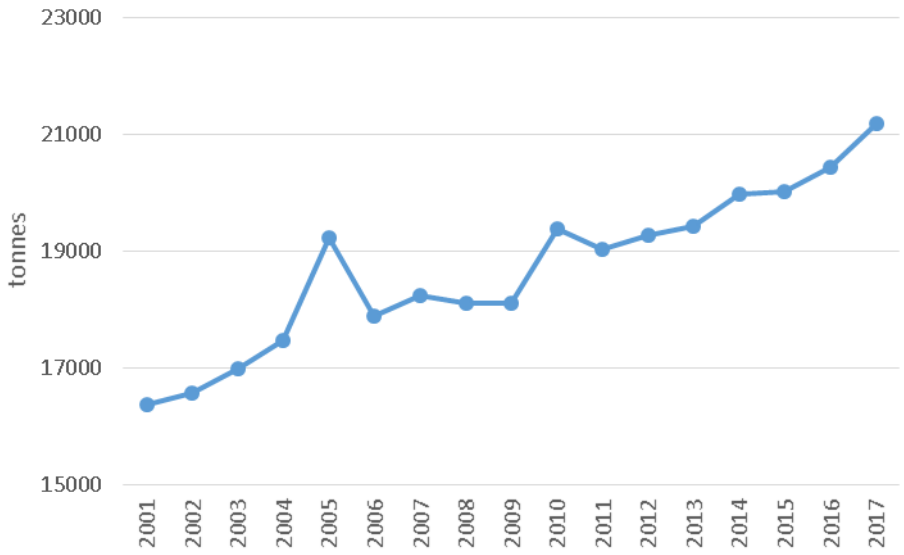

Figure 2.2 *South African mushroom production trend over time (SAMFA, 2018)*

Like any commercial mushroom producing country or region in the world, South African farmers are faced by various challenges. Not only are white button mushroom farming expensive when considering input costs (relative to other mushroom cultivation methods), there are also high maintenance and transport costs to consider. Of major concern during production are diseases and pests that have a significant impact on yield, quality and production volumes. Economically important diseases include amongst others mycoparasitic species such as *Trichoderma* spp., *Lecanicillium* spp., *Mycogone* spp., as well as *Cladobotryum* spp., (Kertesz & Thai, 2018).

2.2.3. APPLIED MUSHROOM BIOLOGY

Mushroom biology can be described to include aspects related to the growth and reproduction of the crop but are interchangeably called *applied mushroom biology*. Three main components make up this concept i.e. mushroom science, mushroom biotechnology (Chang, 1993), and mushroom bioremediation (Figure 2.3). The overall aim of applied mushroom biology can be described to address three basic problems which include shortage of food, diminishing quality of human health and pollution of the environment. Cultivation of white button mushrooms have an impact on the sustainability of natural resources as well as the environment associated with activities in and around production facilities. Environmental pollution includes the smell of the composting process as well as that of raw materials such as manure, water usage, and proliferation of flies, potential contamination of freshwater bodies by waste runoff, and lastly the use of peat during casing as a non-renewable resource. Air, water and soil pollution by agriculture and industrial wastes are causing growing concern that gives rise to accelerating environmental legislation.

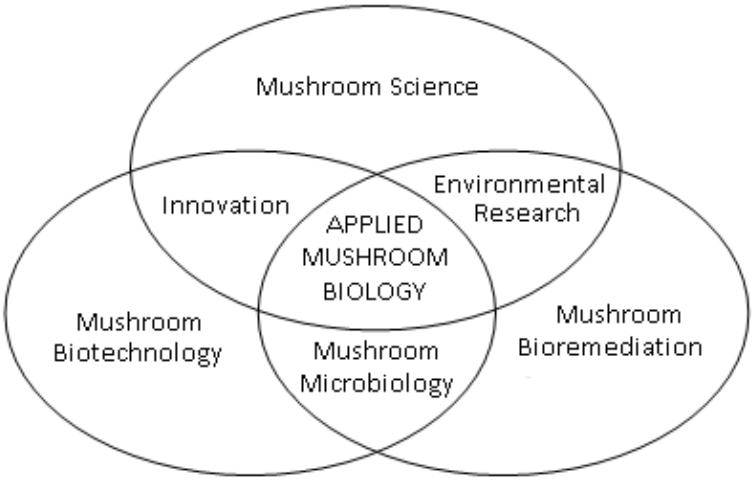

Figure 2.3 *Components of applied mushroom biology (Adapted from Cheung, 2008).*

The discipline concerned with principles and practices of mushroom cultivation is known as *mushroom science* (Chang & Miles, 1982). Systematic investigation provides facts, which are necessary to establish principles. Such investigation evolves through the practical aspects of mushroom cultivation as well as scientific studies. The consistent production of high-quality yields necessitates both practical experience as well as scientific knowledge (Cheung, 2008). In South Africa the responsibility for food safety and quality assurance is shared among several authorities including the Department of Agriculture, Land Reform and Rural Development (DAFF), Department of Health (DoH) and Department of Trade and Industry and Competition. All agricultural sectors must comply with legislative frameworks and current food safety legislation, over and above being certified to a retailer-specific voluntary standard (Dzingirayi & Korsten, 2015).

2.3. MICROBIAL DIVERSITY OF FRESH WHITE BUTTON MUSHROOMS

2.3.1. DETECTION AND IDENTIFICATION

In contrast to other fresh produce, information on the microbiological profile of fresh mushrooms is limited (Venturini *et al*., 2011). Bacteria, fungi and yeast can be found on the surfaces of mushrooms. Populations of residential microorganisms are unique in mushrooms and contribute to the development, maturation and eventual decay of their fruitbodies. Various microorganisms are found naturally on fresh produce and as a result form part of the epi- and endophytic microflora[1]. Depending on various

[1] In nature, living plants are colonised by plurality of microorganisms. The degree of microbial colonization of living host plants varies according to the species. When the microorganisms colonise a host plant and the host tissue is apparently healthy, the relationship between the microorganism and its host may range from latent pathogenesis to mutualistic symbiosis. The microorganisms may be epiphytes, endophytes, or latent pathogens. Generally, endophytic microorganisms are present within tissues of most parts of host plants in contrast to epiphytes which are found on the outside or on the surface of their hosts.

contributing factors, such as season and climate, presence and population profile consistency may vary. Microbial population shifts can be much more consistent and stable on mushrooms due to the presence of a "controlled" growing environment, storage conditions etc. The interior of produce such as fruits, vegetables as well as mushrooms are considered to be sterile, but pathogenic microorganisms can be internalized in edible parts during uptake of contaminated water as an example (Wright *et al*., 2017).

MALDI-TOF MS, which finds its function in analyzing the protein composition of a bacterial cell, has emerged as a new technology for identification in compliment to genetic validation methods (Lv *et al*., 2016). It is reported that most signals of prokaryotic MALDI-TOF MS spectra are divided from abundant proteins within cells (Sauer & Kliem, 2010), an example of this are the ribosomal proteins that are theorized to co-evolve with the ribosomal RNA, which generally mirror the respected phylogeny (Matte-Tailliez *et al*., 2002). Not only is this method of identification applicable to bacterial cells, but more recently the performance of MALDI-TOF MS for classification or identification of foodborne yeast isolates have been described. As one of the first Blättel *et al*. (2013) applied MALDI-TOF MS for species discrimination of environmental, particularly foodborne, *Saccharomyces* strains. In addition, Moothoo-Padayachie *et al*. (2013) proved that MALDI-TOF MS generated fingerprints can be used for differentiation of industrial and laboratory *Saccharomyces cerevisiae* (Desm.) Meyen 1838 strains. As an appropriate platform for reliable classification and identification, Pavlovic *et al*. (2014) suggested the use of MALDI-TOF MS for foodborne yeast isolates. The proof of principle whereby MALDI-TOF MS is used for specifically bacterial colony species-determination was already shown a few years ago (Claydon *et al*., 1996; Holland *et al*., 1996; Krishnamurthy *et al*., 1996). Despite this, MALDI-TOF MS has not been widely used in environmental/food microbiological studies as an identification technique due to described difficulties with reproducibility of the results with different MALDI-TOF MS mass spectrophotometer instruments and with variations in cultivation conditions as well as occasional limits on availability of reference data sets (Timperio *et al*., 2017).

Genotypic methods of identifying microorganisms is widely used and are mostly based on polymorphism of the 16S rRNA genes in bacteria and ITS rRNA genes in fungi and yeasts. Sequencing of these genes has generally become accepted as the reference method for species identification (Mellmann *et al*., 2008; Foschi *et al*., 2017). Genotypic identification methods rely on the use of an extensive and comprehensive quality-controlled databases, such as GenBank, EMBL or RefSeq (Clarridge *et al*., 2004; Harmsen *et al*., 2002). Identifying microorganisms by gene sequencing is objective and does not require a viable sample or any tedious culturing techniques (Tang *et al*., 1998). Considering bacteria, the gene target that is most used is 16S rRNA, which is an ~1500 base pair gene segment that codes part of the 30S ribosomal unit (Bosshard *et al*., 2003). It is also acceptable to use only partial 16S rRNA sequencing as a faster and more accurate method to identify aerobic and anaerobic bacteria (Simmon *et al*., 2006). A limitation of the 16S rRNA sequence is the inability to

discriminate among all bacterial taxa (Hall *et al*., 2003; Patel *et al*., 2004). For bacterial isolates which may share complete sequence identity, alternative gene targets such as *rpoB*, which encodes the β-subunit of the bacterial RNA polymerase can be used to discriminate among taxa (La Scola *et al*., 2006). Like bacteria, there are optimal gene targets used to identify fungi and yeast. The most used genetic regions include internal transcribed spacer regions ITS1 and ITS2, which are variable regions located between conserved genes encoding 18S, 5.8S and 28S rRNA (Pourjafar *et al*., 2013). Microbial identification using gene sequencing has enabled recognition of new microorganisms by better defining taxonomical relationships and have increased the understanding of microbial pathogenesis (Reller *et al*., 2007).

2.3.2. CULTIVABLE MICROBIOME ASSOCIATED WITH WHITE BUTTON MUSHROOMS

Commercial mushroom production depends largely on different microbiological processes to occur in the substrates used for proliferation of and fruit body formation by mushroom mycelia (Kertesz & Thai, 2018). In a similar way, the post-harvest quality of mushrooms is affected by microorganisms present and introduced onto the harvested product (Zhang *et al*., 2018). It is therefore important to understand the microbiological properties of both mushrooms and the substrates in which they are cultivated. The literature focus will be mostly on mushrooms, but the interconnectivity between the various components within the production chain contribute to the population composition and microbial dynamics on the mushrooms themselves (Siyoum *et al*., 2016).

2.3.2.1. Bacteria

The presence of high bacterial populations on fresh white button mushrooms is a major factor that significantly reduces the quality by causing brown, blotchy appearance. A direct correlation can also be drawn between the initial microbial load of fresh mushrooms and the rate of postharvest deterioration (Beelman *et al*., 1989). Doores *et al*. (1987) reported that during postharvest storage at 13°C, bacterial populations increased from an initial load of 7 log cfu/g to almost 11 log cfu/g over a 10-day storage period. The authors also reported that deterioration in quality indicated by maturity and colour measurement was directly affected by bacterial load.

Pseudomonads is the most prominent bacteria found on mushrooms. Species most associated with commercial mushrooms are, *Pseudomonas reactans*, *P. agarici*, *P. gingeri* and *P. tolaasii*. The latter can cause brown stains when counts of this bacterium exceed 6 log cfu/cm^2 (Preece & Wong, 1982; Soler-Rivas *et al*., 1999). Other bacteria that have been reported on fresh postharvest mushrooms include the isolation of *Campylobacter jejuni* (0.9 to 1.5%) by Doyle & Schoeni (1986) as well as Whyte *et al*. (2004). Siyoum *et al*. (2016) reported that three phyla were most abundantly identified on fresh button mushroom samples i.e. *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes*. Species isolated included *Sphingobacterium multivorum* from the phylum *Bacteroidetes*, *Microbacterium*

foliorum from the phylum *Actinobacteria*, as well as *Alcaligenes faecalis* and *Ewingella Americana* from the phylum *Proteobacteria*, respectively. Venturini *et al*. (2011) reported that the second highest most prevalent bacteria isolated next to *Psuedomonas* spp., was the family *Enterobacteriaceae*. Although these authors did not study this bacterial species in detail it has previously been reported that *Ewingella americana* is responsible for internal stipe necrosis of *A. bisporus* (Inglis *et al*., 1996; Reyes *et al*., 2004). Other members of the *Enterobacteriaceae* is coliforms which have also been reported present in white button mushrooms. González-Fandos *et al*. (2000) reported low counts (<2.0 log cfu/g) of thermotolerant coliform bacteria and the absence of *E. coli* in *A. bisporus.* Lactic acid bacteria were reported to be present in low numbers (~ 2 log cfu/g) by Venturini *et al.* (2011). Higher counts (~ 8 log cfu/g) of these bacteria have been detected in other types of mushrooms such as truffles (Nazzaro *et al*., 2007; Parentelli *et al*., 2007).

2.3.2.2. Fungi

In comparison to bacteria, fungal- and yeast-specific studies are not readily available throughout literature, specifically referring to characterization of different species on white button mushrooms. The genus *Paecilomyces* and *Penicillium* was most isolated from fresh button mushrooms in a study performed by Siyoum *et al*. (2016), specific species include, *Penicillium brevicompactum* Dierckx 1901 and *Penicillium toxicarium* I. Miyake 1940. Other fungal species forming part of the less frequently isolated species include *Trichoderma longibrachiatum* Rifai 1969, *Cladosporium cladosporioides* (Fresen.) G.A. de Vries 1952 and *Didymella fabae* G. J. Jellis & Punith. 1991 (Rossouw & Korsten, 2017). Mycoparasitic fungal pathogens also form part of the fungal biosphere of mushrooms, the isolates of significance are discussed in section 2.3.

2.3.2.3. Yeasts

To the author's knowledge, limited scientific information exist and could be found in literature with regards to the types and prevalence of yeasts associated with white button mushrooms. Siyoum *et al*. (2016) isolated two yeast species from fresh button mushrooms, *Cystofilobasidium infirmominiatum* (Fell, I.L. Hunter & Tallman) Hamam., Sugiy. & Komag. 1988 and *Rhodotorula mucilaginosa* (A. Jörg.) F.C. Harrison 1928. Another yeast commonly associated with fresh produce, including mushrooms is *Candida* spp. (Nguyen-the & Carlin, 1994). Yeast presence, including microbial population shifts and dominance within the fresh mushroom microbiome, become relevant during post-harvest storage and transport (Nasiri *et al*., 2017).

2.4. FOOD SAFETY AND PROPAGATION OF WHITE BUTTON MUSHROOMS

2.4.1. AGRICULTURAL SUBSTRATES AND CONTAMINATION OF FRESH PRODUCE

Fresh mushrooms have the potential to carry food-borne bacteria introduced from different sources. In the case of cultivated mushrooms, contamination with pathogenic bacteria is possible during different

stages of mushroom production, especially from the substrate which they are grown on (Venturini *et al*., 2011). The contamination potential is specifically high where potentially contaminated raw materials are introduced into the process (Pardo *et al*., 2017). Mushrooms are generally considered a safe product, but occurrences of pathogenic bacteria in commercial fresh mushrooms samples have been reported (Venturini *et al*., 2011). Modern mushroom production is becoming increasingly sophisticated as well as a mechanized industry due to the growing competition in the market between growers and retailers. Despite this, the industry is still based on the same biological principles (Levanon & Danai, 2013) of traditional mushroom cultivation on a smaller scale. White button mushrooms can be grown on various mediums due to their ability to degrade lignocellulosic substrates and can be produced on various natural materials and by-products from agriculture, woodlands, animal husbandry and manufacturing industries (Rinker, 2002). In almost all agricultural growing and packing operations, the potential exists for foodborne pathogens to cross contaminate the mushroom raw materials. This section explores the production processes as well as some common practices employed during white button mushroom production. Food safety concerns associated with production raw materials and practices are also considered.

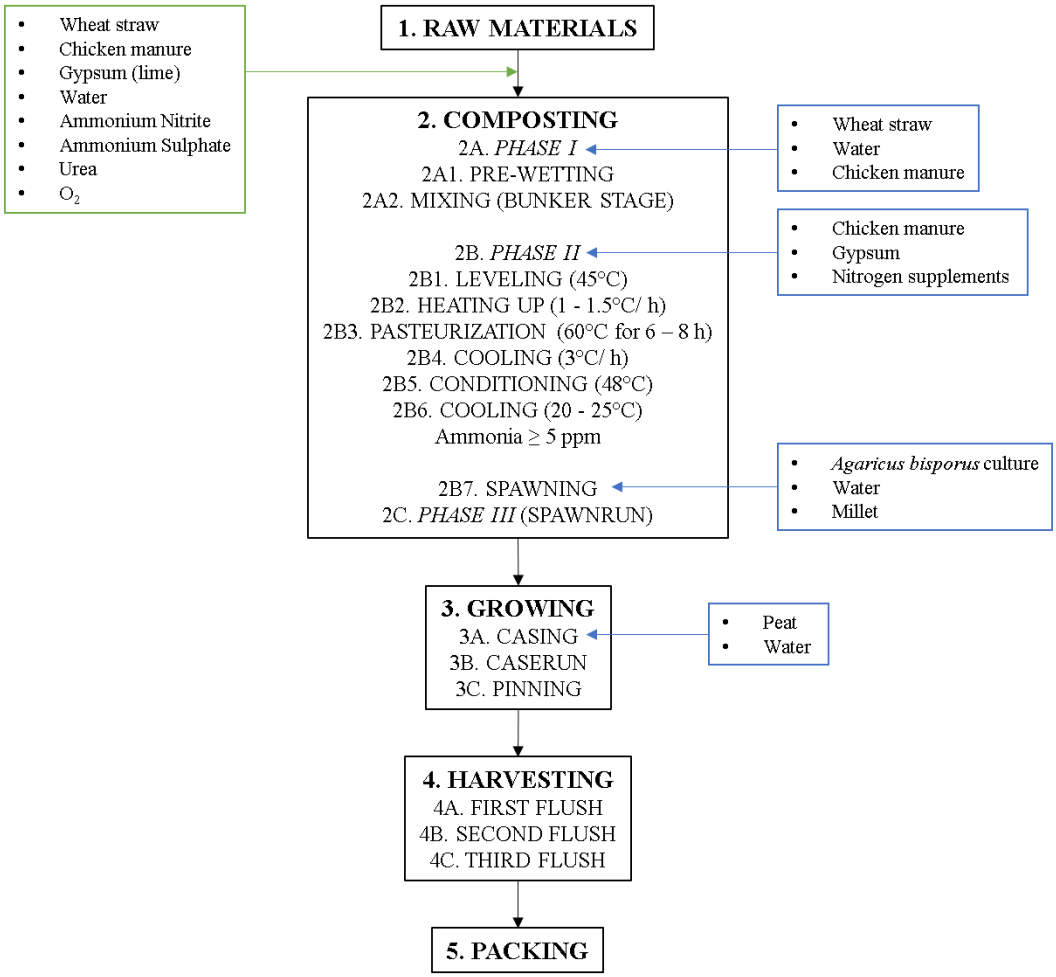

Figure 2.4 *General scheme for the cultivation of mushrooms (Own depiction)*

2.4.1.1. Compost

Compost or substrate production is an important aspect in the multi-faceted process of mushroom cultivation, which include carefully controlled steps (Figure 2.4). Chicken and horse manure are extensively used in mushroom compost (Straatsma *et al*., 1993; Colak, 2004). Depending on farm size and type of operation, composting often occurs near growing and packing areas, which increases the potential likelihood of contamination directly from these raw materials to intermediate or final products. Making use of phase II or phase III compost are usually determined by infrastructure and on-farm growing practices (Haug, 2018). Most human pathogens that are associated with mushrooms originate in the gut of animals (*Salmonella*, *Campylobacter*) or in substrates such as peat (*Listeria*, *Clostridium*), concerns exist that manures used in compost and ingredients used in the preparation of casing soils may serve as potential routes for contamination of mushrooms. Horse and poultry manure, which serves primarily as nitrogen sources in mushroom compost, are potential sources of *Salmonella* spp., *E. coli* O157:H7, *Listeria monocytogenes* and *Campylobacter jejuni* (Atwill, 2005; Himathongkham & Riemann, 1999; Himathongkham *et al*., 2000; Heuvelink *et al*., 1996). Initially, during phase I of the compost preparation process, mixing, pre-wetting and wetting of the raw materials (straw, manure, gypsum etc.) takes place, where-after the mixture is stacked in long windrow piles and left for composting, turning the contents periodically (Iiyama *et al*., 1994). Composting ensures microbiological breakdown of materials to enable availability of nutrients to mushrooms later during the growing phase, but the increase in temperature also plays a vital part in the selection of beneficial microorganisms and elimination of potential pathogenic organisms (Li *et al*., 2019).

During more modern manufacturing practices, this step could happen in bunkers or indoor tunnels. Microbial activity is high in Phase I and there is enormous oxygen utilization (Miller *et al*., 1995). Hereafter controlled aerobic composting for six days at 45°C is followed (Colak, 2004). During Phase II, the compost is pasteurized and conditioned by heating at 60°C for six to 8 hours ("peak heating") in an attempt to eliminate any residual pathogens (human and mycoparasitic) and create a selective growing environment (Sinden & Heuser, 1953; Wuest, 1977; Fermor & Grant, 1985; Overtjins, 1998) for the growth of beneficial microorganisms (Straatsma *et al*., 1993; Colak, 2004). Chikthimmah (2006) showed that *L. monocytogenes* as well as *Salmonella* spp. can grow in sterile or pasteurized substrate. In contrast, studies conducted by Penn State Mushroom Research Center has demonstrated that commercial Phase II pasteurization temperatures can achieve at least 7-log reductions in *Salmonella* and *L. monocytogenes* populations within the compost substrate. It is described that some pathogenic cells associated with animal waste products become acclimatized to some stressful environments, potentially cross-protecting them against subsequent heat treatments, such as composting and pasteurization (Chen *et al*., 2017).

Because of the microbiological reduction steps during the composting process, compost as a growth medium should not be a significant hazard for contamination of mushrooms. But compost is only pasteurized and not sterilized, which means heat-adverse and spore forming organisms can survive and continue further in the growing process (Kurtzman, 2010). A study performed by Weil *et al*. (2013) however showed that most pathogenic organisms including *L. monocytogenes*, *E. coli* O157:H7, total coliforms, Enterobacteriaceae, and *Salmonella* spp. are destroyed during Phase II composting. After the initial composting process, the temperature is lowered to 45 to 48°C for a two to three days for conditioning. Once ammonia levels in the air drops below 5.0 ppm and the pH value are lower than 7, compost is considered ready for spawning. Phase II compost is then inoculated or seeded with spawn randomly at 0.5% (v/w) and incubated for 12 days to be fully colonized by the mycelium (Straatsma *et al.*, 1991). Different cereal grains are used as a carrier for the mushroom mycelia e.g. wheat, rye or millet. As soon as mycelium is developed on these grains they are referred to as spawn (Chang & Hayes, 1978; Chang & Miles, 1989). The compost seeded with spawn is called spawn run compost or Phase III compost. At this stage the compost is very sensitive to contamination by competitor microorganisms than could potentially be harmful to the mushroom mycelia (Savoie *et al*., 2016).

2.4.1.2. Casing

Colonized substrate must be covered by a nutrient poor layer (casing layer), that creates a conducive environment for the formation and the development of fruiting bodies. The microbiological, physical and chemical properties of a material are important components to consider for this material to be suitable for use as a casing matrix. From the microbiological point of view, beneficial bacteria in the casing are known to initiate transformation of vegetative mushroom mycelium to reproductive phase (Urayama, 1961; Eger, 1963; Hayes *et al*., 1969; Hume and Hayes, 1972 cited in Hayes 1979). Moisture retention by the casing material and availability of this moisture from the casing layer for elongated periods is an important physical characteristic that should be met. Chemical properties of the casing should also be suitable for the growth of the mycelium and fruiting body formation, this could include pH (7.3 to 7.8), being free from adverse elements such as heavy metals etc. (Rainey *et al*., 1987). In contrast to the beneficial role of the casing layer during commercial mushroom production, evidence suggest that this substrate layer present a significantly greater risk than compost, when considering transference of potential foodborne pathogens directly onto mushroom surfaces. Current commercial mushroom growing practices do not employ a process to sterilize casing substrates prior to use on the mushroom beds as mushroom formation (fruit body initiation) cannot occur in sterilized casing materials (Long & Jacobs, 1974). Although steam treatment technology is available for treatment of casing soils, it is not a common practice due to extra costs and anecdotal evidence that it causes reduced mushroom yields (Chikthimmah & Beelman, 2005).

Various materials can be used as a casing layer, the most common of these being peat (Gülser & Peksen, 2003). Peat is naturally formed from partial decomposition of plant matter in waterlogged soils, referred to as peat bogs. Peat is readily used as a casing material due to its unique characteristics of absorbing and releasing water (Hayes, 1993). Other advantages to the use of this material is its microbiological properties as well as the fact that peat is free of most pathogens (Ingratta & Blom, 1979), although the potential for association does still exist (Highmore *et al*., 2017). Commercial mushroom producers in South Africa used topogenous peat (or reed-sedge peat) as a casing layer to produce mushrooms, which are usually harvested from wetlands (Eicker and van Greuning., 1989). However, wetlands are valuable natural ecosystems assisting in preservation of ecological balances and as an example play vital roles in filtering pollutants from ground water. Peat extraction (mining) is not regulated by law in South Africa (Grundling & Grobler, 2005). Some South African mushroom producers import peat from other countries such as Ireland and the Netherlands (SAMFA, 2018). Alternative materials used as casing layers for white button mushroom production include coir and wood bark. Coir is a by-product from the coconut fiber industry and has high water absorption, retention and good release capabilities (Kemp, 1990). Wood bark is a by-product of the paper industry and can produce good mushroom yields but have a few problems associated with it, making it a non-viable option for use during commercial mushroom production (Eicker and van Greuning., 1989). Various other alternative casing materials have been considered by researchers and industries, but none show the production capabilities of peat.

2.4.1.3. Pathogens in agricultural substrates and contamination of fresh produce

Listed in Table 2.1 are the major pathogens that are potentially present internally or superficially on fresh produce.

Table 2.1 *Foodborne pathogenic microbes associated with fresh produce, incubation times in human infection, symptoms they cause and possible sources*

Pathogen	Incubation period	Infectious dose and symptoms	Significant sources
Bacillus cereus	6 to 15 h diarrheal type 0.5 to 6 h emetic	*Diarrheal type:* Watery diarrhea, abdominal cramps *Emetic:* vomiting, occasional abdominal cramps and/or diarrhea. Dose $>10^6$ cfu	Soil, starchy grains
Escherichia coli O157:H7	24 to 48 h	Severe abdominal cramps and diarrhea which is initially watery but becomes grossly bloody. Hemorrhagic colitis. >100 cfu	Manure from ruminants, sewage, raw beef
Listeria monocytogenes	1 to 90 weeks	Flu-like symptoms that may develop into septicemia, meningitis and encephalitis. Still birth or abortion in pregnant women. $> 10^4$ cfu	Manure, sewage, soil, silage
Salmonella	24 to 48 h	Nausea, vomiting, abdominal cramps, diarrhea, fever and headache. $>10^3$ cfu	Manure, soil, wild and domestic animals, sewage. Raw meat especially poultry.
Staphylococcus aureus	1 to 6 h	Nausea, vomiting, diarrhea and dehydration, loss of appetite, severe abdominal cramps and mild fever. <103 cfu	Foods prepared with hand contact which require no additional cooking, milk and dairy products, meat, poultry eggs.

Some of the main food-borne pathogens in general and, relating to fresh mushrooms are *E. coli* O157:H7, *S. aureus, Salmonella* spp. and *L. monocytogenes*. The presence of some of these or associated organisms have been widely used as indicators for a possible risk that may be associated with a food product e.g. enteric bacteria such as coliforms, *E. coli* and Enterobacteriaceae are extensively used as an indicator of fecal contamination and *Staphylococcus* presence may be indicative of general human handling (De Keuckelaere *et al*., 2015). The same principles can be applied to the mushroom industry as well.

a) Pathogenic *Escherichia coli*

The gastrointestinal tract of humans and animals are normal habitats for nonpathogenic *E. coli* (Roussel *et al*., 2017). Pathogenic *E. coli* on the other hand have acquired virulence factors which makes it able for them to infect and cause disease in the gastrointestinal and urinary tracts as well as the central nervous system (Huttner *et al*., 2017). The pathogenic strains can be broadly sub-divided into five categories, based on the condition that they cause. All the pathogenic strains infect via similar mechanisms, eventually colonizing the intestinal mucosal cells (Zeng *et al*., 2017). The five categories or groups include: Enterotoxin producing ETEC and EaggEC, EIEC which invades the epithelial cells and EPEC as well as EHEC which adhere to the cell, modifying the cell's activity. All strains potentially pose health risks but the strain that is of most concern is EHEC, especially *E. coli* O157:H7 (Weiner, 2007). The high virulence of this strain may be attributed to the production of verotoxin and verocytotoxin, which are Shiga-like toxins. The human kidney is a great site for infection as they are richly coated in receptors for the attachment of *E. coli* O157:H7 (Bielaszewska *et al*., 2018). When this pathogen infects the kidney through toxic infection, it may be accompanied by renal failure, known as HUS syndrome. Other types such as O111, O145, O113, O103, O91 exist and pose equal threat to infection and outbreak (Bower, 1999; McAllister *et al*., 2016). All *E. coli* that produce or can produce toxin or have toxigenic genes are collectively categorized as Shiga-toxin *E. coli* (STEC). The main source from which *E. coli* can be obtained is usually the manure of ruminants and sewage (Chase-Topping *et al*., 2008). It was previously found that ETEC, EIEC, and EAggEC do exist on contaminated vegetables (Robins-Browne, 2007; Scavia *et al*., 2008), which are some of the leading causes of diarrhea in infants. Pathogenic *E. coli* may either be food-borne or water-borne and are able to transmit between people through contact (Chang *et al*., 2015).

b) *Salmonella* spp.

A major human pathogen which is involved in outbreaks related to fresh produce is *Salmonella* (Callejón *et al*., 2015). It is a Gram-negative, rod shaped bacillus that belongs to the same family as *E. coli*. Over 2700 serovars are included in the genus *Salmonella* and 200 of these are most associated with human illness (Taylor *et al*., 2015). Of these, *Salmonella typhimurium* and *Salmonella enteritidis* are the most common (Liu *et al*., 2013). Salmonella may also be found in the intestines of humans,

animals and poultry (Mughini-Gras *et al*., 2014), but those isolated from vegetables are a lot less common (Liu *et al*., 2013). Multi-drug resistance is a great concern as the distribution of these organisms is throughout various food chains. The reason for such resistance may be the practice of using antibiotics as animal growth promoters (Mather *et al*., 2013). Another reason is the over prescription of antibiotics and then the failure to complete the course, as this encourages the emergence of these resistant organisms (Kelly *et al*., 2004). Salmonella have a similar route of infection to that of *E. coli* onto vegetables, which is through fecal contamination, food-handling and cross-contamination (Jacobsen & Bech, 2012). It is interesting to note that the *Salmonella* spp. isolated from vegetables are usually less virulent than those isolated from clinical sources (Herikstad *et al*., 2002; Olsen *et al*., 2001; Sivapalasingam *et al*., 2004).

c) *Listeria monocytogenes*

Listeria monocytogenes, unlike other normal enteric bacteria, have adapted to not only survive in the host but also in a non-host environment. It is a common contaminant of vegetables, particularly root crops; partly due to its widespread presence in nature (Embil *et al*., 1984; Lianou & Sofos, 2007; Swaminathan, 1988). These pathogens usually infect with the result of severe illness and/or death and its virulence is mostly underestimated (Drevets *et al*., 2008). Like various other human pathogens, the environmental isolates of *L. monocytogenes* are less virulent to those recovered from clinical cases such as listeriosis (Chan *et al*., 2007). Johannessen *et al*. (2002) isolated *L. monocytogenes* from one sample in contrast to Samadpour *et al*. (2006) which detected the bacterium in 5% of their mushroom samples. Similarly, Rivera *et al*. (2010) detected *L. monocytogenes* in fresh mushroom samples collected for their study, which confirms potential association and proliferation of this bacteria on mushroom ascocarps.

d) *Staphylococcus aureus*

Staphylococcus aureus have previously been detected on ready-to-eat vegetable salads, fresh produce and it is particularly associated with food handlers (Castro *et al*., 2016). *Staphylococcus aureus* does not compete well with other microorganisms on the surface of fresh produce, thus the likelihood that spoilage by other non-pathogenic microorganism will precede the accumulation of high numbers of this pathogen is more likely (Giaouris *et al*., 2015). There has been an outbreak of staphylococcal food-borne illness associated with canned mushrooms (Le Loir *et al*., 2003). The growth and toxin production occurred prior to the processing of the mushrooms. There was no apparent visual degradation, but this may be because the mushrooms were held under ambient room temperatures in plastic bags containing salt (www.fda.gov). The conditions in the bags became anaerobic and the natural spoilage microbiota was inhibited and there was selection for *S. aureus*. The mushrooms are then processed through thermal processing, but as the produced toxins are heat stable, it did not inactivate or destroy the toxin (Le Loir *et al*., 2003; Hennekinne *et al*., 2012).

2.4.1.4. Food safety concerns associated with fresh mushrooms

Mushrooms are food that may be consumed cooked or raw, processed, whole or even semi-prepared, various scientific studies around post-harvest safety and quality are conducted to ensure that mushrooms are safe for consumption in all its forms (Zhang *et al*., 2018). There have been no reported outbreaks associated with the consumption of fresh mushrooms. However, there have been several reports of human pathogens isolated from mushrooms. Organisms such as *L. monocytogenes*, *Salmonella* spp. and *E. coli* have been previously found on fresh mushrooms. Cultivation practices, packaging and even storage respectively play a large role in raising the concern of a safe and quality consumer product. In South Africa, mushrooms can be sold under the name of the farm or under the name of house brands of big retailers. If a food safety related outbreak would arise, the retailer is held responsible and may lose a lot of money as well as its credibility with the public (Uyttendaele *et al*., 2015; Wallace *et al*., 2018). A recent example in a related industry could be described by the Listeria outbreak in the processed food (meats) industry in South Africa, with the company *Enterprise* implicated as the origin of the outbreak (National Institute for Communicable Diseases, 2017). Events like this reflects badly on the industry, but the company has redeemed itself by re-inventing its brand in 2019 and are back in business after a time period where all products were removed from retail. The point being, that such a travesty should have been prevented, systems and procedures must have been in place to ensure all possible safety precautions, it is therefore important that there be a suitable measure of quality and safety control for fresh mushrooms that are sold whole and sliced (Jacxsens *et al*., 2015). Table 2.2 summarizes food safety related outbreaks and pathogen isolation from button mushrooms over the last 40 years. Information and a definitive history of contaminated mushroom recalls is very limited within literature for both white button and other edible mushrooms. It is unspecified if a recall was initiated for every outbreak listed in Table 2.2.

Table 2.2 *Food safety related outbreaks and pathogen isolation from button mushrooms*

Food safety organism	Product type	Region/ Country	Reference
Clostridium botulinum	Canned mushrooms	Canada	Canadian Food Inspection Agency (2005)
	Preserved mushrooms	Netherlands	Notermans *et al*., 1989
Campylobacter jejuni	Fresh retail mushrooms	Midwestern U. S	Doyle & Schoeni, 1986
Listeria monocytogenes	Fresh retail mushrooms	Pacific Northwest	Samadpour *et al*., 2006
	Fresh retail mushrooms	Netherlands	Van Netten *et al*., 1989
	Fresh retail mushrooms	Georgia, USA	Georgia Dept. of Agriculture (2003)
	Fresh retail mushrooms	US	Heisick *et al*., 1989
	Fresh retail mushrooms	China	Chen *et al*., 2018
	Fresh retail mushrooms	Minneapolis USA	Heisick *et al*.,1989
Salmonella spp.	Fresh retail mushrooms	Pacific Northwest	Samadpour *et al*., 2006
	Fresh mushrooms	Ireland	Food Safety Authority Ireland, 2001; Meikle, 2001
Staphylococcus aureus	Canned mushrooms	China	Beelman *et al*., 1990; Hardt-English *et al*., 1990
	Fresh mushrooms	Norway	Johannessen *et al*.,2002

Except for the two studies in Table 2.2 which confirmed pathogens from canned mushrooms (Canadian Food Inspection Agency, 2005; Beelman *et al*., 1990; Hardt-English *et al*., 1990), all other positive identifications of related food-borne pathogens were confirmed from fresh or packaged mushrooms

collected at market or retail level. All isolations and identifications were performed in laboratory facilities using enrichment for the specific pathogen, selective and differential media plate cultures and confirmation through PCR and sequencing. Food-borne illness resulting from the consumption of contaminated food, is dependent on several factors (Callejón *et al*., 2015). First and foremost, the produce must be contaminated with the pathogen and must be able to survive and grow to infective dose levels and subsequently these infective doses must be present at the time of consumption (Bhunia, 2018). The abuse of temperature and growth is not always necessary for food-borne illness to occur. The conditions that are needed for the survival and growth of pathogenic microorganisms are influenced by the type of microbe, the produce item, environmental conditions that are present in the field, handling and then subsequently the storage of the product (Yeni *et al*., 2016).

Most pathogens will only survive and will not enumerate on the surface of most fresh produce after harvest. In some cases, the levels of pathogens present may even decline after harvest. When the epidermal barrier of produce is broken, by inflicting surface wounds through bruising and punctures, attack, survival and enumeration of food-borne pathogenic organisms are notably enhanced (Olaimat & Holley, 2012). The transmission of pathogens from their source to the food product is of critical importance as it is through this process that these microorganisms could enter the production chain. The greatest risk associated is direct fecal contamination of fresh produce just before consumption (Gorny *et al*., 2002; Mukherjee *et al*., 2007). This is not the only form of contamination and other sources such as irrigation water, or water used to water the mushroom beds, manure amended soil or not completely sterilizing the mushroom compost are commonly encountered (Hutchison *et al*., 2008; Islam *et al*., 2004).

2.5. ANTIBIOTIC RESISTANCE RISK RELATED TO MICROBIAL POPULATIONS ASSOCIATED WITH FRESH PRODUCE OR AGRICULTURAL ENVIRONMENTS

Modern medicine depends on the effective use of antibiotics to treat and prevent various infections such as skin and soft tissue infections, urinary tract infections etc. It is also well documented that bacteria can develop resistance to certain antibiotics. Over the past few years it is observed that the pipeline of new antibiotics is declining, and bacterial resistance frequencies have continued to rise world-wide (Banin *et al*., 2017). In the last two decades, driven by overconsumption and injudicious use of clinical antibiotics, and the ongoing evolution as well as spread of mobile genetic resistance elements, increasing numbers of multidrug resistant (MDR) and even extremely drug-resistant (XDR) bacterial pathogens have emerged. Important bacterial pathogens have been identified by The World Health Organization (WHO), which should be prioritized during research to prevent drug resistance and help develop effective antibiotic treatments. The list contains 12 bacteria and bacterial families including among other *Enterobacteriaceae, S. aureus* and *Salmonella* spp., which are all commonly associated

with fresh or minimally processed produce (WHO, 2017). One of the modes by which bacteria exert antimicrobial resistance (AMR) is through the ability to form biofilms. Mechanisms underlying resistance exhibition are complex and are described by Hall & Mah (2017), which includes the interaction of antibiotics with biofilm matrix components. Resistance does not usually depend on a single aspect but rather on a combination of several mechanisms that manifests within microbial populations. Another concept described within the broader theme of resistance is the mechanism of persistence. This can be described as a transient tolerance state in which the antibiotic tolerance is non-genetic and persists within a susceptible population (Van den Bergh *et al*., 2017).

Previous studies have identified antibiotic resistant and persistent bacteria on vegetable products at harvest or at the retail level (Schwaiger *et al*., 2011; Raphael *et al*., 2011). Fecal material from food animals, humans, and animals often contain bacteria that are resistant to some antibiotics (Yost *et al*., 2011). It is reported that extensive use of antimicrobials in agriculture expose antimicrobial-resistant bacteria to humans through contaminated food products or vice versa (Silbergeld *et al*., 2008). It is documented that antibiotic resistant bacteria have been identified in animal waste, wastewater, river sediments, farmland soil and raw materials potentially used in mushroom production processes (Micallef *et al*., 2013). Antimicrobial resistant bacteria may be disseminated to the environment through farm waste and may reach humans through the consumption of contaminated foods of animal origin, water, and vegetables (Da Costa *et al*., 2013). Leafy greens and similarly mushrooms could be contaminated with antibiotic-resistant bacteria from animal and human sources during production and harvesting (Holvoet *et al*., 2013). Consumption of fresh produce, particularly raw, unprocessed fresh produce, represents a route of direct human exposure to resistant microorganisms. With the increasing foodborne illness associated with fresh produce, there is a lot of emphasis on good agricultural practices to verify that farms are producing fresh produce in the safest means possible (Kilonzo-Nthenge & Mukuna, 2018).

2.6. FUNGAL DISEASES OF ECONOMIC IMPORTANCE ON WHITE BUTTON MUSHROOMS

2.6.1. MYCOPARASITIC PATHOGENS

Most mycoparasitic fungal pathogens associated with mushroom production are of economic importance (Table 2.3). These pathogens are significant due to their effect on yield and quality of mushrooms. Four main fungal diseases, Green mould (*T. aggressivum*), Dry Bubble (*L. fungicola*), Cobweb (*C. dendroides*) and Wet Bubble (*M. perniciosa*) are annually responsible for considerable disease outbreaks worldwide (Largeteau & Savoie, 2010). Occurrence may lead to serious losses; in worst cases the entire crop can be lost. Estimated losses in western countries each year due to *L. fungicola* and *Trichoderma* sp. alone account for approximately 25% of the total production value

(Soković and van Griensven, 2006). Because mushrooms as host and the described pathogens are all fungal organisms, management of these diseases can be challenging and, in some instances, problematic (Bhatt and Singh, 2000).

Table 2.3 *Summary of the four main fungal pathogens of economic importance on commercially cultivated white button mushrooms in South Africa*

Disease	Causal Pathogen	Inoculum Source	Symptomology	References
Cobweb	*Cladobotryum dendroides*	Airborne conidia from infected beds/ soil	Dense white to pinkish patches on casing, brown spots on mushroom caps	Adie *et al.*, 2006; Back *et al.*, 2010; Grogan, 2000; Fletcher & Gaze, 2008; Fletcher *et al.*, 1989; Mckay *et al.*, 1999; Potočnik *et al.*, 2010.
Dry Bubble	*Lecanicillium fungicola*	Conidia in dust, on flies, infected beds, contaminated casing	Undifferentiated tissue with liquid droplets in early symptom expression, distorted caps and stalks, spots on caps	Berendsen *et al.*, 2010; Fletcher & Gaze, 2008; Fletcher *et al.*, 1989; Romaine *et al.*, 2002.
Green Mould	*Trichoderma aggressivum*	Spores from infected tissue or soil	Dense white mycelium on growing beds at spawn run; turns dark green during sporulation	Fletcher & Gaze, 2008; Muthumeenakshi & Mills, 1995; Samuels *et al*., 2002.
Wet Bubble	*Mycogone perniciosa*	Conidia/aleuriospore from infected tissue, contaminated casing or soil	Small dark brown droplets of liquid on undifferentiated mushroom tissue, white fluffy patches on casing material	Fletcher & Gaze, 2008; Fletcher *et al*., 1989.

2.6.1.1. *Cladobotryum dendroides*

Cobweb (soft mildew) is caused by *C. dendroides* (Bull.) Gams (syn. *Dactylium dendroides* (Bull.) Fr.) (teleomorph *Hypomyces rosellus* (Alb. & 9 Schwein) Tul. (Howard *et al*., 1994; Grogan, 2000). Growth of coarse mycelia covering the affected mushrooms characterizes the disease (Fletcher *et al*., 1989). Affected mushrooms eventually turn brown and rot, whilst the cobweb mycelium turns pink in color (Adie *et al*., 2006; Howard *et al*., 1994). *Cladobotryum dendroides* is a soil inhabiting fungus with simple or branched conidiophores carrying two- or three-celled hyaline conidia (Howard *et al*., 1994). Spores are dispersed by air, water splash, debris, infected casing material or pickers (Fletcher *et al*., 1989; Fletcher & Gaze, 2008; Howard *et al*., 1994). Well controlled hygiene and the use of fungicides are control measures that can be used for Cobweb (Fletcher *et al*., 1989). With resistance of *C. dendroides* becoming a concern, research on the complex interactions between fungicide resistance, fungicide persistence in mushroom casing and on-farm disease management have received considerable attention over the last couple of years (Grogan, 2000; Chakwiya *et al*., 2019).

2.6.1.2. *Lecanicillium fungicola*

This disease is caused by the pathogen *Verticillium fungicola* (Preuss) Hassebr. 1936, which was renamed as *L. fungicola* (Zare & Gams, 2008). Common names for the disease, such as brown spot, fungus spot, verticillium spot, split stipe and dry bubble all describe a symptom that results from infection by *V. fungicola* and the different stage of disease development (Howard *et al*., 1994). When

the young mushroom primordium is infected during an early stage of development, growth is disrupted and a ball-like mass is formed (Howard *et al*., 1994). Later infection causes imperfections in mushroom form with partially differentiated caps or distorted stipes and tilted caps (Fletcher *et al*., 1989). When mature caps are infected, cinnamon-brown spots develop, which will eventually develop into a necrotic lesion (Wuest & Bengtson, 1982). Sources of inoculum include infected casing soil, ventilation air within growing rooms, sciarid and phorid flies, hands and clothing of pickers as well as infected equipment (van Griensven, 1988). Best practices to control dry bubble disease is using chemicals, cultural and good sanitation practices (Gea *et al*., 2005). As with *C. dendroides*, almost all populations of *L. fungicola* are resistant to benzimadazole fungicides (Fletcher *et al*., 1989). Procloraz manganese gives good control of dry bubble and there are no records of fungicide resistance (Fletcher *et al*., 1989), but it has been reported that decreased sensitivity can occur to this fungicide (Gea *et al*., 2005).

2.6.1.3. *Trichoderma* spp.

Green mould disease are caused by several *Trichoderma* species, *T. harzianum* Rifai (teleomorph *Hypocrea vinosa* Cook), *T. koningii* Oudem (teleomorph *H. ceramic* Ellis & Everh.) and *T. viride* (Pers.) Fr. (teleomorph *H. rufa* (Pers.) Fr.) (Howard *et al*., 1994), of which *T. aggressivum* is the most virulent. Two populations of *T. aggressivum* are responsible for disease, *T. aggressivum* f. *aggressivum* (Ta4) and *Trichoderma aggressivum* f. *europaeum* (Savoie & Mata, 2003). Vegetative mycelium of this fungus is septate, hyaline and appears white during first colonization, but turns grey-green when conidia develop (Howard *et al*., 1994). As a soil and organic matter inhabiting fungus it can be dispersed by air, water as well as mechanical means such as equipment and pickers (Howard *et al*., 1994). Good hygiene measurements and good composting practices should be employed to control and eliminate this pathogen.

2.6.1.4. *Mycogone perniciosa*

Wet bubble disease of mushrooms is caused by the fungus *M. perniciosa* (Magnus). This disease is known to affect mushrooms on a global scale, wherever mushrooms are cultivated (Fletcher *et al*., 1995). *Mycogone perniciosa* produce copious flocculent mycelium on most substrata (Smith, 1924). Mycelium are initially white, hereafter it changes to a light brown colour (Fletcher *et al*., 1995). The first spores to appear on infected mushrooms are phialoconidia, which originates on the edge of *M. perniciosa* mycelium (Smith, 1924). When nutrient levels are high when this fungus is cultivated *in vitro*, these are also the first spores produced (Van Griensven, 2000). Conidia are produced when a constriction forms near the apex. The portion beyond the constriction continues to enlarge until the long cylindrical spore is abdicated (Smith, 1924). *Agaricus bisporus* can be infected by *M. perniciosa* at any stage of development, which results in tumorous masses of undifferentiated tissue. Most infections occur at the stage of pinning (Umar *et al*., 2000). Pathogen spread are easily achieved by various vectors, the primary source being contaminated casing material (Fletcher *et al*., 1994).

Dispersal of spores can occur via water (Fletcher & Ganney, 1968), pickers and by mechanical implements (Fletcher *et al*., 1994). Sciarid flies can also spread or disperse small conidia in cases of severe infection (Dielemann-Van Zaayen, 1976). Control of wet bubble disease is challenging, and eradication of the disease has up until now proven unsuccessful. The most common control measure employed in South Africa and some other places in the world is salting patches of identified infection. Salted areas are covered by paper cups to prevent further spread. Chemical control is also practiced, with chemicals such as benomyl (Fletcher, 1975), thiabendazole (Eicker, 1984; 1990) and prochloraz (Fletcher *et al*., 1983), being the most used in the industry.

2.6.2. DETECTION AND IDENTIFICATION OF MYCOPARASITIC FUNGAL PATHOGENS

The occurrence and severity of mycoparasitic mushroom pathogens on commercial and small farms differ according to effectiveness of sanitary and hygiene management systems applied (Carrasco *et al*., 2017). In order to prevent severe outbreaks, development of a method for early detection is necessitated, after which effective management and control of the disease on commercial farms could be deployed (Bhatt & Singh, 2000). One of the most important means of control prevention, early detection then the elimination of the primary sources of the pathogen (Van Griensven, 1988; Fletcher *et al*., 1994; Oei, 2003), whilst farm hygiene, chemical and environmental control remain essential components of a disease control strategy (Fletcher & Ganney, 1968; Muthumeenakshi & Mills, 1995). A rapid, accurate and sensitive method for early detection of mushroom pathogenic fungi is essential for effective disease management and is a prerequisite for studies of environmental and cultural factors related to disease occurrence. Techniques employing polymerase chain reaction (PCR) provide the most sensitive means of detecting fungal pathogens. Methods such as PCR used to detect fungi in various natural environments have successfully been based on internal transcribed spacer (ITS) sequences (Färber *et al*. 1997; Ristiano *et al*. 1998; Böhm *et al*. 1999; Meyer *et al*. 2001; Romaine *et al*. 2002; Bonants *et al*. 2003; Landeweert *et al*. 2003; Meyer *et al*. 2006). A combination of direct and nested-PCR could be proposed to unequivocally identify and detect the mushroom disease pathogens in various substrates.

A molecular PCR-based platform proven to exhibit superior amplification, detection and quantification of positively amplified sequences is droplet digital PCR (ddPCR) (Hindson *et al*., 2013). Challenges with "environmental" samples (such as those obtained from mushroom production systems) are often complex due to variation in sample matrices and may contain PCR inhibitors. PCR inhibition is among the biggest impediment for adopting molecular tools in environmental applications (USEPA, 2012a). The more robust ddPCR improves PCR inhibition concerns and utility of molecular quantification in such samples. Increased resistance to inhibition may also allow analysis of unpurified or larger volumes of DNA extracted indirectly or directly from samples. The former improves cost and inconsistent recovery efficiency associated with DNA purification procedures. The latter applies especially in the detection and purification of rare targets, such as found in pathogens. Nevertheless, it should be noted

that ddPCR may not improve inhibition caused by DNA sequestration or severe inhibition and total molecular drop-out where the target remains unamplified (Nixon *et al*., 2014).

Droplet digital PCR employs a technique of partitioning reactions in picoliter droplets which allows ddPCR to have lesser interference with PCR inhibitors and be able to handle larger amounts of input DNA, compared to traditional PCR methods (Sanders *et al*., 2013). PCR inhibitors function by DNA sequestration (i.e. bind DNA making it unavailable for amplification) or reducing PCR amplification efficiency (i.e. interfere with the polymerase-DNA machinery), both of which increase Cq values and lead to underestimation in qPCR (Cao *et al*., 2015). However, reduced amplification efficiency could still yield positive end-point PCR, albeit with lower fluorescence signals, and therefore does not affect ddPCR quantification if amplification still occurs and that the lowered fluorescence signal is not below the detection threshold. The basis of ddPCR is to absolutely quantify target DNA concentration in a DNA sample, while that of qPCR is to extrapolate DNA concentration using an external DNA standard (Kim *et al*., 2014; Hindson *et al*., 2013). This means that reference DNA templates must be prepared prior to quantification in qPCR assays. Although primer/ probe sets, targeting a wide variety of microorganisms are available, the accessibility of these sets is often limited due to the difficulty associated with obtaining the reference DNA templates. However, ddPCR obviates the preparation of reference DNA templates, which is often expensive, laborious and time-consuming if they are not readily available (Cao *et al*., 2015). Another important advantage of ddPCR would be less variability among laboratories and studies, due to the nature of absolute quantification without external calibrators. qPCR uses spectrophotometric determination of DNA concentration to prepare quantitative calibrators, which often cause errors in DNA quantification (Kim *et al*., 2014). Such spectrophotometric methods will quantify all nucleic acids that absorb at 260 nm, so DNA/ RNA impurities in the reference material will also be included in the quantification (Cao *et al*., 2015). The higher precision, especially at low target concentrations, and higher run-to-run repeatability produced by ddPCR compared to qPCR were consistent with the binary nature of digital PCR quantification (Morisset *et al*., 2013; Sanders *et al*., 2013; Whale *et al*., 2012).

Various studies have shown several advantageous characteristics of ddPCR, however, there are three important limitations unique to ddPCR. The first limitation relates to the narrower dynamic range afforded by ddPCR, as compared to qPCR. The upper quantification limit (UQL) of ddPCR is determined by the number of droplets per reaction and is generally several orders of magnitude lower than that of qPCR. The second limitation is that the analytical sensitivity of ddPCR varies, i.e. sensitivity increases with the number of droplets per reaction. Consequently, detection limits vary from reaction to reaction. This limitation may be turned into a potential advantage in that multiple reactions from the same sample can be merged retrospectively during data analysis to increase the assay's sensitivity, which could be useful for measuring rare or dilute targets during pathogen detection (Cao

et al., 2015). A third and last consideration for ddPCR relates to high concentrations of total DNA which may interfere with partitioning. The general recommendation is to have <66 ng total DNA per reaction without pre-treatment (i.e. restriction enzyme) of DNA (Bio-Rad). Dilution or pre-treatment should be considered if the environmental sample is rich in biomass as are those with high abundance of Eukaryotic DNA (Cao *et al*., 2015).

2.7. CONCLUSION

A worldwide shift towards fresh wholesome products was observed within the last decade, in contrast to the consumption of processed foods. This trend has inevitably put pressure on retailers and primary suppliers to offer high quality products to their customers. Through this demand, special challenges are faced by the commercial mushroom industry to stay economically viable, deliver products of consistent quality and meet consumer volumes and safety requirements of markets. Industry growth has been supported by consumer preference, whilst fresh, whole mushrooms remain a popular product. To cultivate mushrooms on a commercial scale, high demand exists for input products. These are resources such as chicken manure, water and other raw materials used in substrate production. From a food safety perspective, it is well known that contamination of fresh produce such as mushrooms can occur at any stage during production. Particularly when working with materials that inherently have a higher risk of food-borne pathogen association (animal derived products). There is a growing global concern over antibiotic resistance in microbial populations potentially associated with fresh produce and related industries. *Salmonella*, *E. coli* and *Listeria* populations in agriculture and food processing environments are a major point of concern due to recent food-related outbreaks in South Africa and the USA. Food commodities such as fresh, whole or sliced mushrooms that are handled extensively and do not go through a decontamination step, might pose a longer-term human and environmental health threat. Persistent or transient pathogenic organisms can therefore contaminate mushrooms if proper hygiene management is not practiced throughout the production chain. From a production and economic perspective, mushrooms are affected by several fungal, bacterial and insect pests. The most effective way of dealing with pathogens and pests, has to date, been the use of pesticides. With consideration to maximum residue levels, pesticide legislation and limited number of commercially available pesticides on mushrooms, resistance of pest populations become a concern. It could be argued that an integrated health management system be a better option with the effective identification of problem areas on farm as well as prevention of spread and disease outbreaks. This could be achieved by pro-active farm monitoring using molecular detection and identification strategies. Through research and development of a holistic mushroom quality and safety management system, a multi-pronged approach may be advised to regulatory authorities, retail and the commercial mushroom industry to help ensure both food security and safety through economically viable, white button mushroom production.

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CHAPTER 3

MICROBIAL LOAD AND FOOD SAFETY OF WHITE BUTTON MUSHROOMS

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ABSTRACT

The microbial load and presence or absence of foodborne pathogens on commercially grown white button mushrooms is of importance in terms of food safety assurance and quality control. The purpose of this study was to establish the microbial load of fresh white button mushrooms, with the focus on potential presence or absence of foodborne pathogens within the *Agaricus bisporus* microbial population. Freshly picked and packaged white button mushrooms were sampled (432 samples) from two commercial farms over a two-year period. The total microbial load (bacteria, yeasts and fungi) was determined by means of standard viable counts. Possible presence of well-known foodborne pathogens, *Escherichia coli* O157:H7, *Salmonella* spp., *Listeria monocytogenes* and *Staphylococcus aureus* were determined using selective enrichment. Presumptive pathogens were further confirmed using molecular methods, including MALDI-TOF MS, PCR and sequencing. Total microbial counts ranged from 5.2 to 12.4 log cfu/g, with the genus Pseudomonas being the most frequently isolated (45.37%). No *E. coli* O157:H7 or *L. monocytogenes* could be detected from any of the samples tested. Considering current food safety guidelines in South Africa for ready-to-eat fresh produce, coliform counts exceeded the guideline value for allowable microbial loads as specified for fruit and vegetables. It is proposed, based on the research done in this study and literature, that the specifications for microbial loads on fresh healthy mushrooms should reflect the natural microbial load at the point of harvest and at the point prior to sale. Freshly harvested, healthy, quality mushrooms can therefore carry an average total microbial load of 8.9 log cfu/g and that packed, whole, ready-to-consume mushrooms a load of 9.8 log cfu/g. The average fresh mushroom microbial load (4.5 log cfu/g bacteria, 1.5 log cfu/g fungi and 2.7 log cfu/g yeasts).

3.1. INTRODUCTION

The concept of food safety has received considerable attention over the last few decades, especially regarding fresh fruit and vegetables (Ares *et al*., 2007). Globally production and consumption of edible fresh mushrooms have also steadily increased (Boa, 2004; Royse, 2001, Higgins *et al*., 2017), especially in the last few years with volumes of 9,646,813 tons recorded in 2012 compared to 10,790,859 tons in 2017 (FAOSTAT, 2018). *Agaricus bisporus* (J.E. Lange) Imbach 1946, commonly known as white button mushrooms, is globally the most cultivated and consumed mushroom type (Largeteau & Savoie, 2010). In South Africa, white button mushrooms constitute a small but important group of "vegetables" (grouped

according to guideline No.R.364, Department of Agriculture, Forestry and Fisheries, 2013), with local production (2012-2018) averaging 19,000 tons per annum (SAMFA Production Statistics, 2019).

Fresh produce, including white button mushrooms are known to harbour large microbiological populations (Leff & Fierer, 2013). The presence as well as persistence of these organisms within the microbiome of fresh produce is important since it provides a better understanding of pathogens to colonise if introduced within the food chain (Berg *et al*. 2014). Previous associations of foodborne pathogenic bacteria have been well documented for fresh produce but to a lesser extent for mushrooms. It is well known that contamination of fresh produce with pathogenic foodborne pathogens is possible during any stage of production (Oliveira *et al*., 2010; Bhunia, 2018). However, it is recent that advances are being made in understanding the interaction of these pathogens with produce-associated surface microbial communities (Critzer & Doyle, 2010). In the case of mushrooms, the most important potential source of contamination is from the substrate on which they are grown, watering during production cycles as well as handling during harvesting and packing (Venturini *et al*., 2011; Bartz *et al*., 2017).

Although no reported disease outbreaks have been associated with the consumption of fresh mushrooms in South Africa, detection of foodborne pathogens on mushrooms such as *Listeria* spp. have been reported in the United Kingdom (UK) (Personal communication - Peter J McClure, Unilever, Research and Development, UK, 2014), Canada (Canadian Inspection Agency, 2011; 2012a; 2012b; 2014) as well as the United States of America (U.S.A) (United States Food and Drug Administration, 2015). In the case of the latter the products were recalled. *Salmonella* spp. (Samadpour *et al*., 2006a; Jiang *et al*., 2018) and *E. coli* have been reported in raw, loose and packed mushrooms during a market study in Italy (FSAI, 2006; Ramos-Villarroel *et al*., 2015). Surface properties of fresh mushrooms provide ideal conditions for the survival and proliferation of microorganisms, especially bacteria, due to high moisture content and water activity (≥0.98) as well as a near neutral pH (Martinez-Carrera *et al*., 1998; Jiang *et al*., 2017). There are various factors that contribute to the survival of microorganisms on fresh produce; these include nutrient availability, UV radiation, competition from other microorganisms and desiccation (Whipps *et al*., 2008; Alegbeleye *et al*., 2018). It is known that pathogenic bacteria such as *Escherichia coli* O157:H7 and *Salmonella enterica* can survive on produce for long periods of time (Olaimat *et al*., 2012) and *Listeria* spp. and *Staphyloccocus* spp. under refrigeration or cold conditions (Bardsley *et al*., 2019).

The considerable consumption rate of fresh mushrooms (raw and processed) and the potential of foodborne pathogen associated disease hazards make it an important topic to investigate and ensure that the product is safe for consumption. The current national guideline for ready-to-eat fresh fruit and vegetables stipulates

a maximum yeast and fungal count of log 4 with no acceptable or defined range for total bacterial counts (Department of Health, 2000). Work presented in this chapter is aimed at establishing a microbial dynamism for freshly harvested and packaged white button mushrooms within well-established commercial farms in South Africa. Results will be evaluated in comparison to the current microbiological guidelines as the only official document for fresh fruit and vegetables. Total microbial loads and -diversity will be studied during different mushroom growth and production phases (pre-harvest & post-packaging). The presence and absence of foodborne pathogens, including *E. coli* O157:H7, *Salmonella* spp., *S. aureus* and Thermotolerant coliform bacteria will be analysed from all experimental samples.

3.2. MATERIALS AND METHODS

All methods and techniques employed during experimental work conducted in the laboratory and during the collection of samples on farm visits, were conducted aseptically and according to Standard Operating Procedures (SOP's) set forth in ISO regulated standards (SANS:8199)

3.2.1. SAMPLING

Two experimental designs were employed to assess the microbial load of mushrooms and test for presence or absence of selected foodborne pathogens within samples collected during different production phases of commercial mushroom farming operations. In order to determine microbial shifts on mushrooms between production and harvesting/packaging (Experiment 1), a total of 432 samples, consisting of 250 g commercial punnet units (12 to 16 mushrooms per unit depending on size), were collected before harvest and after packaging over the course of a year. Sampling was done during three stages of production (1st, 2nd and 3rd breaks). Mushroom samples were selected at the commercially ready-to-harvest stage, based on uniformity of shape, size, and maturity as well as being devoid of mechanical defects or browning, according to the national commercial standard for white button mushrooms (DAFF, Agricultural Products Standards Act, No. 119 of 1990, Regulation No. R.364). Eight samples were picked aseptically before harvest, from eight growing beds (mushrooms pooled from a single growing bed, constitutes one sample) and placed into punnets. To further determine population shifts of culturable microorganisms, the same mushroom batches were monitored for a 12-week-period from full bud growth through harvesting, packing up to cold storage (3°C ± 2°C). A total of 192 samples were collected in 12 different mushroom growing rooms, using a completely randomised design. To determine microbial succession within growing cycles i.e. from 1st break until the 3rd break, five individual mushroom growing rooms were sampled (Experiment 2). In total, 240 samples were procured and analysed over a 15-week-period. A systematic sampling design

was used, and the experiment was repeated on each farm. The farms were in the same province and followed similar production practices. Both farms are considered large commercial scale production units compliant with at least GlobalG.A.P. Integrated farm assurance standards and practices, farming on both steel shelves and wooden trays, using Phase II and III compost with peat as casing materials.

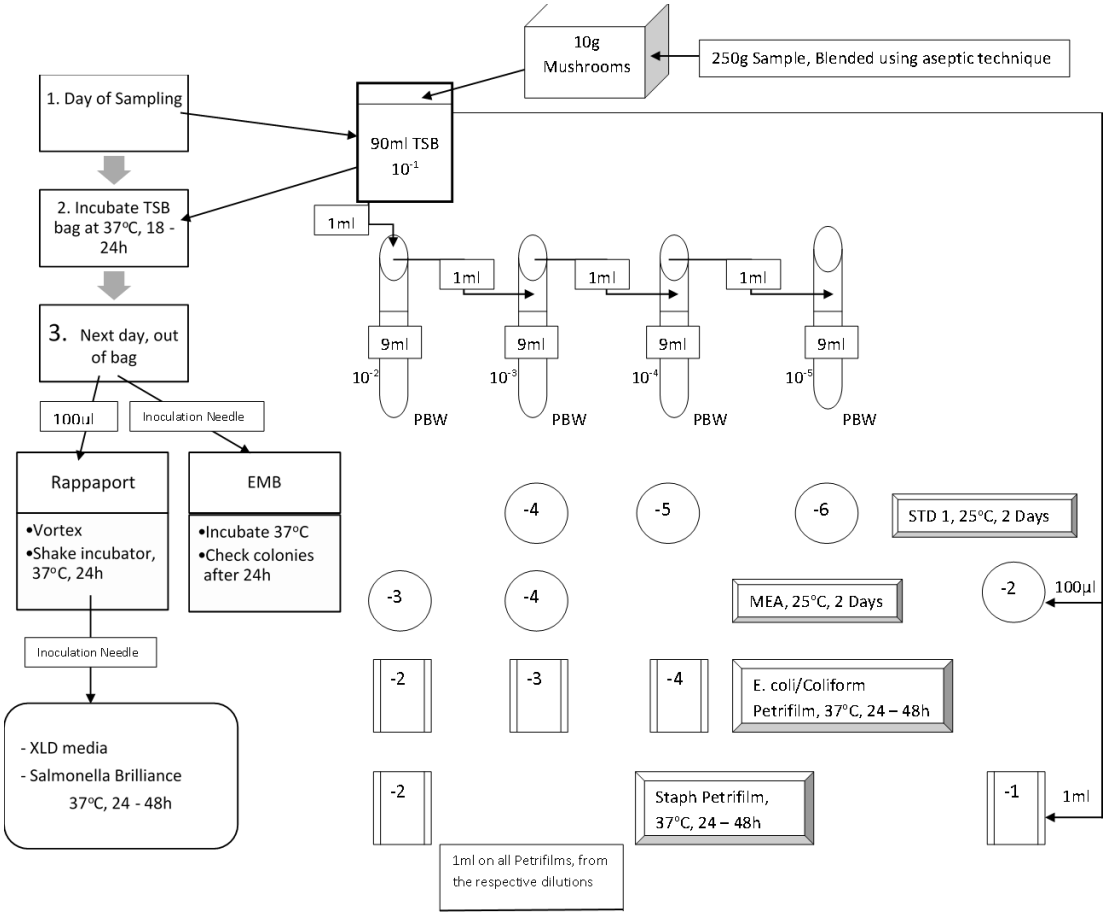

Figure 3.1 *Mushroom sample processing flow and protocol overview up to first colony count and isolations.*

3.2.2. SAMPLE PROCESSING AND ISOLATIONS

An overview and summary of the initial sample processing and handling up to first isolation, can be viewed in Figure 3.1. Each sample (250 g) was placed within an aseptic (75% ethanol rinsed and air-dried) stainless-steel beaker. The mushrooms were blended using a handheld, ethanol sanitized food-blender. From the blended sample, 10 g were weighed off into a sterile stomacher bag containing 90 mL sterilised tryptone soy broth (Merck-Biolab, Johannesburg, South Africa). Contents of the bag were homogenised in a Stomacher® Circulator 400 (5 min at 230 rpm). Standard serial dilutions were made using buffered peptone water (Merck-Biolab) and aliquots were spread-plated onto standard one agar for bacteria and malt

extract agar for yeasts and moulds (Merck-Biolab). All colonies were counted to determine total viable counts where after the most frequently occurring (by number and frequency on different media plates) colonies were isolated and further identified with matrix-assisted laser desorption/ionisation time-of-flight mass spectrophotometry (MALDI-TOF MS), PCR and sequencing. At least one representative colony for each organism and morphology type were selected from cultures throughout the study to ensure as comprehensive as possible diversity representation of the entirety of culturable microorganisms.

To screen for foodborne pathogens, diluents were plated on selective media after a 24 to 72 h enrichment step at 37°C: Eosin methyl blue (Merck-Biolab, Johannesburg) and xylose lysine desoxycholate (Merck-Biolab) for *E. coli* O157:H7. Salmonella brilliance (Oxoid Ltd, England) for *Salmonella* spp., Listeria selective media (Merck-Biolab) for *L. monocytogenes* and Baird Parker medium (Merck-Biolab) for *S. aureus*. In addition, Staph Petrifilm™ and *E. coli*/coliform Petrifilm™ (3M, Johannesburg) were used for general *E. coli* spp. and coliform enumeration. On various selective media, typical colonies were noted for presence or absence according to media specifications and identified using MALDI-TOF MS. All functional isolates were purified and preserved at -70°C using glycerol solutions of 10% (fungi) and 55% (bacteria) as described by Hubalek, (2003).

3.2.3. IDENTIFICATION OF ISOLATES USING MALDI-TOF MS

Purified bacterial cultures were transferred in duplicate directly to the MALDI-TOF MS target plate (Bruker), and overlaid with the α-cyano-4-hydroxycinnamic acid matrix (Bruker). The target plate was subsequently analysed using Bruker MicroFlex LT MALDI-TOF MS in conjunction with Bruker Biotyper Automation Software and library. The MALDI-TOF MS was calibrated prior to use with the bacterial standard supplied by Bruker. Duplicate score values (SV) were recorded; SV were used to determine the accuracy of identification. An SV of between 1.999 and 1.700 was used to identify the genus name of the organism, and a value of above 2.0 was used to determine the genus and probable species of an organism. SV within the range of 2.300 to 3.000 were considered as conclusive species identification. Growth and storage times were standardized for all colonies.

3.3. RESULTS

3.3.1. Microbial load assessment on mushrooms

Bacterial counts ranged from 2.5 to 6.2 log cfu/g, fungal counts between 0.0 and 2.7 log cfu/g and yeasts from 1.0 to 4.3 log cfu/g for each of the mushroom samples analysed. Average total culturable microbial counts over the entirety of the study ranged from 5.2 (min) to 12.4 (max) log cfu/g, with the average total load being 8.7 log cfu/g (Table 3.1). At the point of harvest, fresh first break mushrooms showed a total

load of 5.2 to 10.1 log cfu/g, and when packed 8.0 to 10.1 log cfu/g. Similarly, total microbial loads for second and third break mushrooms at harvest were determined to be 7.6 to 10.2 log cfu/g and 8.8 to 11.5 log cfu/g respectively and correspondingly, 8.3 to 10.5 log cfu/g and 9.5 to 12.4 log cfu/g when packed. When viewed in the context of the interactive effect that exists between mushroom production and dependent measures i.e. growth cycle - break; farm sampled; room sampled; production phase - at harvest or packed it correlated to the isolation trend expected. The determined thermotolerant coliform load ranged from 2.54 to 3.71 log cfu/g (Table 3.1).

3.3.2. Microbial succession between different production stages

Variable bacterial counts were observed during the progression from 1st to 3rd break and between each of the five rooms sampled. Bacterial counts during 1st break ranged from 5.4 to 6.2 log cfu/g for pre-harvest and 5.6 to 6.1 log cfu/g packaged mushrooms. Bacterial counts during 2nd break fluctuated between 5.7 and 6.0 log cfu/g for pre-harvest and 5.7 to 6.2 log cfu/g for packaged mushrooms. Bacterial counts throughout 3rd break ranged from 5.6 to 6.1 log cfu/g for pre-harvest mushrooms and 5.9 to 6.1 log cfu/g for packaged mushrooms. The mean bacterial count was calculated to be 5.8 log cfu/g for both the pre-harvest and packaged mushroom samples. Yeast counts of pre-harvest and packaged mushrooms showed variable progression over time for each of the five rooms sampled. Yeast counts during 1st break was found to be in the range of 2.8 to 3.9 log cfu/g for pre-harvest and 2.9 to 4.0 log cfu/g for packaged mushrooms (Table 3.2). During the 2nd break, yeast counts showed a range varying from 2.3 to 3.9 log cfu/g for pre-harvest mushrooms and 3.5 to 3.9 log cfu/g for packaged mushrooms. Yeast counts throughout 3rd break ranged from 2.3 to 4.2 log cfu/g for pre-harvest mushrooms and 3.6 to 4.2 log cfu/g for packaged mushrooms. The mean yeast counts across the different breaks progressed from 3.5 log cfu/g for pre-harvest up to 3.7 log cfu/g for packaged mushrooms. The thermotolerant coliform load ranged from 2.5 to 3.7 log cfu/g (Table 3.1). No correlation could be found with reference to the progression of coliform load from 1st to 3rd break. Farm 1 showed a higher coliform load throughout all production cycles (3 log cfu/g), and between pre-harvest and packaged samples within each cycle in contrast to Farm 2 (2.5 log cfu/g). Low counts of thermotolerant coliform bacteria and the absence of *E. coli* O157:H7 were similarly described by Gónzalez-Fandos *et al*. (2000). Bacterial, yeast and coliform counts differed significantly between mushroom growing rooms.

3.3.3. Food safety microorganisms

Most of the bacteria isolated were mainly Gram-negative, including members of the family *Enterobacteriaceae*. Some of these organisms are considered indicators of potential faecal contamination in food. However, their presence cannot automatically be associated with the incidence of enteropathogens.

Table 3.1 *Bacteria, yeast, fungal and thermotolerant coliform counts on fresh white button mushrooms*

			Microbial counts (log cfu/g) mean ± standard deviation [a]							
			Farm 1				Farm 2			
	Production phase	Production cycle (Break)	Bacteria	Thermotolerant coliforms	Yeasts	Fungi	Bacteria	Thermotolerant coliforms	Yeasts	Fungi
Room 1	Pre-harvest	1st	2.5 ± 2.7 |G	2.5 ± 0.3 |EFGH	1.0 ± 1.9 |G	1.7 ± 1.1 |BC	5.6 ± 0.7 |ABCDE	2.7 ± 0.3 |DEFG	3.5 ± 0.5 |ABCD	ND
		2nd	4.9 ± 0.2 |DEF	2.8 ± 0.3 |CDEFG	1.8 ± 1.1 |FG	1.6 ± 0.9 |BCD	6.2 ± 0.6 |A	2.8 ± 0.3 |DEFG	4.1 ± 0.5 |A	ND
		3rd	5.4 ± 0.3 |ABCDEF	2.9 ± 0.2 |CDEF	4.0 ± 0.4 |AB	1.8 ± 0.3 |B	5.9 ± 0.5 |AB	2.8 ± 0.2 |CDEFG	3.3 ± 1.4 |ABCD	ND
	Post-packaging	1st	4.7 ± 1.9 |F	2.6 ± 0.2 |DEFG	2.9 ± 2.4 |CDEF	0.4 ± 0.8 |EF	5.7 ± 0.8 |ABCD	2.4 ± 0.6 |GHI	3.8 ± 0.6 |ABC	ND
		2nd	4.8 ± 0.3 |EF	2.7 ± 0.2 |DEFG	2.5 ± 1.4 |DEF	1.9 ± 0.8 |B	5.6 ± 0.4 |ABCDEF	2.8 ± 0.4 |DEFG	3.6 ± 0.6 |ABC	ND
		3rd	4.9 ± 0.3 |CDEF	2.8 ± 0.2 |CDEFG	3.2 ± 0.6 |ABCD	1.7 ± 0.2 |B	5.5 ± 2.3 |ABCDEF	3.0 ± 0.5 |CDE	3.9 ± 0.6 |AB	ND
Room 2	Pre-harvest	1st	5.4 ± 0.8 |ABCDEF	3.1 ± 0.1 |BCD	2.9 ± 0.9 |BCDE	0.9 ± 1.3 |DE	5.9 ± 0.4 |ABC	2.5 ± 0.4 |FGH	3.8 ± 0.6 |ABC	0.5 ± 1.3 |EF
		2nd	5.2 ± 0.4 |BCDEF	3.6 ± 0.5 |AB	3.4 ± 0.7 |ABCD	1.9 ± 0.8 |B	5.9 ± 0.4 |ABC	2.7 ± 0.3 |DEFG	3.7 ± 1.6 |ABC	ND
		3rd	5.7 ± 0.4 |ABCD	3.7 ± 0.4 |A	4.3 ± 0.4 |A	1.5 ± 1.1 |BCD	6.1 ± 0.6 |A	2.9 ± 0.3 |CDEFG	3.8 ± 0.3 |ABC	ND
	Post-packaging	1st	5.5 ± 0.5 |ABCDEF	2.9 ± 0.2 |CDEF	2.5 ± 1.6 |DEF	1.0 ± 1.1 |CDE	5.9 ± 0.6 |ABC	1.9 ± 1.2 |I	3.8 ± 0.4 |ABC	ND
		2nd	5.1 ± 0.4 |BCDEF	3.1 ± 0.3 |BCD	3.2 ± 0.6 |ABCD	1.9 ± 0.5 |B	5.6 ± 0.3 |ABCDEF	1.9 ± 1.2 |I	2.0 ± 2.2 |EFG	ND
		3rd	5.6 ± 0.3 |ABCDEF	3.3 ± 0.3 |ABC	4.2 ± 0.6 |A	2.7 ± 0.7 |A	5.9 ± 0.4 |AB	2.0 ± 1.3 |HI	3.8 ± 0.3 |ABC	ND

Total (average) microbial load: **8.7 ± 0.1 log cfu/g**

Total microbial load range (min-max): **5.2 - 12.4 log cfu/g**

[a] For each column, mean values followed by statistical t-grouping according to analysis with a two way-ANOVA showing interaction effect between farms, pre-harvest, post-packaging, rooms and production cycles (breaks). Same letter/combination of letters in each column do not differ significantly from each other at a $p < 0.05$ significance level. ND: not detected, counts below detection limit.

Table 3.2 *Comparison of fresh white button mushroom microbial counts of bacteria and yeasts for different growing rooms*

			Microbial counts (log cfu/g) mean ± standard deviation									
	Production phase	**Production cycle (Break)**	**Room 1**	**Room 1 Average**	**Room 2**	**Room 2 Average**	**Room 3**	**Room 3 Average**	**Room 4**	**Room 4 Average**	**Room 5**	**Room 5 Average**
Bacteria	**Pre-harvest**	**1st**	5.56 ± 0.14	5.77 |B	5.42 ± 0.09	5.74 |B	5.72 ± 0.20	5.84 |AB	5.93 ± 0.29	5.78 |B	6.24 ± 0.50	6.08 |A
		2nd	5.78 ± 0.27		5.86 ± 0.32		5.93 ± 0.37		5.71 ± 0.28		6.00 ± 0.23	
		3rd	6.05 ± 0.44		5.71 ± 0.38		5.81 ± 0.42		5.60 ± 0.23		6.14 ± 0.27	
	Post-packaging	**1st**	5.87 ± 0.24		5.62 ± 0.17		5.78 ± 0.19		5.64 ± 0.19		6.06 ± 0.37	
		2nd	5.65 ± 0.67		5.94 ± 0.32		6.05 ± 0.32		5.76 ± 0.40		6.15 ± 0.24	
		3rd	5.92 ± 0.42		5.89 ± 0.29		5.80 ± 0.19		6.07 ± 0.52		5.89 ± 0.19	
Yeast	**Pre-harvest**	**1st**	2.84 ± 0.15	3.70 |AB	3.85 ± 0.36	3.47 |B	3.87 ± 0.75	3.67 |AB	3.74 ± 0.43	3.87 |A	3.79 ± 0.46	3.92 |A
		2nd	3.94 ± 0.39		2.29 ± 0.77		3.65 ± 0.26		3.83 ± 0.41		3.88 ± 0.72	
		3rd	3.75 ± 0.54		2.26 ± 1.05		2.41 ± 1.27		4.17 ± 0.39		3.96 ± 0.44	
	Post-packaging	**1st**	2.94 ± 1.13		3.55 ± 0.23		3.73 ± 0.39		3.68 ± 0.31		4.04 ± 0.30	
		2nd	3.67 ± 0.23		3.46 ± 0.14		3.75 ± 0.42		3.65 ± 0.28		3.91 ± 0.35	
		3rd	3.89 ± 0.52		3.63 ± 0.29		3.86 ± 0.38		4.16 ± 0.43		3.95 ± 0.62	

[a] For each column, mean values followed by statistical t-grouping according to analysis with a two way-ANOVA showing interaction effect between farms, pre-harvest, post-packaging, rooms and production cycles (breaks). Same letter/combination of letters in each column do not differ significantly from each other at a $p < 0.05$ significance level.

Presumptive positive *E. coli* O157:H7, *Salmonella* spp., *S. aureus* and *L. monocytogenes* were identified from selective media plates - 36% on eosin methyl blue media identified as presumptive positive *E. coli* O157:H7. In addition, 22% and 5% of bacterial colonies present on brilliance salmonella and xylose lysine deoxycholate was identified as presumptive positive *Salmonella* spp. Viable *S. aureus* were identified 22% of the time. Presumptive *L. monocytogenes* was isolated from 26% of samples tested. Two bacterial colonies were confirmed positive as *E. coli*, originating from the at harvest 2nd break mushrooms. No *E. coli* O157:H7 was identified or could be confirmed. *Salmonella* species were isolated from 2nd break at harvest (1 colony), 3rd break at harvest (1) and 3rd break packed (5) mushrooms. A further five isolates were confirmed positive by MALDI-TOF MS for 2nd break at harvest (2), 3rd break at harvest (2) and 3rd break packed (1) mushroom samples. No presumptive isolations from Listeria selective media were confirmed positive. Out of the presumptive positive *S. aureus* isolates, one isolate was confirmed as *S. aureus*, this isolate originated from the hand of a mushroom packer (Chapter 4), with an isolated cell count of Log 2.1 cfu/mL. Confirmed *S. aureus* was not isolated from mushrooms. *Salmonella enterica* was isolated and identified from packed mushroom samples. Presence of *Salmonella* spp. was confirmed between pre-harvest and packaged mushrooms.

3.4. DISCUSSION

To minimize the impact of potential pathogens that affect human health, has been a persistent challenge in the food industry globally (Kim *et al*., 2016). Two important control points for managing potential foodborne pathogen contamination include the production environment and handling, both during and after harvest. Fruits and vegetables (including mushrooms) are susceptible during growth and harvest to natural contamination (Pao *et al*., 2012). Inadequate and improper handling during harvest, post-harvest processing, packaging and distribution may further promote the growth and spread of undesirable microorganisms. Due to the nature of an unprotected structure, *A. bisporus* are quite perishable and prone to physical and microbial damage. Fresh mushrooms are an ideal medium for microbial growth due to their high water and moisture content as well as their neutral pH (Martinez-Carrera *et al*., 1998).

The mushrooms investigated in this study reflected a relatively moderate to high total microbial load when compared to other studies. Reyes *et al.* (2004) reported mesophilic aerobic loads of 7.9 log cfu/g in a study to determine the prevalence of *Enterobacteriaceae* species specifically *E. americana* and the pathogenic potential of the isolated strains on commercial packed/retail mushrooms. Venturini *et al*. (2011) indicated that the total microbial load on *A. bisporus* from packed/retail samples ranged between 6 and 8 log cfu/g in a study to determine the safety and quality of various mushroom species. Similarly, European studies investigating the microbial load of fresh mushroom species reported that the mean counts of mesophilic aerobic microorganism in *A. bisporus* ranged from 7 to 8 log cfu/g (Donzellini *et*

al., 2018). A recent study by Siyoum *et al.* (2016) described microbial succession on healthy mushrooms at the point of harvest, which represented a 4 to 5 log cfu/g representative of a climax bacterial community. Microbial loads could according to the previous authors reportedly increase to log 7 to 9 cfu/g depending on the storage conditions. From data generated in this study and by comparison in literature, it was shown that a healthy, whole mushroom, considered safe to consume can carry a total microbial load ranging between 5.2 log cfu/g and 12.4 log cfu/g, depending on the relation between farm, production phase, and -break as well as growing room conditions from which the mushrooms are harvested. In a study performed by Doores *et al*. (1987), bacterial counts were determined between different breaks of pre-harvested white button mushrooms, the authors reported counts from 6.3 to 7.2 log cfu/g. Therefore, microbial loads found in this study were thus higher at post-packaging compared to the at-harvest stage. Load differences between the former and latter were however not significant, which means that microbial loads reflected by packaged mushrooms are comparable to loads found on mushrooms at the "point-of-harvest". Microbial loads found on fresh mushrooms showed similarities and were comparable between the different growing rooms studied. It could be deduced from the results obtained that the manufacturing and production conditions in South African mushroom farms are comparable to other leading mushroom producing countries in the world. Microbial quality of white button mushrooms evaluated in this study are very similar to mushrooms in comparable pre- and post-harvest conditions from which fresh raw mushrooms were sampled. Despite the increasing production and consumption of mushrooms, very limited data about microbiological quality, specifically for the local industry are available. The results generated in this study could provide better insight into the dynamics of microbial loads as mushrooms progress through the various stages during "on-farm" production.

Two *E. coli* species were confirmed positive from mushroom samples, in contrast to results obtained in a study performed by Strapp *et al*. (2003) in which no positive isolations were reported. In a similar study the authors Venturini *et al*., 2011, tested for *E. coli* contamination on fresh mushrooms but also confirmed the absence of this pathogen from the 202 samples. In European studies, mean reported counts of *Enterobacteriaceae* ranged between 3.2 and 4 log cfu/g. The microbial indicators including coliforms observed on *A. bisporus* sold in mass retail shops exceeded the reference values as stipulated by Region Tuscany as acceptable for raw foods (Donzellini *et al*., 2018). Despite similar results obtained in this study for the range of coliform counts isolated from fresh mushrooms, under the South African guidance document, *Guidelines for environmental health officers on the interpretation of microbiological analysis data of food*, states that fresh produce should not contain more than 200 coliforms per gram and no *E. coli* should be present per gram and 25 gram respectively in order to ensure safe food (Department of Health, 2000). The South African guideline is equally or sometimes more strict than other international and European guidelines (FSAI Guidance Note No. 3, 2001; Commission Regulation (EC) No 2073/2005). Despite this, there are mushroom-producing countries

which has not set a limit on microbial counts in freshly cultivated mushrooms. These products are subsequently always considered fit for consumption, both cooked and raw, in the absence of macroscopic alterations. Microorganisms of the *Enterobacteriaceae* family like *Escherichia*, usually spread through faecal contamination, indicating possible presence of enteric pathogenic bacteria more efficiently than the broad group *Enterobacteriaceae*. However, previous research, including this study has demonstrated the absence of the enteric pathogenic microorganisms in freshly cultivated mushrooms contaminated by *Enterobacteriaceae* and mesophilic aerobic microorganisms (Venturini *et al*., 2011). These microorganisms can be considered quality indicators and the high levels of both in some samples could raise concerns about the consumption of food items in the raw form, as sometimes done in the case of white button mushrooms.

Coliform bacteria include a large group of bacteria that occur naturally in the environment, but also associate with humans and animals alike (Paruch & Maehlum, 2012). These organisms can therefor commonly occur in soil and water sources such as borehole and surface water. Faecal coliforms are also referred to as "indicator organisms", as their presence indicate that a contamination pathway and conditions exist that could favour the presence and proliferation of other pathogenic bacteria (Gerba, 2009). Compared to a study by Gonzalez-Fandos *et al*. (2000), the coliform loads found in this study were higher than ~2 log cfu/g found by these authors on fresh mushrooms. Detection of coliform bacteria on fresh mushrooms could be related to hygienic handling conditions or to sanitation practices such as water quality assurance, raw material sterilization and proper separation of different processes during production (this also forms part of basic good farming practices). Composting and pasteurization of the final compost before mushroom spawn are introduced for cultivation, may play a large role in the reduction and survival of coliforms in the production process. Temperatures of 60 to 85ºC has the potential to lower coliform numbers, therefor if this process is successful in reducing colony numbers, re-introduction onto mushrooms and beds could be because of other factors during cultivation (Reyneke *et al*., 2018). Colony counts increased between mushrooms analysed before harvest and after packaging, which means prolific progression took place, but coliform growth could in some instances be inhibited by high population numbers of competitor microorganisms (Kotzekidou *et al*., 2016).

Sivapalasingam *et al*. (2004) reported that among 103 (54%) produce-associated outbreaks with known pathogens, 62 (60%) were caused by bacterial pathogens, of which 30 (48%) were caused by Salmonella. Environmental conditions and cultivation methods employed (chicken manure used as raw material in compost manufacturing) on mushroom farms increases the possibility of *Salmonella* spp. exposure to the mushroom growing environment and production system due to untreated poultry manure being stored in bulk on commercial mushroom farms. It is well documented that *S. enterica* is associated with chickens and poultry production (Foley *et al*., 2011). There are various other papers reporting the presence of *Salmonella* spp. on fresh mushrooms, one such example was a study done by

Samadpour *et al*. (2006a) where commercial mushrooms were tested for *Salmonella* spp. in Seattle (USA). In their study, prevalence was confirmed in 7% of all the mushrooms tested out of 100 samples. The authors used a PCR assay with ≥ 98% sensitivity and specificity for presence or absence detection but did not elucidate the species detected in their experiment.

Although no *L. monocytogenes* was detected in any of the samples analysed during this study, the presence of *Listeria* spp. in food may be indicative of unsanitary conditions (McLauchlin, 1997). If conducive circumstances exist for any environmental *Listeria* spp. within a food production or processing system, this may also prove favourable for *L. monocytogenes*, increasing the likelihood for this pathogen to also proliferate and potentially contaminate raw or processed food (Wilkin *et al*., 2015; Heisick *et al*., 1995). Detection and presence of Listeria on mushrooms is not uncommon and can originate through the propagation phase from organic substrate under warm (10 to 20ºC) and humid (relative humidity 85%) conditions (Murray *et al*., 2015). Samadpour *et al*. (2006b) reported detection of *L. monocytogenes* in 1% of mushroom samples analysed during their study, obtained from various retail stores in Seattle, WA. Results from thesis work performed by Chikthimmah (2006), testing the proliferation of artificially inoculated food pathogenic organisms on whole and sliced mushrooms, showed that whole mushrooms do not significantly support growth of *L. monocytogenes* compared to sliced or processed mushrooms.

When considering food security and microbiological profiles of fresh produce, in this case white button mushrooms, food safety principles and guidelines, should be based on a comprehensive and integrated scheme, comprising the entire food chain from the farm until consumption. Work performed in this chapter should be considered to assist farmers and regulating authorities control the maintenance of mushroom quality and safety during production, handling, processing, packaging, and distribution. Critical points in the mushroom production process that have a direct influence on the microbiological diversity and loads within the system are recommended for careful monitoring. Examples of these points could be identified as composting and compost conditioning (including pasteurization) as well as physical handling and processing practices of mushrooms during and after harvest.

3.5. CONCLUSION

Although association of potential human pathogens, such as *S. aureus* and *Salmonella* spp. have been confirmed in this study, it is unlikely that a single strategy will be successful in eliminating presence of pathogens in production systems. A multi-pronged approach should be considered by regulatory authorities, retail and the industry based on effective implementation of a science-based regulation. This must be supported by effective enforcement and good agricultural practices in the commercial mushroom industry. Furthermore, adhering to good manufacturing practices during packaging or

minimal processing, storage and distribution may reduce the hazards of foodborne pathogens. Similar integrated management models have been implemented successfully in alternative fresh produce production systems and it is likely that such an approach will also work for risk minimization of human pathogenic bacteria on fresh white button mushrooms. The experimental data provides evidence to enable adjustment of the current guidance document for total microbial loads of fresh mushrooms based on scientific work and the use of novel technologies. We therefore argue that a more scientifically justified guidance document be developed to ensure more realistic regulations for the industry, based on indicator organisms not being present on mushrooms, absence of *Salmonella* spp., *E. coli* O157:H7 and *Listeria* spp. and a more realistic total microbial load of bacteria, yeast and fungi to be monitored after packaging. Mushrooms tested in this study is comparable in safety and organism load to mushrooms tested in similar studies in other countries. An aspect which was not directly considered during this study was water activity (Aw). Since unbound water, available in food products support growth of microorganisms, higher microbial counts could be attributed to a higher Aw. In future studies, this variable could provide insight into the microbial activity found on mushrooms, especially during post-harvest processes.

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CHAPTER 4

STAPHYLOCOCCUS SPECIES AND ANTIBIOTIC RESISTANCE MONITORING ON COMMERCIAL MUSHROOM FARMS

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Duvenage, S., Rossouw, W., Villamizar-Rodríguez, G., du Plessis, E. & Korsten, L. 2019. Antimicrobial resistance characterization of *Staphylococcus* spp. isolated from white button mushrooms and mushroom handlers. *International Journal of Food Microbiology* (Submitted 20 October 2019).

ABSTRACT

Most freshly consumed produce receives minimal processing after harvest and is often eaten raw. Food-related pathogen contamination can represent a serious risk to the consumer. In the case of freshly picked white button mushrooms, the fruit are picked and packed by hand, in addition mushroom surfaces are very susceptible to microbial contamination due to the lack of a protective layer. *Staphylococcus aureus* and *Staphylococcus epidermidis* are known to naturally associate with humans in nasal passages and on hands, which can be a contributing factor when considering transference, between body surfaces (hands) and handled food products. This chapter aims to determining the potential presence of *Staphylococcus* spp. on freshly picked and packed white button mushrooms as well as on hands of mushroom pickers and packers. Furthermore, antimicrobial resistance profiling of all isolates obtained throughout the study will serve to characterise potential risks associated with on-farm methods and practices of harvesting and packaging. From 432 mushroom samples and 150 handlers' hand samples tested, a total of 59 Staphylococcal isolates were identified. Isolates were divided into the following species groups, *S. epidermidis*, *Staphylococcus equorum*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Staphylococcus saprophyticus*, *Staphylococcus sciuri*, *Staphylococcus succinus*, *Staphylococcus warneri*, *Staphylococcus xylosus* and *S. aureus*. In total six isolates were found to be multidrug resistant, including *S. epidermidis* (2), *S. equorum* (2), *S. haemolyticus* (1) and *S. hominis* (1). The majority of *Staphylococcus* spp. (84.8%) were resistant to the antibiotic penicillin, with 13.6% resistant to cephalosporins, 5.1% resistant to antimycobacterials and lincosamide and 3.4% resistant to tetracyclines and macrolides. This study clearly showed the potential association of *Staphylococcus aureus* with fresh mushrooms and the ability of various strains within the Staphylococcus genus to exhibit antibiotic resistance characteristics.

4.1. INTRODUCTION

In recent years, awareness specifically regarding *Staphylococcus aureus* as a potential microbial contaminant in fresh produce has increased considerably (Saifullah *et al*., 2018). Mushrooms are subjected to handling by pickers and packers alike during harvesting, sorting and packing. This aspect is applicable to most commercial mushroom farms in the South African mushroom industry where automation during production is not yet common practice. Possible contamination and cross-contamination of mushrooms by workers and by contact surfaces such as sorting tables, transport crates

etc., may pose a latent risk along the supply chain, but can also increase the chances for pathogen association with the final product (Chajęcka-Wierzchowska *et al*., 2015; Lowy, 1998). From a food safety and clinical perspective, *Staphylococcus* species in general can be transient organisms, with the ability of causing infection in susceptible hosts. As a result, it is important that growers and packers should be aware of the risks associated with these microorganisms. Farming and production facilities also need to implement essential precautionary steps to prevent contamination of fresh produce during growing and handling.

Recent studies have shown that coagulase-positive and coagulase-negative staphylococci can be associated with food and can cause staphylococci-associated foodborne illness (Osman *et al*., 2015; Veras *et al*., 2008). Evidence suggest that coagulase-negative *Staphylococcus* spp. have in recent years started to show a higher frequency of pathogenicity (Gillespie *et al*., 2009). Mainly two species of *Staphylococcus* are significant in their interactions with humans, *S. aureus* and *S. epidermidis*. *Staphylococcus aureus* is a natural colonizer of humans and are primarily found in nasal passages but may also be found in other regions of the body such as the pharynx, perineum, axillae and on the skin (mainly on the hands, chest and abdomen), with 10 to 20% of adults persistently colonized and 30 to 50% of healthy people colonized (Noble *et al*., 1967; Wertheim *et al*., 2005, Krismer *et al*., 2017). *Staphylococcus aureus* can grow in a wide range of temperatures, from 6 to 48°C with the optimal temperature for growth being 35 to 37°C. *Staphylococcus aureus* require 10^{-3} to 10^{-8} cells for infection to develop (Sewell, 1995; Schmid-Hempel & Frank, 2007; Schmid-Hempel, 2011) and toxin production were detected to be between 10 to 46°C (Smith *et al*., 1982, Nakagawa *et al*., 2017). Similarly, *S. epidermidis* is a skin inhabitant of humans, and are more frequently associated with opportunistic clinical infections as opposed to food related infection or food poisoning (Mack *et al*., 2007, Byrd *et al*., 2017).

It is not common practice for fresh mushrooms to be washed prior to use in salads or for fresh consumption as washing affects the visual appeal and texture of mushrooms. This implies that there can be no significant microbiological reduction between final production (packaged/ retail) and ingestion should any pathogenic contamination persist on the fruiting bodies (Ssemanda *et al*., 2018). Another contributing factor to proliferation of microbial pathogens on fresh, ready-to-eat produce is post-harvest temperature abuse, such as cold chain interruptions during storage and transport or even post sales (Wolf-Hall & Nganje, 2017). Contamination of fresh produce with *Staphylococcus* spp. during production, picking or packing can result in attachment, colonisation and rapidly increasing microbial loads capable of producing high enough titres that can produce toxin levels that can affect human health. No foodborne related outbreak/s from fresh mushrooms contaminated by Staphylococci species have been reported, except in the case of processed (canned) mushrooms (Levine *et al*., 1996). In canned products different conditions and treatments play a role during processing. Survival and

growth of *Staphylococcus* spp. can occur at temperatures where mushrooms are grown (16 to 18°C), processed (12°C) and stored (5 to 8°C). It is important to note that any health risk associated with *Staphylococcus* spp. is only related to ingestion of toxin/s produced by these pathogens when growing and colonizing food surfaces. The aim of this study was to determine prevalence and association of Staphylococci to critical stages within the mushroom production process. Individual species characterization and establishment of any potential antimicrobial resistance associated with these isolates.

4.2. MATERIALS AND METHODS

4.2.1. SAMPLING

Presence of *Staphylococcus* spp. on mushrooms and hand samples were monitored on a weekly basis for 15 weeks. Mushroom samples were sampled at the same commercial farming facilities, using the same methods of sampling as described in Chapter 3 (pg. 50). Before-harvest mushrooms were not handled by pickers nor by packers and were aseptically harvested. Packed mushrooms were handled by pickers and packers prior to sealing the punnet and transportation to the laboratory. In addition, hand swabs were also collected weekly, with five samples per week in the growing rooms (pickers) and five samples per week in the packhouse (packers). A total of 75 pickers' hands and 75 packers' hands were sampled, yielding a total of 150 hand samples for analysis. Hand samples were collected by swabbing the dominant hand of the picker or packer using a wetted Copan Venturi Trans-system (Copan, Italy) according to standard hand swab procedures (ISO 18593, 2004).

4.2.2. SAMPLE PROCESSING AND ISOLATIONS

Mushroom samples were processed using the same method described in Chapter 3 (pg. 51) for isolation and enumeration of potential Staphylococci species. Swab samples were placed into 9 mL buffered peptone water (Merck, Johannesburg), vortexed to aid in dissociation of cells from the swab tip, serially diluted and transferred to agar plates. The growth medium used for isolation and enumeration of potential *Staphylococcus* spp. isolates from both swabs and mushrooms samples in this study were Baird Parker agar plates as well as 3M™ Petrifilm™ *Staph* Express Count Plates (3M, Johannesburg) incubated at 37 °C ± 2°C for 24 to 48 h. Plates were enumerated and isolations were made based on presumptive positive colonies. All colonies with typical morphological characteristics (black, convex and shiny with clear lecthinase production zones and opaque precipitation zones due to lipase activity) for *S. aureus* and all other black colonies were isolated from the selective chromogenic Baird Parker agar (Osman *et al*., 2015). On the Staph express count plates different colony colours appeared of which, red, violet and black colonies were selected as presumptive *S. aureus*. If more than one presumptive colony morphology was present per sample the colonies were regarded as separate isolates and all were isolated for further identification. Colonies were subsequently plated (using quadrant

streaking for single colonies technique) onto standard one nutrient media (Merck-biolab, Johannesburg), incubated for 24 h at 37ºC and then identified using matrix-assisted laser desorption/ionisation time-of-flight mass spectrophotometer (MALDI-TOF MS) combination with the Bruker Biotyper software and database (Standing *et al*., 2013) as described in the previous chapter (pg. 52).

4.2.3. PHENOTYPIC ANTIMICROBIAL ASSESSMENT

A total of 59 isolates were subjected to phenotypic antimicrobial susceptibility testing using the Kirby-Bauer disk diffusion method (Bauer *et al*., 1966). Each isolate was cultured in 9 mL of brain heart infusion broth (Merck, Johannesburg) and incubated for 24 h at 37 °C; subsequently, suspensions were plated onto Mueller-Hinton agar plates (Merck-biolab). Disk diffusion test was employed to determine the susceptibility to: cefoxitin (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), erythromycin (15 µg), gentamicin (10 µg), oxacillin (1 µg), penicillin (10 µg), rifampicin (5 µg), oxytetracycline (30 µg), teicoplanin (30 µg) and vancomycin (30 µg) (Mast Diagnostics, United Kingdom). Antimicrobial susceptibility was measured according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2018). Inhibition zone diameters were measured, compared to CLSI breakpoint tables and recorded as susceptible or resistant. All isolates demonstrating intermediate resistance were classified as susceptible, in order to avoid overestimation of resistance (Ta *et al*., 2014). Strains resistant to three or more antimicrobial classes were defined as multidrug resistant.

4.3. RESULTS

4.3.1. Species profile and distribution throughout the production chain

A total of 59 staphylococcus isolates were obtained from 582 samples collected. These isolates were grouped into ten different species-specific groups including coagulase-negative (*S. epidermidis*, *S. equorum*, *S. haemolyticus*, *S. hominis*, *S. saprophyticus*, *S. sciuri*, *S. succinus*, *S. warneri*, *S. xylosus*) and coagulase-positive (*S. aureus*) species (Becker *et al*., 2014). Isolate descriptions, including their antimicrobial resistance profiles as well as source of isolation are outlined in Table 4.1. Species occurrences for each of the four stages within the mushroom production process are graphically displayed in Figure 4.1. Population profiles revealed that *S. succinus* was isolated from hands of pickers (*n= 6*), packers (*n= 4*) as well as mushrooms before picking (*n= 4*) and after packing (*n= 8*), compared to *S. equorum* isolates which were mainly from hands of pickers (*n= 5*) and packers (*n=6*) but also from packed mushrooms (*n= 1*). It was observed that the diversity of *Staphylococcus* species on the hands of mushroom packers were greater than that observed on the hands of the mushroom pickers. *Staphylococcus epidermidis* was detected from one packer's (*n=1*) and one picker's (*n=1*) hand, this species was found to be exclusively associated with the hands of mushroom handlers in contrast to *S. xylosus*, which was only isolated from mushrooms before picking (*n=5*) and after packing (*n=3*). Data suggest that *S. saprophyticus* present on pre-harvest mushrooms were carried over onto the hands of

pickers during harvesting as mushrooms before harvest that were aseptically removed from the beds revealed *S. saprophyticus* isolates (*n=9*), and three pickers' hands (*n=3*) were also contaminated with this species. A similar pattern was observed for *S. hominis*, which was initially found on the hands of mushroom packers (*n=1*) and later also detected on packaged mushrooms (*n=1*). *Staphylococcus aureus* and *S. heamolyticus* were only found on hand samples (*n=1* for both isolates), where both isolations were from packer's hands. Only one *S. sciuri* isolate was obtained from a packed mushroom sample (*n=1*). Lastly, *S. warneri* was isolated from a packer's hand (*n=1*).

4.3.2. Antimicrobial resistance within the isolated *Staphylococcus* species profile

From all the isolates which were subjected to antibiotic testing, 10% of organisms were considered multidrug resistant (Table 4.1). Six *Staphylococcus* spp. (10%; n=59) were found to be resistant to more than three antimicrobial classes which led to their description of being multidrug resistant. Expressed resistance to at least one antimicrobial agent was found in 86.4% (n=51) of isolates. Penicillin resistance were found in 84.8% of isolates identified from mushrooms and associated handlers. Out of 50 Penicillin resistant isolates, 35 were also simultaneously resistant to oxacillin. One *S. hominis* isolate was resistant to erythromycin and eight different *Staphylococcus* spp. were resistant to cefoxitin, with an additional three isolates resistant to oxytetracycline (Table 4.1). Cefoxitin resistance were observed in 13.6% of the staphylococci isolates. All strains resistant to cefoxitin were in addition, resistant to oxacillin as well as penicillin. In this study, 37.5% of methicillin-resistant staphylococci were also resistant to rifampicin (3/8 isolates), 25% were also resistant to tetracycline and clindamycin (2/8 isolates) and 12.5% were also resistant to erythromycin (1/8 isolates).

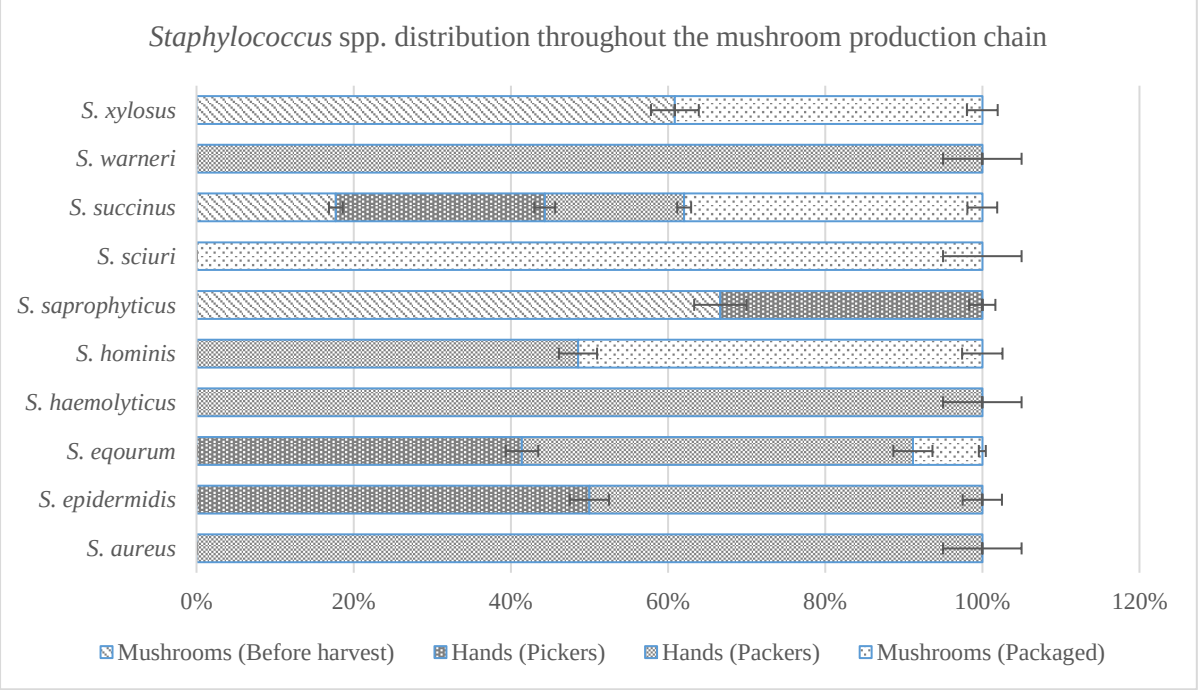

Figure 4.1 *Staphylococcus prevalence and isolation frequency within the production chain*

Table 4.1 *Phenotypic antimicrobial susceptibility profile of Staphylococcus isolates subjected to Kirby-Bauer disk diffusion testing*

Species	Source	MDR* (Y/N)	Number of classes resistant to	Antibiotic class/es	Number of antibiotics resistant to	Resistance pattern
Staphylococcus aureus	Packer's hand	N	1	Penicillin	1	PG30
Staphylococcus epidermidis	Picker's hand	Y	3	Cephalosporins, Penicillin, Antimycobacterials	4	TOX30 - OX1 - PG10 - RP5
Staphylococcus epidermidis	Packer's hand	Y	4	Cephalosporins, Penicillin, Antimycobacterials, Tetracycline	5	TOX30 - OX1 - PG10 - RP5 - T30
Staphylococcus equorum	Packer's hand	N	0	None	0	None
Staphylococcus equorum	Packer's hand	N	0	None	0	None
Staphylococcus equorum	Picker's hand	N	1	Penicillin	2	OX1 - PG10
Staphylococcus equorum	Packer's hand	N	1	Penicillin	2	OX1 - PG10
Staphylococcus equorum	Picker's hand	N	1	Penicillin	1	PG10
Staphylococcus equorum	Packer's hand	N	0	None	0	None
Staphylococcus equorum	Packed mushrooms	N	0	None	0	None
Staphylococcus equorum	Picker's hand	Y	3	Cephalosporins, Lincosamide, Penicillin	4	TOX30 - CD2 - OX1 - PG10
Staphylococcus equorum	Picker's hand	N	0	None	0	None
Staphylococcus equorum	Packer's hand	N	0	None	0	None
Staphylococcus equorum	Picker's hand	Y	3	Cephalosporins, Lincosamide, Penicillin	4	TOX30 - CD2 - OX1 - PG10
Staphylococcus equorum	Packer's hand	N	0	None	0	None
Staphylococcus haemolyticus	Packer's hand	Y	4	Cephalosporins, Macrolides, Lincosamide, Penicillin	5	TOX30 - E15 - OX1 - PG10 - T30
Staphylococcus hominis	Packer's hand	Y	3	Cephalosporins, Penicillin, Antimycobacterials	4	TOX30 - OX1 - PG10 - RP5
Staphylococcus hominis	Packed mushrooms	N	2	Macrolides, Penicillin	2	E15 - PG10
Staphylococcus saprophyticus	Picker's hand	N	1	Penicillin	2	OX1 - PG10
Staphylococcus saprophyticus	Before-harvest mushrooms	N	1	Penicillin	2	OX1 - PG10
Staphylococcus saprophyticus	Before-harvest mushrooms	N	1	Penicillin	1	PG10
Staphylococcus saprophyticus	Before-harvest mushrooms	N	1	Penicillin	2	OX1 - PG10
Staphylococcus saprophyticus	Before-harvest mushrooms	N	1	Penicillin	2	OX1 - PG10

Species	Source	MDR* (Y/N)	Number of classes resistant to	Antibiotic class/es	Number of antibiotics resistant to	Resistance pattern
Staphylococcus saprophyticus	Before-harvest mushrooms	N	2	Cephalosporins, Penicillin	3	TOX30 - OX1 - PG10
Staphylococcus saprophyticus	Picker's hand	N	1	Penicillin	2	OX1 - PG10
Staphylococcus saprophyticus	Picker's hand	N	0	None	0	None
Staphylococcus saprophyticus	Before-harvest mushrooms	N	1	Penicillin	2	OX1 - PG10
Staphylococcus sciuri	Packed mushrooms	N	2	Cephalosporins, Penicillin	3	TOX30 - OX1 - PG10
Staphylococcus succinus	Before-harvest mushrooms	N	1	Penicillin	1	PG10
Staphylococcus succinus	Packed mushrooms	N	1	Penicillin	2	OX1 - PG10
Staphylococcus succinus	Packer's hand	N	1	Penicillin	2	OX1 - PG10
Staphylococcus succinus	Before-harvest mushrooms	N	1	Penicillin	2	OX1 - PG10
Staphylococcus succinus	Picker's hand	N	1	Penicillin	1	PG10
Staphylococcus succinus	Picker's hand	N	1	Penicillin	2	OX1 - PG10
Staphylococcus succinus	Packed mushrooms	N	1	Penicillin	2	OX1 - PG10
Staphylococcus succinus	Picker's hand	N	1	Penicillin	2	OX1 - PG10
Staphylococcus succinus	Packed mushrooms	N	1	Penicillin	2	OX1 - PG10
Staphylococcus succinus	Picker's hand	N	1	Penicillin	2	OX1 - PG10
Staphylococcus succinus	Packer's hand	N	1	Penicillin	2	OX1 - PG10
Staphylococcus succinus	Packer's hand	N	1	Penicillin	2	OX1 - PG10
Staphylococcus succinus	Packed mushrooms	N	1	Penicillin	2	OX1 - PG10
Staphylococcus succinus	Picker's hand	N	1	Penicillin	1	PG10
Staphylococcus succinus	Packer's hand	N	1	Penicillin	2	OX1 - PG10
Staphylococcus succinus	Packed mushrooms	N	1	Penicillin	1	PG10
Staphylococcus succinus	Packed mushrooms	N	1	Penicillin	1	PG10
Staphylococcus succinus	Before-harvest mushrooms	N	1	Penicillin	2	OX1 - PG10
Staphylococcus succinus	Before-harvest mushrooms	N	1	Penicillin	1	PG10
Staphylococcus succinus	Packed mushrooms	N	1	Penicillin	1	PG10
Staphylococcus succinus	Picker's hand	N	1	Penicillin	1	PG10
Staphylococcus succinus	Packed mushrooms	N	1	Penicillin	1	PG10

Species	Source	MDR* (Y/N)	Number of classes resistant to	Antibiotic class/es	Number of antibiotics resistant to	Resistance pattern
Staphylococcus warneri	Packer's hand	N	1	Tetracycline	1	T30
Staphylococcus xylosus	Before-harvest mushrooms	N	1	Penicillin	2	OX1 - PG10
Staphylococcus xylosus	Before-harvest mushrooms	N	1	Penicillin	1	PG10
Staphylococcus xylosus	Before-harvest mushrooms	N	1	Penicillin	1	PG10
Staphylococcus xylosus	Before-harvest mushrooms	N	1	Penicillin	2	OX1 - PG10
Staphylococcus xylosus	Before-harvest mushrooms	N	1	Penicillin	2	OX1 - PG10
Staphylococcus xylosus	Packed mushrooms	N	1	Penicillin	2	OX1 - PG10
Staphylococcus xylosus	Packed mushrooms	N	1	Penicillin	2	OX1 - PG10
Staphylococcus xylosus	Packed mushrooms	N	1	Penicillin	2	OX1 - PG10

*MDR = Multi-drug Resistant

4.4. DISCUSSION

Literature suggest that food handlers and workers are frequently implicated in unsafe food handling practices, especially when considering microbial contamination of ready-to-eat foods (Clayton & Griffith, 2004; Manning, 1994; Rajagopal & Strohbehn, 2013; Sneed *et al*., 2004). Although a broad scope of food safety organisms is concerned in this statement, the same holds true for any potential Staphylococcal species. Contamination of food with microorganisms during the processing chain can be due to handlers as asymptomatic carriers of pathogenic organisms, especially bacteria such as *S. aureus* (McEvoy *et al*., 2004; Todd *et al*., 2008). Equipment and surfaces that have not been effectively cleaned or which have remained wet between cleaning and use, could also serve as a direct route for contamination of ready-to-eat foods such as fresh mushrooms (Evans *et al*., 2004; Gill *et al*., 2001). Although *Staphylococcus aureus* is the most well-known and commonly associated of this genus with food safety concerns, other *Staphylococcus* species can also infect susceptible hosts. Recent studies suggest that both coagulase-positive and -negative staphylococci have the ability to cause staphylococci-associated foodborne illness (Osman *et al*., 2015; Veras *et al*., 2008) In this study, these organisms were detected and confirmed in mushroom production facilities, as well as on the hands of mushroom handlers (pickers and packers). It is important that mushroom production facility management as well as workers that handle fresh produce be aware and understand the risks that are associated with food safety related organisms, especially food-associated *Staphylococcus* species.

A clear distinction can be made between *Staphylococcus* spp. population compositions during the different stages of production compared to the packed product. The results indicate that *S. succinus* as well as *S. saprophyticus* originally reside on mushroom surfaces and are also naturally found on hands of pickers. Although transference was not directly studied, the potential for these two species to be introduced to new surfaces, could explain higher concentrations on hands of pickers during harvesting and subsequent persistence to some of the sampling areas further down the processing chain. According to trends generated by the data in this study, it is likely that *S. equorum* are introduced onto mushroom surfaces by pickers and further handling by packers could contribute to the higher bacterial concentrations. This species continues to reside on packaged mushrooms in low concentrations. *Staphylococcus epidermidis* is part of the normal skin flora of humans (Peters, 1988) and were only found on the hands of pickers and packers; no isolates were confirmed on mushrooms. Similarly, in an extensive study of the mushroom microflora, Siyoum *et al*. (2016) did also not isolate *S. epidermidis* from fresh mushrooms.

Furthermore, from the results it is evident that both *S. aureus* and *S. warneri* have the potential to be introduced onto the final product as the only detection for these two species were on the hands of mushroom packers. Isolation and identification of *S. aureus* and *S. warneri* within the food production process, confirms a potential hazard associated with fresh mushrooms (Prokopowich & Blank, 1991).

Staphylococcus aureus is a known foodborne pathogen linked to persistence and presence in fresh or processed food and *S. warneri* rarely cause disease but may occasionally cause infection in immuno-compromised patients (Kamath *et al*., 1992). *Staphylococcus saprophyticus*, *S. succinus* and *S. xylosus* were mainly associated with mushrooms and could not readily be associated with hands. A study by Chajęcka-Wierzchowska *et al*. (2015) has shown the presence of *S. xylosus*, *S. epidermidis* and *S. saprophyticus* in ready-to-eat products from animal origin. Mushrooms are cultivated on compost that is comprised mainly of wheat-straw, chicken manure, leachate and agricultural lime. Chicken manure as a raw component before the compost is pasteurized, are known to be a habitable environment for antimicrobial resistant bacteria (Graham *et al*., 2009a). The authors Graham *et al*. (2009a) also concluded that typical storage of chicken manure was not enough to eliminate antimicrobial resistant *Staphylococcus* spp. Compost used to produce mushrooms is normally pasteurized between 60°C and 75°C for approximately 13 days during the composting process. Fontes *et al*. (2013), described that during the process of milk pasteurization within similar temperature ranges, coagulase-negative staphylococci were able to survive. Therefore, presence of staphylococci are not necessarily eliminated from mushroom compost during the pasteurization process and the potential exist that developing mushroom fruiting bodies could be exposed to these organisms during cultivation. This theory supports findings from this study of *Staphylococcus* spp. before harvest on the mushroom samples tested, which could be explained as possible remnant organisms originally associated during the composting process.

Factors which could affect and possibly allow for survival of microorganisms throughout pasteurization could include that various *Staphylococcus* spp. have the innate ability to survive temperatures of up to 80ºC (Montanari *et al*., 2015), or due to inhomogeneous heating (cooler pockets still exist in the compost) during the pasteurization process as well as the possibility of contamination after pasteurization when compost is handled and growing rooms are filled with the freshly prepared substrate. In addition, previous research has demonstrated that the use of antimicrobials in the chicken rearing industry has been linked to the presence of antimicrobial resistant organisms present in farm workers as well as the growing environment (Graham *et al*. 2009b). On mushroom farms, chicken manure is stored on the farm close to the composting area and therefore these bacteria can spread within the mushroom growing environment, not necessarily through contact contamination but by airflow as well. Staphylococci can exist at levels of 2.2×10^3 cfu/m^3 which equates to roughly 41% of total bacterial species distribution in the inhalable portion of a chicken bioaerosol (Nonnenmann *et al*., 2010). Furthermore, flies have previously been reported to increase human exposure to antimicrobial resistant bacteria (Graham *et al*., 2009b). Combined, all these factors can contribute to potential on-farm spread of *Staphylococcus* species, including antimicrobial resistant staphylococci as well as multidrug resistant staphylococci species.

Considering hand-hygiene and Staphylococcal occurrence found on mushroom handlers' hands, the authors Grice & Segre (2011), describe the small contribution of Staphylococcaceae to the total bacterial population on hands, which are mostly dominated by Proteobacteria and Bacteriodetes respectively. However, hands are considered vectors in the spread of foodborne disease, mainly because of poor personal hygiene – it is estimated that up to 97% of foodborne illnesses may be attributed to contamination through handling of fresh food. A study performed, to evaluate the efficacy of hand washing and sanitary practices by ready-to-eat food handlers in South Africa, showed that an unsatisfactory level of hand hygiene was prevalent among these workers (Lambrechts *et al*. 2014). The presence of *Staphylococcus* spp. is not frequently reported on fresh mushrooms and therefore it is assumed that these bacteria are not commonly associated with mushrooms. However, mushrooms are subjected to extensive handling (mostly bare hands) by pickers and packers alike during harvesting and packing.

As an extension to the occurrence of *Staphylococcus* species on hands and mushrooms alike, the status of antimicrobial resistant microorganisms in the agricultural environment is becoming a major concern in the global food industry and in public health (Osman *et al*., 2017). Prevalence and association of staphylococci in animal products like meat and cheese are well established from literature (Casaes Nunes *et al*., 2016; Chajęcka-Wierzchowska *et al*., 2015; Fontes *et al*., 2013; Klimiene *et al*., 2016; Osman *et al*., 2017). Trends are becoming more apparent through which show correlations between association of antimicrobial resistant organisms to humans (mostly food handlers), the food produced (Casaes Nunes *et al*., 2016; Chajęcka-Wierzchowska *et al*., 2015; Fontes *et al*., 2013; Klimiene *et al*., 2016; Osman *et al*., 2017, 2015) and worker's direct living/ working environments (Gandara *et al*., 2006). With the inherent ability of food providing a viable means of transmission for microorganisms, resistant bacteria are further aided in their spread of resistance by using such contaminated food as vector (Chajęcka-Wierzchowska *et al*., 2015). Limited knowledge and evidence are however available for antimicrobial resistance and prevalence of *Staphylococcus* spp. on ready-to-eat foods and associated food handlers (Chajęcka-Wierzchowska *et al*., 2015). Seven of the ten *Staphylococcus* spp. identified during this study were found to be associated with raw mushrooms; these included *S. equorum*, *S. hominis*, *S. saprophyticus*, *S. sciuri*, *S. succinus* and *S. xylosus*. However, *S. aureus*, *S. epidermidis*, *S. equorum*, *S. haemolyticus* and *S. warneri* were predominantly associated with hands of mushroom pickers and packers. Both *S. aureus* and *S. epidermidis* are naturally associated with the human microbiome and these species as well as other coagulase-negative staphylococci might pose an important health concern in terms of antimicrobial resistance. It is evident that a strong association has been identified between the former and latter staphylococci species and hands of mushroom handlers, which warrants improved personal hygiene implementation, training and subsequent enforcement of these practices. These isolates can contribute to illness of the handlers as well as susceptible consumers. Previous studies on specifically stone fruit has shown the potential survival of *S. aureus* even through

refrigerated storage (Collignon & Korsten, 2010) as well as the survival and proliferation of these bacteria on contact surfaces (Duvenage & Korsten, 2016). When staphylococcal infection is identified in humans, doctors usually treat patients with Penicillin, oxacillin and methicillin antibiotics as first line of defence. A similar trend can be described in alternative food industries for the identification of Penicillin resistant *Staphylococcus* spp., Fontes *et al*. (2013) found that 94% of isolates from chicken and beef as well as 78.5% of staphylococci isolates from soft cheeses were resistant to penicillin. Clinical *S. aureus* and coagulase-negative staphylococci isolates from Spain were found to be 89.3% and 78.9% resistant to penicillin. The level of phenotypic resistance in this study are comparable to other studies of similar nature. Cefoxitin resistance of food isolated strains determines methicillin resistant coagulase-negative Staphylococcus phenotype which are resistant to all β-lactam antimicrobials, including penicillin, isoxazolyl penicillins (Chajęcka-Wierzchowska *et al*., 2015). The relation between methicillin, oxacillin and penicillin resistance has been studied previously (Bhargava & Zhang, 2012). In addition, Chajęcka-Wierzchowska *et al*. (2015) found a relation between methicillin, oxacillin and penicillin resistance with resistance to rifampicin, clindamycin and tetracycline.

Fresh produce such as ready-to-eat mushrooms could be contaminated with antibiotic-resistant bacteria from animal or human origin during production, harvesting and preparation for retail (Holvoet *et al*., 2013). As confirmed in this study, consumption of fresh produce, especially raw, unprocessed, fresh produce may represent a route of direct exposure of consumers to resistant microorganisms such as certain *Staphylococcus* species. With a global increase in foodborne related illness associated with fresh produce, a lot of emphasis should be placed on good agricultural practices that verify the safety and quality of final products produced. Considering mushroom production, this emphasis is on three critical on-farm areas which include production, processing, storage. Various *Staphylococcus* species were isolated from all three areas investigated; therefore, it might not be feasible to suggest entire elimination of these organisms through sanitation management practices. Due to the nature of mushroom production, a systems approach might be better suggested where sanitary procedures and overall hygiene will be of utmost importance.

4.5. CONCLUSION

Human handling of mushrooms is a vital part in the commercial production process, especially during picking and packaging. Mushrooms harbour a great diversity of microorganisms and handling by workers affect and influences the final microbial load and population compositions on product surfaces. Furthermore, the risk of introducing pathogenic microorganisms due to unhygienic handling and potential cross-contamination issues will always remain. Various steps could be considered by industry to mitigate the associated risks involved, one of which will be the emphasis of activities and practices surrounding the handling and storage of raw materials used in compost manufacturing. It was

determined that *Staphylococcus* species present on pre-harvest mushrooms and species introduced by pickers, less frequently persist long enough to occur on packed mushrooms, in contrast to *Staphylococcus* species mainly introduced by packers which are more likely to be associated with the final product entering cold storage. The presence of antimicrobial resistance within the Staphylococcus population, confirmed from mushroom production facilities, further highlight the importance of good personal hygiene. The packing process and hygiene practices employed during this stage of production should be carefully considered and controlled to ensure a safe final product for consumers. A thorough understanding of the interactions between production, processing and distribution, including the behaviour of microbiological contaminants are needed if farms and production managers are to effectively achieve high levels of sanitation. To determine the total risk to consumers, prevalence of potentially harmful pathogens in raw materials used during mushroom production, specifically animal-based could be considered in conjunction with the final product as it appears on retailer shelves.

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CHAPTER 5

MICROBIAL DIVERSITY OF WHITE BUTTON MUSHROOMS

ABSTRACT

The microbiological profile present on commercial white button mushrooms is important when considering food safety assurance and when determining the potential of spoilage and quality of the final consumer product. Considering an important aspect such as food safety, most outbreaks can be attributed to microbial imbalances or lack of diversity within or on the associated host surface and residing microbial population. The purpose of this chapter is to determine the microbial profile on fresh, whole white button mushrooms cultivated and commercialized in South Africa. Population diversity (bacteria, yeasts and fungi) were determined through isolation of representative microbial colonies using both standard viable plating (selective and general mediums) and rapid detection methods. Representative isolates were characterized into 96 different environmental as well as food pathogenic species, using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), PCR and sequencing for identity determination and validation. Most frequent isolations of significance were *Pseudomonas* spp. (45.37%), *Rhodotorula mucilaginosa* (84.31%), and *Penicillium brevicompactum* (66.67%) representing the most observed bacterial, yeast and fungal groups respectively. MALDI-TOF MS as a standalone method for rapid and accurate detection of specifically bacterial cultures were evaluated against traditional genotypic identification methods such as 16S rRNA sequencing. Data indicated that the MALDI-TOF MS can be established as an accurate, inexpensive and fast identification method, and can be recommended for routine detection of purified bacterial and yeast isolates from food-based origins.

5.1. INTRODUCTION

Fresh produce harbours transient as well as residential microbes (Simon *et al*., 2005; Kumar *et al*., 2017). These microbial populations play an important role in maintaining product quality by preventing the establishment of pathogenic organisms (Pradhan *et al*., 2018). Fresh mushroom surfaces in general are an ideal medium for microbial growth, as they are rich in moisture; water activity of 0.98 or higher and have a neutral pH (Martinez-Carrera *et al*., 1998). Mushrooms can therefore harbour a diverse microbiological profile (Venturini *et al*., 2011), but in contrast to other fresh produce and products, information on the microbial profile of fresh mushrooms, especially white button mushrooms are still limited due to variations in cultivation, handling and storage conditions. Current trends in nutrition and food technology are increasing the demand on food microbiologists to ensure safe food supplies (Mandal *et al*., 2011; King *et al*., 2017). The analysis of various foods for the presence of pathogenic and spoilage microorganisms is standard practice for assurance of food safety and quality (Doyle, 2001; Korada *et al*., 2018).

Beside the potential association of foodborne pathogenic microorganisms, it is important to understand the microbial community dynamics, or microbiome, on mushrooms from a health and hygiene

perspective. As a result of a generally short shelf-life and increasing trends of raw consumption, the indirect impact on human health due to exposure to non-pathogenic microorganisms should also be considered – such as the potential of introducing new commensal bacteria into the human gastrointestinal system (Leff & Fierer, 2013). As mentioned, the cultivation dynamics, harvesting, handling and storage methods used during mushroom production differ from that of other fresh produce.

Various methods can be used for the identification and quantification of microorganisms (Walsh *et al*., 2018). Classic, culture-dependent methods are well established approaches for the detection and enumeration of bacteria, fungi and yeasts (Kraková *et al*., 2018). Sometimes these methods are defined as "gold-standard" methods and are internationally accepted (Jasson *et al*., 2010). Such methods can be time consuming and tedious, especially when identifying pathogens on time sensitive sources such as food and especially fresh mushrooms (Hausdorf *et al*., 2013). Identification methods and analysis are migrating away from standard viable methods to more rapid procedures. The category of rapid methods may include a type of assay that gives instant or real time results, but on the other hand can also be a modification of an existing procedure that reduces the assay time (Kraková *et al*., 2018). Such methods not only deal with detection and enumeration, but also with the characterization of isolates by making use of technologies and principles from the fields of microbiology, chemistry, biochemistry, biophysics and serology (Boening & Tarr, 1995; Yongsheng *et al.* 1996; Westerman *et al*., 1997; Groisman & Ochman, 2000; Shah *et al*., 2003; Naravaneni & Jamil, 2005; Biswas *et al*., 2008).

Making use of full or partial DNA sequences to identify microorganisms is a practice that is readily used in biological science (Petti *et al*., 2005). There are various conserved parts in the genomes of bacteria, yeasts and fungi constituting genes or gene regions characteristic and unique to each species or group of organisms (Washburne *et al*., 2018). These genes are used as genetic markers for several reasons including presence in virtually all respective microbes and being large enough for informatic analysis (Patel, 2001). Several studies have revealed that rRNA gene sequencing provides genus identification in most of the cases (> 90%), less so for species identification (65 to 83%) and leaving 1 to 14% of isolates remaining unidentified (Drancourt *et al*., 2000; Mignard & Flandrois, 2006; Woo *et al*., 2003). This method of identification is still highly regarded and is useful in classification of microorganisms (Janda & Abbott, 2007). Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) is a novel progression in the field of plant pathology for identification of microorganisms. The technology is reliant on the protein profiles obtained directly from isolates (Krishnamurthy *et al*., 1996; Holland *et al*., 1996). As a rapid, cost effective and accurate method of identification, this platform is mostly used in clinical microbiology, but advancements have ensured the identification of yeasts and fungi in addition to bacteria on which the technology was based (Alanio *et al*., 2011; Ferreira *et al*., 2011; Kroumova *et al*., 2011). The application of equipment such as the MALDI-TOF MS, for identification of microorganisms in an ecological context are not yet well

established (Hausdorf *et al*., 2013). The aim of this study was to use a combination of standard microbiological, molecular and rapid techniques to isolate, identify and describe the most commonly occurring microorganisms on and within white button mushrooms. Additionally, comparison was also drawn between traditional identification techniques such as genotypic database identity confirmation through sequencing and mass spectrometric identification of purified cultures using MALDI-TOF MS.

5.2. MATERIALS AND METHODS

5.2.1. SAMPLING

Mushroom samples were sampled at the same commercial farming facilities, using the same methodology of sampling as described in Chapter 3 (pg. 50).

5.2.2. SAMPLE PROCESSING AND ISOLATIONS

Mushroom samples were processed using the same methods and microbiological techniques described in Chapter 3 (pg. 51) for isolation and enumeration of the total viable microorganism population on mushrooms. To ensure purity of isolates for further experimentation, the method of quadrant streaking for single colonies were used for both bacteria and yeasts, whereas single spore isolations were performed for fungal colonies. To eliminate or reduce the influence of different growth and storage times on the obtained spectra, all colonies were transferred and grown in batches, so that all bacteria were grown for the same amount of time and were stored for a maximum of 2 days at 4°C before MALDI-TOF MS analysis. A total of 734 isolations were made and processed for identification.

5.2.3. IDENTIFICATION OF ISOLATES USING MALDI-TOF MS

Similarly, as described for the food safety isolates in Chapter 3 (pg. 52), selected bacterial and yeast isolates which form part of the natural microbiome of fresh mushrooms were identified using MALDI-TOF MS in combination with the Bruker Biotyper software and database (Standing *et al*., 2013). Only bacterial cultures were evaluated using MALDI-TOF MS as the database for fungal and yeast isolates were not available and optimized at the time of analysis.

5.2.4. DNA EXTRACTIONS

DNA extractions were performed for all isolates in this study, even for bacterial cultures that were identified with the MALDI-TOF MS. Single-spore purified fungal isolates, transferred to malt extract agar were sampled for DNA extraction after five days of growth by dry-scraping culture mycelia from the agar surface into extraction tubes prepared with silica-beads (Biospec Products Inc.). Purified bacterial and yeast cultures were inoculated in 9 mL Tryptone Soy Broth (Merck-Biolab) and were incubated statically as overnight cultures at 25°C. Culture suspensions were spun down in Eppendorf tubes to obtain cellular pellets. Supernatant were discarded and pellets allowed to air-dry within a

laminar flow cabinet before resuspension in lysis reagent from the respective DNA extraction kits. DNA was extracted from bacteria using the Quick-GDNA miniprep kit (ZymoResearch) and the Nucleospin® Plant II DNA Extraction kit (Macherey-Nagel) for yeasts and fungi.

5.2.5. POLYMERASE CHAIN REACTION

Depending on the organism either 16S primers (27 Forward 5'-GAGTTTGATCCTGGCTCAG-3' / 1492 Reverse 5'-TACGGYTACCTTGTTACGACTT-3') (Weisburg *et al*., 1991) or ITS primers (1 Forward 5'-TCCGTAGGTGAACCTGCGG-3' / 4 Reverse 5'-TCCTCCGCTTATTGATATGC-3') (White *et al*., 1990) were used for sequence amplification. PCR reactions were conducted in 100 µL thin-walled tubes using an Eppendorf 6325 Mastercycler Pro S 96 well Thermal Cycler Vapo Protect (Eppendorf). Each 25 µL bacterial reaction mixture contained 0.3 µL MyTaq™ DNA Polymerase (Bioline), 5 µL MyTaq™ Reaction Buffer (Biolone), 0.3 µL PCR Forward Primer (IDT, South Africa), 0.3 µL PCR Reverse Primer (IDT), 1 µL DNA Template and 16.3 µL ddH_2O (sterile double distilled water). Each 25 µL fungal and yeast reaction mixture contained 0.25 µL MyTaq™ DNA Polymerase (Bioline), 5.25 µL MyTaq™ Reaction Buffer (Biolone), 0.25 µL PCR Forward Primer (IDT, South Africa), 0.25 µL PCR Reverse Primer (IDT), 1 µL DNA Template and 18.8 µL ddH_2O. Thermal cycling for bacterial, fungal and yeast reactions consisted of an initial denaturation at 95°C for 2 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 45 s, and extension at 72°C for 1 min 30 s.

5.2.6. AGAROSE GEL ELECTROPHORESIS

Agarose gels for 16S amplified products were made by adding 1.5 g of Agarose powder (SeaChem) to 150 mL TBE Buffer supplemented, with 15 µL Ethidium Bromide (EtBr). Agarose gels for ITS amplified products were made by adding 3 g of Agarose powder (SeaChem) to 150 mL TBE Buffer, supplemented with 15 µL EtBr. PCR samples were loaded into solidified gels using 3 µL loading dye/buffer and 12 µL of each PCR sample. Gels containing 16S and ITS PCR samples were finally loaded with 5 µL DNA marker/ladder Hyper Ladder IV (Bioline) for ITS amplicons and GeneRuler™ 100 bp Plus Ladder (Thermo Scientific) for 16S amplicons. Respective gels were run at a 100V for 1 h 30 min whereafter DNA bands were visualised using a Vilber Lourmat (Omni-Science CC, Johannesburg) gel imaging system.

5.2.7. SEQUENCING

The 16S rRNA gene primers amplified a 1465 bp DNA segment (DeLuca *et al*., 2017), which meant amplicons with similar band sizes were cut from their gel positions using a sterilized scalpel under UV illumination. A similar procedure was followed for ITS primer amplified DNA with theoretical sizes of 500 to 700 bp (Larena *et al*., 1999). The agarose blocks containing DNA were placed in 1.5 mL Eppendorf tubes. DNA was recovered from the agarose through a DNA purification step, using the

Zymoclean™ Gel DNA Recovery Kit (Zymo Research). Purified DNA was sequenced by Inqaba Biotechnical Industries (South Africa) using the 27-Forward Primer and ABI Big Dye V3.1. Sequencing reaction clean-up was done with Zymo sequencing clean-up kit. Injection was on ABI 3500 XL, POP7 with a 50 cm array. Bioinformatic and comparative analysis of all the samples were carried out online at (http://www.ncbi.nlm.nih.gov). A Basic Local Alignment Search Tool (BLAST) search was performed for each gene sequence using the NCBI gene bank database (Marchler-Bauer *et al*., 2002; Pruitt *et al*., 2005).

5.3. RESULTS

5.3.1. MICROBIAL RICHNESS OF WHITE BUTTON MUSHROOMS

The microbial diversity observed on white button mushrooms during this study, showed a larger variety of bacteria compared to yeast and fungi. The most isolated yeast and bacteria, identified with MALDI-TOF MS, consisted of 29 species, contributing a combined average of 81.55% (599 isolates) of the total population recovered from mushroom samples. The greatest diversity is contained in the remaining 62 species contributing 18.45% (135 isolates) to the total population of less frequently isolated organisms. Fungal isolates added a further five species to the overall population richness identified from mushrooms. During this study, 734 isolations were made and characterized into 96 different bacterial, yeast and fungal species (Table 5.1 and Table A1 in Appendix A).

5.3.1.1. Bacterial isolates of significance

Various species (*n=32*) of Pseudomonas were isolated from mushrooms throughout this study. *Pseudomonas extremorientalis* were the most isolated bacterium from mushrooms and constituted 12.20% of the total bacterial isolations. *Raoultella ornithinolytica* were identified as the second most common species to occur on fresh mushrooms, with an identification rate of 10.52% of the total bacterial population. The bacterium *Microbacterium oxydans* were isolated third most often with a population contribution of 9.86%. These three species combined are representative of >30% of the total bacterial population isolated from mushroom samples. The rest of the bacterial species listed in Table 5.1 contribute towards the remaining diversity of the bacterial group, with each representing, in descending order, a smaller portion of occurrence from the samples analysed.

Ewingella americana is noted as a species of interest. Various bacterial species isolated during the work performed in this study are classified as Gram-negative bacteria, also including members of the family Enterobacteriaceae, of which *E. americana* associate. This species was not isolated very often, only 5.18% of the time and most commonly from post-harvest mushrooms (Table A1, Appendix A).

Table 5.1 *Most frequently isolated bacterial species from white button mushrooms, during different production stages, identified using the matrix-assisted laser desorption/ionisation time-of-flight mass spectrophotometer (MALDI-TOF MS)*

MALDI-TOF MS isolate identification	Percentage (%) occurrence	MALDI-TOF MS Identification accuracy (3.00 = 100% Accurate)	16S BLAST Result (Confirmed species)	Similarity Score %	Pre- / Post-harvest isolation
Pseudomonas extremorientalis	12.20	2.07 ± 0.07	*Pseudomonas extremorientalis*	95	48% Pre / 52% Post
Raoultella ornithinolytica	10.52	2.43 ± 0.12	-	-	41% Pre / 59% Post
Microbacterium oxydans	9.86	2.06 ± 0.15	*Microbacterium oxydans*	96	22% Pre / 78% Post
Enterobacter cloacae	5.68	2.27 ± 0.13	*Enterobacter cloacae*	89	35% Pre / 65% Post
Pseudomonas tolaasii	5.35	2.23 ± 0.11	*Pseudomonas tolaasii*	99	32% Pre / 68% Post
Pseudomonas orientalis	5.02	1.87 ± 0.07	*Pseudomonas orientalis*	97	23% Pre / 77% Post
Microbacterium maritypicum	4.18	2.05 ± 0.17	*Microbacterium maritypicum*	96	14% Pre / 86% Post
Pseudomonas rhodesiae	4.01	1.94 ± 0.09	*Pseudomonas rhodesiae*	98	25% Pre / 75% Post
Staphylococcus succinus	3.84	1.77 ± 0.09	*Staphylococcus succinus*	98	13% Pre / 87% Post
Pseudomonas antarctica	3.67	2.04 ± 0.07	*Pseudomonas antarctica*	97	41% Pre / 59% Post
Pseudomonas kilonensis	3.67	1.85 ± 0.08	*Pseudomonas kilonensis*	97	18% Pre / 82% Post
Pseudomonas chlororaphis	3.34	1.87 ± 0.08	*Pseudomonas chlororaphis*	98	30% Pre / 70% Post
Microbacterium liquefaciens	2.83	1.91 ± 0.12	*Microbacterium liquefaciens*	90	18% Pre / 82% Post
Enterobacter asburiae	2.50	2.22 ± 0.16	*Enterobacter asburiae*	98	13% Pre / 87% Post
Pseudomonas azotoformans	2.50	2.03 ± 0.06	*Pseudomonas azotoformans*	97	33% Pre / 67% Post
Pseudomonas frederiksbergensis	2.34	1.93 ± 0.09	*Pseudomonas frederiksbergensis*	96	36% Pre / 64% Post
Citrobacter freundii	2.17	2.32 ± 0.17	-	-	54% Pre / 46% Post
Staphylococcus equorum	2.17	1.99 ± 0.11	*Staphylococcus equorum*	99	0% Pre / 100% Post
Serratia marcescens	2.17	2.26 ± 0.14	*Serratia marcescens*	99	46% Pre / 54% Post
Pseudomonas marginalis	1.99	1.99 ± 0.11	*Pseudomonas marginalis*	97	17% Pre / 83% Post
Salmonella spp.	1.99	2.39 ± 0.12	*Salmonella enterica*	97	50% Pre / 50% Post
Cedecea neteri	1.83	2.04 ± 0.15	*Cedecea neteri*	97	55% Pre / 45% Post
Citrobacter braakii	1.83	2.41 ± 0.10	-	-	9% Pre / 91% Post
Pseudomonas mandelii	1.50	2.12 ± 0.07	*Pseudomonas mandelii*	99	44% Pre / 56% Post
Staphylococcus saprophyticus	1.49	1.89 ± 0.12	*Staphylococcus saprophyticus*	74	44% Pre / 56% Post
Staphylococcus xylosus	1.34	2.04 ± 0.29	*Staphylococcus xylosus*	99	63% Pre / 37% Post

5.3.1.2. Yeast isolates of significance

In total three species of yeast were frequently isolated from fresh white button mushrooms, representing the entire diversity of yeast that was identified in this study. The most abundant yeast species isolated was *Rhodotorula mucilaginosa* (A. Jörg.) F.C. Harrison 1928 (Table 5.3) with an isolation percentage of 84.31%. During this study *R. mucilaginosa* was only isolated from post-harvest mushrooms. *Cystofilobasidium infirmominiatum* (Fell, I.L. Hunter & Tallman) Hamam, Sugiy & Komag. 1988, was the second most frequently identified species of yeast with a 14.02% occurrence. Post-harvest isolations were significantly more than on before-harvest mushrooms, but this species was identified on

mushrooms from both production phases. The genus *Candida* Berkhout (1923) *Candida* spp., were non-conclusively identified up to species level, but were the third and least isolated yeast on mushrooms. With an identification frequency of 1.67%, on both pre- and post-harvest mushrooms, but significantly more from pre-harvest mushrooms.

5.3.1.3. Fungal isolates of significance

Five species of significance were commonly identified and isolated, identifying these isolates as the most dominant fungi found on samples analysed. Included in the group of different fungal species, was *Penicillium brevicompactum* (Dierckx, 1901) which was found to be dominant or most frequently isolated, 66.67% overall, but occurrence was observed to be more readily on post-harvest mushrooms. *Trichoderma longibrachiatum* (Rifai, 1969) represented 13.33% of the fungal species in contrast and was equally like to be isolated from pre- or post-harvest mushrooms. *Penicillium toxicarium* (Miyake, 1940), *Cladosporium cladosporioides* (Fresen.) (G.A. de Vries, 1952) and *Didymella fabae* (G.J. Jellis and Punith, 1991) each represented 6.67% of the all fungal cultures and was only found on post-harvest or packaged mushrooms (Table 5.3).

Table 5.2 *Most frequently isolated yeast and fungal species from white button mushrooms, different production stages, identified using ITS sequencing and BLAST analysis*

ITS BLAST Result (Confirmed species)	Percentage (%) occurrence	Similarity Score %	Pre- / Post-harvest isolation
Yeast			
Rhodotorula mucilaginosa	84.31	97	100% Post
Cystofilobasidium infirmominiatum	14.02	98	34% Pre / 66% Post
Candida spp.	1.67	98	78% Pre / 22% Post
Fungi			
Penicillium brevicompactum	66.67	99	40% Pre / 60% Post
Trichoderma longibrachiatum	13.33	99	50% Pre / 50% Post
Penicillium toxicarium	6.67	99	100% Post
Cladosporium cladosporioides	6.67	99	100% Post
Didymella fabae	6.67	99	100% Post

5.3.2. MALDI-TOF MS COMPARED TO 16S SEQUENCING

Comparison of the MALDI-TOF MS identification results with correlating 16S-rDNA BLAST results for different isolates are listed in Appendix A (Table A2). The highlighted cells indicate isolates, where both replicates were identified as the same organism by the MALDI-TOF MS and 56.41% of the colony identities were the same as BLAST results of 16S DNA. Highlighted cells with an asterisk before the isolate number indicate isolates which were identified as the same organism for both replicates by the MALDI-TOF MS, but the identity did not match any of the identifications given by the BLAST search

results. The non-highlighted cells indicate isolations that were identified by the MALDI-TOF MS as two different organisms. When an isolate was given two different identities, the identity with the highest score value given by the MALDI-TOF MS, gave a higher or equal BLAST search maximum identity percentage 74.29% of the time, and a lower BLAST maximum identity percentage 25.71% of the time. A total percentage of 20.51% of the bacteria identified by the MALDI-TOF MS did not correlate with the 16S BLAST results. Of the 20.51% it was determined that 81.25% of the replicates were the same organism using MALDI-TOF MS analysis, and 18.75% were not.

5.4. DISCUSSION

Research to understand and control the microbiological risks associated with the consumption of fresh fruits and vegetables has examined many environments in the farm-to-fork continuum (Ottesen *et al*., 2013). An important data gap, that remains mainly poorly studied is the "baseline" microbial populations that are associated with fresh mushrooms, either endemically or in response to environmental/ variable production pressures. Microorganisms are an integral part of the surface composition of fresh produce. Microbiome studies have the potential to lead to shifts in knowledge regarding potential biocontrol strategies, biocontrol products and post-harvest biology (Droby & Wisniewski, 2018). Microbiological diversity research of surface dynamics could enhance the understanding of ecosystems present on pre- and post-harvest produce, in which the microbiome plays an essential role in the health and physiology of for example mushrooms after they are harvested.

Due to the large amount of species isolated and the sake of brevity, only the most dominant and influential organisms will form part of the discussion. This does not imply that any of the other organisms, irrespective of their contribution to the total population, are not important. The list of microorganisms listed in this chapter, as far as can be determined, is the most comprehensive collective of organisms described from fresh white button mushrooms in a single study. Potential foodborne microorganisms are included in the results section, for comprehensiveness of this chapter but their prevalence and significance are included in greater detail in Chapter 3. *Staphylococcus* species constituted a large portion of the bacterial microorganisms isolated from mushrooms, but as such an important species group and considering their role in phytosanitary and food safety implications, the diversity, transference and potential antibiotic resistance of each species were already discussed in Chapter 4.

Siyoum *et al*. (2016) performed work on the progression of microorganisms throughout the production chain and described a similar trend to what was found in this chapter on mushroom fruiting bodies. Fungi and yeasts have contributed a lower presence and diversity (dominated by *Penicillium* spp.) while bacteria were dominant and showed significant diversity. In contrast to both our study and that by

Siyoum *et al*. (2016), Chikthimmah (2006) described larger fungal loads of ~6 log cfu/g on freshly harvested mushrooms compared to yeasts and bacteria. The genus Pseudomonas has been well described as one of the most important groups of bacteria present on pre-harvest and packaged mushrooms (Siyoum *et al*., 2016). It has also been shown that *A. bisporus* has the highest Pseudomonas counts in comparison to other mushroom species (Venturini *et al*., 2011). Interactions of some pseudomonads such as *P. tolaasi*, *P. fluorescens* and *P. putida* (also isolated in this study) can have beneficial or negative effects on mushroom production or disease control (Frey-Klett *et al*., 2011). *Pseudomonas putida*, for instance is well known for the essential role it plays in stimulating and contributing to mushroom fruit body formation during earlier stages of mushroom production (Noble *et al*., 2009). Literature describes these bacteria as an essential part of mushroom development (Burton and Noble, 1993; Fett *et al*., 1995; Wells *et al*., 1996). Some of these bacteria have an important role to play within the casing medium. They help stimulate the fruiting body initiation of the mushroom through pin establishment (formation of visible mushroom fruiting body). The casing layer therefore constitutes the region where the pseudomonads are most found (Fett *et al*., 1995). This was well established in this study as the frequency and amount of Pseudomonas isolated from mushroom samples, increased during advanced (later) stages of the production process. It has also been shown that, some pseudomonads as well as other non-pathogenic bacteria play an important role in the eventual spoilage and quality degradation of harvested mushrooms. Pseudomonads may be either saprophytic or pathogenic; of which the latter may cause symptoms such as discoloration on the cap of the mushrooms. *Pseudomonas tolaasii* have been implicated in the production of an extracellular toxin, tolaasin that manifests itself in brown blotch disease of *A. bisporus* (Hutchison & Johnstone, 1993). *Pseudomonas tolaasii* is also implicated in post-harvest decay as well as browning of mushrooms (Wells *et al*., 1996). Some *Pseudomonas* spp. are responsible for mummy disease of mushrooms. Another Pseudomonad, *Pseudomonas agarici* are responsible for drippy gill disease of mushrooms (Young, 1970).

Limited information is available for yeasts association with fresh mushrooms as well as the potential role played in spoilage of the final food product. Yeast isolates may not be as important in their role on cultivated or freshly harvested mushrooms as opposed to their role later in the distribution chain and their contribution to spoilage of packaged mushrooms. Although this study did not investigate the dynamism of microbial populations on mushrooms much later in the value chain i.e. retail samples, it is known from literature that yeasts become more dominant under conditions that might not favour beneficial surface microorganisms, such as modified atmosphere packaging, or packaging which will allow for an anaerobic environment. Although *R. mucilaginosa* have not been described as a dominant yeast species on fresh white button mushrooms, being isolated only on post-harvest mushrooms is supported by literature, that this species is most readily associated with fresh post-harvest fruits and vegetables (Deligios *et al*., 2015). *Cystofilobasidium infirmominiatum* can be classified as a

pectinolytic yeast that is frequently found in cold environments (Cavello *et al*., 2017). This yeast species is also well known for post-harvest association on the surfaces of fresh fruits and has also been described as an antagonist to various pathogenic fungal species during cold storage (Garat *et al*., 2010). Considering the species contribution of *C. infirmominiatum* to the total microbial diversity and the nature of its association with especially fungal species, this organism will affect the microbial population composition on mushroom surfaces. Further investigation at a retail level will need to be considered to determine the true relevance and effect of this yeast species on post-harvest mushroom microbial profiles. Lastly, the third yeast isolated belongs to the genus *Candida* Berkhout (1923) which in turn is classified under the order *Saccharomycetales*. *Candida* spp., are widespread in natural and artificial habitats, being damp and wet with a high level of organic material, including organic acids and ethanol, low and high temperatures, high salt and sugar osmolality (Robinson, 2014). The environmental conditions created during commercial production of mushrooms are therefore very suitable for the proliferation and growth of *Candida* spp. Literature indicates that members of this genus also contribute to growth and development of *A. bisporus* (Kertesz & Thai, 2018). Similarly, the authors Siyoum *et al*. (2016), also isolated various *Candida* spp. in their study describing microbial succession in white button mushroom production systems, but they only isolated and described *Candida* spp. on compost and casing materials tested. Therefore, results from this chapter confirms that *Candida* spp. can also associate with mushrooms.

One of the organisms that was frequently isolated, *P. brevicompactum* has been implicated in the cause of mushroom worker's lung and originates in the compost tunnels during the fermentation of compost (Van den *et al*., 1993). This information supports findings in this chapter, as this organism was found to be present before mushrooms were harvested but results also indicated that persistence on to post-harvest mushrooms is possible as *P. brevicompactum* was isolated more on mushrooms after harvest. Similarly, from literature, *C. cladosporioides* have previously been isolated and described from the compost on which mushrooms are cultivated (Fergus, 1978), but this is in contrast to findings from this study, as this organism was only detected on post-harvest mushrooms and not on mushrooms while still in the growing rooms on beds. This could mean that *C. cladosporoides* has not been previously known from literature to associate with mushrooms themselves, or this finding could indicate to contamination during or after harvest. Further investigation will be necessary to confirm. *Penicillium toxicarium* is implicated as a non-bacterial foodborne pathogen to produce toxin in rice (Goldblatt, 1969), but the pathogenicity on mushrooms are still unknown. As a collective, the genus Penicillium are most associated with post-harvest pathogens, or as spoilage organisms (de W Blackburn, 2006). This was confirmed by the post-harvest isolations which were made for all *Penicillium* spp. in this study. *Trichoderma longibrachiatum* is a fungus that is commonly found on wheat straw that is used in the preparation of compost for mushroom production, it is more so found in the production of exotic mushrooms than white button mushrooms (Velazquez-Cedeno, *et al*., 2004).

Lastly, *Didymella fabae* is commonly known as "Pod and Bean leaf spot", causing necrotic brown lesions on field and broad bean crops (Biddle & Cattlin, 2007). This fungus is still mostly unknown on white button mushrooms as a pathogen or to cause any significant adverse impact on mushroom cultivation. It can be seen from literature and other sources that most of the fungal isolates which were isolated from white button mushrooms originates in a stage before harvest, usually the compost. With the available information, it could perhaps be theorised that these organisms (specifically some of the fungal species) are only transferred to mushrooms during the stages of mycelial colonization of the compost or perhaps even more so during pin and fruit body formation. Origins of these fungal species are mostly from plant material, such as wheat straw. From this study it was also observed that the presence of fungal isolates, such as those described, are not always according to a distinct pattern or association with a specific stage or phase of production. Theoretically, their presence on mushrooms may vary depending on their presence in the compost medium on which the mushrooms are grown or hygiene and sanitary practices employed during later stages of production, such as picking, packaging and storage.

Making use of full or partial DNA sequences to identify microorganisms is an emerging practice that is readily used in biological science fields (Petti *et al*., 2005). There are various conserved parts in the genomes of bacteria, one of these being a housekeeping gene, 16S rRNA. This gene is used as a genetic marker for several reasons including its presence in virtually all bacteria as well as being large enough for informatics analysis (Patel, 2001). Various studies have revealed that 16S rRNA sequencing provides genus identification in most of the cases (> 90%), less so for species identification (65 to 83%) and leaving 1 to 14% of isolates remaining unidentified (Drancourt *et al*., 1995; Mignard & Flandrois, 2006; Woo *et al*., 2003). This method of 16S rRNA identification is highly useful with regards to bacterial classification (Janda & Abbott, 2007). MALDI-TOF MS also makes use of the 16S ribosomal proteins when identifying bacterial samples (Davies, 2010). This makes this technology directly relatable and comparable to traditional methods such as sequencing to confirm identity isolates. Various authors from alternative studies, have shown that whole cell MALDI-TOF MS, similar to the method used in experimental work from this chapter, can be used as an efficient tool to identify and characterize isolates which originate from specific microbiomes (Singhal *et al*., 2015). With minimal optimisation and calibration of reference isolates, the MALDI-TOF MS analysis was able to accurately identify and assign 86.21% of the isolates with a correct species ($x \geq 2.0$) identification, which also correlated with BLAST comparisons ($E < 0.5$). The isolates which correlated with the BLAST search results, consisted of 3% having a 'non-reliable' score value, 52% a 'probable genus identification', 32% a 'secure genus identification' and 13% a 'highly probable species' score value. Although the crop from which isolates were made differs, similar results were obtained by Hausdorf *et al*. (2013), who used MALDI-TOF MS to characterize the cultivatable microbial community within a spinach

processing plant. The authors also stated that the MALDI-TOF MS system showed comparable results in their study to conventional 16S rRNA gene sequencing methods.

Not all analyses and identifications were correct, when compared to 16S sequencing, during the first analysis performed using the MALDI-TOF MS. Inaccurate identifications showed a probable or secure genus that differed from the 16S BLAST identity search results 50% of the time. Based on these results, the MALDI-TOF MS seems to be a less accurate method to identify and characterise isolates, but any test based on biochemical traits will exhibit a larger degree of inaccuracy when used to identify microbes isolated from environmental samples, as the diversity of microbes in these habitats are enormous (Torsvik *et al*., 2002). A consideration that should be mentioned when bearing in mind accuracy of results in comparison to an online database such as GenBank is that it is an open access database and continual free submission of DNA sequences onto the database leads to its continuous integration with new entries, revision and even removal of misidentified records. Evidence of mistakes within the database exist and it is recommended that phylogenetic analysis should be carried out with reference or type strains as benchmarks (Timperio *et al*., 2017). The results also showed a 43.75% rate by which the correct score was given for a 'non-reliable' identification and a 6.25% rate of incorrectly assigning 'non-reliable' identification. As a result, two main causes could be described or associated with most of the inaccurate identifications obtained in this study. The first being the absence of an adequate reference spectrum in the BioTyper database, this is fortunately an easy correction which could be achieved by software and database updates. The second cause is a failure to obtain enough protein signal in order to build a spectrum that can be compared to the BioTyper database. Failure to obtain a spectrum can be explained either because of the structural properties of the cell wall of some bacteria (Gram-positive bacilli being a prototypical example) or fastidious growth of some isolates which yield only small amounts of colonies that can be harvested for protein extraction purposes (Bizzini *et al*., 2011). Like other studies, overall data has shown that routine isolates could be identified at accuracy levels greater than 85% with good reproducibility (Seng *et al*., 2009; Eigner *et al*., 2009). Therefore, further expansion of the database of the instrument and optimization of extraction protocols for difficult-to-treat samples will undoubtedly increase the accuracy of identification by the MALDI-TOF MS and the diversity of species that might be efficiently identified in future applications.

This research chapter were concluded using traditional methods of culturing and microorganism isolation as basis for composing species diversity compositions. Although the MALDI-TOF MS has shown to be a promising platform for rapid identification compared to sequencing, pure cultures are still needed as starting point for the analysis. Advancements such as Meta-omics (metagenomics, metatranscriptomics, metaproteomics and metametabolomics) technologies are increasingly becoming more popular methods to analyse microbial populations. Applying these technologies to post-harvest studies have not yet gained a lot of popularity, but as more research in these fields will be done the

understanding of pre- and post-harvest biocontrol systems, foodborne pathogens and post-harvest physiology will be revolutionized (Droby & Wisniewski, 2018). The role of the microbiome and species diversity in mushroom health, productivity and to a lesser extent cultivar development should be considered as much as the fruiting body itself.

5.5. CONCLUSION

From this study a comprehensive cultivatable diversity has been described for microbial populations found on whole white button mushrooms before and after harvest. Evidence from literature and scientific trials, suggest that mushrooms do not have an innate or natural association with any specific microorganism group or species. Therefore, microorganisms isolated from mushrooms are the result of saprophytic or transient species which are accumulated from the casing and compost mediums, contaminants resulting from environmental sources during cultivation, and those attained during the processes of handling, packing and storage. The population of microorganisms isolated and described in this study form part and make up the microbiome of fresh white button mushrooms as found between morphological maturity and packaging post-harvest. This diversity found could be described as the apex microbial community as the relevance of these organisms to the overall quality and safety of mushrooms are at a critical stage of production. MALDI-TOF MS has been established as an accurate, inexpensive and fast identification method, and can be recommended for routine detection of purified bacterial and yeast isolates from food-based origins. Our results indicated that MALDI-TOF MS is equally and in some instances more accurate than partial 16S rRNA and ITS gene sequencing for species identification. An important consideration for the continual success of this technique will be accurate and ongoing expansion of the MALDI-TOF MS database which will enhance the utility of this methodology for the identification of unknown bacterial, yeast and even fungal pathogens. Future studies of a similar nature may consider a polyphasic approach combining MALDI-TOF MS and gene sequencing, which can grant a higher discriminatory ability during identity verification of hard-to-identify microbial isolates.

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CHAPTER 6

MOLECULAR DETECTION OF FUNGAL MUSHROOM PATHOGENS

METHOD DEVELOPMENT, PRIMER DESIGN AND OPTIMISATION

ABSTRACT

Four major fungal diseases of economic importance on white button mushrooms were included in this chapter; compost green mould (*Trichoderma aggressivum*), dry bubble (*Lecanicillium fungicola*), cobweb (*Cladobotryum dendroides*) and wet bubble (*Mycogone perniciosa*). This chapter explores and highlights the development of molecular methods for accurate detection and quantification of fungal specific DNA fragments from various starting materials most found on mushroom farms. A nested polymerase chain reaction (PCR) test on different mushroom casing and compost material was developed to unmistakably identify the wet bubble disease pathogen. This includes the isolation of DNA from various mushroom samples and the design of a primer set based on internal transcribed spacer (ITS) sequences. A portion of the ITS region of the ribosomal DNA operon was amplified using universal primers. Primer sets for pathogen specific detection of green mould, dry bubble and cobweb were adapted from literature to enable repeatable and accurate detection of these organisms together with *M. perniciosa* during commercial implementation of a farm-based monitoring system. In order to confirm specificity, primers were analysed using the BLAST program, and sequenced isolates were used to evaluate the efficacy of the primer sets. Other isolates, including known fungal contaminants and direct DNA isolations from mushroom tissue, were included in the evaluation process. Recurrent positive amplification of pathogenic DNA confirmed method sensitivity and repeatability for each of the pathogens of interest. The primer pairs and methods described in this chapter will serve as basis for a rapid and accurate protocol to detect and monitor pathogen presence on commercial mushroom farms.

6.1. INTRODUCTION

White button mushrooms *Agaricus bisporus* (J.E. Lange) Imbach 1946, are susceptible to a wide range of bacterial, fungal and viral diseases (Fletcher *et al*., 1989). A few mycoparasitic species of fungal pathogens are of economic importance when considering large-scale commercial cultivation of white button mushrooms. These pathogens can, to varying extents, affect the yield and quality of mushrooms produced. The four main fungal diseases under consideration include, green mould (*Trichoderma aggressivum* Samuels & W. Gams 2002), dry bubble (*Lacanicillium fungicola* (Preuss) Zare & W. Gams 2008), cobweb (*Cladobotryum dendroides* (Bull.) W. Gams & Hooz. 1970) and wet bubble (*Mycogone perniciosa* (Magnus) Delacr. 1900), which are annually responsible for considerable disease outbreaks worldwide (Largeteau & Savoie, 2010). Mushroom crops can be infected through various pathways by these pathogens, including contaminated or dusty air circulated through air-ducts, flies that transmit the pathogen within and between growing rooms, picking personnel using poor personal hygiene, dirty picking trays as well as equipment exposed to infected material. Primarily suspected inoculum sources of pathogens on most mushroom farms are contaminated casing material as well as compost produced in the mushroom cultivation process from various raw materials. Spores of some of these pathogens can survive for more than three years in favourable conditions in various substrates and

matrixes which make these sources of recurring infection if gone undetected (Fletcher & Ganney 1968; Bhatt & Singh 2000).

One of the most important considerations for control is eliminating primary on-farm origins of potentially harmful pathogens (Van Griensven 1988; Fletcher *et al*. 1994; Oei 2003). This is becoming increasingly difficult by means of traditional sanitation methods as many countries in the world, are increasingly using less chemical crop protection agents, as the availability and permissibility of these products for use in mushroom cultivation are also in decline. Consequently, mushroom cultivation will lose the ability to use regulated chemical crop protection. This means that early and accurate recognition of pathogens will become crucial for effective management decisions and subsequent control strategies. Such an approach will have to form part of good hygiene management as well as early detection and monitoring of pathogen (diagnostics) protocols which are needed as means to alternatives for a holistic crop protection approach (Baars *et al*., 2013).

Techniques employing PCR provide the most sensitive means of detecting pathogens without having to isolate or culture such organisms, as these traditional methods are sometimes inaccurate and slow to provide information for disease management purposes. Many PCR methods used to detect fungi in various natural environments have successfully been based on internal transcribed spacer (ITS) sequences (Färber *et al*. 1997; Ristiano *et al*. 1998; Böhm *et al*. 1999; Meyer *et al*. 2001; Romaine *et al*. 2002; Bonants *et al*. 2003; Landeweert *et al*. 2003; Meyer *et al*. 2006). Polymerase chain reaction-based amplification of pathogen-specific gene regions have been previously described for DNA extracted from pathogens causing green mould (Chen *et al*. 1999), dry bubble (Romaine *et al*. 2002) and cobweb disease (no reference available). Method development in this chapter were performed and optimised on three PCR-based platforms. Traditional, 1st generation PCR were used for primer design, specificity testing and implementation of *Mycogone* spp. primer sets, where after all four fungal pathogen primer sets were transferred and optimised on 2nd generation quantitative real-time PCR (qPCR) technology. Successful validation of qPCR methodology for each pathogen allowed commencement of commercial implementation and monitoring (Chapter 7), whilst 3rd generation PCR technology were introduced and optimised only a few years after mushroom farm-based screening in South Africa was under way.

The qPCR platform is commonly used and known for its role in studies involving RNA interference, quantification of gene expression and specifically pathogen detection (Navarro *et al*., 2015). An initial concern is that due to simultaneous quantification and detection of amplicons in the same tube, sensitivity of assays run on qPCR could be compromised in detecting the lowest possible pathogen number. Two main approaches exist for qualitative (showing the presence or absence of the DNA sequence of interest) or quantitative pathogen analysis. The first employs non-specific probes such as

SYBR® green dye (used in this study) and the other with species/ site specific probes such as TaqMan® (Livak *et al*., 1995). These reactions are usually performed in a closed-tube system, eliminating the need for post-amplification manipulation, reducing any possible contamination. The main advantage of qPCR over traditional PCR assays is that the starting DNA concentration can be determined with high accuracy and sensitivity (Longo *et al*., 1990; Costa, 2004; Kaltenboeck & Wang, 2005). Furthermore, 3rd generation droplet digital PCR (ddPCR) were investigated as the most advanced PCR-based platform on which primer function and pathogen detection could be established. Droplet digital PCR provides a new method for accurate quantification of DNA copy number by amplifying single DNA target molecules in many separate droplets. Distribution of target DNA molecules among reactions follows Poisson statistics, which means most reactions contain either one or zero target DNA molecules (Kalinina *et al*., 1997; Vogelstein & Kinzler, 1999; Zhong *et al*., 2011). Compared to qPCR, ddPCR technology has several advantages which include, absolute quantification of target nucleic acid without reliance on rate-based measurements (C_q and C_T values) and the need for calibration curves (Hindson *et al*., 2011; Pinheiro *et al*., 2012). It also has high sensitivity and precision for low-copy-number target nucleic acids, making this platform ideal for detection and identification of fungal pathogens present in low concentrations within mushroom production systems (Diehl & Diaz, 2007; Hayden *et al*., 2013; Hindson *et al*., 2011; Sanders *et al*., 2011; Whale *et al*., 2012).

The purpose of this study therefor included the development of a nested-PCR reaction that could be used to identify and detect wet bubble disease causal agents in various complex substrates. Primer sets and amplification protocols for detection of *T. aggressivum*, *Cladobotryum* spp., *Lecanicillium* spp., were adapted and optimized to work in conjunction to those designed for *Mycogone* spp. When working towards an applied method that will have the potential to be implemented within a real-world industry, the goal would also include ensuring a standardized protocol and methodology that will accommodate accurate and repeatable detection of these four fungal pathogens.

6.2. MATERIALS AND METHODS

6.2.1. FUNGAL ISOLATES

For the development of the *Mycogone* primers set, named MYC5 (forward primer) and MYC2R (reverse primer), a set of 11 verified *Mycogone* spp., isolates were selected. These isolates were obtained from major button-mushroom-producing farms in South Africa (Table 6.1). The fungal isolates were obtained by direct isolation from symptomatic host material onto malt extract agar (MEA) (Merck-Biolab, Johannesburg) amended with 250 mg chloramphenicol (Caps Pharmaceuticals SA). Cultures of *Hyphomyces perniciosus* from the Centraalbureau voor Schimmelcultures, the Netherlands

(CBS658.82), and the Plant Protection Research Institute, South Africa (PPRI 5784), were used as comparative standards in all tests.

Table 6.1 *Mycogone perniciosa isolates used for selection and validation of the MYC5 and MYC2R primers*

Isolate code	Origin	Genbank accession number
WB2-2	Blue Hills	FJ904624
WB2-4	Mushroom Cordon Blue	FJ904625
WB3-2	Highveld Mushroom	FJ904626
WB3-3	Highveld Mushroom	FJ904627
WB5-1	Bonbello/White River	FJ904628
WB6-1	Bonbello/White River	FJ904629
WB7-1	Denny Mushroom Deodar Farm	FJ904630
WB14	Medallion Mushrooms	FJ904631
WB17	Country Mushrooms	FJ904632
PPRI 5784	Plant Protection Research Institute, South Africa	FJ904633
CBS 648.82	Centraalbureau voor Schimmelcultures, The Netherlands	FJ904634

Table 6.2 *Trichoderma aggressivum, Lacanicillium* spp., *Cladobotryum* spp., *and Mycogone* spp., *isolates used for method optimization and validation*

Isolate name	Symptom	Source/ origin	Collection Reference
Trichoderma aggressivum var. aggressivum	Green mould disease	Pennsylvania, USA	CBS 100528
Trichoderma aggressivum var. europaeum	Green mould disease	Research Station, Belgium	MES 13004
Trichoderma aggressivum var. europaeum	Green mould disease	Netherlands	MES 13067
Cladobotryum spp.	Cobweb disease	Penn State University, USA	ACC 314
Cladobotryum mycophilum	Cobweb disease	DPI Victoria, AUS	AMC 00107
Lecanicillium fungicola var. aleophilum	Dry bubble disease	Penn State University, USA	ACC 123
Lecanicillium fungicola var. aleophilum	Dry bubble disease	Sydney University, AUS	DAR 43407
Leacanicillium fungicloa var. aleophilum	Dry bubble disease	Sydney University, AUS	DAR 42124
Mycogone perniciosa	Wet bubble disease	Penn State University, USA	ACC 176
Mycogone perniciosa	Wet bubble disease	Belgium	MES 11240

During optimization of existing primer sets for detection and identification of *T. aggressivum*, *Lecanicillium* spp., and *Cladobotryum* spp., representative isolates from these pathogen groups found on commercially grown button mushrooms were used (Table 6.2). All isolates were obtained from the International Mushroom Fungal Pathogen culture collection and were cultured as well as maintained on MEA (Merck-Biolab).

6.2.2. DNA PREPARATIONS

Fungal cultures and mushroom tissue: DNA were extracted with the DNeasy® Plant Mini Kit (Qiagen, Valencia, California) for all isolates included in Table 6.1. The kit was also evaluated for rapid yield of enough and good quality DNA from diseased mushroom tissue as well as pure fungal cultures as starting material. Small pieces of diseased mushroom tissue were dissected – half of isolated material were plated onto MEA media plates and the other half (±50 mg wet weight) were used for direct DNA extraction. Fungal growth was removed from 10 to 14-day old purified *M. perniciosa* cultures on MEA

to provide up to 50 mg wet weight mycelia as starting material for DNA extraction. The fungal material was broken up in DNeasy API buffer, using ceramic beads and a FastPrep Instrument (20 s at a revolution speed of 5.5 m/s). Extractions were performed using the DNeasy® Plant Mini Kit according to the manufacturer's instructions, but the elution volume was reduced to 75 µL. Successful extractions were confirmed using a 1.0% (w/v) agarose gel in TBE buffer (Waalwijk *et al*. 1996).

Casing soil and compost: The SoilMaster DNA extraction kit (Epicentre® Biotechnologies, Madison, WI) was used for isolations from complex substrates, such as casing soil and compost according to the manufacturer's protocol with additional modifications as follows: A 100 mg sample was placed in a 2 mL screw cap Eppendorf tube with two ceramic beads (1/4 inch). Soil DNA Extraction Buffer (375 µL) and 2 µL Proteinase K were added. Tissue was disrupted using the FastPrep Instrument (20 s at a revolution speed of 5.5 m/s). Soil lysis buffer (75 µL) was added and the sample vortexed briefly. DNA was eluted from the tube filter only once with 100 µL AE buffer. The standard protocol was otherwise used.

Swab and pure culture: Single-spore purified fungal isolates, transferred to MEA were sampled for DNA extraction by dry-scraping culture mycelia after five days of growth from the agar surface into extraction tubes prepared with silica-beads (Biospec Products Inc.). For pathogen detection from environmental samples, flocculated swab samples were taken and used directly for DNA extractions. Genomic DNA was extracted using a QIAGEN DNeasy® Plant Mini Kit (WhiteSci). The experimental procedure followed was according to the quick-start bench protocol supplied with the kit. DNA was dissolved in 100 µL of elution buffer in the last step of the protocol and frozen at −20ºC. The DNA concentration for each sample was determined using a Fluorometer Qubit® 2.0 (Invitrogen).

6.2.3. CONVENTIONAL PCR, PRIMER DESIGN AND GEL ELECTROPHORESIS

When designing molecular assays such as those described in this chapter, a comprehensive workflow is demanded with careful consideration of not just the primers themselves but also of the amplicon uniqueness, structure, and location – with the aim of creating an optimal primer/ amplicon combination. Figure 6.1 gives an overview of the process and steps followed during primer design and adaptation of existing primers onto the various PCR platforms utilized during this study. For detection of *M. perniciosa* from diseased mushroom tissue, and verification of pure cultures, the MYC5 primer was used together with the ITS4 primer (White *et al*. 1990). For casing soil, compost and complex samples, two-step PCRs were performed as follows: in the first amplification step, primer ITS1 and primer ITS4 were used. Amplicons of the first PCR step were then used as template for the second amplification using primer pair MYC5 and MYC2R. These primers amplify a DNA segment, nested within the first PCR amplification region. The reaction mixtures and cycling programs are identical. Amplified DNA fragments were visualised on a 1.25% (w/v) agarose gel in TBE buffer (Waalwijk *et al*. 1996).

Design of 24-base oligonucleotide primer MYC5 (5'-CTA TGT GAA CCT TAC CAT TCT GTT-3') and 20-base oligonucleotide primer MYC2R (5'-CGT TCA AAG ATT GGA TGA TT-3') was based on the base pair sequences of amplified ITS regions. Primers were synthesised by Integrated DNA Technologies, Inc. (IA, USA). PCR reactions were performed in 50 µL volumes, each reaction containing 1 µL template DNA, primers i.e. MYC5 (10 pmol) and ITS4 (15 pmol), 5 µL 10×buffer (supplied with Taq polymerase), 200 µM of each dCTP, dGTP, dATP and dTTP (TaKaRa) and 0.5 U Taq polymerase (TaKaRa). Following an initial denaturation step of 95°C for 2 min, 35 PCR cycles were performed in an Eppendorf thermocycler using the following conditions: a denaturation step of 93°C for 30 s followed by annealing at 61°C for 45 s and extension at 72°C for 90 s, followed by a final extension of 72°C for 7 min. Water mixed with purified DNA extracted from mushrooms (*A. bisporus*) and clean water were always included, both as negative controls. In order to confirm the specificity of the primers, analysis was done using the BLAST program (National Centre for Biotechnology Information) (Altschul *et al*. 1990). The sequenced isolates, MP5 and MP11 were used to evaluate the efficacy of the primer sets. Other isolates, including known fungal contaminants and direct DNA isolations from mushroom tissue, were included in the evaluation process. All tests were repeated five times to ensure consistency and repeatability of results. In some instances, DNA originating from the same sample, either directly isolated from the mushroom tissue or first cultured for 10 to 14 days on MEA, was used. Polymerase chain reaction analysis was conducted on all the isolates described.

Primers adapted and used for *Trichoderma aggressivum* included Th-F (5'-CGGTGACATCTGAAAAGTCGT-3') / Th-R (5'-TGTCACCCGTTCGGATCATCCG-3') as described by Chen *et al*. (1999). Primers for *Lecanicillium* spp. were used as described by Romaine *et al*. (2002) for targeting the genome of *Lecanicillium fungicola* (Vfa), Vfa-F (5'-CGGCAGGTATGTTTGCACAATC-3') / Vfa-R (5'-CCGCACCTTACGATTAGAAGTC-3'). *Cladobotryum* spp. primer sets consisted of CLADO1F (5'-TCCTGTTTTCTCTTAGCGGAAT-3') / CLADO1R (5'-CCGTCCCCCAACACCAT-3') (Unreferenced primer set). PCR reactions were conducted in 100 µL thin-walled tubes using an Eppendorf 6325 Mastercycler Pro S 96 well Thermal Cycler Vapo Protect (Eppendorf, Germany). Each 25 µL fungal reaction mixture contained 0.25 µL MyTaq™ DNA Polymerase (Bioline, Johannesburg), 5.25 µL MyTaq™ Reaction Buffer (Bioline), 0.25 µL PCR Forward Primer (IDT, South Africa), 0.25 µL PCR Reverse Primer (IDT), 1 µL DNA Template and 18.8 µL ddH_2O (double distilled water). Thermal cycling for reactions consisted of an initial denaturation at 95°C for 2 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 45 s, and extension at 72°C for 1 min and 30 s. The amplified DNA fragments were visualised on a 1.25% (w/v) agarose gel in TBE buffer (Waalwijk *et al*. 1996).

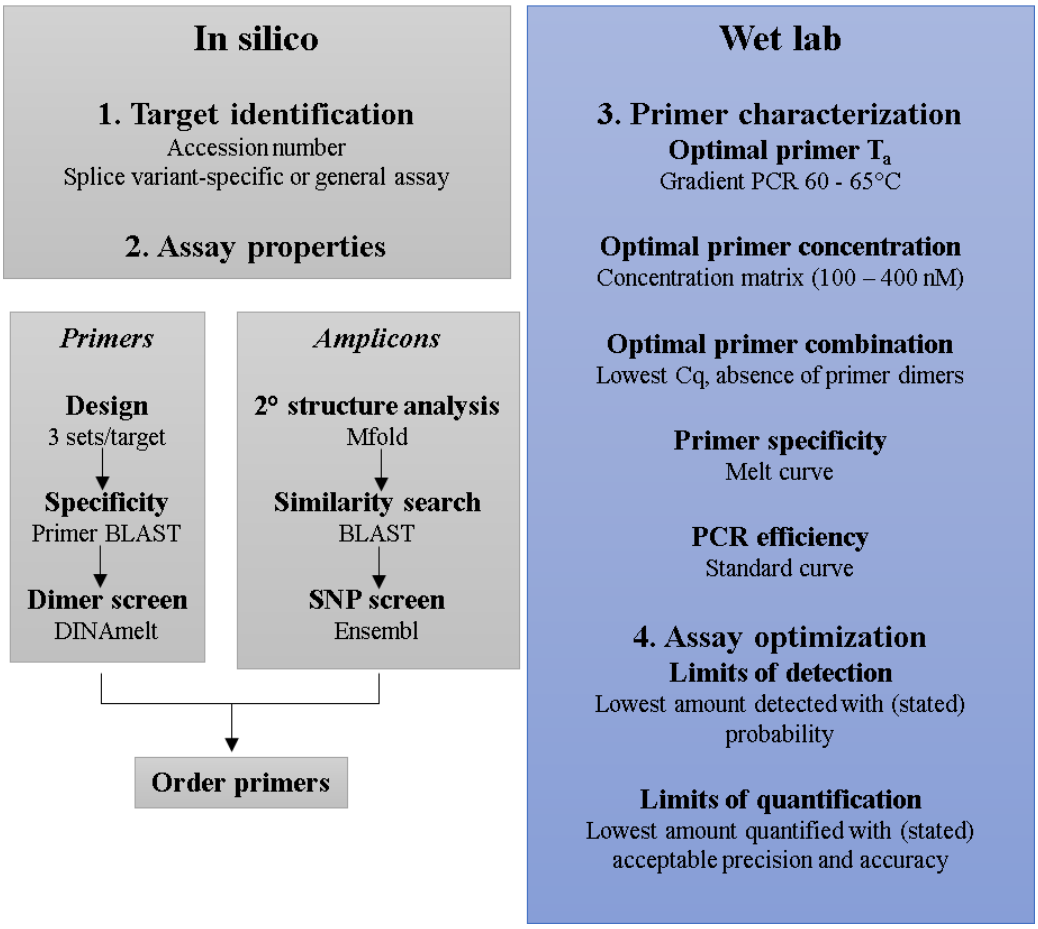

Figure 6.1 *PCR primer design workflow (Adapted from Bustin & Huggett, 2017). The in-silico workflow was employed during design and optimisation of Mycogone spp. primers and the wet lab methodology were mainly used during optimisation and transference of primer sets to digital PCR platforms.*

6.2.4. qPCR WITH SYBR® GREEN DYE

Real-time or quantitative PCR (qPCR) reactions were conducted in 100 µL thin-walled tubes using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Johannesburg). Each 25 µL reaction mixture contained 12.5 µL SYBR® Premix Ex Taq™ II (Tli RNaseH Plus), 1 µL PCR Forward Primer (10 µM), 1 µL PCR Reverse Primer (10 µM) 5 µL Template (<100 ng) and 5.5 µL ddH_2O. Thermal cycling consisted of an initial denaturation at 95°C for 2 min followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 45 s, and extension at 72°C for 1 min 30 s. Following amplification, melting curves were acquired on the FAM channel using 1°C steps with a hold of 50 s at each step from 65 to 95°C. When required, samples were analysed by 1.25% agarose gel electrophoresis with the addition of EtBr nucleic acid stain (Sigma-Aldrich, Johannesburg) using standard methods and procedure.

6.2.5. DROPLET DIGITAL PCR (ddPCR)

Detection and quantitation of fungal isolates by ddPCR was experimented with, using the same primers as in end-point PCR reactions described earlier. The ddPCR assays were carried out in 20 µL reaction

mixtures containing Bio-Rad ddPCR master mix, 1.0 µM of each primer and 1 µL of DNA. Reactions were loaded into eight-well cartridges and droplets were generated by processing with 70 µL of droplet generation oil using the Bio-Rad QX100™ Droplet Generator. Following droplet generation, the water-in-oil droplets were transferred using a multichannel pipette to a single 96 well PCR plate (Eppendorf, Johannesburg) which was heat sealed with the foil plate sealer (Bio-Rad) and placed in a Bio-Rad CFX96 thermocycler (ramping speed at 2.5ºC/s) for amplification. The PCR amplification was performed with a thermal profile of denaturation at 95ºC for 10 min, followed by 40 cycles at 94ºC for 30 s, 57ºC for 60 s followed by 98ºC for 10 min.

Experimental design consisted of two to three biological replicates for each primer set. For each biological replicate, at least two technical replicates of each PCR reaction were run. A full experiment containing all replicates and controls were analysed on a single (typically 96-well) plate. Quantitative PCR differentiated data were analysed using the CFX Manager™ software (Bio-Rad Laboratories, CA, USA), whereas ddPCR data was analysed in QuantaSoft™ software (Bio-Rad) following manufacturer's recommendation. Frequency of accepted droplets during ddPCR reactions were monitored and all wells with <10,000 accepted droplets were excluded from the analysis. Positive droplets were discriminated from negative droplets by applying a fluorescence amplitude threshold above the negative droplets in NTC wells. Target concentrations in copy number per mL reaction were automatically calculated by analysis software.

6.3. RESULTS

6.3.1. TRADITIONAL PCR - *MYCOGONE* SPECIES DETECTION

The consistency of correctly amplified results proves sensitivity of MYC5 and MYC2R primers in detecting the presence of *Mycogone* in various matrices (data not shown). Most importantly, it has been shown that these primers do not react with any other fungal genera often present during mushroom production. This was confirmed by PCR and BLAST searches. Extracting good-quality DNA directly from any fungal material, using the DNeasy® Plant Mini DNA and SoilMaster extraction kits, makes routine diagnosis of *M. perniciosa* more cost and time effective. This is considered critical, particularly considering management strategies for wet bubble disease. Successfully extracting fungal DNA directly from complex matrixes such as casing material has been achieved. Moreover, the DNA extracted can be used with very specific and robust *Mycogone* spp., nested primers during a detection analysis.

Amplification reactions, when using the MYC5-ITS4 primer set, yielded a PCR amplicon of approximately 560 bp (Figure 6.1). Results obtained from previously sequenced isolates, including the comparative standards CBS658.82 and PPRI5784, confirmed identities of positively amplified DNA

based on sequence data during every PCR reaction (Figure 6.2). The BLAST analysis done using the primer specific for *M. perniciosa* (MYC5) showed no significant similarities. Isolates of unknown identity were tested, and PCR results compared to the source from which they were isolated. In all cases, the isolates were *M. perniciosa*, which was also consistent with the cultural morphology and growth rate characteristics for this organism on duplicate culture plates. In all assays, no reactions were obtained from the *A. bisporus* and water starting material controls. The same results were obtained for all five replications (data not shown).

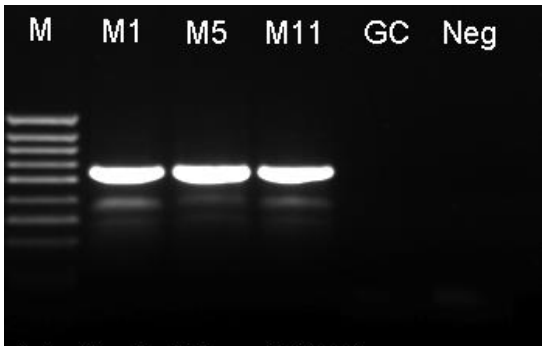

Figure 6.2 *DNA amplification products from primers ITS4 and MYC5. Sequenced Mycogone perniciosa isolates, M1, M5 and M11. GC denotes a fungal DNA control and Neg is a negative water control. The first lane is a 100 bp ladder.*

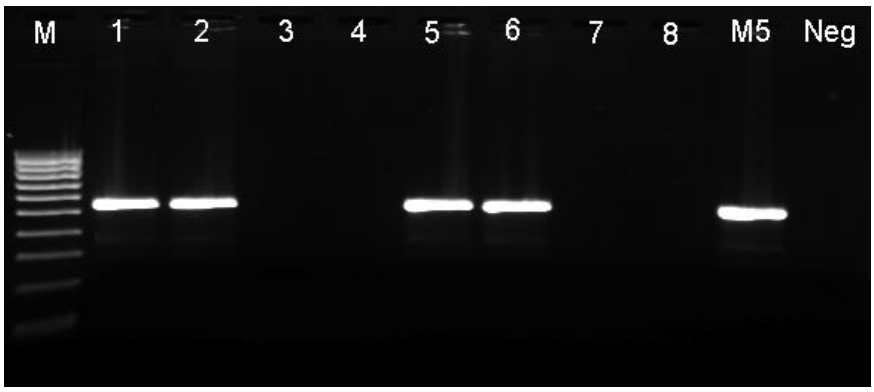

Figure 6.3 *Use of species-specific primer (MYC5-ITS4) on DNA from mushroom tissue and casing soil samples.*

6.3.2. qPCR AMPLIFICATION (ASSAY SENSITIVITY AND SPECIES DETERMINATION)

Table 6.3 shows the sensitivities of detection for all mushroom pathogens by qPCR where within multiple amplification reactions on average 30 copies for each respective pathogen were obtained. Plotting calibration curves based on dilutions of the standards and Cq values resulted in slopes ≥1.368 and R^2 values of ≥0.999 for all assays (Figure 6.4).

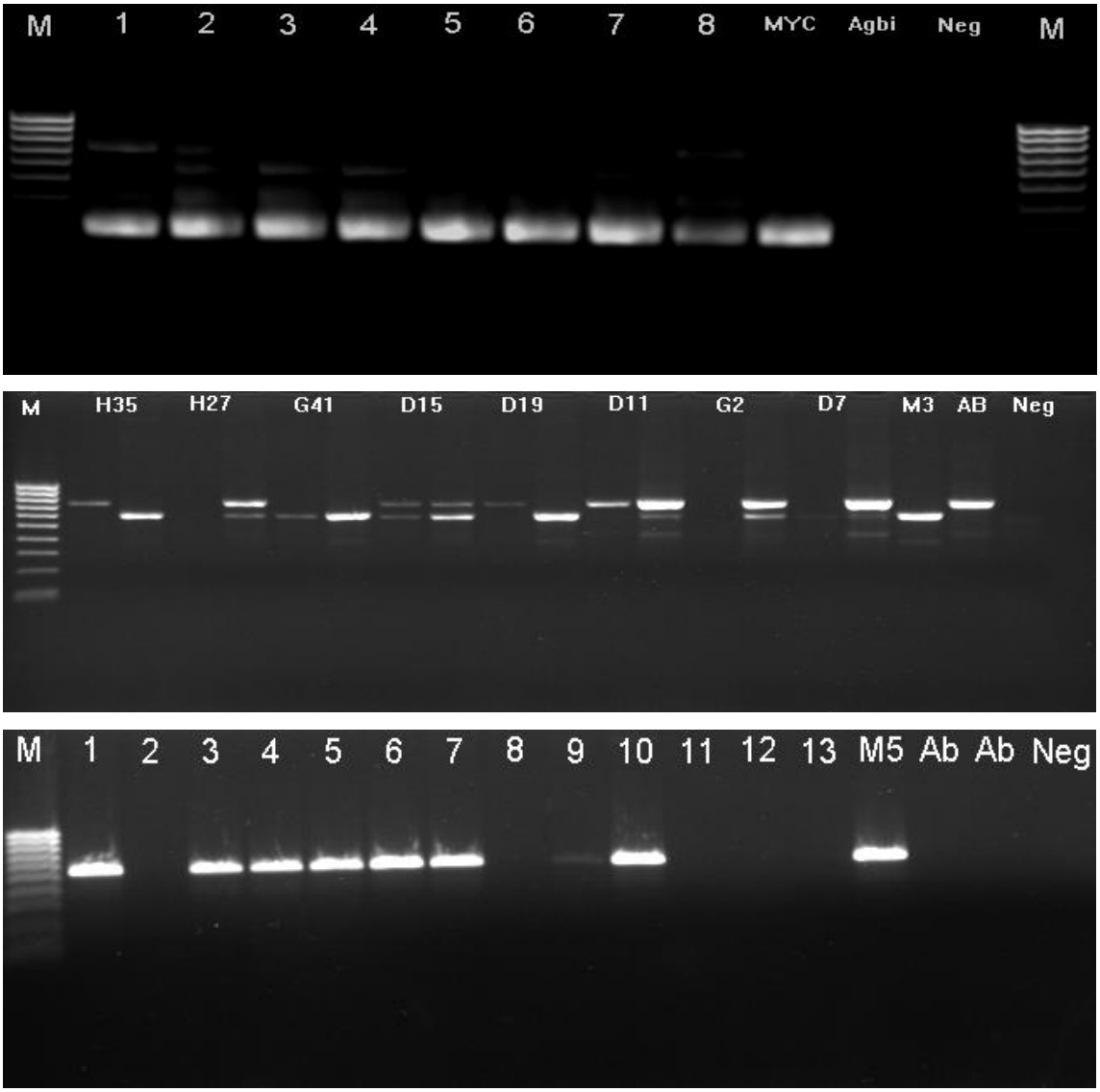

Figure 6.4 *Use of nested PCR primer set (MYC5-MYC2R)*

Table 6.3 *qPCR detection and quantitation of fungal pathogen DNA standards*

Trichoderma aggressivum[a]	Cq		***Cladobotryum spp.***[a]	Cq		***Lecanicillium spp.***[a]	Cq		***Mycogone spp.***[a]	Cq	
	Mean	SD		Mean	SD		Mean	SD		Mean	SD
1 x 10^6	29.58	0.663	**1 x 10^6**	33.05	0.560	**1 x 10^6**	34.96	0.183	**1 x 10^6**	24.78	0.296
1 x 10^5	32.05	0.061	**1 x 10^5**	35.0	0.049	**1 x 10^5**	36.27	0.225	**1 x 10^5**	30.21	0.921
1 x 10^4	34.22	0.109	**1 x 10^4**	37.04	0.078	**1 x 10^4**	37.7	0.127	**1 x 10^4**	34.79	0.387
1 x 10^3	36.68	0.316	**1 x 10^3**	39.04	0.398	**1 x 10^3**	39.04	0.494	**1 x 10^3**	40.12	0.742
H_2O	0.0	0.0	**H_2O**	0.0	0.0	**H_2O**	0.0	0.0	**H_2O**	0.0	0.0

a Values reflecting copy numbers per 25 µL reaction based on serial dilutions of extracted pathogen DNA by concentration. Data represents the means ± standard deviations of each dilution tested in triplicate from a representative experiment.

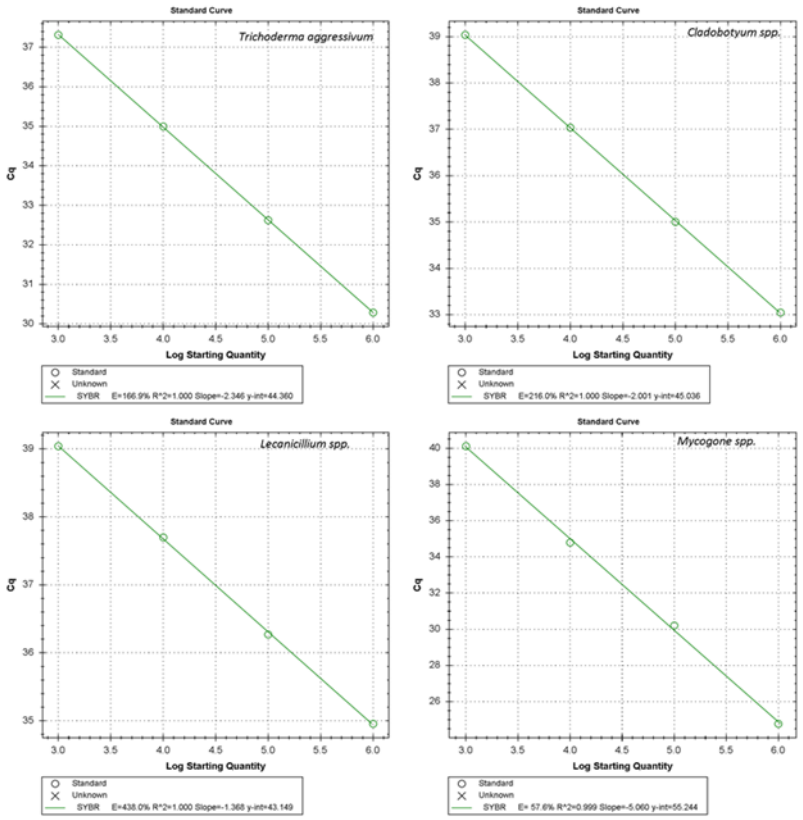

Figure 6.5 *Calibration curves of qPCR assays run with standards (positive controls) for each mushroom pathogen. Trichoderma aggressivum (top left panel), Cladobotryum species (top right panel), Lecanicillium species (bottom left panel), and Mycogone species (bottom right panel). The data correspond to a representative experiment performed in triplicate.*

Analytical specificity of qPCR showed that positive amplification of pathogen specific target regions is possible irrespective of the source of DNA, i.e. from pure culture or the environment. However, all assays were only performed after a DNA extraction protocol, in order to prevent unanticipated inhibition by diverse matrices especially if the sample was not obtained from pure culture. The assays produced distinct melting profiles (Figure 6.5). Melt curve analysis yielded melting temperature (T_m) values varying from 70 to 90°C, with reproducible results for each species. Each pathogen group had a characteristically shaped melt-profile, the analysis proved to be very reproducible in replicate studies when different isolates of the same species were used, which is indicative that little sequence heterogeneity exists in the region targeted for each assay.

6.3.3. ddPCR AMPLIFICATION

Sensitivities of adapted ddPCR assays were comparable with qPCR when using the same primers as both assays obtained positive signals down to as low as 10 copies of diluted standard DNA. An initial

difference was observed by the need to dilute samples with more than 100,000 copies of the target amplicon. Copy numbers detected by using ddPCR amplification of serially diluted DNA positives are shown in Table 6.4. A visual representation of positive and negative ddPCR droplet reactions for diluted pathogen DNA with minimum threshold for droplet positivity is shown in Figure 6.6. Primer set optimisation included an annealing temperature gradient PCR. A thermal gradient PCR from 55 to 61°C were run to check for the annealing temperature which would best separate the positive droplets from the negative. Little difference was observed in the negative droplet amplitudes (~10 000) between all the temperatures. At lower annealing temperatures (55 to 57.3°C) the amplitude distribution of the positive droplets was relatively high. From 59.8 to 60.6°C the amplitude distribution of the droplets showed a slight decline and therefore the annealing temperature used during subsequent analyses was 57°C. It was observed that the amount of starting template DNA added per reaction can also affect the degree of droplet population separation. Although the input masses for positive DNA samples of each pathogen was relatively high, additional ddPCR experimentation during this study has shown that positive amplification is possible with DNA input concentrations as low as 0.105 µg/µL. However, the optimal input DNA mass ranged between 95 to 100 µg/µL, subsequent analyses were performed using this range guideline for starting DNA concentrations.

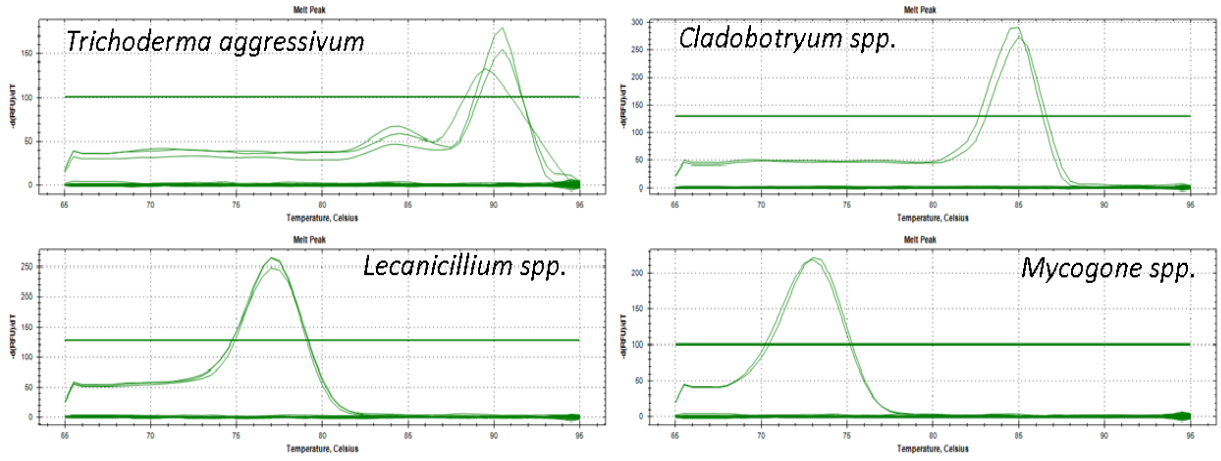

Figure 6.6 *Melt curve analysis after qPCR amplification. Trichoderma aggressivum (top left panel), Cladobotryum species (top right panel), Lecanicillium species (bottom left panel), and Mycogone species (bottom right panel). Blank reactions without any DNA were used as negative controls.*

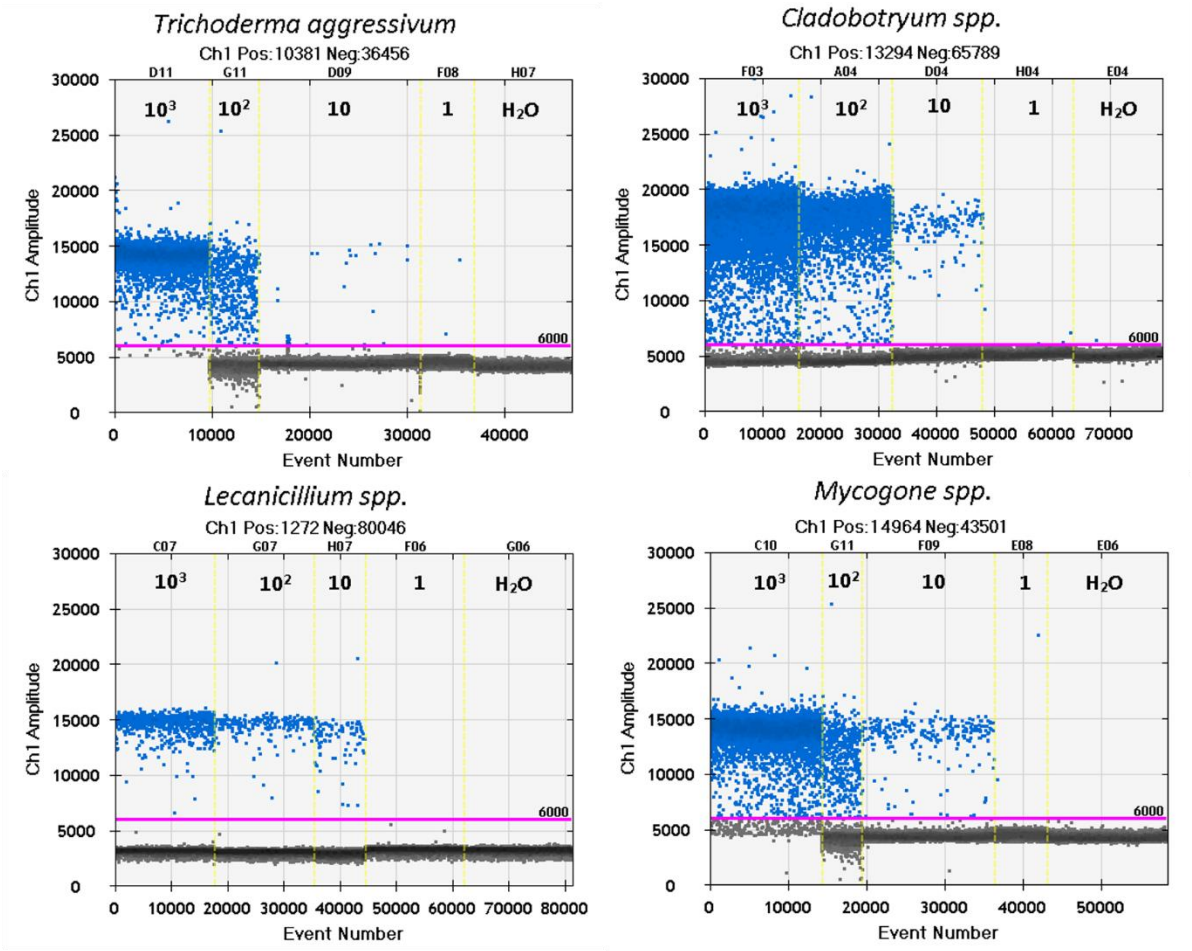

Figure 6.7 *ddPCR droplet reactions for mycoparasitic pathogens of Agaricus mushrooms, Trichoderma aggressivum (top left panel), Cladobotryum species (top right panel), Lecanicillium species (bottom left panel), and Mycogone species (bottom right panel). Minimum threshold for droplet positivity is shown with a pink line. Positive droplets in blue and negative droplets in gray-scale are shown for all pathogen reaction dilution standards (10^3 to 10^0) and water as negative control.*

Table 6.4 *ddPCR detection and quantitation of mushroom pathogen DNA standards*

Trichoderma aggressivum[a]	ddPCR value[b]		*Cladobotryum spp.*[a]	ddPCR value[b]		*Lecanicillium spp.*[a]	ddPCR value[b]		*Mycogone spp.*[a]	ddPCR value[b]	
	Mean	**SD**		**Mean**	**SD**		**Mean**	**SD**		**Mean**	**SD**
1 x 10^3	7200	150	**1 x 10^3**	1085	115	**1 x 10^3**	66.2	6.4	**1 x 10^3**	4730	170
1 x 10^2	122	26.5	**1 x 10^2**	260	31.4	**1 x 10^2**	13.5	0.12	**1 x 10^2**	122	19.4
10	5.6	0.19	**10**	11.9	0.15	**10**	1.7	0.0	**10**	18.1	0.17
1	0.4	0.0	**1**	0.45	0.0	**1**	0.0	0.0	**1**	0.5	0.0
H_2O	0.0	0.0	**H_2O**	0.0	0.0	**H_2O**	0.0	0.0	**H_2O**	0.0	0.0

[a] Values reflecting copy numbers per reaction based on serial dilutions of extracted pathogen DNA by concentration.

[b] Copies per 25 µL reaction detected. Data represent the mean ± standard deviations of each dilution tested as three replicates from a representative experiment.

6.4. DISCUSSION

As proof-of-principle and method development for quicker and more accurate detection of four fungal pathogens of commercial importance to white button mushroom production, molecular assays using PCR technology were designed. These amplification reactions were evaluated for the ability to qualitatively amplify mushroom pathogens present in the environment and on mushrooms sampled from mushroom farms. Primers could be considered as a critical aspect to successful amplification in any PCR assay, as their properties control specificity and sensitivity that make PCR-based methods so useful in molecular diagnostics. Poor design and failure to optimize reaction conditions could also lead to reduced technical precision and false positive or negative detection of amplification targets (Bustin & Huggett, 2017).

Currently, wet bubble disease is symptomatically diagnosed when symptoms appear during mushroom production within growing rooms. Identification is primarily done by visual identification (morphologically) yielding low efficacy and accuracy; therefore, a more consistent and sensitive method were needed to positively detect and identify *M. perniciosa*. Initial development, with traditional PCR considered low concentrations of DNA obtained from swab samples or other starting materials such as mushrooms or casing problematic, due to inherent properties of this technology having difficulty in amplifying very low starting concentrations of potentially contaminated DNA. The advancement of more sensitive methods later during the study qPCR and ddPCR, proved this initial challenge of low template concentration void. Casing soil or peat may be considered complex matrixes, usually with a low proportion of pathogen DNA in the total community DNA extracted, as well as high amount of co-extracted contaminants (e.g. humic acids) can reduce the amplification efficiency (Wilson 1997; Oros-Sichler *et al*. 2006). Diluting or purifying crude DNA before PCR might be effective in eliminating inhibitors but might also critically lower fungal template concentrations. Direct PCR using the traditional method during development of primer sets suitable for amplification of *Mycogone* spp., initially failed to amplify the fragment of interest within the total DNA extracted from casing soil and compost samples. Where amplicons could be obtained, they were often rather faint. The use of a two-step PCR with nested primers were expected to increase both the sensitivity and the specificity of the PCR significantly which were theorized to overcome problems with relatively impure DNA extracts and low pathogen numbers. Therefore, a nested- PCR, based on the initial amplification of 560 bp of the ITS regions with a universal primer combination (ITS1/ ITS4) was developed, followed by a subsequent amplification with MYC5/ MYC2R. By means of this PCR approach distinct ITS fragments of 280 bp could be reproducibly generated for casing soil and compost samples (Figure 6.3). The application of the information contained within the ITS region of rDNA for diagnostic purposes is well documented (Rehner & Uecker 1994; Waalwijk *et al*. 1996; Jacobs & Rehner 1998; Martinez-Culebras *et al*. 2000; Weiland & Sundsbak 2000). Further, PCR-based techniques for diagnosis and differentiation of pathogens have proven to be very useful. The MYC5 primer was designed to detect

M. perniciosa in conjunction with the ITS4 primer in a PCR test from diseased mushroom samples and pure fungal cultures. The positive PCR identification of all sequenced isolates proved that this assay is robust and reliable, since the PCR results obtained using this primer set were in accordance with the described sequence data. This was further confirmed by the positive identification of the Centraalbureau voor Schimmelcultures (CBS658.82) and Plant Protection Research Institute (PPRI 5784) cultures of *M. perniciosa*. This experiment produced a workable primer set, confirmed to be accurate and sensitive enough for consistent amplification of *Mycogone* spp. pathogens within the laboratory. Reliability and robustness within a commercial setup when used as a tool for disease detection still need to be proven. Disease monitoring and detection over a five-year period from samples to be collected on mushroom farms (Chapter 7), will establish longer term efficiency or shortcomings of the designed assay.

Initial design and adaptation of primer sets to qPCR and ddPCR were subject to considerations of assays which will be run under standard and constant PCR conditions, consisting of a short melting period, followed by single annealing and elongation in such a way that automation and the running of different assays could be achieved under the same method parameters. Slightly higher hybridisation temperatures were chosen compared to standard 60ºC, as most polymerases work best at temperatures of 72ºC. This also has the advantage of ensuring less polymerisation, which helps shortens the time needed to complete a PCR run (Bustin, 2017). Annealing temperature (T_a) profiles for primer sets can vary widely and if an assay is not sufficiently robust it can perform poorly when not run at the optimal T_a for each of the primer sets. Primers used in this study has shown to have an optimal temperature range with similar Cqs (average ΔCq = 0.69) between 56 to 58ºC. PCR reactions performed at temperatures outside of this optimum i.e. 60ºC showed less robustness resulting in significantly lower Cqs from the optimum (average ΔCq = 8.13). In support of having a robust assay, specificity is just as important during reactions, where the melt curves reveal single peaks which are indicative of the assays remaining specific in the temperature range tested (Banowary *et al*., 2018). Melt curves for data for pathogen assays showed distinct peaks that were obtained consistently during consecutive reactions for each of the pathogens. Sensitivities for qPCR assays were evaluated by performing a dilution series of positive control DNA. Generating standard curves using dilutions of template as well as determining the slope from linear regression of a plot of Cq (y-axis) vs log [quantity] is the best way to determine amplification efficiency (Bustin, 2010). Sensitivity is not dependent on Cq value, in fact it is the ability of an assay to reliably amplify and detect low copy number targets.

An overall limitation of molecular detection from conventional to digital PCR is the lack of distinction between live and dead spores/cells as described by Cangelosi & Meschke (2014), without the addition of supplementary tests and analysis (discussed and addressed in more detail in Chapter 7). But as opposed to microscopy or culturing, which can be time consuming and requires proficiency in the

detection of parasite morphology, quantitative PCR assays, such as ddPCR, is very useful in high throughput screening scenarios such as that found or prosed on commercial mushroom farms for disease detection. Two areas of optimization were considered during method transfer from traditional PCR to digital PCR assays as these became more important compared to method sensitivities increasing; 1) template DNA concentrations, 2) annealing temperature and temperature gradients. Droplet digital PCR results from three different concentrations of the sample DNA were assessed. The concentration of DNA was chosen with the aim of obtaining an identical number of both positive and negative droplets with good separation between them during amplification. The lowest template concentrations resulted in the highest amplitude difference between the positive and the negative droplets, this was also observed by Gutiérrez-Aguirre *et al*. (2015) in their study using ddPCR when detecting bacterial pathogens.

Observations from doing experiments on both platforms confirmed that ddPCR does not need standard curves during analysis and for quantification purposes (Abachin *et al*., 2018). The ddPCR methodology quantified the absolute number of copies added to the reaction by partitioning the PCR volume and by using Poisson distribution estimations (Hindson *et al*., 2011; Pinheiro *et al*., 2012; King *et al*., 2017). In addition, partitioning of individual reactions into a picolitre droplet scale, allows ddPCR to have reduced interference with PCR inhibitors present. Due to the potential that most samples taken in future implementation of these methods will contain contaminating material from the environment in which they are collected, this principle bestows a significant advantage when considering accuracy or trueness of these tests. Yang *et al*. (2014) observed that without the need to run calibration samples, this reduces the bias introduced by the qPCR method in assays. Overall sensitivities of the ddPCR assays were comparable with qPCR when using the same primers in the amplification of positive controls. The characteristic melt curves were reproducible consistently from run to run, during replicate studies. Equally important, amplification reproducibility was maintained across different strains of the same pathogen genus. The ddPCR showed better performance, compared to qPCR, during the quantification of low target numbers molecules and this would be an advantage in samples containing a low number of the target pathogen (Rajapaksha *et al*., 2018). Environmental samples taken from mushroom farms normally contains low pathogenic fungal counts. A limitation of ddPCR as compared with qPCR is the need to dilute samples with ≥100,000 copies of the target DNA (Henrich *et al*., 2012; Wilson *et al*., 2015). This is because overloading the assay may affect the efficient partitioning of standards into reaction droplets which may in turn result in a loss of linearity at high template concentration (Personal communication – BioRad technical representative).

From this chapter, the developed assays were able to detect available strains of the targeted organisms using conventional- and digital PCR-based methods. Pathogen-specific DNA targets showed no cross-

reactivity and could be clearly differentiated between possible contaminating DNA such as that of mushrooms and other environmental microorganisms.

6.5. CONCLUSION

A highly sensitive and easy-to-adopt digital PCR method have been developed and validated for organism specific detection and quantification of *Trichoderma agressivum*, *Cladobotryum* spp., *Lecanicillium* spp. and *Mycogone* spp. Through adaptation of a previously developed qPCR assays and primer development/adaptation, the potential and applicability of ddPCR to target mycoparasitic fungal pathogens have been shown. This technology will enable mushroom farmers/growers to identify critical areas of contamination and infection early in production; enabling them to act in a preventative rather than a curative approach in disease control. As digital PCR is adaptable to multiplexing (Zhong *et al*., 2011; Wood-Bouwens & Ji, 2018), a quadruplex ddPCR assay is foreseeable to simultaneously detect all four pathogens using different fluorophores combined with the primers described in this study. As this is an initial demonstration of the promising utility of ddPCR in pathogen detection and identification, applications requiring unbiased and consistent quantitative results as well as future field case studies are needed to further evaluate its full spectrum of merits and limitations.

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CHAPTER 7

MUSHROOM DISEASE DETECTION AND FARM BIOSECURITY

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ABSTRACT

Mushrooms are susceptible to various diseases of which the causal agents may be bacterial, fungal or viral. Four mycoparasitic fungal diseases are of economic importance on white button mushrooms, *Agaricus bisporus*, in the South African commercial mushroom industry and include; compost green mould (*Trichoderma aggressivum*), dry bubble (*Lacanicillium spp.*), cobweb (*Cladobotryum spp.*) and wet bubble (*Mycogone spp.*). Molecular test methods have been developed, optimized and used on commercial mushroom farms to detect the presence of these pathogens prior to disease or symptom expression. The use of standard microbiological methods and advanced PCR-based identification techniques provided a diagnostic platform for accurate and reliable monitoring. Mycoparasitic pathogen presence were monitored in the main process areas on 13 farms over a period of five years. Samples (n = 912) were collected from three thematic areas i.e. composting, preparation and production areas for which presence or absence of pathogens were determined as well as comparative viability of detected organisms using culturing techniques. Results showed that all four fungal pathogens were molecularly detected in the three production areas monitored, although *T. aggressivum* and *Cladobotryum spp.* were the most frequently identified. Isolation trends also showed that all pathogens were most frequently detected and isolated from production areas, especially growing rooms. The development and implementation of a rapid molecular detection method have proven to be a vital instrument in assisting farmers control mushroom disease occurrence more effectively by acting as an early warning system within the production process.

7.1. INTRODUCTION

Commercial strains of cultivated mushrooms, specifically *A. bisporus* (J.E. Lange) Imbach 1946, exhibit an attractive morphology and texture ensuring popularity amongst consumers but are susceptible to a wide range of biotic and abiotic disease-causing agents and factors (Fletcher *et al*., 1989). Biotic agents such as bacteria, fungi, viruses, nematodes, mites and insects can cause damage to mushrooms directly and/or indirectly, depending on the organism. Mycoparasitic fungal pathogens are of major economic importance to the cultivation of button mushrooms. These pathogens have a reputation due to their effect on the production yield and the quality of the mushrooms produced (Savoie *et al*., 2016). These fungi are commonly encountered in compost and casing soils and predominantly act as both competitor and parasitic moulds, which in turn can affect spawn run or fruit body development of white

button mushrooms at various stages of crop growth. The research focus of this chapter was specifically on the four major (most common) fungal pathogens found within the commercial mushroom cultivation industry in South Africa. Diseases and causal species investigated included, Green mould (*Trichoderma aggressivum* Samuels & W. Gams 2002), dry bubble (*Lacanicillium* spp.), cobweb (*Cladobotryum* spp.) and wet bubble (*Mycogone* spp.), responsible for considerable disease outbreaks worldwide (Largeteau & Savoie, 2010). Outbreaks may lead to serious crop losses of up to 10-100% depending on the severity of infection. The estimated crop losses in western countries each year due to *L. fungicola* and *T. aggressivum* alone account for approximately 25% of the total production value in these countries (Soković & van Griensven, 2006).

Trichoderma aggressivum emerged as an aggressive compost mould during the mid-1980s and 1990s (Williams *et al*., 2003; O'Brien *et al*., 2017). If freshly spawned compost got contaminated with *T. aggressivum*, it could cause disease that decimated mushroom production (Grogan, 2008). Dry bubble disease is commonly found in all major mushroom producing countries. Many mushroom farmers struggle to produce a fourth flush of mushrooms because of the susceptibility and incidence of later flushes to dry bubble disease (Staunton *et al*., 1999; McGee *et al*., 2017). Cobweb disease of white button mushrooms is caused by several *Cladobotryum* species (Adie *et al*., 2006; Back *et al*., 2010). The causal agent of this disease may be found globally in areas where mushrooms are cultivated (Carrasco *et al*., 2017). Wet bubble disease has been known for many years and has been reported worldwide wherever mushrooms are cultivated (Brady & Gibson, 1976; Fu *et al*., 2016). The major characteristics for each of the four diseases and pathogens of interest are summarized in Table 7.1.

Table 7.1 *Summary of the four main fungal pathogens of economic importance on commercially cultivated white button mushrooms*

Disease	Most Common Causal Pathogen	Inoculation Source	Symptomology	References
Cobweb	*Cladobotryum dendroides*	Airborne conidia from infected beds/ soil	Dense white to pinkish patches on casing, brown spots on mushroom caps	Adie *et al.*, 2006; Back *et al*., 2010; Grogan & Gaze, 2000; Fletcher & Gaze, 2008; Fletcher *et al.*, 1989; Mckay *et al.*, 1999; Potočnik *et al.*, 2010.
Dry Bubble	*Lecanicillium fungicola*	Conidia in dust, on flies, infected beds, contaminated casing	Undifferentiated tissue with liquid droplets in early symptom expression, distorted caps and stalks, spots on caps	Berendsen *et al*., 2010; Fletcher & Gaze, 2008; Fletcher *et al.*, 1989; Romaine *et al.*, 2002.
Green Mould	*Trichoderma aggressivum*	Spores from infected tissue or soil	Dense white mycelium on growing beds at spawn run; turns dark green during sporulation	Fletcher & Gaze, 2008; Muthumeenakshi *et al*., 1994; Samuels *et al*., 2002.
Wet Bubble	*Mycogone perniciosa*	Conidia/aleuriospore from infected tissue, contaminated casing or soil	Small dark brown droplets of liquid on undifferentiated mushroom tissue, white fluffy patches on casing material	Fletcher & Gaze, 2008; Fletcher *et al*., 1989.

Detection of these pathogens are difficult as disease presence are usually only noticed when symptoms are visually expressed on growing beds and mushrooms alike. Early detection of pathogens on growing

beds or in production and preparation areas will allow farmers and growers the opportunity to act in a preventative, rather than remedial manner when managing farm biosecurity and disease spread. A molecular diagnostic platform was developed and optimized in Chapter 6 for pathogen specific detection of the four fungal pathogens identified for on-farm monitoring. In this chapter implementation of the PCR-based method for commercial farm monitoring and pathogen detection was employed and used in conjunction to traditional culture-based detection methods. The ddPCR method described in the previous chapter was optimised towards the end of the five-year monitoring period, as access to this technology platform was only available in the department during the final stages of this research. As part of suggested continuation of the project, farm monitoring using the ddPCR method is not included in this research chapter's results. The major aims of this chapter were 1) to successfully implement the developed molecular testing methods described in Chapter 6, 2) to monitor disease presence and prevalence on commercial mushroom farms and identify any isolation patterns for each organism , 3) identify the main on-farm areas for persistence of pathogens, 4) compare methods in terms of viability of pathogens, which will indirectly indicate possible risk associated for further infection once a positive identification has been made from samples.

7.2. MATERIALS AND METHODS

7.2.1. SAMPLING

Thirteen commercial and smallholder mushroom farms, forming part of the South African Mushroom Farmers' Association, were monitored twice a year over a period of five years. A summary and overview of the health check sampling and analysis process can be viewed in Figure 7.1. Sample collection were according to a set protocol ensuring uniformity and non-bias during collection of samples (UP-SOP/GT012). The sampling areas considered were critical spaces and points during production activities most likely to harbour pathogens and which have influence on processes further down the production chain. Some of the samples were also collected in locations suggested by the grower/farm manager. A total of 912 samples were collected by swabbing the sampling areas in duplicate according to standard swab procedures (ISO 18593, 2004). On the sampling spot/area, the first swab taken was a DNA Flocked Swab (MicroRheologics), and the second swab taken on the same sampling area was a wetted cotton/ "dry" swab (Copan). Each Flocked swab was placed in a sterile 1.5 mL Conical screw cap microtube (WhiteSci) containing silica beads. Cotton swabs were placed in their original casing after sampling for transport and further processing at the laboratory. All samples were clearly marked with the location, date and sample number. Sampling areas on farms were divided into three broad thematic categories i.e. composting area, preparation area and production area (Table 7.1). On-farm functions such as casing, spawning, mushroom growing etc. were then classified into their characteristic functional groups and representative samples were collected in each corresponding assortment.

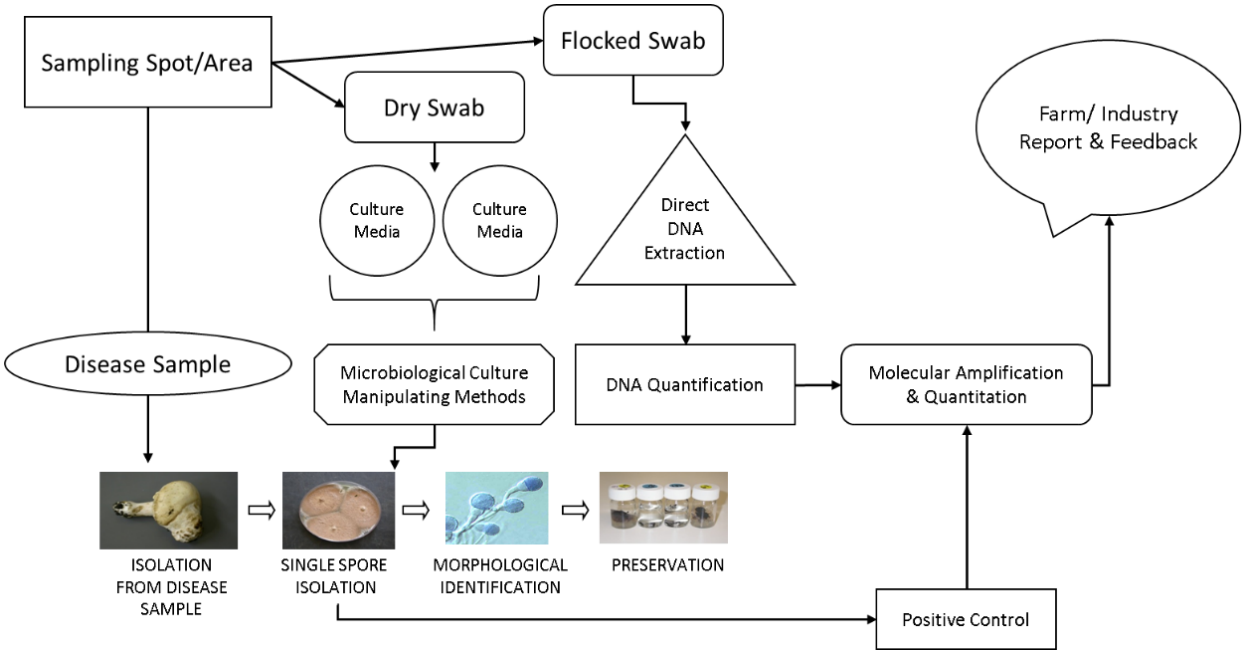

Figure 7.1 *Mushroom pathogen health check process overview*

Table 7.2 *Typical sampling areas during a "Farm Health Check"*

On-farm categories and areas	Primary sampling function	Alternative* sampling function
Composting area		
One composite sample for each	Spawn hopper	Spawning conveyor
	Compost emptying hopper	Tunnel emptying equipment
	Conveyor belts	Conveyor rollers
	Compost trailer	Compost conveyor
Preparation area		
One composite sample for each	Compost hopper	Compost bunker
	Spawn hopper	Spawn dispenser
	Head filler machine	Relevant filling equipment
	Head filler main belt	Relevant equipment used
	Compost leveller	Relevant equipment used
	Peat shed floor	Peat bags
	Machinery used for mixing peat	Peat mixing shovels
	Ruffling machine	Relevant equipment used
	Peat transport belt	Casing buckets
Production area		
One composite sample for each, performed in three different growing rooms	Floor	-
	Walls	Room filling doors
	Beds	Growing bags
	Air duct/s	Air louvers

*Commercial and smallholder farms usually differ in farming practices, techniques and degree of automation of certain processes. Therefore, a primary sampling spot/area has been listed for each category (usually found on bigger/automated farms) as well as an alternative if the primary is not available or has been modified (usually on smaller farms).

7.2.2. SAMPLE PROCESSING, ISOLATIONS AND DNA EXTRACTION

On arrival at the laboratory (usually within 3 to 4 hours after sampling), dry swabs were placed in 9 mL buffered peptone water (Merck, Johannesburg), vortexed and plated onto a suitable growth medium such as malt extract or potato dextrose agar, amended with 250 mg/L Chloramphenicol (Caps Pharmaceuticals SA, Johannesburg) and left to incubate at room temperature (25°C) for a maximum of 14 days. Fungal isolations were made based on characteristic growth patterns and physical appearance of cultures on agar plates (morphological identification). Isolates were single-spore isolated and purified. After five days of pure culture growth, samples were prepared for DNA extraction by aseptically dry-scraping mycelia from the agar surface into extraction tubes prepared with silica-beads (Biospec Products Inc., Johannesburg). In contrast, flocked swabs were used directly in the DNA extraction process as starting material. Genomic DNA was extracted using a QIAGEN DNeasy® Plant Mini Kit (WhiteSci, Johannesburg). The experimental procedure followed was according to the quick-start bench protocol supplied with the kit. DNA was dissolved in 100 µL of elution buffer in the last step of the protocol and frozen at -20ºC. DNA concentrations of all extraction samples were determined using a Fluorometer Qubit® 2.0 (Invitrogen, Johannesburg).

7.2.3. PCR AND GEL ELECTROPHORESIS

7.2.3.1. Traditional PCR

Depending on the organism, primers used for *T. aggressivum* included Th-F (5'-CGGTGACATCTGAAAAGTCGT-3') / Th-R (5'-TGTCACCCGTTCGGATCATCCG-3') as described by Chen *et al*. (1999). Primers for *Lecanicillium spp*. comprised those described by Romaine *et al*. (2002) for targeting the genome of Vfa, Vfa-F (5'-CGGCAGGTATGTTTGCACAATC-3') / Vfa-R (5'-CCGCACCTTACGATTAGAAGTC-3'). *Cladobotryum spp*. primer sets consist of CLADO1F (5'-TCCTGTTTTCTCTTAGCGGAAT-3') / CLADO1R (5'-CCGTCCCCCAACACCAT-3'). Primer sets for *Mycogone spp.* included ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as described by White *et al*. (1990) / MYC5 (5'-CTATGTGAACCTTACCATTCTGTT-3') as described in Chapter 6. PCR reactions were conducted in 100 µL thin-walled tubes using an Eppendorf 6325 Mastercycler Pro S 96 well Thermal Cycler Vapo Protect (Eppendorf, Johannesburg). Each 25 µL fungal reaction mixture contained 0.25 µL MyTaq™ DNA Polymerase (Bioline, Johannesburg), 5.25 µL MyTaq™ reaction buffer (Biolone), 0.25 µL PCR forward primer (IDT, Johannesburg), 0.25 µL PCR reverse primer (IDT), 1 µL DNA Template and 18.8 µL ddH_2O (sterile, double distilled water). Thermal cycling for reactions consisted of an initial denaturation at 95°C for 2 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 45 s, and extension at 72°C for 1 min 30 s.

7.2.3.2. Gel electrophoresis

Agarose gels for visualization of amplified PCR products were made by adding 3.0 g of Agarose powder (SeaChem, Johannesburg) to 150 mL TBE Buffer, supplemented with 15 µL ethidium bromide (EtBr).

PCR samples were loaded onto solidified gels using 3 µL loading dye/buffer and 12 µL of each PCR sample. In addition to the amplicons, 5 µL DNA marker/ladder Hyper Ladder IV (Bioline) was loaded and run on each gel. Respective gels were run at a 100 V for 1 h 30 min whereafter DNA bands were visualised using a Vilber Lourmat (Omni-Science CC, South Africa) gel imaging system.

7.2.3.3. qPCR

Real-time reactions were conducted in 100 µL thin-walled tubes using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Johannesburg). Each 25 µL reaction mixture contained 12.5 µL SYBR® Premix Ex Taq™ II (Tli RNaseH Plus), 1 µL PCR Forward Primer (10 µM), 1 µL PCR Reverse Primer (10 µM) 5 µL Template (<100 ng) and 5.5 µL ddH_2O. Thermal cycling consisted of an initial denaturation at 95°C for 2 min followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 45 s, and extension at 72°C for 1 min 30 s. Following amplification, melting curves were acquired on the FAM channel using 1°C steps with a hold of 50 s at each step from 65 to 95 °C. Only when required, samples amplified using RT-PCR were analysed by 1.25% agarose gel electrophoresis with the addition of EtBr nucleic acid stain (Sigma-Aldrich, Johannesburg) using standard methods.

7.3. RESULTS

7.3.1. METHOD IMPLEMENTATION

By using a molecular detection protocol a much higher detection sensitivity can be achieved compared to manual culturing or plating methods. Exposing previously developed molecular methods (Chapter 6) to samples taken from an on-farm environment, a few aspects were evident during implementation and evaluation of results obtained from swab samples early on during the study. All pathogens studied are of fungal nature, which means these organisms have cell walls that could impede the efficient lysis of cells or spores and liberation of DNA, which could lead to false-negative PCR results. Fifteen instances of such false negatives were detected during earlier monitoring for both *Cladobotryum* spp. and *Mycogone* spp. Pathogen growth on corresponding culture plates made it possible to identify these occurrences (data not shown) and indicate which negative results should have been positive for pathogen presence. Sampling from a production/ agricultural environment also collects other organisms (transient or indigenous) which could influence a false positive during molecular amplification. As part of method validation, random amplification bands for each organism were isolated and purified throughout the study, sequenced and compared to genetic databases, to verify primer accuracy and sensitivity. It was established in the first year of monitoring, and at various consecutive intervals as the study progressed that primer discrimination was above a >99% confidence interval for each pathogen.

7.3.2. PATHOGEN ISOLATION AND IDENTIFICATION PATTERNS

Data trends and isolation patterns described for pathogens are not only applicable to an on-farm scenario for each of the individual farms monitored but can be extrapolated and could therefor also be

representative of pathogen trends within the button mushroom industry of South Africa. The most frequently detected and isolated pathogens from farm monitoring, highest to lowest, were *T. aggressivum*, *Cladobotryum* spp., *Lecanicillium* spp. and then *Mycogone* spp. Each of these pathogens were molecularly detected in all three production areas monitored (Figure 7.2). As a visual observation during monitoring, the latter mentioned two pathogens have also shown less recurrent disease symptoms (physical manifestation) compared to green mould and cobweb disease in growing rooms on active mushroom producing beds. A trend that is observed from the data, show that for all four pathogens there is an increase in detection frequency from the composting process up until production (growing rooms) i.e. the isolations became more frequent as the production process aged or progressed. A higher isolation/ detection rate in this instance could infer a higher concentration or pathogenic load within a given area or location, as the probability for isolation correlates directly with the number of pathogenic units in a sampling area. Results indicate that the highest probability of molecular and culture-based detection for all four pathogens are from production areas, which includes the growing rooms, associated equipment and infrastructure. This means pathogens are more regularly present in this area compared to the other two areas which are also sequentially earlier in the production process. Molecular identification trends for all pathogens show an accumulation or build-up in presence as the production process proceeds from start to finish, until a maximum pathogenic load is reached for each species within the growing areas. This was also observed when DNA extractions were performed, as extracted and amplified DNA concentrations for each pathogen were usually higher from swab samples in the growing area compared to other sampling areas.

7.3.3. MOLECULAR DETECTION VS. PLATING METHODS

When considering positive amplification frequency during PCR for each pathogen, trends show a significant difference in the amount of times pathogens are detected by molecular isolation compared to culturing. Obtaining a positive identification result for pathogen presence using PCR-based methods was found to be on average seven times as likely as detecting any of the four pathogens through a culture-based method. Culture data show the same trend when comparing pathogen frequencies, proving *T. aggressivum* the most frequently cultured, then in descending order, *Cladobotruym* spp., *Lecanicillium* spp., and *Mycogone* spp. By comparing culture data to individual area isolations, it is shown that *T. aggressivum* and *Cladobotryum* spp., are the only two pathogens that occur or were isolated in living form for all three production areas. *Lecanicillium* spp. and *Mycogone* spp. living isolates were only obtained from production areas. For each pathogen, the highest number of culture isolations from all areas monitored, were the growing rooms.

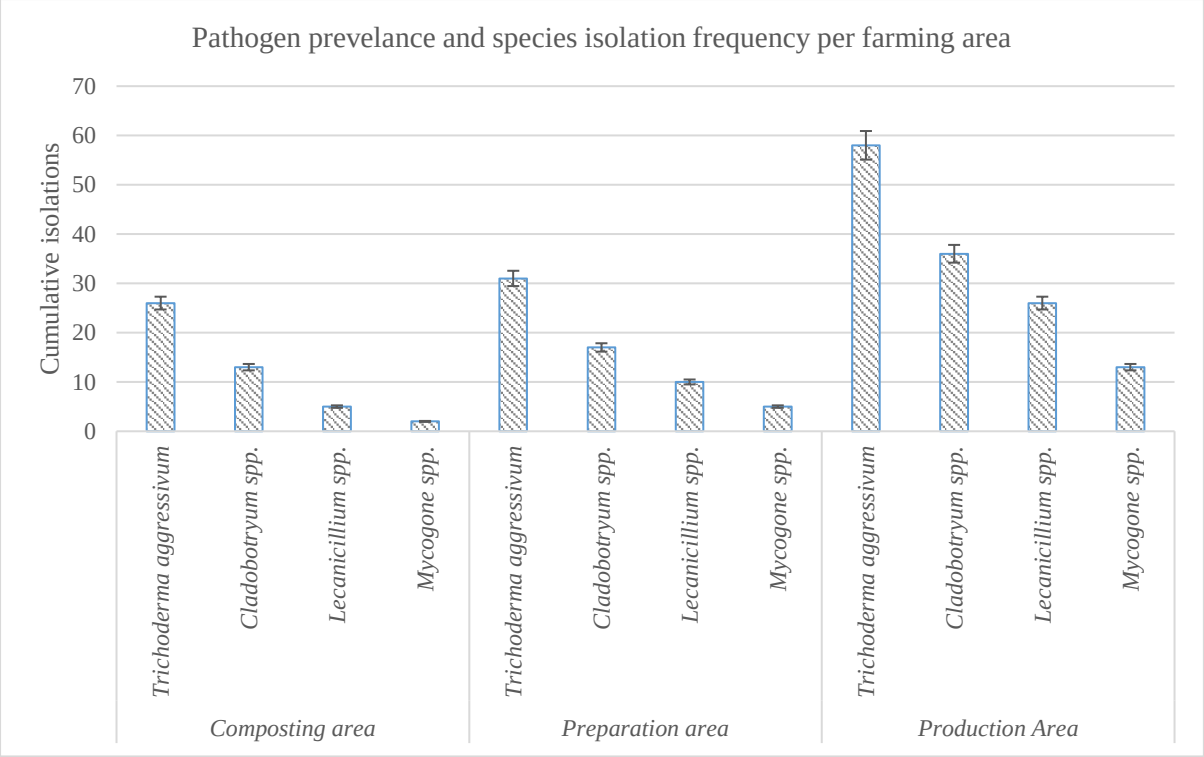

Figure 7.2 *On-farm isolation prevalence for the four investigated mycoparasitic pathogens on commercial white button mushroom farms per process location.*

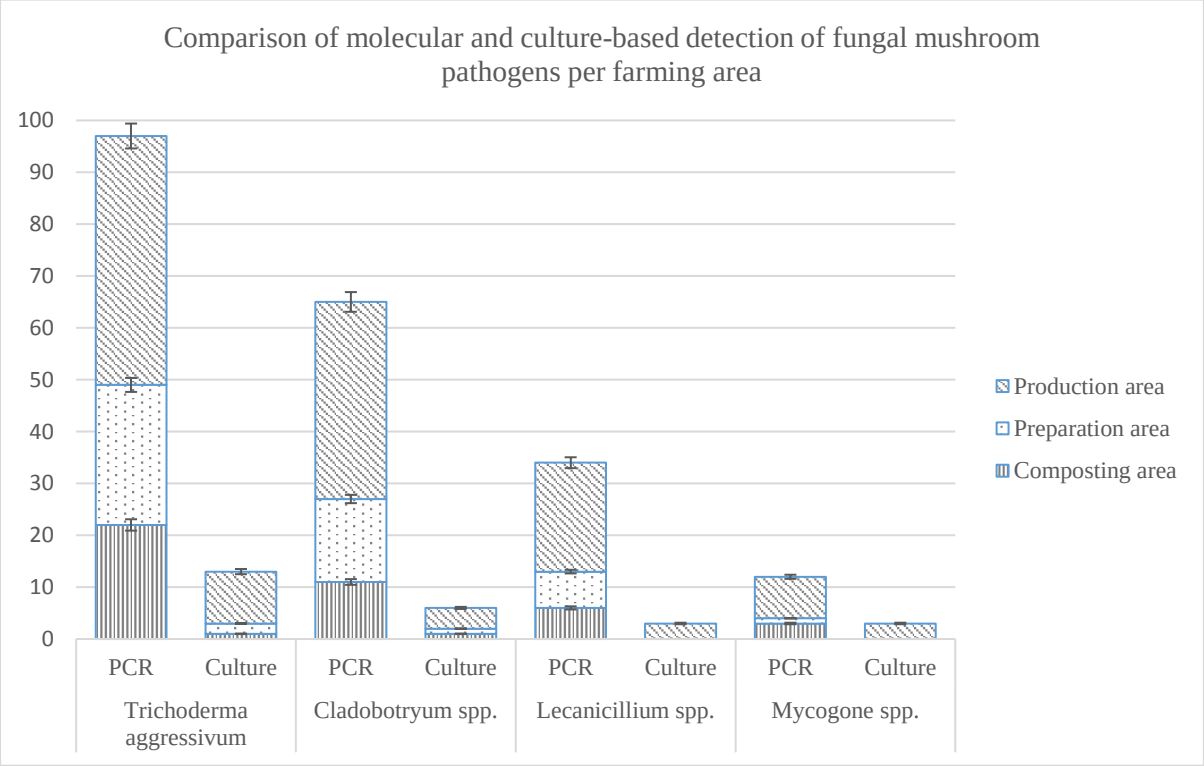

Figure 7.3 *Molecular vs. culture-based detection comparison of fungal mushroom pathogens per farming area to establish the biotic state of inoculum.*

7.4. DISCUSSION

Disease challenges and crop losses before harvest have a considerable impact on the final yield of white button mushrooms (Largeteau & Savoie, 2010). As a related aspect to the work performed in this chapter, the farm "Health Check" initiative is working towards establishing a holistic approach to farm health through assessment of various biotic and abiotic factors in conjunction to rapid DNA-based techniques. Applying a molecular method such as a PCR assay to detect pathogen specific DNA fragments from samples collected in environments found within mushroom production facilities, are a quick and accurate way of establishing identity as well as spatial presence or absence for these organisms (Jang *et al*., 2019). As a rapid technique, PCR technology can be used with greater specificity and sensitivity, compared to conventional detection methods (Rajapaksha *et al*., 2019). Establishing exact genetic characteristics of microorganisms from environmental surfaces or spaces, requires removal and consideration of possible inhibitors and contaminants during laboratory preparation and testing (Bellemare *et al*., 2018). The sampling and DNA extraction method used in this study to prepare samples for testing, have shown adequate removal and elimination of most inhibitors and possible contaminants collected from sampling surfaces. Although the methodology was designed to eliminate as much as possible variable factors which could possibly affect amplification, there may still exist an unlimited number of environmental contaminants that have not yet been identified. The method application for mycoparasitic detection of the four major fungal pathogens affecting white button mushrooms may therefore not be similarly effective in alternative environments as optimization during implementation was specifically established for on-farm/ mushroom production facility assessment.

Growing areas present a greater risk when considered as a source of contamination on mushroom farms, compared to other areas of production. Although the aim of this study did not include monitoring transference of pathogens between production areas, observational patterns indicating movement of some of these pathogens from the preparation areas into growing rooms were evident. As the most frequent isolations for all four pathogens were made from growing rooms, it could indicate that this area and the conditions within growing areas are conducive to pathogen proliferation and survival (Savoie *et al*., 2016). These conditions include substrate or growth medium in the form of mushroom compost, casing and host mycelia; conditions of high humidity as well as temperatures in the range of 18 to 28ºC. The processes of composting and preparation exhibit conditions that will favour pathogen persistence but have inherent variability as well as process steps and factors which can be considered only partially favourable, depending on the circumstance, for pathogen transference and proliferation i.e. high composting temperatures, occasional exposure to sunlight, incomplete pasteurisation etc. Growing areas present an additional threat to farm-biosecurity due to the constant movement that takes place during cultivation by workers and equipment moving between rooms and the rest of the farm areas (Kredics *et al*., 2010).

Isolations were consistently higher for each of the pathogens in consecutive production areas, which indicate that raw materials and compost are not necessarily the main contributing factors when introduction of pathogens into the growing process are considered. After composting, the final product is pasteurized to lower or minimize any transference of foodborne and fungal pathogens to any successive processes (Gurtler *et al*., 2018). Data trends indicate that in the preparation process, pathogenic presence is detected to such an extent that higher isolation values are obtained compared to the composting process. This could also implicate potential inefficient pasteurization or treatment that may allow inoculum present in compost to survive and proliferate. Peat as a raw material is introduced into the production process in the preparation area, and as standard practice are not pasteurized or cleaned before being used (Siyoum *et al*., 2016). Sphagnum peat has been associated as a source of contamination for some, but not all the pathogens, *T. aggressivum* and *Cladobotryum* spp. have a greater ability to persist in casing mediums, even as a secondary contaminant (Chakwiya *et al*., 2019).

In a similar trend to molecular identifications and isolations, culture-based detection of the four pathogens studied showed that (in descending order) *T. aggressivum, Cladobotryum* spp., *Lecanicillium* spp. and *Mycogone* spp. were most frequently isolated in a viable form during sampling. Correlation data of viable isolations against molecular identifications, give a more realistic oversight in terms of the risk that can be linked to each area. Data showed that most identifications or detections were of a non-living nature, as 89% of positive identifications were only detected by molecular methods and had no associated fungal growth on culture medium. The results further show that composting and preparation areas, barely contribute any isolation value or risk of contamination to the overall process as the isolations made were less than 0.7% of all samples taken in these areas. Pathogens such as *Lecanicillium* spp. and *Mycogone* spp. were not detected in a vegetative state in the composting and preparation areas. Culture-based data support the trend exhibited in the molecular data, which is that the major biosecurity risk on white button mushroom farms are the growing areas, including the rooms, tools and equipment as well as physical infrastructure. On average a total of 11% of samples taken during a "health check" showed positive molecular identification as well as growth of a pathogen, of which 78% of these samples (the 11%) were localized to growing rooms.

Compared to physical isolation frequencies, the molecular data seems overstated compared to conventional plating results, indicating a positive result may be obtained much more often (over-estimation of target organism). It can be argued that molecular isolations from samples most likely included dead spores, inactive mycelial fragments, or even pathogenic DNA fragments in conjunction to any living cells. The lower detection/ isolation rate by conventional methods could also be explained when considering the ecological dynamics within an environmental space (Rajapaksha *et al*., 2019). Successfully isolating and culturing microbial pathogens from swab samples could be influenced by various factors. Viability potential of the pathogenic unit, population characteristics of other

microorganism within the same sample and the susceptibility of pathogens to be grown on artificial culture media should also be considered. This is indicative that the molecular method reflects a positive isolation result even if the source or parent material could be of a non-viable nature. Although frequent positive identifications were made during direct amplification from swab samples, one of the most apparent limitations of PCR techniques comes from the lack of differentiation between viable and non-viable cells (Gerard & Cangelosi, 2014). This is because DNA is always present whether the cell is alive or dead. Reverse transcriptase PCR (RT-PCR) was developed to counteract this lack of discrimination and can differentiate viable cells (Sheridan *et al*., 1998). This technique was not employed within this study but could be considered as part of a continual improvement process during further development of the pathogen monitoring program.

Lecanicillium spp. and *Mycogone* spp. were only detected in viable form from growing beds, a reason being a lack of conducive environmental conditions and favourable growth factors within these areas. Spores of *Lecanicillium* for example can remain viable for more than a year in soil (Cross & Jacobs, 1968) and, if present on a mushroom farm, *L. fungicola* spores can survive for 7–8 months under dry conditions (Fekete, 1967). Therefore, once *L. fungicola* has occurred on a farm, there is likely to be a reservoir of inoculum on the site, and this inoculum will serve as a source of infection for following crops if poor hygiene is maintained as standard practice. It is very unlikely that the spores of *L. fungicola* will survive pasteurisation temperatures during composting, although the primary source of contamination is postulated to be from casing material, but it is also improbable that *L. fungicola* proliferates saprophytically in peat, as its growth is inhibited by the microbial community in this substrate (Cross & Jacobs, 1967). Moreover, anaerobic conditions and low pH are unfavourable for *L. fungicola*. It is described for *M. perniciosa* that this pathogen only appears in association with *A. bisporus*, as there exists a metabolic relationship between mushroom and fungal mycelia – the former affecting sporulation and growth of the latter (Mamoun *et al*., 2000).

Traditional methods allow quantification of pathogenic loads through viable cell or spore counts, but as the aim was to develop and successfully implement a rapid detection method, culturing and counting techniques are tedious and time consuming. Therefore, molecular determination of pathogenic load can be achieved indirectly through target concentration determination after amplicon generation. The techniques and methodology used during farm monitoring in this study did not consider the concentration of pathogens present during detection, only presence or absence. As a transitional objective from this research, further development of third generation molecular techniques and methods should be considered on ddPCR technology (described in Chapter 6), which will allow pathogenic load detection during every amplification reaction performed. Droplet digital PCR (ddPCR) is an emerging nucleic acid detection method that provides absolute quantitation of target sequences without relying on the use of standard curves (Henrich *et al*., 2012; Abachin *et al*., 2018). Following PCR amplification,

enumeration of both fluorescing and non-fluorescing droplets is performed, allowing the absolute quantification of target molecules present in the original sample.

7.5. CONCLUSION

Incorporation of a molecular detection and identification method as part of a continuous on-farm biosecurity and disease monitoring protocol, has shown potential to help farmers identify areas of pathogen persistence and patterns of disease spread in different production areas. Conventional methods have demonstrated a reliable and resourceful tool for determining environmental and production system contamination with pathogens. Current techniques demonstrate reproducible sensitivity and are generally much quicker than conventional methods. During the research, no single approach satisfied all criteria for quick, effective, reproducible and sensitive results and there are still innovation and development necessary to establish a robust monitoring system for absolute mitigation of threats to mushroom farm biosecurity. Implementation of a pathogen specific DNA-based method in conjunction with traditional morphological/ culture-based techniques during "Farm Health" checks has proven an effective and rapid method for mycoparasitic fungal detection in the three main areas of mushroom production. For each of the four pathogens, individual farm monitoring has also shown definite patterns of persistence and disease expression in these key production areas. Results showed similar trends on all farms monitored, which could be combined to signify a biosecurity threat status for the local white button mushroom industry in South Africa. *Trichoderma aggressivum* and *Cladobotryum spp*. were most detected and confirmed. Although prevalence of *Mycogone spp*. and *Lecanicillium spp*. was lower, all pathogens were established through physical isolation and culture purification from diseased material and swab samples collected. Through observation of the localities and areas where pathogens were most commonly detected, it may be emphasized that general facility and plant hygiene should remain top priority to minimize spread of potential pathogens. Effective hygiene practises will lessen the risk of pathogen association, accumulation and spread to other portions of the production process. Future research could include identification of potential indicator organisms to any of the mycoparasites, that will show positive correlation and association within production processes or areas. Quantification of mycoparasite contamination and occurrence by measuring infective unit concentration per sample, will characterize probable threats by establishing realistic control limits compared to actual detected disease manifestation within growing rooms. Raw material and seasonal variances affecting compost quality and growth medium preparation conditions could also be monitored in conjunction to on-farm disease occurrence. Growth proliferation or suppression of mycoparasites could be affected by biological vigour of the host crop as a direct correlation to growing-bed condition and the subsequent influence on mushroom health.

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CHAPTER 8

GENERAL DISCUSSION AND CONCLUSION

The global production and consumption of edible mushrooms have increased significantly over the last seventeen years, from 4 189 812 tons in 2000 to 10 790 859 tons in 2016 (FOASTAT, 2018). The continuous and increased performance of the mushroom industry world-wide, is a direct consequence of increasing demand, as well as the improvement and mechanisation of production systems (Chang, 1993; Royse, 1997). Understanding the prevalence and persistence of microorganisms within the microbiome of fresh mushrooms is an important aspect in disease control (Kertesz & Thai, 2018). Some of these microorganisms are important in food safety and mushroom mycoparasitic pathogens during production. Most of the time, outbreaks can be attributed to microbial imbalances or lack of diversity within the associated host surface and residing microbial populations (Rossouw & Korsten, 2017). Microbial community composition of mushrooms can rapidly change and be influenced by a range of environmental factors, such as temperature, pH, moisture availability and environmental pathogen load (Leff & Fierer, 2013). To understand trends associated with occurrence and persistence of potentially harmful organisms within a production system, it becomes important to consider the microbial diversity and pathogen presence in a more holistic manner.

The purpose of this study was to assess the safety of mushrooms linked to production of white button mushrooms within South Africa. The second was to define, the microbial diversity associated with whole, fresh mushrooms and how these populations are affected by changes in the production processes. Focus was specifically given to possible transference and persistence of *Staphylococcus* spp., within the production chain, linking antimicrobial resistance to the most prominent species identified. Lastly, was using and applying next generation molecular technology to detect and identify mycoparasitic pathogens on commercial mushrooms farms and monitor variation in pathogen occurrence over time. Although there are more advanced methods for detection, identification and quantification currently described in literature and available on the market, the methods used during this study were the most relatable, applicable and cost-effective at the time of the study.

Experimental data showed that freshly harvested mushrooms displayed an average total microbial load of 8.9 log cfu/g and packed ready-to-eat mushrooms a load of 9.8 log cfu/g. The average fresh mushroom microbial load break-down being 4.5 log cfu/g bacteria, 1.5 log cfu/g fungi and 2.7 log cfu/g yeasts. Representative isolates were characterized into 96 different environmental and food-associated species, using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), PCR and sequencing for identification and validation. Most frequent isolations included *Pseudomonas* species (45.37%), *Rhodotorula mucilaginosa* (A. Jörg.) F.C. Harrison 1928 (84.31%), and *Penicillium brevicompactum* Dierckx 1901 (66.67%) representing dominant isolates in the bacterial, yeast and fungal groups respectively. The total mushroom microbiome was considered and determination of microbial change during production stages showed that there were no significant changes in microorganism numbers associated with mushrooms as the production stages progressed.

This means that the initial hypothesis of microbial load differences between pre-picked, picked and packaged mushrooms of different cycles (breaks) were disproved. Variations in isolation quantities of the same species of organisms exist for mushrooms sampled during different stages and locations.

Prevalence and persistence of foodborne pathogens within the various production stages were studied on mushrooms and hands of mushroom handlers. All samples processed confirmed an absence of *Salmonella enterica*, *E. coli* O157:H7 and *Listeria monocytogenes* on fresh mushrooms evaluated. As an addition to the concept of mushroom food safety, Staphylococci were considered in areas and stages when mushrooms are extensively handled during production. It was shown that *Staphylococcus* species present on pre-harvest mushrooms and on mushrooms after picking, are less likely to persist throughout the rest of the processing chain onto packed mushrooms. However, this contrasts with *Staphylococcus* species introduced by packers which are more likely to be associated with the final product and cold storage. Considering the latter, it was confirmed that pathogenic species such as *Staphylococcus aureus* and *Staphylococcus epidermidis* were present on the hands of mushroom packers and that transference to the final product is possible within the current production practices employed by commercial farms. Improvement of hand hygiene habits during production and packaging practices should be considered, together with an efficient monitoring system to further mitigate any future risks for contamination of final product.

A big challenge within the global mushroom industry relates to crop losses as a result of mushroom pathogens (Soković & van Griensven, 2006). The focus here, was to develop and utilize test methods and equipment available in the department, to detect and identify fungal pathogens prior to disease expression. As a secondary objective, an industry-specific health status was to be established over time. Making use of next generation strategies such as qPCR and ddPCR the initial development and application was based on internally transcribed spacer (ITS) regions of the mycoparasitic pathogens. This approach has enabled the successful development and use of genus- and in some instances species-specific molecular primer sets, to detect and investigate the diversity within certain mushroom pathogenic groups. Consideration of different DNA extraction methods and products available on the market during the optimization phases, has allowed the ability to perform a direct extraction of PCR-amplifiable genetic material from swab-samples taken on various surfaces. The developed detection method was validated to still be sensitive enough to successfully detect the presence of pathogens when sampled from matrixes containing various environmental and target non-specific contaminants. This easy-to-adopt PCR method evolved from initial tests, utilizing conventional (1st generation) PCR up to droplet digital (3rd generation) PCR for organism specific detection and quantification of *Trichoderma aggressivum* Samuels & W. Gams 2002, *Cladobotryum* spp., *Lecanicillium* spp. and *Mycogone* spp. As far as could be determined, this detection method, specifically the combination of microorganisms evaluated during a farm health evaluation is first of its kind in the global mushroom industry. The

concept of molecular detection as an early warning system and on-farm monitoring tool was presented at the 2016 International Society for Mushroom Science conference which was held in Amsterdam, Netherlands (Rossouw *et al.*, 2016).

Disease prevalence was monitored through persistent and systematic sampling on different farms practicing varying production and farming strategies. *Trichoderma aggressivum* and *Cladobotryum* spp. were most detected and confirmed within different localities on farms. The prevalence of *Mycogone* spp. and *Lecanicillium* spp. was low although these pathogens have been confirmed through physical isolation and culture purification from diseased material and swab samples collected from growing beds. A trend in species occurrence and detection were visible on a yearly cycle. The most frequent occurrence of all four pathogens on most farms and throughout the industry were in the months of January to March, from May to July, and again during November to December, with the rest fluctuating throughout the remainder of the year. Very sparse literature is available, describing industry specific trends and isolations for all four pathogens, therefor the observations made during this study constitutes new knowledge and research data, specifically tailored to the South African commercial mushroom industry. An example of a similar study is described by Carrasco *et al*. (2016) specifically for *Cladobotryum* spp. in Spain, but the authors observed a different pattern during isolations and detection patterns for this pathogen. In their study the most widespread infection was registered in autumn (44% of crops infected) and winter (37%), while the incidence was lower in spring (18%) and summer (28%). Throughout the duration of this study, the development and application of the described detection methods has proven to be an invaluable tool in assisting farmers to control disease more effectively. Farmers are implementing this detection technique as part of their on-farm monitoring and sanitation schemes. Through continual and consistent assessment, it will be possible to detect persistence of specific pathogens within production systems and even national production areas. All four target pathogens were isolated and found across southern Africa, but certain farms and localities have shown to be more prone to re-infection by the same pathogens than others.

8.1. SUGGESTIONS FOR FUTURE RESEARCH

1. Food safety orientated studies and supporting literature are limited on the dynamics within the packhouse during the final processing and handling of mushrooms and implementation of controls within the cold chain process to detect and monitor potential introduction and elimination of disease-causing microbes associated with mushroom products.
2. An in-depth investigation into the prevalence and persistence of harmful pathogens associated with raw materials, such as wheat-straw and chicken manure, used during mushroom production up to and in conjunction with the final product as it appears on retailer shelves.

3. Further development of next generation technologies such as digital PCR methods to enable multiplexing (e.g. quadruplex ddPCR assay) of all four primer sets for simultaneous detection and quantification of mycoparasitic pathogens. The application of this method will need to be evaluated through additional field case studies to further evaluate the full spectrum of merits and limitations. New primers for each of the pathogens could be considered to further advance specificity and accuracy of the diagnostic tests performed.
4. Although it was not the focus of this study, direct methods may be considered in future work for the detection of the non-viable portion of the mushroom microbiome. However, diagnostic test methods utilized by regulatory authorities mainly relies on traditional standard methods and will most likely continue to be used in the immediate future. Methods should stay current and comparable as to provide a measurable quantification which are fit-for-purpose.

8.2. CONCLUSION

It was hypothesised that foodborne pathogens would occur more readily after harvest on the picked mushrooms due to the handling by workers during picking and packaging. Results from this study showed that this hypothesis was not true in all circumstances and that the overall pathogenic presence are likely to occur before or after harvest. It was also anticipated that human associated *Staphylococcus* species would be introduced onto mushrooms as they were being handled during picking and packing. Transference and occurrence of different staphylococcus species throughout the production chain, from before harvest to after packing, were shown to occur as a result of natural or manufacturing-induced (e.g. raw materials) association with mushrooms or even the hands of the handlers. *Staphylococcus aureus* were confirmed on the hands of a packer, which still affords the possibility of contamination of the final product. No *E. coli* O157:H7, *L. monocytogenes* or *S. enterica* were identified or confirmed from any of the samples collected during this study (mushrooms, swabs or water). Considering food safety guidelines in South Africa for ready-to-eat fresh produce, coliform counts found on mushrooms tested, exceeded the guideline value for fresh fruit and vegetables (Department of Health, 2000). It is proposed, based on results from this and similar studies, locally and internationally, on the microbiological safety of commercial mushrooms, that the specifications for microbial loads on fresh healthy mushrooms be revised to reflect and be evaluated at realistic levels.

Besides food safety and indicator microorganisms, species diversity of the most associated microorganisms with fresh, whole mushrooms were evaluated. It was shown that a well proved and established method (MALDI-TOF MS) could be applied as a novel technique to characterise and confirm identity of isolated colonies. A fungal identification database was not available at the time of experimentation on the MALDI-TOF MS, therefor only bacterial and yeast cultures could be identified

using this method. Sequencing of strategic isolates served as reference and confirmation of consistency and accuracy during analysis, which showed that as a stand-alone method of identification the MALDI-TOF MS will reliably identify organisms on a species level, 82% of the time. Results from this research correlate to similar studies, which show that routine isolates can be identified at accuracy levels greater than 85% with good reproducibility. Therefor the applicability of the MALDI-TOF MS as a rapid method of culture identification was proved and developed successfully for future implementation in the mushroom industry. Most frequently isolated microorganisms were bacteria, yeasts and least of all fungi. The yeast and fungal populations showed little species diversity in contrast to the large variety of bacterial species characterised. From the microbiome compiled in this study, various species isolated were also confirmed by other studies, but new species were additionally identified and described. As a research contribution and as far as could be determined, the microbial populations described in this study are the most comprehensive for white button mushrooms in comparison to other literature and scientific findings of a similar nature. Updated and newer methods for determining richness and population composition have also since been accepted as standard practice. These methods use molecular and genetics-based principles to allow complete and rapid community fingerprinting as well as phylogenetic classification of microorganisms.

A highly sensitive and easy-to-adopt next generation PCR method has been developed and validated for organism specific detection and quantification of *T. aggressivum*, *Cladobotryum* species, *Lecanicillium* species and *Mycogone* species. This methodology together with the technology employed have enabled mushroom farmers/growers to identify critical areas of contamination and infection early in production; helping them to act in a preventative rather than curative manner when controlling disease. As this study included an initial demonstration of the promising utility of ddPCR in pathogen detection and identification, applications requiring unbiased and consistent quantitative results as well as future field case studies are needed to further evaluate its full spectrum of merits and limitations. The most prevalent pathogens detected on commercial farms in South Africa were *T. aggressivum* and *Cladobotryum* spp., isolated typically from growing rooms already containing mushrooms. It was considered that both living and non-living isolations should likewise be compared when conducting a health check as molecular techniques do not necessarily have the ability to specify if a positive result originated from a living or "infectious" source or not. Results showed a trend indicating that most positive identifications (89%) by molecular technique were of a non-living, non-infectious nature. Therefor as a consideration for future farm monitoring through molecular methods, a probability or statistical indication of potential risk for infection could be incorporated and assigned to positive results based on correlational data such as that described in Chapter 7.

As it is unlikely that a single strategy will be successful in eliminating all the challenges associated with sustainable and regulatory compliant mushroom production, a multi-pronged approach may be

considered by regulatory authorities, retail and the industry in future. Such an envisaged approach should include effective implementation of scientific research and continual development, supported by effective enforcement and regulatory control, including farmer "self-regulation" through good agricultural production practices. Aspects and concepts from the research findings have the potential to contribute to the development of a holistic mushroom quality and safety management programme, ultimately ensuring improved product quality and safety for consumers. This study has mainly contributed to the advancement of knowledge and the understanding of specifically microbial dynamics of white button mushrooms during different productional phases, including pathological considerations for continued industry viability.

8.3. REFERENCES

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APPENDIX A

Table A1 ***Less frequently isolated bacterial species from white button mushrooms, during different production stages, identified using the matrix-assisted laser desorption/ionisation time-of-flight mass spectrophotometer (MALDI-TOF MS)***

MALDI-TOF MS isolate identification	**Percentage occurrence (%)**	**MALDI-TOF MS Identification accuracy (3.00 = 100% Accurate)**	**16S BLAST Result (Confirmed species)**	**Similarity Score %**	**Pre- / Post-harvest isolation**
Ewingella americana	5.18	2.44 ± 0.13	*Ewingella americana*	97	43% Pre / 57 % Post
Pseudomonas migulae	5.18	1.99 ± 0.06	*Pseudomonas migulae*	99	72% Pre / 28% Post
Pseudomonas brassicacearum	4.47	1.96 ± 0.03	*Pseudomonas brassicacearum*	96	83% Pre / 17% Post
Pseudomonas gesardii	4.47	2.06 ± 0.04	-	-	34% Pre / 66% Post
Pseudomonas corrugata	3.71	1.89 ± 0.13	-	-	100% Pre
Pseudomonas fluorescens	3.71	2.01 ± 0.12	*Pseudomonas fluorescens*	95	80% Pre / 20% Post
Pseudomonas koreensis	2.94	1.78 ± 0.27	*Pseudomonas koreensis*	96	75% Pre / 25% Post
Pseudomonas putida	2.94	1.88 ± 0.23	*Pseudomonas putida*	95	25% Pre / 75% Post
Staphylococcus sciuri	2.94	1.85 ± 0.13	*Staphylococcus sciuri*	97	50% Pre / 50% Post
Serratia liquefaciens	2.94	2.33 ± 0.14	*Serratia liquefaciens*	91	25% Pre / 75% Post
Brevibacterium ravenspurgense	2.23	1.99 ± 0.09	-	-	100% Post
Chryseobacterium indologenes	2.23	1.81 ± 0.07	-	-	34% Pre / 66% Post
Klebsiella pneumoniae	2.23	2.48 ± 0.02	*Klebsiella pneumoniae*	99	34% Pre / 66% Post
Microbacterium phyllosphaerae	2.23	1.87 ± 0.15	-	-	100% Post
Pseudomonas libanensis	2.23	1.96 ± 0.11	-	-	34% Pre / 66% Post
Pseudomonas synxantha	2.23	2.06 ± 0.05	-	-	34% Pre / 66% Post
Pseudomonas thivervalensis	2.23	1.97 ± 0.04	-	-	34% Pre / 66% Post
Staphylococcus epidermidis	2.23	1.99 ± 0.03	*Staphylococcus epidermidis*	97	100% Post
Staphylococcus hominis	2.23	1.89 ± 0.47	*Staphylococcus hominis*	97	100% Post
Citrobacter koseri	1.47	2.29 ± 0.17	-	-	100% Post
Enterobacter amnigenus	1.47	2.19 ± 0.32	*Enterobacter amnigenus*	97	50% Pre / 50% Post
Escherichia coli	1.47	2.23 ± 0.14	-	-	100% Pre
Lactobacillus mali	1.47	1.43 ± 0.06	*Lactobacillus mali*	98	100% Post
Ochrobactrum tritici	1.47	2.10 ± 0.17	-	-	100% Post
Providencia rettgeri	1.47	2.10 ± 0.17	-	-	50% Pre / 50% Post
Pseudomonas aeruginosa	1.47	2.32 ± 0.03	-	-	50% Pre / 50% Post
Pseudomonas lundensis	1.47	1.72 ± 0.30	*Pseudomonas lundensis*	97	100% Post
Staphylococcus warneri	1.47	2.08 ± 0.06	*Staphylococcus warneri*	98	100% Post
Bacillus circulans	0.76	1.86 ± 0.00	-	-	100% Pre
Bacillus muralis	0.76	1.58 ± 0.00	*Bacillus muralis*	81	100% Pre
Bacillus vallismortis	0.76	1.65 ± 0.00	-	-	100% Post
Brevibacterium casei	0.76	2.26 ± 0.00	-	-	100% Post
Cedecea lapagei	0.76	2.11 ± 0.00	-	-	100% Post
Citrobacter gillenii	0.76	2.26 ± 0.00	-	-	100% Post
Corynebacterium mycetoides	0.76	1.86 ± 0.00	-	-	100% Post
Chryseobacterium scophthalmum	0.76	2.04 ± 0.00	-	-	100% Pre
Enterobacter aerogenes	0.76	2.50 ± 0.00	-	-	100% Post
Flavobacterium saccharophilum	0.76	1.74 ± 0.00	-	-	100% Post

MALDI-TOF MS isolate identification	Percentage occurrence (%)	MALDI-TOF MS Identification accuracy (3.00 = 100% Accurate)	16S BLAST Result (Confirmed species)	Similarity Score %	Pre- / Post-harvest isolation
Kocuria carniphila	0.76	1.76 ± 0.00	-	-	100% Post
Kocuria palustris	0.76	1.99 ± 0.00	-	-	100% Post
Lactobacillus coryniformis	0.76	1.29 ± 0.00	-	-	100% Post
Lactobacillus equi	0.76	1.33 ± 0.00	*Lactobacillus equi*	92	100% Post
Microbacterium hydrocarbonoxydans	0.76	1.89 ± 0.00	-	-	100% Post
Microbacterium saperdae	0.76	1.77 ± 0.00	-	-	100% Post
Micrococcus luteus	0.76	1.74 ± 0.00	*Micrococcus luteus*	96	100% Post
Ochrobactrum anthropic	0.76	1.87 ± 0.00	-	-	100% Post
Pseudomonas agarici	0.76	2.13 ± 0.00	-	-	100% Pre
Pseudomonas brenneri	0.76	1.87 ± 0.00	-	-	100% Post
Pseudomonas graminis	0.76	1.88 ± 0.00	*Pseudomonas graminis*	96	100% Pre
Pseudomonas mendocina	0.76	1.99 ± 0.00	-	-	100% Post
Pseudomonas poae	0.76	1.80 ± 0.00	-	-	100% Post
Pseudomonas trivialis	0.76	1.88 ± 0.00	-	-	100% Pre
Pseudomonas umsogensis	0.76	1.72 ± 0.00	-	-	100% Pre
Pseudomonas vancouverensis	0.76	1.83 ± 0.00	*Pseudomonas vancouverensis*	97	100% Pre
Pseudomonas veronii	0.76	1.68 ± 0.00	-	-	100% Pre
Raoultella terrigena	0.76	2.31 ± 0.00	-	-	100% Pre
Rhizobium radiobacter	0.76	2.12 ± 0.00	-	-	100% Post
Rhodococcus erythropolis	0.76	1.75 ± 0.00	-	-	100% Pre
Sphingobacterium multivorum	0.76	1.97 ± 0.00	-	-	100% Post
Staphylococcus aureus	0.76	2.43 ± 0.00	*Staphylococcus aureus*	98	100% Post
Staphylococcus haemolyticus	0.76	1.79 ± 0.00	*Staphylococcus haemolyticus*	95	100% Post
Streptomyces griseus	0.76	1.41 ± 0.00	*Streptomyces griseus*	80	100% Post

Table A2 Comparison of MALDI-TOF MS identification with the matching 16S-rDNA BLAST results obtained for various isolates

Isolate Number	MALDI-TOF MS Identification	MALDI-TOF MS Score Value	16S BLAST Result	16S Max. Identity (%)
*65	*Lactobacillus equi*	1.325	*Sphingobacterium composti*	92
66	*Pseudomonas extremorientalis*	2.099	*Pseudomonas extremorientalis*	78
66	*Pseudomonas marginalis*	1.991	*Pseudomonas marginalis*	78
68	*Microbacterium phyllospaerae*	1.907	*Pseudomonas phyllospaerae*	96
68	*Microbacterium hydrocarbonoxydans*	1.893	*Microbacterium hydrocarbonoxydans*	96
73	*Pseudomonas antarctica*	2.244	*Pseudomonas Antarctica*	97
75	*Pseudomonas graminis*	1.875	*Pseudomonas graminis*	96
76	*Pseudomonas koreensis*	1.83	*Pseudomonas koreensis*	96
76	*Pseudomonas vancouverensis*	1.829	*Pseudomonas vancouverensis*	97
79	*Pseudomonas orientalis*	1.91	*Pseudomonas orientalis*	97
79	*Pseudomonas fluorescens*	1.876	*Pseudomonas fluorescens*	95
82	*Pseudomonas marginalis*	2.049	*Pseudomonas marginalis*	98
82	*Pseudomonas antarctica*	1.991	*Pseudomonas antarctica*	98
83	*Pseudomonas extremorientalis*	2.096	*Pseudomonas extremorientalis*	75
83	*Pseudomonas gessardii*	2.044	*Pseudomonas gessardii*	81
85	*Pseudomonas gessardii*	2.045	*Pseudomonas gessardii*	98
85	*Pseudomonas antarctica*	1.995	*Pseudomonas antarctica*	98
*86	*Micrococcus luteus*	1.738	*Pseudomonas extremorientalis*	96
88	*Pseudomonas orientalis*	1.889	*Pseudomonas orientalis*	97
88	*Pseudomonas kilonensis*	1.827	*Pseudomonas kilonensis*	97
91	*Pseudomonas antarctica*	2.118	*Pseudomonas antarctica*	98
91	*Pseudomonas extremorientalis*	2.147	*Pseudomonas extremorientalis*	98
92	*Pseudomonas chlororaphis*	1.888	*Pseudomonas chlororaphis*	95
92	*Pseudomonas orientalis*	1.795	*Pseudomonas orientalis*	95
97	*Pseudomonas orientalis*	1.909	*Pseudomonas orientalis*	97
97	*Pseudomonas kilonensis*	1.866	*Pseudomonas kilonensis*	98
101	*Pseudomonas extremorientalis*	1.902	*Pseudomonas extremorientalis*	99
101	*Pseudomonas tolaasii*	1.872	*Pseudomonas tolaasii*	99
102	*Pseudomonas gessardii*	2.053	*Pseudomonas gessardii*	98
102	*Pseudomonas extremorientalis*	2.008	*Pseudomonas extremorientalis*	99
105	*Pseudomonas rhodesiae*	1.8	*Pseudomonas rhodesiae*	98
111	*Pseudomonas brassicacearum*	1.948	*Pseudomonas brassicacearum*	96
*112	*Lactobacillus mali*	1.469	*Microbacterium trichothecenolyticum*	75
*114	*Lactobacillus mali*	1.388	*Microbacterium esteraromaticum*	98
128	*Staphylococcus equorum*	1.987	*Staphylococcus equorum*	99
130	*Enterobacter asburiae*	1.904	*Enterobacter asburiae*	98
131	*Raoultella ornithinolytica*	2.332	*Raoultella ornithinolytica*	99
134	*Citrobacter freundii*	2.504	*Citrobacter freundii*	97
135	*Citrobacter braakii*	2.528	*Citrobacter braakii*	85
136	*Klebsiella pneumoniae*	2.505	*Klebsiella pneumoniae*	99
*141	*Ewingella americana*	2.604	*Rahnella aquatilis*	97
144	*Pseudomonas lundensis*	1.936	*Pseudomonas lundensis*	97
150	*Raoultella ornithinolytica*	2.349	*Raoultella ornithinolytica*	81
154	*Pseudomonas migulae*	2.078	*Pseudomonas migulae*	99
156	*Pseudomonas putida*	2.137	*Pseudomonas putida*	95
156	*Pseudomonas monteilli*	2.115	*Pseudomonas monteilli*	97
163	*Pseudomonas marginalis*	2.103	*Pseudomonas marginalis*	98
163	*Pseudomonas extremorientalis*	2.089	*Pseudomonas extremorientalis*	97

Isolate Number	MALDI-TOF MS Identification	MALDI-TOF MS Score Value	16S BLAST Result	16S Max. Identity (%)
164	*Pseudomonas orientalis*	1.917	*Pseudomonas orientalis*	97
164	*Pseudomonas kilonensis*	1.886	*Pseudomonas kilonensis*	97
165	*Pseudomonas marginalis*	2.106	*Pseudomonas marginalis*	98
165	*Pseudomonas extremorientalis*	2.059	*Pseudomonas extremorientalis*	97
171	*Salmonella spp*	2.493	*Salmonella enterica*	97
174	*Serratia liquefaciens*	2.402	*Serratia liquefaciens*	91
176	*Enterobacter amnigenus*	1.957	*Enterobacter amnigenus*	97
176	*Enterobacter asburiae*	1.842	*Enterobacter asburiae*	98
180	*Pseudomonas antarctica*	2.036	*Pseudomonas antarctica*	98
180	*Pseudomonas extremorientalis*	1.964	*Pseudomonas extremorientalis*	98
181	*Pseudomonas extremorientalis*	2.054	*Pseudomonas extremorientalis*	95
181	*Pseudomonas antarctica*	1.995	*Pseudomonas antarctica*	93
182	*Pseudomonas orientalis*	1.665	*Pseudomonas orientalis*	98
183	*Pseudomonas extremorientalis*	1.986	*Pseudomonas extremorientalis*	97
183	*Pseudomonas marginalis*	1.872	*Pseudomonas marginalis*	97
186	*Pseudomonas extremorientalis*	2.195	*Pseudomonas extremorientalis*	99
186	*Pseudomonas azotoformans*	2.146	*Pseudomonas azotoformans*	97
187	*Pseudomonas orientalis*	1.864	*Pseudomonas orientalis*	97
187	*Pseudomonas chlororaphis*	1.769	*Pseudomonas chlororaphis*	98
189	*Pseudomonas orientalis*	1.945	*Pseudomonas orientalis*	97
189	*Pseudomonas kilonensis*	1.832	*Pseudomonas kilonensis*	97
190	*Raoultella ornithinolytica*	2.62	*Raoultella ornithinolytica*	97
191	*Enterobacter cloacae*	2.582	*Enterobacter cloacae*	89
*196	*Bacillus muralis*	1.583	*Bacillus aerius*	81
253	*Pseudomonas koreensis*	1.406	*Pseudomonas koreensis*	96
259	*Serratia marcescens*	2.307	*Serratia marcescens*	99
D3	*Microbacterium oxydans*	1.91	-	-
*D3	*Microbacterium liquefaciens*	1.899	*Bacillus weihenstephanensis*	90
*D11	*Streptomyces griseus*	1.409	*Enterobacter hormaechei*	80
D12	*Pseudomonas extremorientalis*	2.057	*Pseudomonas extremorientalis*	95
D12	*Pseudomonas marginalis*	2.051	*Pseudomonas marginalis*	95
*D13	*Microbacterium hydrocarbonoxydans*	2.222	*Enterobacter cancerogenus*	77
D16	*Pseudomonas orientalis*	2.051	*Pseudomonas orientalis*	77
D16	*Pseudomonas rhodesiae*	2.029	*Pseudomonas rhodesiae*	76
*D18	*Pseudomonas mandelii*	2.206	*Enterobacter asburiae*	78
D19	*Pseudomonas marginalis*	2.065	*Pseudomonas marginalis*	83
D19	*Pseudomonas antarctica*	2.002	*Pseudomonas antarctica*	83
D21	*Pseudomonas marginalis*	2.065	*Pseudomonas marginalis*	97
D21	*Pseudomonas antarctica*	2.032	*Pseudomonas antarctica*	97
D37	*Staphylococcus succinus*	1.492	*Staphylococcus succinus*	98
D38	*Staphylococcus saprophiticus*	1.747	*Staphylococcus saprophiticus*	74
D40	*Staphylococcus succinus*	1.962	*Staphylococcus succinus*	96
D41	*Staphylococcus xylosus*	2.38	*Staphylococcus xylosus*	99
*W2	*Staphylococcus hominis*	1.352	*Pedobacter roseus*	97
W6	*Pseudomonas orientalis*	1.833	-	-
*W6	*Pseudomonas kilonensis*	1.808	*Clostridium irregulare*	79
*W11	*Streptomyces griseus*	1.409	*Pseudomonas migulae*	99
*W13	*Pseudomonas frederiksbergensis*	2.032	*Lysinibacillus sphaericus*	88
W14	*Pseudomonas chlororaphis*	1.957	*Pseudomonas chlororaphis*	96
W14	*Pseudomonas kilonensis*	1.876	*Pseudomonas kilonensis*	97
W15	*Pseudomonas frederiksbergensis*	2.069	*Pseudomonas frederiksbergensis*	95

Isolate Number	MALDI-TOF MS Identification	MALDI-TOF MS Score Value	16S BLAST Result	16S Max. Identity (%)
W17	*Pseudomonas fluorescens*	1.894	*Pseudomonas fluorescens*	98
W20	*Pseudomonas tolaasii*	2.339	*Pseudomonas tolaasii*	99
W22	*Pseudomonas kilonensis*	1.908	*Pseudomonas kilonensis*	97
W23	*Pseudomonas chlororaphis*	1.918	*Psuedomonas chlororaphis*	95
W23	*Pseudomonas orientalis*	1.865	*Pseudomonas orientalis*	95
W25	*Pseudomonas extremorientalis*	2.07	*Pseudomonas extremorientalis*	95
W28	*Pseudomonas orientalis*	1.805	*Pseudomonas orientalis*	95
*W28	*Pseudomonas kilonensis*	1.803	*Bacillus amyloliquefaciens*	94
W30	*Pseudomonas frederiksbergensis*	1.789	*Pseudomonas frederiksbergensis*	92
W30A	*Pseudomonas frederiksbergensis*	1.789	*Pseudomonas frederiksbergensis*	96
*W31	*Pseudomonas mandelii*	2.063	*Bacillus thuringiensis*	99
W53	*Pseudomonas orientalis*	1.886	*Pseudomonas orientalis*	97
W53	*Pseudomonas kilonensis*	1.779	*Pseudomonas kilonensis*	97
W54	*Pseudomonas chlororaphis*	1.92	*Pseudomonas chlororaphis*	97
W54	*Pseudomonas orientalis*	1.877	*Pseudomonas orientalis*	96

Score Values: 0.000 – 1.699 = not reliable identification, 1.700 – 1.999 = probable genus identification, 2.000 – 2.299 = secure genus identification, probable species identification, 2.300 – 3.000 = highly probable species identification

* Isolates which was identified with the MALDI-TOF, but showed no similarity to any of the results obtained from the 16S-rDNA BLAST

Highlighted cells: Isolates were given only one identity by the MALDI-TOF for both replicates tested
Non-highlighted cells: Isolates were given a different identity for each replicate tested

APPENDIX B

RESEARCH OUTPUTS

PEER REVIEWED JOURNAL ARTICLES

Rossouw, W. & Korsten, L. 2017. Cultivable microbiome of fresh white button mushrooms. *Letters in Applied Microbiology*, 64(2): 164-170.

Duvenage, S., Rossouw, W., Villamizar-Rodríguez, G., du Plessis, E. & Korsten, L. 2019. Antimicrobial resistance characterization of *Staphylococcus spp*. isolated from white button mushrooms and mushroom handlers. *International Journal of Food Microbiology* (20 October 2019).

POPULAR PUBLICATIONS

Rossouw, W., du Plessis, E. & Korsten, L. 2012. Microbiological quality and safety of whole white button mushrooms (*Agaricus bisporus*). *Journal of the South African Mushroom Farmers' Association*, 5-10.

Dzingirayi, G., Rossouw, W. & Korsten, L. 2013. University of Pretoria Mushroom Research: Product Risk Assessment and Farm Health. *Journal of the South African Mushroom Farmers' Association*, 5-7.

Rossouw, W. & Korsten, L. 2014. Hand me that mushroom! Hygiene and sanitation monitoring, with the focus on *Staphylococcus spp*. isolation, identification and progression on the hands of workers and mushrooms. *Journal of the South African Mushroom Farmers' Association*, 17-20.

Rossouw, W., Korsten, L. & van Greuning, M. 2014. Sampioene – verbouing, risiko's en navorsing. *Afgriland Magazine*, March/April Issue: 44-46.

Rossouw, W., Mutengwe, T. & Korsten, L. 2015. Pesticide Residue Levels of Fresh White Button Mushrooms. *Journal of the South African Mushroom Farmers' Association*, 7-10.

Rossouw, W. & Korsten, L. 2015. Part I: Food safety assurance in the mushroom industry. *Journal of the South African Mushroom Farmers' Association*, 10-11.

Rossouw, W., Siyoum, N. & Korsten, L. 2016. Part II: Food Safety Challenges. *Journal of the South African Mushroom Farmers' Association*, 13-14.

Korsten, L., Rossouw, W. & Siyoum, N. 2016. Part III: Food Safety Challenges for the South African Mushroom Industry. *Journal of the South African Mushroom Farmers' Association*, 5-8.

Rossouw, W. & Korsten, L. 2016. Wild and wonderful mushrooms around us. *Journal of the South African Mushroom Farmers' Association*, 10-15.
Photographs: Werner Rossouw, Garikayi Dzingirayi & Lise Korsten

CONFERENCE PRESENTATIONS

Rossouw, W., Duvenage, S. & Korsten, L. *Mushroom disease detection, surveillance and farm health*. Science and Cultivation of Edible Fungi, Baars & Sonnenberg (ed.) © 2016 International Society for Mushroom Science, ISBN 978-90-9029771-2 91.

Rossouw, W. *Food safety in mushroom research*. Presented at the South African Mushroom Farmers' Association Conference 2012, held at Emperors Palace (Gauteng).

Rossouw, W. *Mushroom Farm Health and Disease Diagnostics*. Presented at the South African Mushroom Farmers' Association Conference 2013, held at Salt Rock Hotel (Durban).

Rossouw, W. *Molecular and next generation detection of mushroom pathogens*. Presented at the South African Mushroom Farmers' Association Conference 2015, held at Avianto Conference Venue (Krugers Dorp).

NON-THESIS RELATED OUTPUTS

De Bruin, W., Rossouw, W. & Korsten, L. 2016. Comparison of safe alternative dipping treatments to maintain quality of zucchini. *Journal of Food Quality*, 39(2): 109-115.

Rossouw, W. 2019. Biologies-Geïntegreerde Bemesting. Gee jou somer-aanplantings 'n hupstoot vir 'n meer volhoubare toekoms. *Spilpunt Magazine*, Sept/Oct Issue, 6-8.

Rossouw, W. 2019. Biological considerations for sustainable crop production. *Afgriland Magazine*, Sept/Oct Issue, 16-19.