

CRANFIELD UNIVERSITY

AZLINA BINTI MOHD DANIAL

METABOLITES OF LACTIC ACID BACTERIA (LABs) FROM
MALAYSIAN FERMENTED FOODS AND A *STREPTOMYCES* SP.
FOR CONTROL OF FUNGAL GROWTH AND MYCOTOXIN
PRODUCTION

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PhD THESIS

Academic Year: 2015- 2018

Supervisor: PROF. NARESH MAGAN DSc

Co-supervisor: Dr ANGEL MEDINA-VAYA

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ABSTRACT

This study examined metabolites produced by (a) lactic acid bacteria (LABs) from Malaysian fermented foods and (b) from a *Streptomyces* AS1 strain on the control of human fungal pathogens (*Trichophyton rubrum*, *Aspergillus fumigatus*, *Candida albicans*) and mycotoxigenic spoilage fungi (*Aspergillus flavus*, *Fusarium verticillioides*, *Penicillium verrucosum*, *Penicillium nordicum*). This was followed by (c) identification of the metabolites, (d) examination of the mechanism of action in relation to inhibition of extracellular hydrolytic enzyme activity, fungal growth, sporulation and mycotoxin production and (e) evaluation of the relationship between efficacy of the extracts on colonisation of nails by *T. rubrum*, and that of stored wheat grain by the mycotoxigenic species *P. verrucosum*, to control colonisation and ochratoxin A (OTA) contamination.

A total of 66 LAB strains, isolated from Malaysian fermented foods, were screened against these fungal pathogens. Only four LAB strains had good efficacy in controlling germination, growth and sporulation of the dermatophyte *T. rubrum in-vitro*. The *Streptomyces* AS1 extracts were effective against all the fungal pathogens tested.

More detailed studies with some LAB strains showed that *Lactobacillus plantarum* strain VS1 alone contributed the highest antifungal activity compared to the other three strains examined, alone or in co-culture. Growth of the dermatophyte *T. rubrum* was inhibited significantly ($p < 0.05$) by VS1 supernatant when using the optimized culture conditions. The best culture conditions were found to be an inoculum size of 3%, 48 h incubation time, a temperature of 35°C, pH 6.2 and glucose as the main carbon source. A total of six metabolites were found in the LABs, with the two major metabolites L-lactic acid (19.1 mg/g cell dry weight (CDW)) and acetic acid (2.2 mg/g CDW). The MIC, IC₅₀ and MFC

were 20 mg/mL, 14 mg/mL and 30 mg/mL, respectively. A comparative study showed that the supernatants gave higher efficacy ($p < 0.05$) than the single compound identified in the supernatants and the major compound responsible for the antifungal activity against *T. rubrum* was L-lactic acid. The possible mechanism of action was studied by measuring the effect of different concentrations (0, 4 and 15 mg/mL) on the inhibition of hydrolytic activity of four key enzymes produced by *T. rubrum*. The total and specific activities of alkaline phosphatase, N-acetyl- β -glucosaminidase, keratinase and esterase were decreased when compared to the control after treatment with 15 mg/mL of the supernatant and this paralleled effects on fungal growth. However, the sporulation of the treated *T. rubrum* was similar ($p = 0.05$) to the control at 15 mg/mL but significantly ($p < 0.05$) higher at 4 mg/mL. Subsequent studies on nail media did not detect any alkaline phosphatase, N-acetyl- β -glucosaminidase and keratinase activity from *T. rubrum* spores and mycelia after treatments with 25 and 50 mg/mL supernatants. The supernatants at 25 mg/mL were able to completely inhibited spore viability and mycelial growth after 7 and 14 days incubation, respectively.

For the *Streptomyces* AS1 strain, the ethyl acetate extract was very effective at inhibiting spore germination of *P. verrucosum* when compared to that of *A. fumigatus* and *F. verticillioides*. The efficacy was further confirmed in studies on a conducive semi-solid YES medium in Bioscreen C assays. The increment of time to detection (TTD) at OD = 0.1 for *P. verrucosum* treated with AS1 extract at 0.95 and 0.99 a_w was the highest amongst the three fungi. The IC₅₀ at 0.95 and 0.99 a_w was approximately 0.005 ng/mL and 0.15 μ g/mL, respectively and the MIC at both a_w levels was >40 μ g/mL. In addition, OTA production was totally inhibited at 2.5 μ g/mL AS1 extract at both a_w levels in *in-vitro* assays. Ten metabolites were identified and three of these were present in

concentrations $>10 \mu\text{g/g}$ CDW. These were identified as valinomycin, cyclo(L-Pro-L-Tyr) and cyclo(L-Pro-L-Val). Comparative studies showed that the AS1 ethyl acetate extract which contained a naturally produced mixture of the various compounds significantly ($p<0.05$) inhibited growth and OTA production by *P. verrucosum* than the individual compounds. Temporal studies showed inhibition of the four key hydrolytic enzyme (N-acetyl- β -glucosaminidase, esterase, acid and alkaline phosphatase) activities during growth of *P. verrucosum* and OTA production by $1.25 \mu\text{g/mL}$, at both 0.95 and 0.99 a_w . However, the specific activity of N-acetyl- β -glucosaminidase and acid phosphatase were higher than in the control at 0.95 a_w . The efficacy of different concentrations of AS1 extract were later examined in stored wheat under different moisture content conditions. The AS1 mixture of metabolites significantly ($p<0.05$) inhibited populations of *P. verrucosum* isolated from the treated grain when compared to the controls at 0.90 a_w . However, the AS1 extract did not significantly ($p>0.05$) inhibit populations of the mycotoxigenic species at 0.95 a_w . The production of OTA was not significantly ($p>0.05$) reduced by any of the metabolite mixture naturally produced by the *Streptomyces* AS1 strain.

Keywords:

Bacteria, inhibit, fungal pathogen, human, plant

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LIST OF ABBREVIATIONS

α	<i>Alpha</i>
A.	<i>Aspergillus</i>
Abs.	Absorbance
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
ATP	Adenosine triphosphate
a_w	Water activity
β	Beta
C.	<i>Candida</i>
°C	Degree Celcius
CDW	Cell dry weight
cm	Centimetre
CFU	Colony Forming Unit
CO ₂	Carbon dioxide
Cyclo(L-Pro-L-Tyr)	Cyclo(L-Prolyl-L-Tyrosyl)
Cyclo(L-Pro-L-Val)	Cyclo(L-Prolyl-L-Valyl)
Δ	Delta
Ø	Diameter
DAD	Diode array detector
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EA	Ethyl acetate
EC	European Commission
Erh	Equilibrium relative humidity
et al.	et alia (and others)
EU	European Union
λ_{em}	Emission wavelength

λ_{ex}	Excitation wavelength
<i>F.</i>	<i>Fusarium</i>
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	Ferrous sulphate heptahydrate
FB_1	Fumonisin B ₁
FB_2	Fumonisin B ₂
FLD	Fluorescence detector
FUM	Fumonisin
g	Gram derived into mg, μg
>	Greater than
h	hour
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
3-HPA	3-Hydroxypropionaldehyde
HPLC	High Performance Liquid Chromatography
IC_{50}	Half maximal inhibitory concentration
I_D	Index of dominance
i.e	<i>id est</i> (Latin), translated as “that is”
k	Kilo
kDa	Kilodalton
KH_2PO_4	Potassium dihydrogen phosphate
<i>L.</i>	<i>Lactobacillus</i>
L	Liter
LAB	Lactic acid bacteria
<	Less than

LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LOD	Limit of detection
log	Logarithm
LOQ	Limit of quantification
μ	Micro
μL	Microliter
m	Milli
mN	Millinormality
μm	Micrometer
mL	Milliliter
mm	Millimetre
MFC	Minimum fungistatic concentration
MIC	Minimum inhibitory concentration
min	minutes
M	Molarity
Max.	Maximum
MEA	Malt Extract Agar
MgSO ₄ .H ₂ O	Magnesium sulphate monohydrate
MOPS	3-[N-morpholino]propanesulfonic acid
MRS agar	De Man, Rogosa and Sharpe agar
MRS broth	De Man, Rogosa and Sharpe broth
N	Normality
NA	Nutrient Agar
NaCl	Sodium chloride
Na ₂ CO ₃	Disodium carbonate
NaOH	Sodium Hydroxide
ng	Nanogram
nm	Nanometer

%	Percent
π	pi
$\pm$	Plus minus
OD	Optical density
OPA	Ortho-phthaldialdehyde
OTA	Ochratoxin A
<i>P.</i>	<i>Penicillium</i>
pH	Potential Hydrogen, scale for acidity and alkalinity
ppm	Part per million (mg/kg)
r	Radius
R ²	Coefficient of determination
rpm	Rotation per minute
RPMI	Roswell Park Memorial Institute
SBD	Sabouraud Dextrose
SD	Standard deviation
SE	Standard error
S/N	Signal to noise ratio
sp	Species (singular)
spp	Species (plural)
<i>T.</i>	<i>Trichophyton</i>
UV	Ultra Violet
vs	versus
v/v	Volume over volume ratio
w/v	Weight over volume ratio
WHO	World Health Organisation
YES	Yeast Extract Sucrose
ZEN	Zearalenone
ZnSO ₄ .7H ₂ O	Zinc sulphate heptahydrate

CHAPTER 1

General introduction, literature review and research objectives

1.1 GENERAL INTRODUCTION

Filamentous fungi are ubiquitous and have both beneficial and harmful impacts on ecosystems. While they have been responsible for a range of important biotechnological and pharmaceutical compounds including drugs and enzymes, some are very specialised and can cause significant human, animal and plant infections. There is thus significant interest in identifying novel compounds which may be able to control these fungal pathogens.

Microorganisms also play important roles in natural decomposition processes and this has been adapted for the development of cell factories via fermentation processes to produce high titres of useful compounds such as enzymes or secondary metabolites which can be used to effectively control infectious fungal pathogens. In nature, the activity of microorganisms, because of their production of a battery of extracellular enzymes, helps degrade the nutritional compounds in raw commodities including hemicellulose and cellulose matrices and lignin for their growth. For example, it was postulated that the increase in phenolic content, antioxidant and antimicrobial activity of fermented mung beans, was associated with the presence of the β -glucosidase enzyme. β -glucosidase hydrolyzed the β -glucosidic bond of conjugated phenolics to the corresponding phenols and sugar. The sugar was used as a source of energy and the phenolics were further degraded to phenolic acid which acts as an antioxidant and antimicrobial agent in fermented foods (Sheih et al., 2014; Randhir and Shetty, 2007). Indeed, many fermented foods contain microorganisms which naturally produce preservative compounds. However, the success of the fermentation processes and the improvement of the biological function of fermented foods depends on the starter culture used to ensure that spoilage microorganisms cannot become established. Thus, many food industries use single or mixtures of pure cultures under controlled fermentation condition for the production of a uniform fermented product with the desired characteristics which are safe for the consumer. Some of the pure cultures used for the preparation of fermented foods include non-toxicogenic *Aspergillus oryzae* strains for preparation of soy sauce (Hoang et al., 2016),

Rhizopus for tempeh (Hartanti et al., 2015), *Lactobacillus* for yogurt (Ertem and Cakmakci, 2018) and *Saccharomyces* for wine (Marsit and Dequin, 2015) and bread (Rezaei et al., 2014). In addition, the use of natural fermentation for the production of fermented food is also widely practiced around the world especially as a cottage industry. This uncontrolled fermentation process may affect the final product as the quality of the same fermented food may vary between batches because of the resident microflora which may be present in the production facility. However, despite these potential problems, small scale producers have flourished because of the perceived health benefits of these products (Kuligowski et al., 2017; Sanjukta and Rai, 2016; Shin and Jeong, 2015; see Table 1.1).

Malaysia is rich with various kinds of traditional or naturally fermented foods made from plant and animal sources. Among these are *tapai* (fermented tapioca, fermented glutinous rice), *tempoyak* (fermented durian flesh), *budu* (fermented fish sauce), *pekasam* (fermented fish), *belachan* (fermented shrimps paste) and fermented vegetables. For fermented tapioca and glutinous rice, these are boiled or steamed, mixed with starter cultures, wrapped in leaves from banana or rubber plants and kept at room temperature for 2 days. For other fermented food products, this involves the use of salt and/or rice and water which are mixed together with the food and kept at room temperature in a closed environment for several weeks or months. Numerous microorganisms such as lactic acid bacteria (LABs) have been isolated from indigenous Malaysian fermented foods. Some studies have found bioactive compounds produced by these strains which can be applied to industrial processes. Among these are glutamic acid production by *L. plantarum* MZ-02 (Zareian et al. 2012), bacteriocins produced by *Enterococcus faecium* B1 and *E. faecium* B2 (Moreno et al., 2002) and lactic acid produced by *L. plantarum*, *L. casei*, *Lactococcus lactis* (Mohd Adnan and Tan, 2007).

In summary, the natural fermentation process involves a mixed microbial community with successional changes in the dominant groups of microbial species. The microorganism that initiate the fermentation will continue growing until its by-products inhibit further growth and fermentation. Once initiated, as the ecological conditions change, there is a succession of bacterial activity followed by yeasts and then filamentous fungal colonisation. There has been interest in the utilisation of these beneficial

microorganisms or their metabolites for efficacy in other systems or as control compounds for spoilage microorganisms. Of particular interest are the LABs which are known to produce a range of potentially useful anti-microbial compounds (Schmidt et al., 2018; Lay et al., 2016; Jang et al, 2014). Very few, if any LABs isolated from a range of Malaysian foods, have been screened for efficacy against human and plant fungal pathogens.

Table 1.1 Fermented food products around the world (from Adams and Moss, 2008)

Fermented Food	Ingredients	Geographical Distribution
Belacan	Shrimp	Malaysia
Budu	Anchovy	Malaysia
Busa	Rice, millet, sugar	Turkey
Beer	Barley	Widespread
Cencalok	Shrimp	Malaysia
Cheese	Milk	Widespread
Chicha	Maize and others	South America
Dawadawa	Locust beans	West Africa
Gari	Cassava	Nigeria
Idli/dosa	Rice and black gram	India
Injera	Tef	Ethiopia
I-sushi	Fish	Japan
Kefir	Milk	Eastern Europe
Kenkey	Maize, sorghum	Ghana
Kimchi	Vegetables	Korea
Koko	Maize, sorghum	Ghana
Leavened bread	Wheat	Europe, North America
Lambic beer	Barley	Belgium
Mahewu	Maize	South Africa
Nam	Meat	Thailand
Ogi	Maize, sorghum, millet	Nigeria
Palm wine	Palm sap	Widespread
Pekasam	Fish	Malaysia
Poi	Taro	Hawaii
Puto	Rice	Philippines
Salami	Meat	Widespread
Sauerkraut	Cabbage	Europe, North America
Sorghum beer	Sorghum	South Africa
Sourdough bread	Wheat, rye	Europe, North America
Soy sauce, miso	Soy beans	South East Asia
Tapai	Tapioca / Glutinous rice	Malaysia
Tempeh	Soy beans	Indonesia
Tempoyak	Durian (Fruit)	Malaysia

1.2 LACTIC ACID BACTERIA (LABs)

LABs are a group of microorganisms which produce lactic acid as the sole or major fermentation product of carbohydrates. They are gram positive, catalase negative, non-sporulating, anaerobic but aerotolerant and acid resistant with various temperature optima from 30°C for mesophilic to 42°C for thermophilic strains/species. Some of them are homofermentative and others are heterofermentative. Homofermentative species produce more than 85% lactic acid as the sole end product while heterofermentative ones produce a mixture of lactic acid, carbon dioxide and ethanol or acetic acid. They can have different shapes, from rods (bacilli) to spheres (cocci) (Table 1.2). LABs are a diverse group of bacteria belonging to different genera including *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Bifidobacterium*, *Enterococcus*, *Pediococcus* and *Streptococcus* (Ringø et al., 2018; da Silva Sabo et al., 2014).

In general, LABs grow in environments which are high in nutrient content which provides them with carbohydrates, proteins, fatty acids and vitamins. They are typically found in plant, animal and fermented products of plant and animal origin (Table 1.3). They are also found in humans, and normally colonize the mouth (streptococci), gastrointestinal tract (bifidobacteria, enterococci, lactobacilli) and mucosa of the vagina (lactobacilli) and can survive and grow at low pH levels (about pH 3). For this reason, LABs have long been used for the generation of safe food products by preventing growth of spoilage and pathogenic microorganisms through acidification and production of antimicrobial compounds. The long history of safe consumption of LABs in fermented foods has allowed them to be ‘generally recognized as safe’ (GRAS) status in the food industry (Vosough et al., 2014). Due to this status LABs have also been chosen as potential vehicles for the delivery of antigens to mucosal immune systems (Rottiers and Snoeck, 2018).

Additionally, certain species of LABs have been shown to have probiotic properties. They are able to survive in the harsh environment of the small intestine and colon, able to colonize the gastrointestinal tract and may help eliminate the colonization of pathogenic microorganisms (Campana et al., 2017; Ahmadova et al., 2013a). Besides this, some LABs are considered as spoilage organisms in certain food industries especially for fermented beverages and sugar processing (Sabel et al., 2017; Liu et al., 2017).

Table 1.2 Principle genera of lactic acid bacteria (from Adam and Moss, 2008)

Genus	Cell morphology	Fermentation	Lactate isomer
<i>Lactobacillus</i>	Rods	Homo/hetero	DL, D, L
<i>Lactococcus</i>	Cocci in chains	Homo	L
<i>Leuconostoc</i>	Cocci	Hetero	D
<i>Pediococcus</i>	Cocci	Homo	DL
<i>Streptococcus</i>	Cocci in chains	Homo	L

(Other genera included in the lactic acid bacteria; *Carbobacterium*, *Enterococcus*, *Oenococcus*, *Vagococcus*, *Aerococcus*, *Tetragenococcus*, *Alloiococcus*, *Waisella*)

Table 1.3 Lactic acid bacteria isolated from different sources

Lactic Acid Bacteria	Source	Reference
<i>E. faecium</i>	Cheese	Ahmadova et al., 2013b
<i>E. faecium</i>	Raw bovine milk	Gaaloul et al., 2014
<i>L. plantarum</i>	Papaya	Sabo et al., 2015
<i>L. spicheri</i>	Fermented vegetable	Gautam and Sharma, 2015
<i>L. casei</i>	Sturgeon fish	Ghanbari et al., 2013
<i>L. plantarum</i>	Sturgeon fish	Ghanbari et al., 2013
<i>L. curvatus</i>	Salami	Souza et al., 2015
<i>L. fermentum</i>	Kasseri cheese	Zoumpopoulou et al, 2010
<i>L. rhamnosus</i>	Maize silage	Dogi et al., 2013
<i>L. harbinensis</i>	Cow milk	Delavenne et al., 2014
<i>L. rhamnosus</i>	Goat milk	Delavenne et al., 2014
<i>L. plantarum</i>	Pickled cabbage	Starzyńska-Janiszewska et al., 2014
<i>L. animalis</i>	Calves gut	Tirloni et al., 2014
<i>L. paracasei</i> subsp. <i>paracasei</i>	Calves gut	Tirloni et al., 2014
<i>Lc. lactis</i> subsp. <i>lastis</i>	Milk	Salama et al., 1993
<i>Lc. lactis</i> subsp. <i>cremoris</i>	Milk	Salama et al., 1993
<i>Lc. lactis</i>	Camel milk	Abushelaibi et al., 2017
<i>Lc. lactis</i> subsp. <i>lastis</i>	Sugar cane	Cock and De Stouvenel, 2006
<i>Ln. carnosum</i>	Meat	Budde et al., 2003
<i>P. pentosaceus</i>	Meat	Anastasiadou et al., 2008

Key: *E.* – *Enterococcus*, *L.* – *Lactobacillus*, *Lc.* – *Lactococcus*, *Ln.* – *Leuconostoc*, *P.* – *Pediococcus*

1.3 LACTIC ACID BACTERIA AND PROTECTION AGAINST MYCOTOXIN PRODUCING FUNGAL PATHOGENS

The function of LABs in inhibiting the growth of pathogenic microbes has been demonstrated in many research findings and some of them are listed in Table 1.4. LABs have been shown to suppress the growth of mycotoxin producing fungi (*P. commune*) in cheese and increase the safety of such food products (Cheong et al., 2014; Souza et al., 2015). The relative inhibition was often dependent on the strains of LABs used (Tirloni et al., 2014; Dogi et al., 2013). The inhibition of growth and mycotoxin synthesis by microbial pathogens was often related to low molecular weight substances produced by LABs (Luz et al., 2017; Peyer et al., 2016; Gupta and Srivastava, 2014). The absorption of these substances, such as bacteriocins, onto the cell surface of the target pathogens was shown to suppress the growth of pathogenic microorganisms (Ahmadova et al. 2013b). Although growth inhibition usually eliminates or minimizes the adverse effects of targeted organisms, in certain conditions, growth inhibition increased the synthesis of toxic compounds. For example, the interaction of *L. plantarum* RC009A with *A. parviticus* in maize silage extract was found to increase the production of aflatoxin B₁ in most of the interaction conditions assayed (Dogi et al., 2013). Therefore, there is a need to find LABs strains with both potential for inhibition of growth of the pathogenic fungi and suppress the synthesis of toxic compounds.

Additionally, the protective role of LABs against pathogenic microorganisms has also been associated with the removal of toxic substances from the environment. It has been shown that LABs are able to bind mycotoxins and subsequently remove the toxins from contaminated matrices (Vosough et al., 2014). This can lead to protection of the host from the adverse effects of these compounds (Khalil et al., 2014). The role of LABs in controlling the synthesis of mycotoxins and the removal of these toxins from the environment have been discussed extensively by Dalié et al. (2010) in their review. They postulated that the binding and removal of mycotoxins depended on the number of binding sites available, which involved the hydrophobic interaction between mycotoxins and degraded proteins and carbohydrates of membrane cell peptidoglycan after acid or heat treatment.

Table 1.4 Lactic acid bacteria with antifungal activities (from Dalié et al., 2010)

Lactic acid bacteria	Spectrum
Genus <i>Lactococcus</i>	
<i>Lc. lactis</i> C10	<i>A. parasiticus</i>
<i>Lc. lactis</i>	<i>A. flavus</i> , <i>A. parasiticus</i>
<i>Lc. lactis</i> subsp. <i>diacetylactis</i> DRCI	<i>A. fumigatus</i> , <i>A. parasiticus</i>
<i>Lc. lactis</i> subsp. <i>lactis</i> CHD 28.3	<i>A. flavus</i> , <i>A. fumigatus</i> , <i>Fusarium</i> spp.
<i>Lc. lactis</i> subsp. <i>cremoris</i>	<i>P. expansum</i>
Genus <i>Lactobacillus</i>	
<i>L. acidophilus rhamnosus</i>	<i>A. fumigatus</i>
<i>L. casei</i> subsp. <i>rhamnosus</i>	<i>A. niger</i> , <i>Fusarium</i> spp., <i>Penicillium</i> spp.
<i>L. casei</i> subsp. <i>pseudopantarum</i>	<i>A. flavus</i>
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	<i>P. expansum</i>
<i>L. plantarum</i> VTT E-78076	<i>F. avenaceum</i>
<i>L. plantarum</i>	<i>P. corylaphilum</i> , <i>P. roqueforti</i> , <i>P. expansum</i> , <i>A. niger</i> , <i>A. flavus</i> , <i>F. graminearum</i>
<i>L. plantarum</i> MiLAB 393	<i>F. sporotrichioides</i> , <i>A. fumigatus</i> , <i>A. nidulans</i>
<i>L. plantarum</i> MiLAB 14	<i>P. roqueforti</i> , <i>P. commune</i> , <i>A. nidulans</i> , <i>A. fumigatus</i>
<i>L. reuteri</i>	<i>F. graminearum</i>
<i>L. rhamnosus</i>	<i>Penicillium</i> spp., <i>Aspergillus</i> spp., <i>Fusarium</i> spp.
<i>L. sanfranciscensis</i> CB1	<i>Fusarium</i> spp., <i>Penicillium</i> spp., <i>Aspergillus</i> spp.
Genus <i>Leuconostoc</i>	
<i>Ln. mesenteroides</i>	<i>Penicillium</i> spp., <i>Aspergillus</i> spp.
Genus <i>Pediococcus</i>	
<i>P. acidilactici</i> LAB 5	<i>A. fumigatus</i> , <i>A. parasiticus</i> , <i>F. oxysporum</i> , <i>Penicillium</i> spp.
<i>P. pentosaceus</i>	<i>P. expansum</i>

1.4 ANTIMICROBIAL SUBSTANCES FROM LACTIC ACID BACTERIA (LABs)

Numerous studies have reported the potential of LABs and their metabolites in inhibiting the growth of food-borne pathogenic and spoilage microorganisms such as gram positive and/or gram negative pathogenic bacteria (Gaspar et al., 2018; Lü et al., 2014; Gaaloul et al., 2014), filamentous spoilage moulds (Juodeikiene et al., 2018; Saladino et al., 2016; Cheong et al., 2014; Gupta and Srivastava, 2014) and yeasts (Jørgensen et al., 2017; Nyanzi et al., 2014). The main mechanism has been via the production of metabolites with antimicrobial activity including organic acids, proteinaceous compounds, reuterin, hydrogen peroxide, fatty acid derivatives and phenolic acid. Among these, the first four metabolites have been extensively studied. These are considered in more detail in the following sub-sections.

1.4.1 Organic acids

Lactic and acetic acids are the two main organic acids produced by LABs. Others are citric, malic and succinic acid depending on the strain. Acetic acid is solely produced by heterofermentive LABs while lactic acid is produced by both homo and heterofermentive LABs. The homofermentation and heterofermentation pathway of LABs is shown in Figure 1.1. The concentration of substrate and the pH have a significant effect in the production of organic acids. Previously, with a reducing sugar concentration of 27 g/L and controlled pH (pH 6.2) resulted in the highest production of lactic acid compared to other concentrations of reducing sugar without pH control (Pejin et al., 2017). Furthermore, the presence of sodium acetate in the substrate is essential for antifungal efficacy by LABs. Low concentrations of sodium acetate in MRS medium resulted in a negative antifungal activity of LABs although the lactic acid concentration produced was in a similar range and the pH value was reduced. Antifungal activity was observed against *P. nordicum* (a OTA mycotoxin producer) as the concentration of sodium acetate was increased to 61 mmol/L (Schillinger and Villarreal, 2010).

The use of organic acid mixtures has often been found to give better inhibition than an individual one alone. A promising result was obtained by Tirloni et al. (2014) who examined the antimicrobial activity of a mixture of *L. animalis* and *L. paracasei subsp paracasei* towards a broad range of spoilage and pathogenic bacteria. Apart from the

possibility of the synergistic effects of these lactobacilli, it may also be due to the combined action of lactic and acetic acids produced by these lactobacilli (Fernandez et al., 2017). This broad range of inhibitory activity of the mixture of these two acids was also observed by Jang et al. (2014) in the culture supernatant of *P. pentosaceus*. Apart from acetic acid, high concentrations of lactic acid in *L. plantarum* (Russo et al., 2017) and phenyllactic acid in *L. zeae* (Yoo et al., 2016) have been postulated to contribute to significant inhibition towards *F. culmorum* and *Rhizoctonia solani*, respectively.

The accumulation of organic acids generated by LABs reduces the pH of the surrounding environment. It has been postulated that the undissociated organic acid from the external environment with a low pH will diffuse through the membrane of the target organism. The high pH of cytoplasm favours the dissociation of organic acids which releases protons and acidifies the cytoplasm. This eventually slows down the cell growth because significant energy is expended in neutralizing the internal pH. If the pH decreases too rapidly then growth is completely inhibited (Schillinger and Villarreal, 2010).

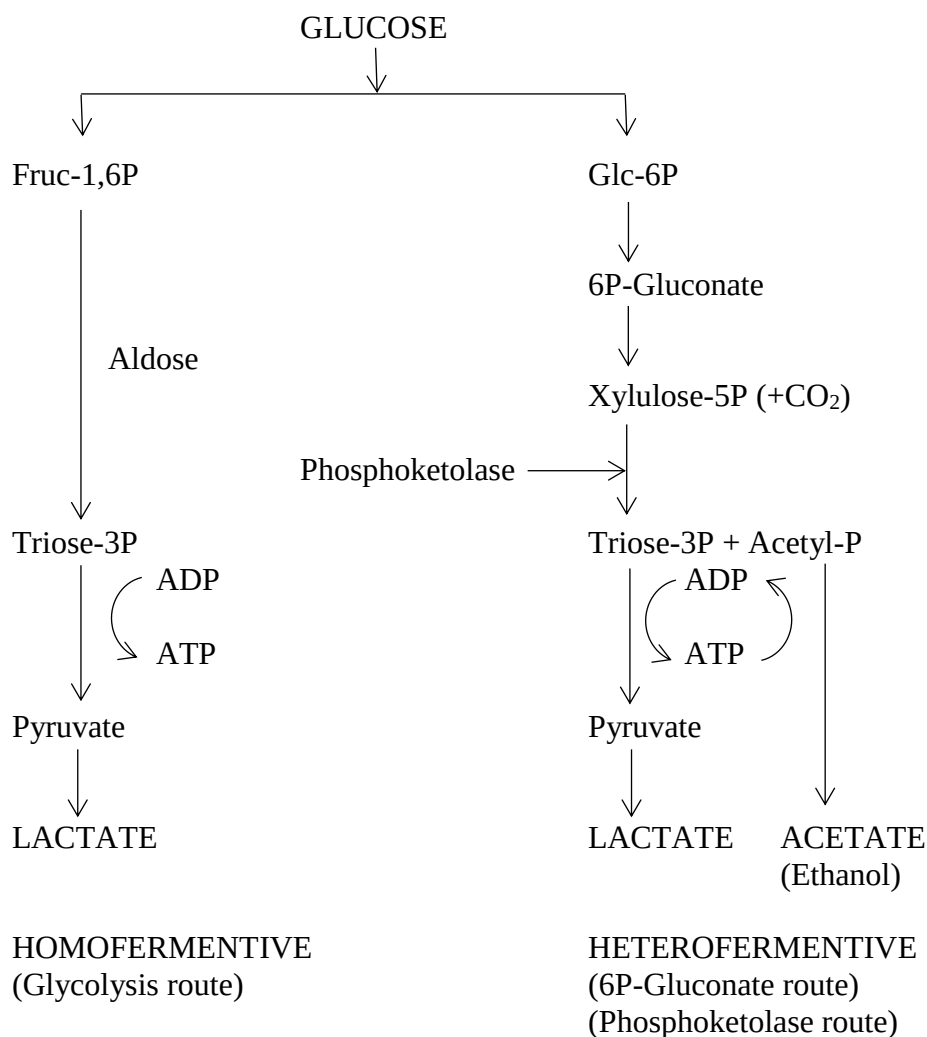


Figure 1.1 Homofermentation and heterofermentation pathways for LABs (from Adams and Moss, 2008).

1.4.2 Proteinaceous compounds

The proteinaceous compounds of LABs which have been extensively studied, are the bacteriocins. They are synthesised in the ribosome and predominantly made up of proteins of various molecular sizes, characteristics and mode of action. They are different from antibiotics as they are primary metabolites of bacteria. The production of bacteriocins starts at an early exponential growth phase until late exponential phase (Souza et al., 2015) while antibiotics are secondary metabolites produced by bacteria . There are four classes of bacteriocins categorized according to their molecular weight, heat stability, primary structure, and molecular organization: (1) Class I is linear (type A)

or globular (type B) peptides, the molecular weight is $\leq 5\text{kDa}$ with approximately 19-38 amino acids; (2) Class II are the thermostable peptides, the molecular weight is $\leq 10\text{kDa}$, with approximately 37-48 amino acids; (3) Class III are heat unstable peptides with the molecular weight of $\geq 30\text{kDa}$ and (4) Class IV which is a complex bacteriocin containing carbohydrate and lipid moieties (da Silva Sabo et al., 2014). However, bacteriocins have a narrow spectrum of antimicrobial activity and are effective against the same species or closely related species, to the producer strain (Souza et al., 2015; Ahmadova et al., 2013b) although some studies disagree with this claim (Lü et al., 2014; Ghanbari et al., 2013). Some LABs produce bacteriocins in a single form (Souza et al., 2015; Lü et al., 2014;) while others produced them in multiple forms (Gaaloul et al., 2014). Bacteriocins with the potential to be used as a biopreservative in food have been isolated, identified and extensively studied including those for meat and milk products (Aspri et al., 2017; Vijayakumar and Muriana, 2017; Souza et al., 2015; Martinez et al., 2015). However, from the various types of bacteriocins that have been studied, only nisin and pediocin PA-1 are commercially employed in the food industry (Zacharof and Lovitt, 2012; Rodríguez et al., 2002).

It was reported, that the production and secretion of bacteriocins requires at least four genes: (1) the structural bacteriocin gene, encoding the prebacteriocin gene which is biologically inactive; (2) the immunity gene, coding an immunity protein which protects the producer from its own bacteriocins; (3) a gene encoding an ABC (ATP binding cassette) transporter necessary for secretion and (4) a gene encoding an accessory protein of unknown function (Gabrielsen et al., 2014; Drider et al., 2006). Most class II bacteriocins are first synthesised in a pre-peptide form or biologically inactive pre-bacteriocin. This compound contains a leader sequence which consists of 18 to 27 amino acids with two glycine residues at its C-terminus. This sequence has a function in preventing the bacteriocins from becoming activated inside the producer cell. The two glycines present in the sequence serve as a recognition signal for the transporter system involving the ABC transporter protein and accessory proteins. Once the pre-peptide has been recognized, the leader amino acid sequence of the bacteriocin is removed and the active peptide/bacteriocin is secreted into the extracellular medium (da Silva Sabo et al., 2014; Ennahar et al., 2000).

The antimicrobial mechanism of action of bacteriocins is related to the permeabilization and pore formation on the cytoplasmic membrane (Wen et al., 2016). Observations using scanning electron microscopy and transmission electron microscopy of *S. aureus* treated with lactocin MXJ 32A found changes in the cell surface of *S. aureus* compared to non-treated cells. The cell surface was changed from having a normal integrated cell wall to irregular and shrinking concave cell wall followed by emergence of perforations and leakage of cytoplasmic compounds (Lü et al., 2014). The formation of pores has also been associated with the release of extracellular β -glucosidase from *Listeria monocytogens* treated with bacteriocin ST71KS (Martinez et al., 2013). Apart from this, other bacteriocins have shown antimicrobial modes of actions through absorption to the cell of the target pathogenic microbes. The absorption of bacteriocin of *L. curvatus* A61 to both sensitive and resistant *Listeria monocytgens* was indicated by Ghanbari et al. (2013) as a first step in its antimicrobial mode of action.

Proteinaceous compounds have also been shown to have antifungal activity. They are active in inhibiting spore germination, hyphal formation and mycelial growth which involves different mechanisms for each phase of the life cycle. The antimicrobial peptide (AMPs LR14) from *L. plantarum*, was suggested to be able to inhibit spore germination and hyphal growth of *Aspergillus niger*, *Rhizopus stolonifer*, *Mucor racemosus* and *Penicillium chrysogenum* due to interference with the spore imbibition process. In addition, elongation of mycelium was hindered by disturbance of the normal turgor induction mechanism and action on the cell wall (Gupta and Srivastava, 2014).

1.4.3 Reuterin

Reuterin (β -hydroxypropionaldehyde) is a metabolic product of glycerol during anaerobic fermentation of *L. reuteri* (Spinler et al., 2008). It is water soluble, stable over a broad range of pH, resistant to heat and has antimicrobial activity towards pathogenic gram positive and gram negative bacteria (Langa et al., 2018), yeasts and filamentous fungi (Vimont et al., 2019) and protozoa (Stevens et al., 2011). The use of reuterin to control gram positive food-borne pathogenic microorganism in milk and fish has been studied. This showed that growth of *Listeria monocytogenes* in both foods kept at different temperature were inhibited by this compound (Montiel et al., 2014; Arqués et al, 2011). Further studies on the gram negative food pathogenic microorganism found that reuterin

was more active against gram negative than gram positive bacteria. However, the activity of reuterin towards gram positive food-borne pathogenic microbes was increased when used in combination with a bacteriocin such as nisin (Arqués et al, 2011). Higher improved efficacy of reuterin was also achieved in combination with diacetyl in acidified (pH 5) milk. Furthermore, addition of glycerol to the growth medium of *L. reuteri* enhanced the inhibitory activity of reuterin (Langa et al., 2018).

There are several hypotheses on the reuterin mode of action, but this is still not fully understood. A recent hypothesis suggested that the aldehyde group of 3-HPA is mainly responsible for the antimicrobial activity of reuterin. The compound reacts with the sulfhydryl group of amino acids and eventually activates the oxidative stress responses (Stevens et al., 2011).

1.4.4 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is produced by most of the LABs under aerobic conditions (Gong et al., 2014). H_2O_2 producing LABs have been isolated from various sources including healthy women vagina (Sgibnev and Kremleva, 2017), contaminated avian blood (Zbrun et al., 2013) and fermented foods (Batdorj et al., 2007). Since most of the LABs are unable to secrete catalase enzymes, they are unable to hydrolyse accumulated H_2O_2 . Therefore addition of catalase into the culture medium under aerated conditions is necessary to prevent inhibition of the producer bacterium (Batdorj et al., 2007). The presence of oxygen is important as it plays an important role in the production of hydrogen peroxide. The effect of aerated conditions on a mixed culture of *L. delbrueckii subsp. lactis* T31 (hydrogen peroxide producer) with *Escherichia coli* and *Listeria innocua* showed stronger inhibition than non-aerated condition (Batdorj et al., 2007). This strong inhibition was postulated to be due to the presence of hydrogen peroxide. Studies have shown the antimicrobial activity of hydrogen peroxide against gram positive and gram negative bacteria and yeasts (Sgibnev and Kremleva, 2017; Cooke et al., 2015; Zbrun et al., 2013; Sookkhee et al., 2001). This activity is due to oxidizing effect of hydrogen peroxide towards bacterial cells and cellular proteins of the targeted organism (Zhu et al., 2017).

1.5 STREPTOMYCES SPECIES AND METABOLITE PRODUCTION

Streptomyces species are gram positive filamentous bacteria which can grow in various ecosystems, including soil (Nguyen et al., 2015), animal faeces (Wang et al., 2014), sea sponges (Jackson et al., 2018), ants (Vieira et al., 2017) and termites (Lee et al., 2018). This genus is considered the largest antibiotic producing one and many of the important antibiotics used in the world were produced by strains of species within this group. The first antibiotic, streptothricin produced by a *Streptomyces* was reported in 1942 and two years later streptomycin was introduced (Watve et al., 2001). These discoveries had a significant impact on *Streptomyces* as it initiated many studies on the development of new antibiotics or metabolites with potential antifungal properties. The antifungal activity of *Streptomyces* was related to the production of extracellular hydrolytic enzymes and/or bioactive compounds which are produced during the exponential and stationary growth phases, respectively.

1.5.1 Secondary metabolites

Most of the previous compounds with antifungal activities produced by *Streptomyces* species were due to the production of secondary metabolites (Lyu et al., 2017), predominantly produced during the stationary growth phase. These metabolites have been isolated, purified and identified. They are from various chemical groups such as phenolic compounds (Tan et al., 2017), cyclic thiopeptide (Mizuhara et al., 2011), aromatic polyketides (Akhter et al., 2018) or organosulphur compounds (Wang et al., 2013). Furthermore, the inhibitory effects have not only been on mycelial growth (Nguyen et al., 2015) but also on spore germination (Saeed et al., 2017; Zhang et al., 2013; Wang et al., 2013). These secondary metabolites exhibit different mechanism of actions against filamentous fungal growth. The inhibition was suggested to be due to antifungal metabolites binding to chitin (Mizuhara et al., 2011) or alterations of the cell membrane structure and thus disruption of the activity of cytoplasmic organelles (Xu et al., 2015). This increased the fragility of the hyphae, causing hyphal bending, irregular branching and swelling leading to bursting and leakage of the cytosolic contents (Xu et al., 2015; Mizuhara et al., 2011).

1.5.2 Hydrolytic enzymes

Streptomyces produce hydrolytic enzymes, not only for growth purposes, but also as one of the defence mechanisms. In particular, chitinase was reported to have some antifungal activity. The crude (Nagpure and Gupta, 2013) and purified (Rashad et al., 2017; Karthik et al., 2015; Han et al., 2009; Hoster et al., 2005,) chitinase inhibited fungal growth by *Aspergillus niger*, *Colletotrichum gleosporioides*, *Penicillium expansum*, *Pythium alphanidermatum*, *Fusarium oxysporum*, *Penicillium chrysogenum* and *Sclerotium rolfsii*. Chitinase hydrolyses the chitin which is an important component of the fungal cell wall, and this enzyme makes them more permeable (Shrivastava et al., 2017; Al-Askar et al., 2015) and thus influences the ability to actively grow. In addition, the antifungal activity of *Streptomyces* was also associated with the secretion of β -1,3-glucanase enzyme (Choudhary et al., 2014; Prapagdee et al., 2008)

Recently, Sultan and Magan (2011) examined a *Streptomyces* strain (AS1) isolated from peanuts. This was found to be competitive and some extracts from the culture were found to be very effective at inhibiting *A. flavus* and aflatoxin production, both *in-vitro* and in stored peanuts. However, this *Streptomyces* and its metabolites have not previously been screened against other spoilage moulds or indeed against any human fungal disease-causing pathogens. In addition, the compounds responsible for the inhibition have also not previously been identified.

The next two sections focus on key human and agrifood fungal pathogens which were finally focused on in the subsequent *in-situ* studies.

1.6 GRAIN SPOILAGE AND MYCOTOXINS

Food and feeds such as cereals, nuts and spices are commonly contaminated with mycotoxigenic fungi and mycotoxins which are a serious problem faced by many countries (Lee and Ryu, 2017). This causes significant losses to producer countries when their exports are rejected because they do not meet the legislative limits, especially in Europe. The Rapid Alert System for Food and Feed shows that up 30% of commodities imported into the EU are rejected because of mycotoxin contamination (RASFF, 2018). Food contaminated with fungi can occur before, during and after harvest, during transportation, drying, storage and food processing due to improper management. Among

the pathogenic fungi which represent the greatest concern in terms of food contamination are species from the genera *Penicillium*, *Fusarium* and *Aspergillus*. Species from these three genera can all produce mycotoxins. Table 1.5 listed the major mycotoxins of worldwide prevalence produced by those genera. A survey conducted in 2014 revealed that more than half of worlds' regions were severely affected by mycotoxins including fumonisin (FUMs), deoxynivalenol (DON), and zearalenone (ZEA) which had increased when compared to 2013 (Kovalsky, 2015). Indeed, a more recent survey by Biomin (Tulln, Austria) has suggested that the level of contamination of feed ingredients with mycotoxins world-wide has increased in 2017. Moreover, the maximum and average ochratoxin A (OTA) concentration in sample from Europe in 2017 was the highest when compared to the other regions (Biomin, 2018). In a different survey by Limay-Rios et al. (2017) for four years, stored wheat in Canada was contaminated with *P. verrucosum* and contained not only OTA, but also citrinin and ochratoxin B (OTB). The higher contamination may in part be due to the impact of climate change where modifications in key environmental parameters and pest diversity may be responsible for an increase in *Fusarium* Head Blight (FHB) and *Septoria tritici* blotch (STB) on wheat and a reduction in yield (Váry et al., 2015). More recently, Medina et al. (2015, 2017) showed that climate change factors such as elevated CO₂, +4°C and drought stress can stimulate the biosynthetic genes in the aflatoxin cluster (*aflD* and *aflR*) and aflatoxin B₁ production by *A. flavus* on maize.

Exposure to highly contaminated ingredients and finished products can result in severe health problems such as cancer, effects on the immune system, damage to DNA and in the worst case scenario can cause death. In 2004, 125 people in Kenya died due to consumption of maize contaminated with high levels of aflatoxin (Centers for Disease Control and Prevention, 2004). More recently, in 2016, a number of children died after consuming maize contaminated with aflatoxins in Tanzania (Kamala et al., 2018).

Table 1.5 Major mycotoxigenic fungal genera and major classes of mycotoxins

Fungal pathogens	Mycotoxin	Commodities/ food contaminated with mycotoxin	References
<i>Penicillium</i>			
<i>P. verrucosum</i>	Ochratoxin A	Cereal grains (wheat, barley, oat, maize), dry bean, peanuts, cereal products, cured meats	Limay-Rios et al., 2017
<i>P. nordicum</i>	Ochratoxin A		Rodríguez et al., 2014
<i>Aspergillus</i>			
<i>A. flavus</i>	Aflatoxin	Maize, peanut, wheat, rice, cottonseed, copra, nuts, egg, milk, fig	Frisvad et al., 2019
<i>A. paraciticus</i>	Aflatoxin		Frisvad et al., 2019
<i>A. ochraceus</i>	Ochratoxin A	Coffee, raisins, grapes, dried fruits, wine	Taniwaki et al., 2018
<i>A. carbonarius</i>	Ochratoxin A		Akbar et al., 2016
<i>A. westerdijkiae</i>	Ochratoxin A		Akbar et al., 2016
<i>Fusarium</i>			
<i>F. verticillioides</i>	Fumonisin	Fumonisin - Maize T-2 and HT-2, Deoxynivalenol, Nivalenol - Maize wheat, oat, barley, rice, rye, feed Zearalenone - Maize, mold hay, feed	Samsudin et al., 2017
<i>F. graminearum</i>	Zearalenone Deoxynivalenol Nivalenol		Dweba et al., 2017
<i>F. langsethiae</i> <i>F. sporotrichioides</i>	T-2 and HT-2		Medina and Magan, 2011
<i>F. culmorum</i>	Deoxynivalenol		Spanic et al., 2018

1.7 CONTROL OF *P. VERRUCOSUM* AND OCHRATOXIN A (OTA) CONTAMINATION OF CEREALS

P. verrucosum is a major producer of OTA in cereals, particularly of wheat, oats, barley, rye and cereal products and is usually found in cool and temperate regions of Northern Europe and Canada (Limay-Rios et al., 2017; Lund and Prisvad, 2003). It is also known as a “storage fungus” because normally it invades and produces OTA after harvest during drying and storage (Duarte et al., 2010). It is a very competitive species and able to grow and produce OTA in wheat grain with relatively low moisture contents (approx. 17-18% or 0.80-0.83 a_w) and over a wide range of temperature (0-35°C) (Sanchis and Magan, 2004). Therefore, minimizing time between harvest and drying to <14.5% moisture content and keeping this level throughout storage is crucial to prevent *P. verrucosum* from become established (Magan et al., 2010) and contamination with OTA above the EU Commission Regulation limit (5 µg/kg unprocessed cereal). However, because grain is often sold on a wet weight basis, batches of dry (<14.5% MC) and wetter grain (16-17%) can be mixed in a grain silo. Depending on the ambient external conditions and changes in temperature this can lead to condensation and moisture migration, especially during the autumn and spring. Poor management of aeration and hygiene (pest infestation) can lead to pockets of moist grain allowing initiation of spoilage and mycotoxin contamination (Limay-rios et al., 2017; Jian et al., 2009; Magan and Aldred, 2007; Magan et al., 2004).

To control spoilage in moist grain for feed use sometimes preservatives based on propionic and acetic acid and their salts and modified atmospheres are used to try and control contamination with mycotoxins such as OTA. There are some limitations as the preservatives are fungistats and very good coverage of the grain is required for efficacy. Untreated pockets or low concentrations can lead to a stimulation of OTA production (Arroyo et al., 2005). Furthermore, the commercial preservative, Rovral, an imidazole fungicide, at concentrations $\geq 50\%$ of the recommended concentration to treat fungi strongly stimulated the production of OTA on wheat kernels although *P. verrucosum* growth was significantly inhibited (Schmidt-Heydt et al., 2013). The use of modified atmospheres with elevated CO₂ has been examined, but >50% CO₂ was necessary for effective control of OTA in stored wheat grain (Cairns-Fuller et al., 2005). Natural products particularly essential oils (garlic, wild oregano, neem, eucalyptus, bay, clove,

cinnamon oil) and phenolic compounds from plants have been found to be effective in inhibiting *P. verrucosum* and OTA production *in-vitro* (Ozcakmak et al., 2017; de Elgue-Culebras et al., 2016; Koteswara Rao et al., 2015; Jeršek et al., 2014; Aldred et al., 2008). However, they have been relatively ineffective in stored grain (Aldred et al., 2008). Furthermore, the application of antagonistic microorganism may not be suitable because of difficulties in application and establishment on stored cereals and possible stimulation of mycotoxin production due to competition (Magan et al., 2010). Recently, Vankudoth et al. (2016) found co-culture with various antagonistic fungal species was able to inhibit *P. verrucosum* growth and OTA production, possibly due to antifungal substances secreted by the antagonistic species. Therefore, the application of antifungal extracts to the grain may be better than the use of viable microorganisms. Previously, the efficacy of antifungal compounds from bacteria to control the growth of *Fusarium* spp., *A. flavus*, *F. oxysporum*, *P. expansum*, *R. solani* and *C. albicans* was reported (Kumar et al., 2013; Smaoui et al., 2012). However, they only examined effects on fungal growth inhibition, not on mycotoxin production or the effect of interacting environmental factors such as a_w x temperature effects on treatments. There has been very little focus on the potential use of antifungal compounds from bacteria for the control of growth of *P. verrucosum* and OTA production *in-vitro* and in stored grain. Thus, there is a need to identify and develop potential alternative compounds which may have applications for the control of this mycotoxigenic species and OTA contamination of stored commodities. The present study screened the bacteria and their antifungal compounds for efficacy against germination of conidia, growth of *P. verrucosum* and OTA production *in-vitro* and *in-situ* in stored grain.

1.8 ANTIFUNGAL RESISTANT OF HUMAN FUNGAL PATHOGENS

The issue of antimicrobial resistance is of international concern because of the serious threat to public health in many regions world-wide (WHO, 2014). The resistance to fungicidal compounds have been reported in relation to human fungal infections including those by *Trichophyton* species (dermatophytes), *Candida albicans* and *Aspergillus fumigatus* responsible for aspergillosis in immunocompromised patients. In fact there was an emergence of non-*C. albicans* infection and the resistance of both *C. albicans* and non-*C. albicans* species against fluconazole and echinocandin has increased

(Cleveland et al., 2012). For *A. fumigatus*, the frequency of resistance to azoles have increased significantly in Manchester, UK since 2004 since the first isolate in Manchester was detected in 1999 (Howard et al., 2009). Other than in the UK, azole resistant *A. fumigatus* strains have also been reported in Switzerland, USA, India and China (Riat et al., 2018; Hurst et al., 2017; Lockhart et al., 2011). In addition to this, the susceptibility of the dermatophyte *T. rubrum* to the antifungal drug has continuously decreased due to the prolonged exposure to sub-inhibitory concentration of this antifungal drug (Ghelardi et al., 2014)

The uncontrolled use of anti-microbial compounds has been identified as one of the factors which has increased the level of resistance. It has been suggested that the over-use of anti-microbials can change the balance of beneficial and harmful organisms and allow resistant strains to become more dominant. This has resulted in resistant microorganisms becoming dominant in a range of ecological niches (Shah, 2010). Development of anti-fungal resistance is an evolutionary process for each specific organism to adapt and survive in such stressed environments

The development of antifungal drug resistant infections has important implications for society and the economy. They impact on health care resources, and the loss of productivity has an impact on economic production. Thus, a built up of resistance to infections can result in longer stays in hospitals, a need for prolonged health treatment and additional resources which increases the medical costs, when compared to prevention strategies using effective antibiotics or antifungal. A report by the Centers for Disease Control and Prevention, (2013) estimated that each case of a patient infected with fluconazole resistant *Candida* required an additional \$6,000.00 to \$29,000.00 in healthcare costs and also required a 3 to 13 days longer stay in hospital.

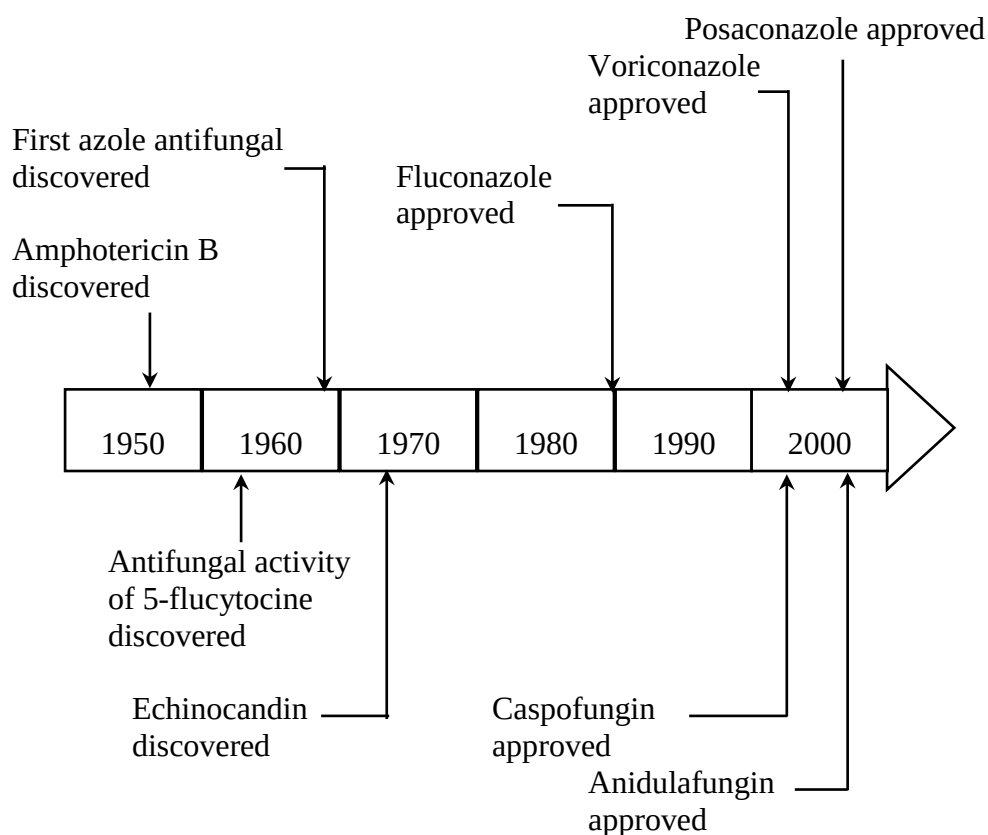


Figure 1.2 Timeline of key antifungal drug development (from Roemer & Krysan 2014)

1.9 FUNGAL DERMATOPHYTES

Fungal dermatophytes are common world-wide and are responsible for skin infections in both humans and animals (Kim et al., 2016; Seebacher et al., 2008; Teklebirhan et al., 2015). Indeed, all age groups can be affected, regardless of whether the patients are immunocompromised or not (Farag et al., 2018; Thomas et al., 2010; Wu et al., 2013). They need a keratin rich surface to grow on and thus predominantly infect skin, nails and hair of humans and animals. In addition, there are reports of deep dermatophytosis in immunosuppressed patients although these cases are relatively uncommon (Wu et al., 2013; Gong et al., 2007). The most common dermatophytic fungal species isolated have been from the *Trichophyton*, *Microsporum* and *Epidermophyton* genera. These dermatophytes can be transmitted from human to human (antrophophilic organisms), soil

to humans on contact (geophilic organisms) or via animals to humans (zoophilic organisms) (Hainer, 2003). The prevalence of people infected with dermatophytic fungi has been continuously increasing. In south-eastern Korea the number of patients with dermatophyte infections between 1979 and 2013 increased from 1,973 to 6,166 per year (Kim et al., 2016). In Italy, the frequency (%) and the number of fungal infections of nails (tinea unguium) and skin (tinea pedis) referred to the Mycology Service of the Dermatology Clinic of Policlinico Hospital - University of Bari, Italy during 1995-2010 increased progressively (Vena et al., 2012).

In addition to this, the susceptibility of the dermatophytes to antifungal drugs has continuously decreased. Recently, Ghannoum (2016) reported the resistance of dermatophytes to azoles had increased to 19% among the worldwide population. Earlier, Ghelardi et al. (2014) and Mukherjee et al. (2003) revealed that the resistance of *T. rubrum* isolated from patients to antifungal drug was increased after prolonged exposure to sub-inhibitory concentration of itraconazole, amorolfine and terbinafine. In addition to this, the terbinafine resistant *T. rubrum* also showed cross-resistance to naftifine, butenafine, tolnaftate and tolciclate (Mukherjee et al., 2003). This is a serious problem, as these drugs are used clinically either for oral (terbinafine and itraconazole) or topical treatment for dermatophyte infections. The cellular target of most of the antifungal drugs are ergosterol biosynthesis (Martinez-Rossi et al., 2008). Those antifungals inhibit enzymes involved in the biosynthetic pathways of ergosterol such as sterol reductase and cholestenol- Δ -isomerase by amorolfine, lanosterol 14- α demethylase by itraconazole and squalene epoxidase by terbinafine, naftifine, butenafine, tolnaftate and tolciclate (Gupta et al., 2013; Mukherjee et al., 2003).

Due to an increase in antifungal drug resistance, many studies have been carried out to try and identify alternative treatments, especially from natural sources. Although many plant extracts with anti-dermatophyte activities have been reported (Li et al., 2015; Khan and Ahmad, 2011; Koroishi et al., 2008; Ghahfarokhi et al., 2004), no clinical studies have been carried out to validate their efficacy. Furthermore, the consistency of the antifungal compounds from plants are generally difficult to maintain because of plant physiological conditions and the effect of agronomic environmental factors for plant growth.

The use of microorganisms particularly lactic acid bacteria (LABs) is one of the promising approaches to overcome the problem of the build up of resistance of dermatophytic fungi to the existing anti-fungal drugs. Previously, the efficacy of fermented foods as a method to control and overcome pathogenic bacterial resistance has been reported. Fox et al. (2015) found that the incidence of antibiotic resistant diarrhea among children prescribed broad spectrum antibiotics showed a reduction after being given 200 g/day probiotic yogurt from the beginning to the end of their antibiotic treatment. These effects have been associated with the presence and increased levels of various bioactive compounds in fermented food such as bacteriocins, free phenolic compounds, gamma-aminobutyric acid (GABA), peptides and enzymes (Limón et al., 2015; Chaves-López et al., 2014; Ahmadova et al., 2013b; Gonzalez-Gonzalez et al., 2013; Torino et al., 2013).

LABs isolated from Malaysian fermented foods have not been previously examined for control of human fungal pathogens, including dermatophytic fungi. Thus, these were screened for efficacy and control of the dermatophyte *T. rubrum* *in-vitro* and *in-situ*. Furthermore, the production of antifungal compounds was optimised and the primary compounds responsible were identified.

1.10 AIM AND OBJECTIVES

The aim of this study was to examine the efficacy of a large collection of lactic acid bacteria (LABs) from Malaysian fermented foods and a *Streptomyces* strain (AS1) against human fungal pathogens and mycotoxigenic spoilage fungi, to identify the antifungal metabolites and mechanism of action, and to carry out studies *in-situ* to examine whether efficacy was maintained in simulated practical conditions. The project was divided into four sequential phases in 5 separate Chapters with specific objectives.

PHASE 1. Screening of lactic acid bacteria (LABs) from Malaysian fermented foods and a *Streptomyces* strain (AS1) for efficacy against human and plant fungal pathogens

- (a) To screen 66 LABs isolated from Malaysian fermented foods against human (*Candida albicans*, *Trichophyton rubrum* and *Aspergillus fumigatus*) and

mycotoxigenic spoilage fungi (*Aspergillus flavus*, *Fusarium verticillioides*, *Penicillium verrucosum*, *Penicillium nordicum*)

- (b) To screen the metabolites produced by a *Streptomyces* AS1 strain isolated from peanuts for efficacy against the human and mycotoxigenic fungal plant pathogens detailed previously

PHASE 2. *In-vitro* efficacy of the best lactic acid bacteria (LABs) and *Streptomyces* AS1 metabolites for control of growth and mycotoxin production by selected pathogenic fungi

- (a) Determine the efficacy of LABs and the *Streptomyces* AS1 metabolites on spore germination and mycelial growth of the target fungal pathogens
- (b) Calculate the minimum inhibitory concentrations (MIC) and IC₅₀ values for the LABs and *Streptomyces* AS1 metabolites with efficacy against mycelial growth using the Bioscreen C system
- (c) To examine the effect of the *Streptomyces* AS1 metabolites on the production of mycotoxins by the target plant fungal pathogens
- (d) To isolate and identify the bioactive compounds produced by the LABs and the *Streptomyces* AS1

PHASE 3. Impact of LAB (*L. plantarum* VS1) and *Streptomyces* AS1 metabolites on mechanism of action in relation to production of extracellular hydrolytic enzymes by the target pathogens

- (a) Studies were initially conducted using a semi-quantitative screen of 19 different enzymes using the bioMérieux kit assay and then using p-nitrophenyl substrates for the key enzymes produced by the different target pathogenic fungi
- (b) Relationship between different concentrations of *L. plantarum* VS1 cell free supernatants on control of growth of *T. rubrum*, sporulation and hydrolytic enzyme production on a keratin based matrix
- (c) Examination of the effect of different concentrations of *Streptomyces* AS1 ethyl acetate extracts on *P. verrucosum* growth, ochratoxin A (OTA) production and hydrolytic enzyme activity on a conductive yeast extract sucrose (YES) agar medium modified to 0.99 and 0.95 a_w

PHASE 4. *In-situ* studies of the effects of bacterial metabolites in controlling (a) the growth of *T. rubrum* on nail matrices (from LAB) and (b) *P. verrucosum* and ochratoxin A production in stored wheat (from *Streptomyces* AS1).

- (a) To examine the efficacy of *L. plantarum* VS1 cell free supernatant on hydrolytic enzyme activity and inhibition of *T. rubrum* growth on nail matrices
- (b) To study the efficacy of *Streptomyces* AS1 ethyl acetate on control of *P. verrucosum* colonisation and OTA production in stored wheat of different initial moisture contents.

Figure 1.2 shows the relationship between the different components of the project and the thesis presentation.

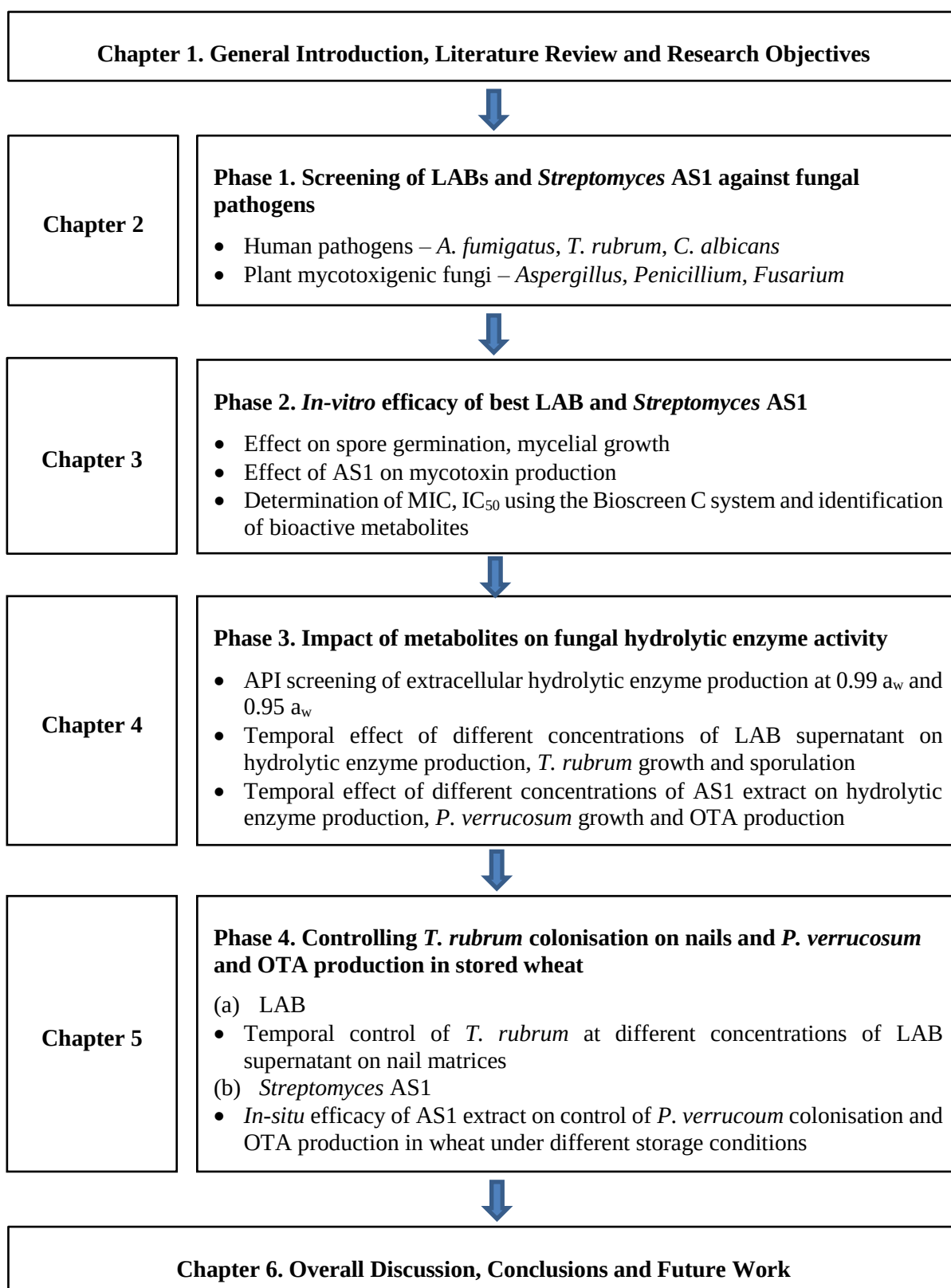


Figure 1.3 Flow diagram to summarise the links between the different phases of research carried out in this project.

CHAPTER 2

Screening of lactic acid bacteria (LABs) from Malaysian fermented foods and a *Streptomyces* strain (AS1) for efficacy against human and plant fungal pathogens

2.1 INTRODUCTION

Lactic acid bacteria (LABs) has long been used in preserving food and received much attention as safe and effective biopreservatives. There are several metabolites produced by LABs which are considered as preservative such as lactic acid, acetic acid, bacteriocins and reuterin. Previous studies have examined the antifungal activities of LABs and/or their metabolites towards both plant and human pathogenic fungi. LABs and their metabolites have been shown to inhibit the growth of *Aspergillus paraciticus*, *Fusarium graminearum*, *P. nordicum*, *A. fumigatus*, *C. albicans* and dermatophyte fungi (Sharma and Srivastava, 2014; Dogi et al., 2013; Guo et al., 2012; Guo et al., 2011; Schillinger and Villarreal, 2010; Kim, 2005). There are some reports of a reduction in mycotoxin production by *F. graminearum* such as that of zearalenone (ZEA) (Dogi et al., 2013). Most of the LABs with antifungal activity were from the *Lactobacillus*, *Pediococcus* and *Lactococcus* genera (Dalié et al., 2010). Many LABs have been isolated from Malaysian fermented foods and some studies have been done on their potential antibacterial properties especially of food-borne pathogens (Hajar and Hamid 2013; Ramasamy et al., 2012; Liasi et al., 2009). Very few studies have focused on their antifungal activities. Furthermore, antifungal activity studies were mainly on non-pathogenic food spoilage fungi such as *Aspergillus niger* and *Aspergillus oryzae* (Muhialdin et al., 2011). Thus, there is a lack of information on whether Malaysian LABs or their metabolites could be effective as preservatives to control spoilage mycotoxigenic fungi of food or human-related pathogenic fungi.

Actinomycete strains, particularly *Streptomyces* species, are known to produce various metabolites with antimicrobial properties. Previous studies by Sultan and Magan (2011) showed that a *Streptomyces* AS1 strain had antifungal properties and was able to control growth of *A. flavus* and inhibit aflatoxin production *in-vitro* and in stored peanuts. The potential efficacy of the *Streptomyces* AS1 against other spoilage mycotoxigenic and pathogenic fungi has not previously been examined.

The objective of this study was to screen (a) 66 lactic acid bacteria isolated from Malaysian fermented foods and (b) the *Streptomyces* AS1 strain isolated from peanuts, for efficacy against human (*C. albicans*, *T. rubrum*, *A. fumigatus*) and mycotoxigenic spoilage fungi (*A. flavus*, *F. verticillioides*, *P. nordicum*, and *P. verrucosum*).

2.2 MATERIALS AND METHODS

2.2.1 Microorganisms assayed for efficacy against fungal and human-related pathogens

A total of 66 lactic acid bacteria (LABs) (Table 2.1) were obtained from the Centre for Functional Food Collection (CFFC), Malaysian Agricultural Research and Development Institute (MARDI), Malaysia. These LABs were isolated from naturally fermented foods (vegetables, fish, rice bran and milk) around Malaysia and were from four genera; *Lactobacillus*, *Leuconostoc*, *Lactococcus* and *Pediococcus*. The *Streptomyces* strain (AS1) previously found to produce metabolites with efficacy against *A. flavus* was also used in the screening against fungal pathogens (Sultan & Magan, 2011). This strain was originally isolated from Egyptian peanuts. A total of seven plant and human fungal pathogens (including 2 strains of *A. fumigatus*) were screened (Table 2.2). These were obtained from the fungal collection of the Applied Mycology Group, Cranfield University and from Gloucester Hospital Trust (*Trichophyton* species).

Table 2.1 List of different lactic acid bacteria used in the screening assays

Lactic acid bacteria	Sources
<i>Pediococcus</i>	
<i>Pediococcus pentosaceus</i> 1151	Rice bran
<i>Pediococcus acidilactici</i> 1489	Rice bran
<i>Pediococcus acidilactici</i> 1328	Rice bran
<i>Pediococcus acidilactici</i> 806	Rice bran
<i>Pediococcus acidilactici</i> 1395	Rice bran
<i>Pediococcus acidilactici</i> 1195	Rice bran
<i>Pediococcus acidilactici</i> 1532	Rice bran
<i>Pediococcus acidilactici</i> 1474	Rice bran

<i>Pediococcus acidilactici</i> 84	Rice bran
<i>Pediococcus acidilactici</i> 1375	Rice bran
<i>Pediococcus acidilactici</i> 1537	Rice bran
<i>Pediococcus pentosaceus</i> 1386	Rice bran
<i>Pediococcus pentosaceus</i> 1410	Rice bran
<i>Pediococcus pentosaceus</i> 1423	Rice bran
<i>Pediococcus acidilactici</i> 1380	Rice bran
<i>Pediococcus acidilactici</i> 1419	Rice bran
<i>Pediococcus pentosaceus</i> 95	Rice bran
<i>Pediococcus acidilactici</i> 1413	Rice bran
<i>Pediococcus pentosaceus</i> 1417	Rice bran
<i>Pediococcus pentosaceus</i> strain MRSD2	Pickled vegetables (<i>Cleome gynandra</i>)
<i>Pediococcus pentosaceus</i> isolate: qz-201	Pickled vegetables (<i>Cleome gynandra</i>)
<i>Lactobacillus</i>	
<i>Lactobacillus acidophilus</i>	Acidophilus milk
<i>Lactobacillus</i> sp	Fermented cocoa
<i>Lactobacillus viridens</i>	Fermented cocoa
<i>Lactobacillus fermentum</i>	Fermented cocoa
<i>Lactobacillus casei</i>	Cultured milk
<i>Lactobacillus bulgaricus</i>	Yogurt
<i>Lactobacillus casei</i>	Cultured milk
<i>Lactobacillus acidophilus</i>	Supplement
<i>Lactobacillus coryniformis</i> 1483	Rice bran
<i>Lactobacillus casei</i>	Coconut sap vinegar
<i>Lactobacillus harbinensis</i>	Coconut sap vinegar
<i>Lactobacillus plantarum</i> strain BJ26-2	Fish sauce (<i>Stolephorus</i> spp.)
<i>Lactobacillus farciminis</i> strain 5-33	Pickled fish (<i>Barbodes schwanenfeldi</i>)
<i>Lactobacillus acidophilus</i> strain AA105	Salted fish (<i>Pennahia</i> spp.)
<i>Lactobacillus plantarum</i> strain TCP008	Pickled shrimp (<i>Metapenaeus lysianassa</i>)

<i>Lactobacillus plantarum</i> strain qz-572-2	Pickled shrimp (<i>Metapenaeus lysianassa</i>)
<i>Lactobacillus plantarum</i> strain L1212	Pickled shrimp (<i>Metapenaeus lysianassa</i>)
<i>Lactobacillus plantarum</i> strain X3-5B	Pickled vegetable (<i>Cleome gynandra</i>)
<i>Lactobacillus futsaii</i> strain YM 0188	Pickled vegetable (<i>Cleome gynandra</i>)
<i>Lactobacillus plantarum</i> strain VN5	Pickled vegetable (<i>Cleome gynandra</i>)
<i>Lactobacillus paralimentarius</i> strain Akhavan.a-Q2	Pickled vegetable (<i>Cleome gynandra</i>)
<i>Lactobacillus plantarum</i> strain DJ-04	Pickled siew pak choy
<i>Lactobacillus plantarum</i> strain 49	Pickled siew pak choy
<i>Lactobacillus plantarum</i> strain B4.3.1	Pickled siew pak choy
<i>Lactobacillus plantarum</i> strain Akhavan-Q3	Pickled siew pak choy
<i>Lactobacillus plantarum</i> isolate isolate 11	Pickled siew pak choy
<i>Lactobacillus plantarum</i> strain SM71	Pickled siew pak choy
<i>Lactobacillus brevis</i> strain Lb14F1	Pickled siew pak choy
<i>Lactobacillus plantarum</i> strain TN635	Pickled siew pak choy
<i>Lactobacillus brevis</i> strain PQ25	Pickled siew pak choy
<i>Lactobacillus plantarum</i> strain VS1	Pickled vegetable (<i>Parkia speciose</i>)
<i>Lactobacillus pentosus</i> strain SM35	Pickled vegetable (<i>Parkia speciose</i>)
<i>Lactobacillus brevis</i> strain SD2S7L1	Pickled vegetable (<i>Parkia speciose</i>)
<i>Lactobacillus plantarum</i> strain SKT109	Pickled vegetable (<i>Parkia speciose</i>)
<i>Lactococcus</i>	
<i>Lactococcus lactis</i> 1057	Rice bran
<i>Lactococcus garvieae</i> 1402	Rice bran
<i>Lactococcus garvieae</i> 1415	Rice bran
<i>Lactococcus</i> spp. SXVIII12(2011	Milk
<i>Lactococcus lactis</i> strain LC (S)	Milk
<i>Lactococcus lactis</i> strain N1 (S)	Milk
<i>Lactococcus lactis</i> subsp <i>lactis</i> 10-1	Milk

<i>Leuconostoc</i>	
<i>Leuconostoc</i> spp.	Soy sauce
<i>Leuconostoc mesenteroides</i> strain CTBRBL226	Shrimp sauce (<i>Metapenaeus lysianassa</i>)
<i>Leuconostoc mesenteroides</i> strain MPL49	Shrimp paste (<i>Metapenaeus lysianassa</i>))
<i>Leuconostoc mesenteroides</i> subsp. <i>dextranicum</i> strain qz-534	Pickled fish (<i>Scomberomorus guttanus</i>)

Table 2.2 List of fungal pathogens used in the screening assays

	Fungal pathogens
1.	<i>Aspergillus flavus</i>
2.	<i>A. fumigatus</i> (Mi26)
3.	<i>A. fumigatus</i> (Mi538)
4.	<i>Penicillium verrucosum</i>
5.	<i>P. nordicum</i>
6.	<i>Fusarium verticillioides</i>
7.	<i>Trichophyton rubrum</i>
8.	<i>Candida albicans</i>

2.2.2 Preparation of bacterial cell and fungal spore suspensions

(a) Preparation of lactic acid bacteria (LABs) cell suspensions and *Streptomyces* AS1 spore suspensions

For the preparation of LAB cell suspensions, each glycerol stock of each LAB strain was inoculated on sterile de Man, Rogosa, Sharpe (MRS) agar and incubated at 30°C for 20 h. After 20 h, 2 mL of sterile sodium chloride (NaCl 0.85%; w/v) was poured onto MRS agar containing the cells, and they were harvested using a sterile spreader and transferred aseptically into sterile 50 mL conical tubes. The cell suspension was then centrifuged at 2000 g, 4°C for 3 min. The NaCl was discarded and fresh sterile 0.85% NaCl was added

and the density of the cell suspension was adjusted with sterile 0.85% NaCl to approximately 1.0 at OD₆₀₀ or 10⁷ to 10⁸ CFUs/mL.

A glycerol stock solution of *Streptomyces* AS1 was inoculated on ½ nutrient agar (NA) and incubated at 25°C for 5 days or until sporulation had occurred. The colonies were flooded with sterile 10 mL of 0.1% (w/v) Tween-80 water and harvested by gently scraping the colony with a sterile spreader to release the spores and this was then transferred aseptically into a sterile 50 mL conical tube and the density adjusted to 1.0 at OD₆₀₀ or approximate 10⁸ spores/mL.

(b) Preparation of fungal spore suspensions

Filamentous fungi: *A. flavus*, *A. fumigatus* and *F. verticillioides* were grown on Malt Extract Agar (MEA; Oxoid Ltd) and *P. nordicum*, *P. verrucosum* on Potato Dextrose Agar (PDA, Oxoid Ltd) for 7 to 10 days or until sporulation occurred at 25°C and 30°C for *A. fumigatus*. *T. rubrum* was grown on Sabouraud Dextrose Agar (SDA; Oxoid Ltd) at 30°C for 14 days (or until sporulation). *C. albicans* was grown on PDA at 30°C for 24 h. The yeast cells and fungal spores were harvested by pouring 10 mL of sterile 0.1% Tween-80/water solution onto the agar surface containing the cells or spores and gently scraping with a surface sterilised glass rod. The cell/spore suspensions were transferred into sterile 50 mL tubes, centrifuge at 2000 rpm for 2 min and the supernatants discarded. Fresh 0.1% Tween-80/water was added. The density of yeast cells was adjusted to 0.5 at OD₆₀₀ or approximately 10⁷ CFUs/mL. The concentration of fungal spores were counted using a haemocytometer (Thoma, Germany) and adjusted to approx. 10⁶/mL with sterile 0.1% Tween-80/water.

2.2.3 Preparation of cell free supernatants (CFS)

The cell suspensions (300 µL) were inoculated into sterile 30 mL of MRS broth and incubated at 30°C for 48 h in static conditions. The cell free supernatants were obtained by centrifugation at 4,000 g for 10 min at 4°C (Thermo Scientific) and the pH was measured.

The *Streptomyces* AS1 spore suspension (200 µL) was inoculated into 200 mL ½ strength sterile nutrient broth (NB) and incubated at 30°C at 200 rpm for 4 days. After 4 day the

supernatant was separated from the mycelium by filtration using Whatman filter paper no 4.

Finally, the supernatant was filter sterilized using a sterile cellulose acetate syringe filter (0.22 μ m, Sartorius, UK) and kept at -20°C until used.

2.2.4 Agar well diffusion assay

Antifungal activity of LAB cell free supernatants was first examined using the agar well diffusion assay. A total of 50 μ L of fungal pathogen spore suspension and cell suspension of *C. albicans* were spread uniformly on solid growth media with sterile bent glass rods and dried at room temperature for 30 min. The wells were cut with a sterile 6 mm cork borer and 50 μ L of CFS was loaded. The plates were then kept at ambient temperature (approx. 25°C) in sterile condition for 3 h to allow the diffusion of CFS into the agar (Tagg and McGiven, 1971). The plates were then incubated at 25°C for *A. flavus*, *P. nordicum*, *P. verrucosum*, and *F. verticillioides* for 48 h; at 30°C for *T. rubrum* for 5 days and *C. albicans* at 30°C for 24 h. The diameter of the clearing zone around the agar wells which indicated fungal inhibition was measured and grouped according to Ghanbari et al. (2013) as followed; -: no inhibition zone, +: 1-5 mm inhibition zone, ++: 5-8 mm inhibition zone and +++: >8 mm inhibition zone. Un-inoculated MRS broth was used as a control. All the experiments were done in triplicate.

2.2.5 Agar overlay screening method

The LABs and *Streptomyces* AS1 cells were inoculated on MRS agar and ½ NA respectively as a 2 cm streak across the middle of the 9 cm Petri plates. The LABs were then incubated at 30°C for 48 h and the *Streptomyces* AS1 at 25°C for 72 h. The plates were then overlaid with a mixture of 7 mL of sterile soft agar and 50 μ L of spore suspension of the target pathogens (10⁶ spores/mL) and incubated at 25°C for 48 h for *F. verticillioides* (Gupta and Srivastava, 2014) and 24 h for *C. albicans*. The soft agar consisted of a mixture of 0.7% agar and 1.5% ME for *Fusarium* and 1.5% PD for *C. albicans*. The area of the clear zone and the percentage inhibition was determined based on the calculation below and scaled according to Magnusson et al. (2003):

$$\text{Area} = \pi (3.14) \times r^2 \text{ (radius of horizontal axis} \times \text{radius of vertical axis)}$$

$$\% \text{ inhibition} = \frac{\text{Area of inhibition zone}}{\text{Area of petri plate}} \times 100$$

Area of petri plate

Scale:

- = no inhibition
- + = no fungal growth at 0.1 - 10% of Petri plate area
- ++ = no fungal growth at 11 - 20% of Petri plate area
- +++ = no fungal growth at >20% of Petri plate area

2.2.6 Dual culture assays

Single colony of the LABs and *Streptomyces* AS1 were inoculated on MRSA and ½ NA, respectively as a 2 cm streak approximately 2 cm from the 9 cm Petri plate edge. After incubation at 30°C for LABs and 25°C for *Streptomyces* AS1 for 48 h, an amount of 5 µL of spore suspension (10⁶ spores/mL) was applied (Sultan and Magan, 2011) at a distance of 3-4 cm from the LABs or *Streptomyces* AS1. *Trichophyton* assays were incubated at 30°C for 20 days, *A. fumigatus* at 30°C for seven days and the other assays at 25°C for seven days. The inhibition was determined based on the fungal colony area and macroscopic interaction between the dual cultures and scored as an Index of Dominance (I_D) developed by Magan and Lacey (1984). The Index scores were: 1) 1:1 - mutual intermingling, 2) 2:2 - mutual antagonism on contact, 3) 3:3 - mutual antagonism at a distance, 4) 4:0 - dominance of one species on contact and 5) 5:0 - dominance of one species at a distance. All the experiments were done with three triplicates and repeated once.

2.2.7 Statistical analysis

Normal distribution of data was checked by Shapiro-Wilk W Test. For data with normal distribution, the general influence of LABs and *Streptomyces* AS1 on fungal colony area was checked using a one way analysis of variance (ANOVA) and Kruskal Wallis Tests (Rank Sums) for non-normally distributed data. Student's t test was further applied to compared mean of fungal colony area treated with different LAB strains and

Streptomyces AS1 for normally distributed data and Wilcoxon method (non-parametric comparison) for non-normal distribution dataset. $p < 0.05$ was accepted as significant difference. The JMP Pro (SAS Institute Inc., Cary, North Carolina, USA) was used in the analysis.

2.3 RESULTS

2.3.1 Screening of lactic acid bacteria for efficacy in the agar well diffusion assay

The final pH of LAB strains supernatants was measured after 48 h incubation (Table 2.3). *Pediococcus*, *Lactobacillus* and *Lactococcus* were effective in reducing the pH. All *Pediococcus* strains, 82% of the *Lactobacillus* and 57% of *Lactococcus* strains showed pH values < 4.1 after 48 h incubation. However, *Leuconostoc* strains were less effective as the lowest pH measured was 4.2. None of the *Pediococcus* and *Lactococcus* strains had pH values > 5.1 except for one strain each of the *Lactobacillus* and *Leuconostoc* strains.

Table 2.3 Final range of pHs of supernatants after 48 h growth at 30°C in static conditions for the different groups of strains examined.

LAB Strains	Final pH		
	3.0 – 4.0	4.1 – 5.0	5.1 – 6.0
<i>Lactobacillus</i> (34 strains)	28 strains	5 strains	1 strain
<i>Pediococcus</i> (21 strains)	21 strains	0	0
<i>Lactococcus</i> (7 strains)	3 strains	4 strains	0
<i>Leuconostoc</i> (4 strains)	0	3 strains	1 strain

Culture supernatants were used as a first assay to examine the potential for inhibition of growth of several pathogenic fungi using the agar well diffusion assay. Figure 2.1 shows an example of inhibition zone around the wells. It was noted that none of the LABs

produced any effect on growth of *A. flavus*, *P. nordicum*, *P. verrucosum*, *C. albicans* and *T. rubrum* (Table 2.4). Only three *Lactobacillus* and two *Pediococcus* strains showed a very weak inhibition to *F. verticillioides* with inhibition zones of between 1-5mm.



Figure 2.1 Example of fungal growth inhibition using the agar well diffusion assay

Table 2.4 Antifungal activity of *Lactobacillus*, *Pediococcus*, *Lactococcus* and *Leuconostoc* strains against several selected fungal pathogens using the agar well diffusion method.

Number of LAB strains	Inhibition scale					
	<i>A. flavus</i>	<i>F. verticillioides</i>	<i>P. nordicum</i>	<i>P. verrucosum</i>	<i>T. rubrum</i>	<i>C. albicans</i>
<i>Lactobacillus</i> (34 strains)	-	+	-	-	-	-
		(3 strains)				
<i>Pediococcus</i> (21 strains)	-	+	-	-	-	-
		(2 strains)				
<i>Lactococcus</i> (7 strains)	-	-	-	-	-	-
<i>Leuconostoc</i> (4 strains)	-	-	-	-	-	-

Key: (-)= no inhibition zone, (+)= 1-5 mm inhibition zone, (++)= 5-8 mm inhibition zone and (+++)= >8 mm inhibition zone. The result is a mean of three replicates. *A. flavus*, *F. verticillioides*, *P. nordicum* and *P. verrucosum* were incubated at 25°C for 48 h, *C. albicans* at 30°C for 24 h and *T. rubrum* at 30°C at 5 days.

2.3.2 Screening of the LABs and *Streptomyces* AS1 for efficacy against fungal pathogens using the agar overlay assay

Screening was further conducted using the agar overlay method against *F. verticillioides* as an indicator strain. *F. verticillioides* was chosen because it showed sensitivity to some LABs when compared to other pathogenic fungi in the initial agar well diffusion assays. Figure 2.2 shows an example *F. verticillioides* growth inhibition around bacterial cultures. With the exception of three LABs (each one from *Lactobacillus*, *Leuconostoc* and *Pediococcus*), all strains were observed to have anti-*Fusarium* activity with a different degree of efficacy (Table 2.5). Among these strains, *Lactobacillus plantarum* VN5, *L. plantarum* VS1, *Pediococcus acidilactici* 84 and *P. pentosaceus* 95 were found to have the strongest inhibitory activity with the inhibition zone areas of 26%, 25%, 23% and 21%, respectively. In contrast, a large number of *Lactobacillus* (23/34), *Lactococcus* (4/7), *Leuconostoc* (3/4) and *Pediococcus* (16/21) strains showed inhibition areas between 11 and 20% only.

Two *Lactobacillus* and two *Pediococcus* strains with the most effective inhibitory activity were selected, and their potential efficacy against *C. albicans* was assayed. Overall, four LAB strains (*L. plantarum* VN5, *L. plantarum* VS1, *P. acidilactici* 84 and *P. pentosaceus* 95) were less effective against *C. albicans* (Table 2.6). However, *C. albicans* was sensitive to *Streptomyces* AS1 with about 20% inhibition of the plate area.

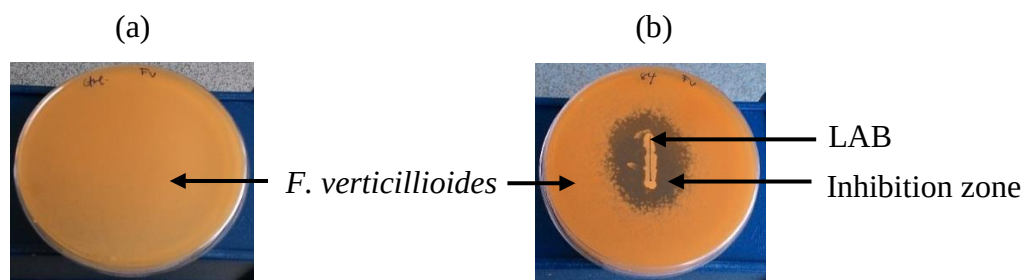


Figure 2.2 Example of the inhibition of *F. verticillioides* growth using the agar overlay assay with a lactic acid bacterium at 25°C. (a) Control, (b) LAB overlay with *F. verticillioides*.

Table 2.5 Determination of the number of LABs showing antifungal effect on MRS agar at 30°C for 48 h before being overlaid with a mixture of *F. verticillioides* spore suspension and soft ME agar and incubated at 25°C for 48 h.

LAB Strains	Inhibition scale			
	-	+	++	+++
<i>Lactobacillus</i> (34 strains)	1/34	8/34	23/34	2/34
<i>Lactococcus</i> (7 strains)	0/7	3/7	4/7	0/7
<i>Leuconostoc</i> (4 strains)	1/4	0/4	3/4	0/4
<i>Pediococcus</i> (21 strains)	1/21	2/21	16/21	2/21

(-) = no suppression, (+) = no fungal growth on 0.1-10% of the plate area per bacterial streak, (++) = no fungal growth on 11-20% of the plate area per bacterial streak, (+++) = no fungal growth on >20% of the plate area per bacterial streak.

Table 2.6 Effect of four selected LABs and the *Streptomyces* AS1 on inhibition of *C. albicans* using the agar overlay method.

LAB strains / <i>Streptomyces</i> AS1	Inhibition scale
<i>L. plantarum</i> VS1	+
<i>L. plantarum</i> VN5	+
<i>P. acidilactici</i> 84	+
<i>P. pentosaceus</i> 95	+
<i>Streptomyces</i> AS1	+++

(-) = no suppression, (+) = no fungal growth on 0.1-10% of the plate area per bacterial streak, (++) = no fungal growth on 11-20% of the plate area per bacterial streak, (+++) = no fungal growth on >20% of the plate area per bacterial streak.

2.3.3 Screening of LAB strains and *Streptomyces* AS1 for control of fungal pathogens using dual-culture assays

L. plantarum VS1, *L. plantarum* VN5, *P. pentosaceus* 95 and *P. acidilactici* 84, were examined in more detail for control of growth of *Aspergillus*, *Penicillium*, *Fusarium* strains and *T. rubrum* using the dual-culture assays. Figure 2.3 compares the relative colonization areas of various pathogenic fungi in the presence of four LABs strains and the *Streptomyces* AS1. The colony area of all the fungi was measured after seven days, with the exception of the *Trichophyton* which grew very slowly and this was done after 20 days. Overall, there was a reduction of colony areas of *A. flavus*, *A. fumigatus* Mi538, *A. fumigatus* Mi26, *F. verticillioides* and *T. rubrum* in the presence of each of the LABs tested except for *A. fumigatus* (Mi538) in the presence of VS1. However, these colony area reductions were not significant ($p>0.05$) when compared to the control and between the LABs. Furthermore, both *P. verrucosum* and *P. nordicum* growth were also unaffected by the presence of all the LABs. In contrast, the reduction of *T. rubrum* colony area was significant ($p<0.05$) when compared to the control. *P. pentosaceus* 95, *Pediococcus acidilactici* 84, *L. plantarum* VS1 and *L. plantarum* VN5 reduced the *T. rubrum* colony area by 99%, 98%, 96% and 89%, respectively.

A significant reduction ($p<0.05$) in colony area (cm^2) of all pathogenic fungi in the presence of *Streptomyces* AS1 were observed. The highest reduction was for *P. verrucosum* (90%) followed by *P. nordicum* (76%), *T. rubrum* (71%), *A. fumigatus* Mi538 (59%), *F. verticillioides* (51%) and *A. fumigatus* Mi26 (45%).

Table 2.7 shows the interaction scores and Index of dominance (I_D) of the LABs and the *Streptomyces* AS1 strain and the pathogenic fungi. Interaction scores give information on the type of macroscopic interactions between the competing strains/species (Magan and Lacey, 1984). I_D is the sum of interaction scores for each competing strain/species. It provides an overall view of the best competitor due to metabolite production or direct colony interactions. The interaction of LABs with competitors were variable with in some cases mutual intermingling, antagonism at a distance or dominance depending on the target pathogenic fungal strain. The LABs were most effective against *T. rubrum* which was dominant at a distance. The LABs were competitive against *Aspergillus* and *Fusarium* species with growth inhibited at a distance. However, *P. pentosaceus* 95 was

uncompetitive against *A. fumigatus* Mi26 where mutual intermingling occurred. Overall, *P. acidilactici* 84, *L. plantarum* VS1 and *L. plantarum* VN5 had similar I_D totals (17) except for *P. pentosaceus* 95 which had a slightly lower I_D total (15) and were less dominant than the *Streptomyces* AS1 (I_D total 35). Figure 2.4 shows the visual growth of fungi in the presence of the LABs. Overall, the presence of the LABs decreased the growth of the *Aspergillus* and *Fusarium* species and *T. rubrum*. There was also an inhibition zone between LABs and the test fungi. Furthermore, *T. rubrum* was strongly inhibited by LABs at a distance and the establishment of the colony was very slow.

The interactions between *Streptomyces* AS1 and each fungal pathogen showed that this strain was the most dominant species (I_D total 35) and inhibited all competitors at a distance. The competitors tended to grow away from this *Streptomyces* AS1 strain. Indeed, the colony area after day three was not increased, especially for *P. nordicum* and *P. verrucosum* (Figure 2.4)

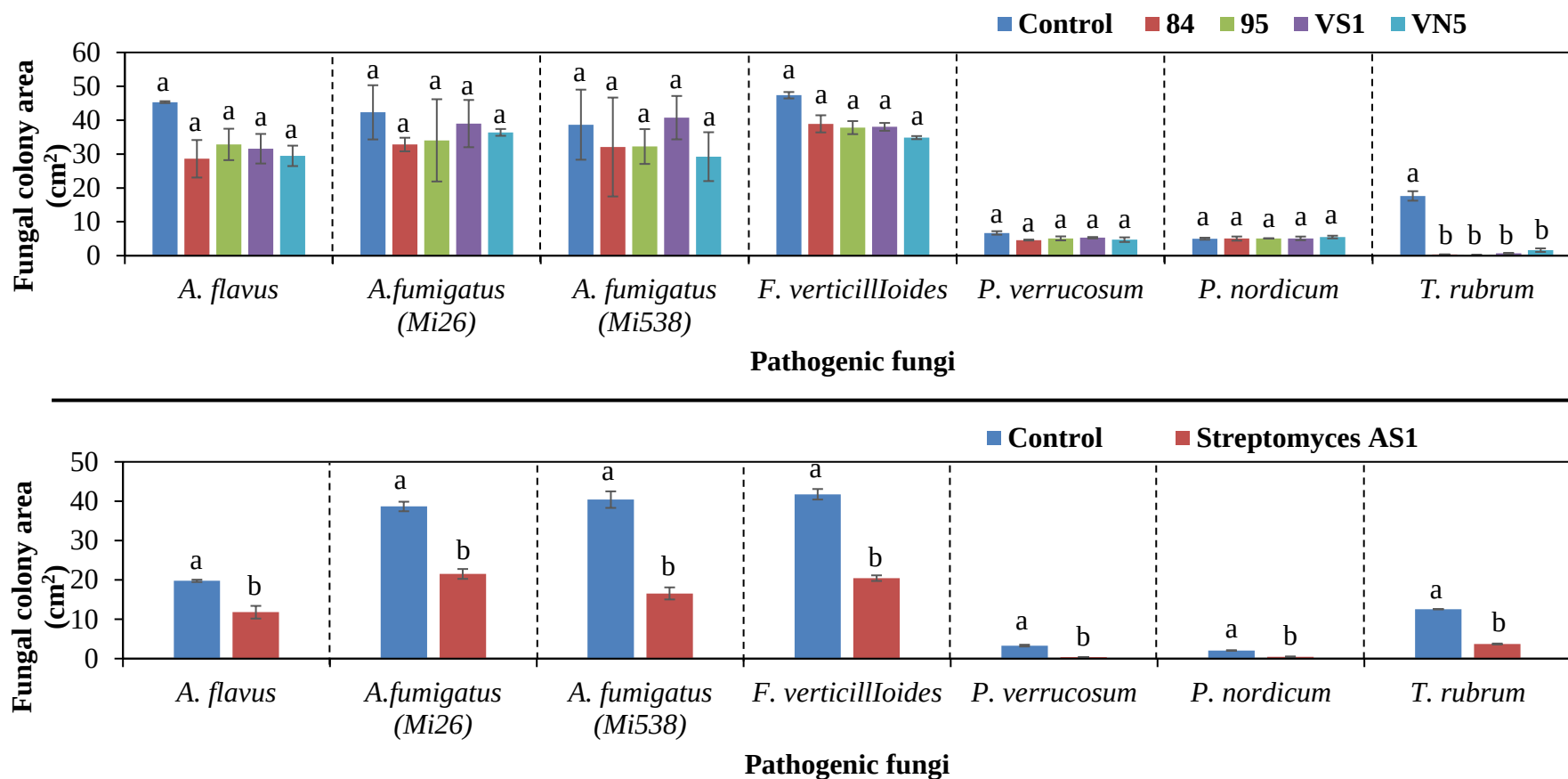


Figure 2.3 Effect of interaction between bacteria (LABs and *Streptomyces* AS1) and fungal pathogens on the fungal colony area. Each value is a mean of three replicates $\pm$ SD. Different letter (a,b) indicates significant difference ($p < 0.05$) within the treatment by Student's t test and Wilcoxon method (non parametric). Key: 84 - *P. acidilactici*, 95 - *P. pentosaceus*, VS1 - *L. plantarum*, VN5 - *L. plantarum*.

Table 2.7 Effect of interaction between bacterial strains and *Streptomyces* AS1 and different pathogenic fungi on the individual scores and the total Index of Dominance (I_D). The first score refers to the LABs /AS1 and the second to the target pathogens.

Bacterial strains	Interaction scores							I _D
	T.r.	A.f.	A.fumi Mi26	A.fumi Mi538	P.n.	P.v.	F.v.	
84	5:0	3:3	3:3	3:3	n.g.	n.g.	3:3	17:12
95	5:0	3:3	1:1	3:3	n.g.	n.g.	3:3	15:10
VS1	5:0	3:3	3:3	3:3	n.g.	n.g.	3:3	17:12
VN5	5:0	3:3	3:3	3:3	n.g.	n.g.	3:3	17:12
AS1	5:0	5:0	5:0	5:0	5:0	5:0	5:0	35:0

Key: 84: *P. acidilactici*; 95: *P. pentosaceus*; VS1: *L. plantarum*; VN5: *L. plantarum*; AS1: *Streptomyces*; A.f.: *A. flavus*; A. fumi: *A. fumigatus*; F.v.: *F. verticilloides*; P.n.: *P. nordicum*; P.v.: *P. verrucosum*; T.r.: *T. rubrum*; n.g.; not enough growth. *T. rubrum* was incubated at 30°C for 20 days, *A. fumi* Mi26 and *A. fumi* Mi538 at 30°C for 7 days and *A. flavus*, *F. verticilloides*, *P. verrucosum* and *P. nordicum* at 25°C for 7 days.

Key (I_D) - Index of Dominance

Key to macroscopic scores:

1:1	Mutual intermingling
2:2	Mutual antagonism on contact
3:3	Mutual antagonism at a distance
4:0	Dominance of one species on contact
5:0	Dominance of one species at a distance

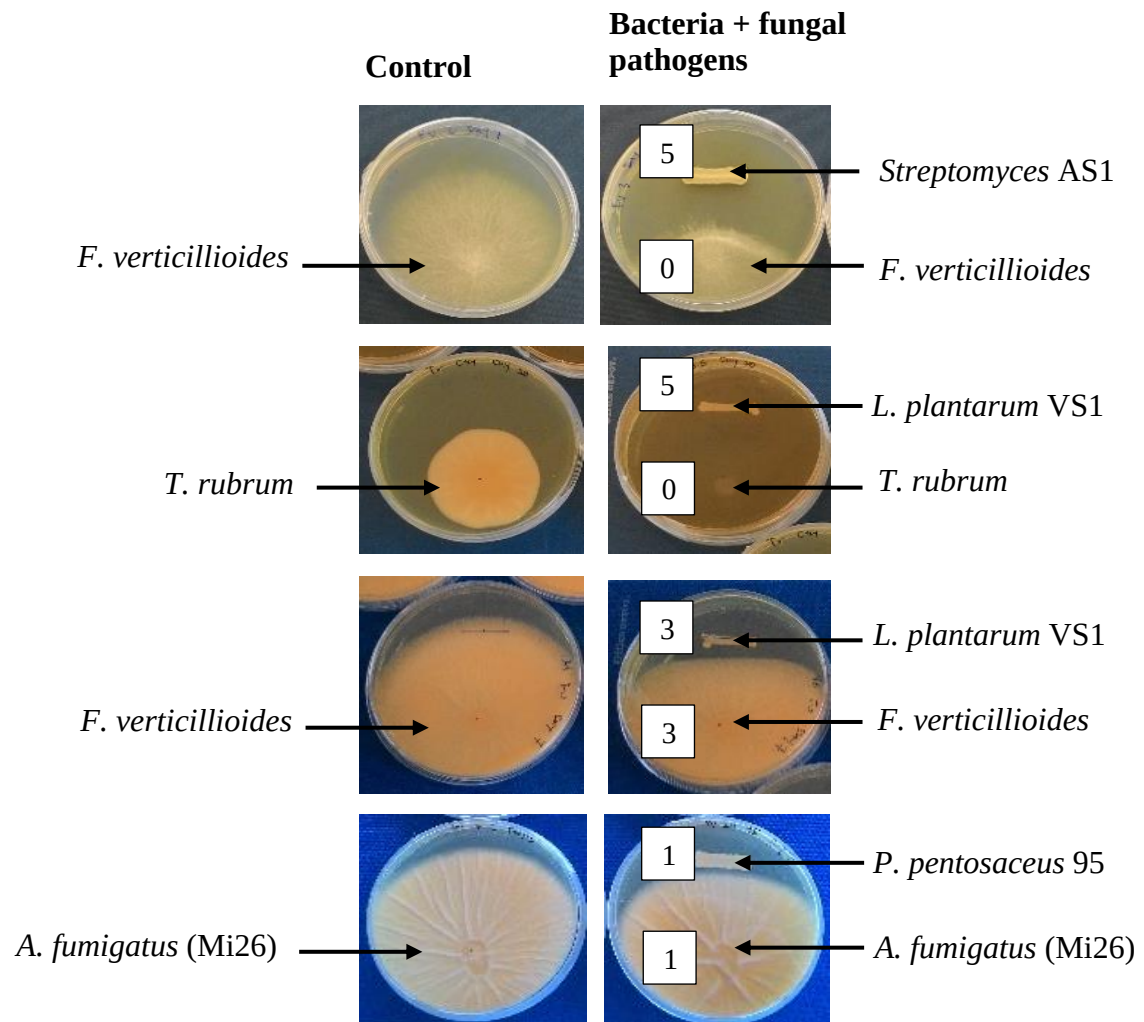


Figure 2.4 Dual culture assay showing examples of different interactions between antagonist (*L. plantarum* VS1, *P. pentosaceus* 95 and *Streptomyces* AS1) and fungal pathogens and the individual Index scores.

2.4 DISCUSSION

In the present study, a total of 66 LAB strains including strains of *Lactobacillus*, *Pediococcus*, *Lactococcus* and *Leuconostoc* isolated from a range of Malaysian fermented foods and a *Streptomyces* AS1 isolated from Egyptian peanuts, were screened for their efficacy against several human and spoilage mycotoxigenic fungal species. Three methods, including the agar well diffusion, agar overlay and dual-culture assays were used to identify potential LABs with anti-fungal properties and the efficacy of *Streptomyces* AS1 against other fungal pathogens not previously screened.

The use of the culture supernatant in the agar well diffusion assay only showed weak antifungal activity against *F. verticillioides* by three *Lactobacillus* and two *Pediococcus* strains. The relatively ineffective anti-fungal activity of the LABs in the present study might be due to the low concentrations of antifungal compounds secreted in the culture supernatant (Kim, 2005), although 79% of the LAB supernatants had low pH values of between 3.0- 4.0. It has been suggested that the presence of organic acids and non-organic acid metabolites in the supernatant contributes to enhanced antifungal activity of LABs. There is also some suggestion that inhibition may not be directly related to pH (Schillinger and Villarreal, 2010).

The growth inhibition was more pronounced when live cells of LABs were used (agar overlay assays). From this study, four LAB strains from *Lactobacillus* isolated from pickled vegetables and *Pediococcus* isolated from rice bran possessed the strongest inhibitory activity against *F. verticillioides*. However, in dual assays these four LABs were not effective. Indeed, most fungal pathogens were unaffected by the LABs in the dual culture assays. The only efficacy was observed for these four LAB strains against the dermatophyte *T. rubrum* with dominance at a distance (5/0). This suggests that the antifungal activity is not only dependent on the LAB strains (Cheong et al., 2014) but also on the target fungal species (Gupta and Srivastava, 2014). Overall, from a total of 66 LAB isolates, only four belonging to *Lactobacillus* and *Pediococcus* strains or 6% had strong antifungal activity. The low level of efficacy of LAB isolates against fungal pathogens has been previously reported by Cheong et al. (2014); Guo et al. (2011); Rouse et al. (2008); Kim (2005) and Magnusson et al. (2003). Overall, they found between 1-4% of strains screened were effective. These previous studies found strains of *Lactobacillus* and

Pediococcus were more effective in controlling fungal pathogens. This was due to the production of a mixture of active compounds such as lactic acid, acetic acid, phenyllactic acid and cyclic dipeptides (Magnusson et al., 2003, Rouse et al., 2008).

Sultan & Magan (2011) showed that *Streptomyces* AS1 had antifungal properties against *A. flavus*. In the present study *Streptomyces* AS1 was screened against other human and spoilage mycotoxigenic fungi and this showed that it has the ability to control a wide range of fungi. *Streptomyces* AS1 generally inhibited all the species tested with an interaction score of “5” and the pathogen score “0” indicating dominance at a distance with the production of anti-fungal metabolites. Previous studies with *Streptomyces* species has been associated with the production of primary and secondary metabolites including hydrolytic enzymes and antibiotic like compounds (Prapagdee et al., 2008; Taechowisan et al., 2005)

2.5 CONCLUSIONS

Overall, the best four LABs have been identified for further study against the dermatophyte *T. rubrum*. In addition, the *Streptomyces* AS1 had a broad spectrum of antifungal activity and strongly inhibited all the fungal pathogens examined.

CHAPTER 3

***In-vitro* efficacy of the best lactic acid bacteria (LABs) and *Streptomyces* AS1 metabolites for control of growth and mycotoxin production by selected pathogenic fungi**

3.1 INTRODUCTION

There has been significant interest in the use of natural primary and secondary metabolites from microorganisms to control fungal pathogens which cause diseases of humans, and contaminate food and feed (Guo et al., 2011; Faheem et al., 2015; Dogi et al., 2013). Thus, there has been interest in the utilisation of actively growing natural microorganisms to competitively exclude fungal pathogens or by using their naturally produced metabolites for inhibiting the germination, growth and toxin production by such pathogens. It is also important to conserve the efficacy of these bioactive compounds where they may be applied.

Lactic acid bacteria (LABs) and *Streptomyces* species produce both primary and secondary metabolites with antifungal activity. Some have been found to inhibit spore germination and mycelial growth (Schillinger and Villarreal, 2010; Wang et al., 2013). However, sometimes the target pathogens have been shown to develop resistance to potentially fungicidal compounds (Chowdhary et al., 2013). Furthermore, many studies have screened novel metabolites for efficacy against mycelial growth, with less emphasis on control of toxin production or in relation to different interacting environmental conditions (Yan et al., 2004). Because of strict legislation on maximum contamination levels for mycotoxins in food raw and processed commodities, especially cereals, there is interest in the identification of natural compounds which may control or reduce contamination. Surveys of food raw commodities have also shown that while frequency of contamination with a mycotoxin may remain the same over time, sometimes the concentrations may significantly increase. For example, 9% of soybeans were positive for zearalenone (ZEN) in 2014 and 2015. However, the mean concentration of ZEN increased from 19 to 48 ppb over this period (Biomin 2015; 2016).

Research on screening, isolation and identification of novel compounds with antifungal activities from LABs and *Streptomyces* strains are on-going. These bacteria isolated from different sources and regions have resulted in the discovery of potentially novel anti-microbial compounds (Gupta and Srivastava, 2014; Xu et al., 2015; Yekkour et al., 2015; Magnusson et al., 2003; Řezanka et al., 2004; Wang et al., 2014;). There is particular interest in LABs or

actinomycetes which can produce compounds which have more generic applications against different spoilage microorganisms.

The findings from the first phase of the present study showed that several LABs and the *Streptomyces* AS1 strain could successfully inhibit the growth of several human and plant pathogenic fungi such as *P. verrucosum*, *F. verticillioides*, *A. fumigatus* and *T. rubrum*. However, less information is available on whether they would control growth and more importantly mycotoxin production. It is hypothesized that LABs and *Streptomyces* AS1 produced metabolites with antifungal activities that could reduce growth and mycotoxin production. Several questions have been addressed.

1. What is the effective concentration of LABs and *Streptomyces* AS1 metabolites that can inhibit the growth of these fungal pathogens?
2. Can these bioactive metabolites inhibit or reduced the production of mycotoxins?
3. What is/are the bioactive compounds produced by the LAB strains and *Streptomyces* AS1 that are responsible for the efficacy observed?

The objectives of this research were as follows:

1. Determine the efficacy of LABs and the *Streptomyces* AS1 metabolites on spore germination and mycelial growth of the target fungal pathogens
2. Calculate the minimum inhibitory concentrations (MIC) and inhibitory concentrations which control 50% of germination/growth (IC₅₀) values of the LABs and *Streptomyces* AS1 metabolites for control of growth
3. To examine the effect of *Streptomyces* AS1 metabolites on the production of mycotoxins by the target mycotoxigenic fungal pathogens
4. To isolate and identify the bioactive compounds produced by the LABs and the *Streptomyces* AS1 strain

3.2 MATERIALS AND METHODS

I: Lactic acid bacteria

3.2.1 Preparation of fungal spore suspensions

This has been described previously in Section 2.2.2(b)

3.2.2 Preparation of modified MRS (mMRS) broth culture

Modified MRS (mMRS) broth consists of mycological peptone 10 g/L, 'Lab-Lamco' powder 8 g/L, yeast extract 4 g/L, glucose 20 g/L, dipotassium hydrogen phosphate (K_2HPO_4) 2 g/L, magnesium sulphate ($MgSO_4 \cdot 7H_2O$) 0.2 g/L and manganese sulphate ($MnSO_4 \cdot 4H_2O$) 0.05 g/L. Some ingredients such as sodium acetate and triammonium citrate that have antifungal properties were omitted. The pH was adjusted to 6.2 with 1N hydrochloric acid (HCl) prior to sterilisation at 121°C for 15 min.

3.2.3 Effect of LABs metabolites on the spore germination of *T. rubrum*

Sterilized MRS agar was poured into Petri plates (90 mm diameter) and after the agar was solidified this was overlaid with a disc of sterile cellophane sheet (90 mm diameter). A mixture of four LABs (*L. plantarum* VS1, *L. plantarum* VN5, *P. acidilactici* 84 and *P. pentosaceus* 95) were inoculated on MRS agar previously overlaid with sterile cellophane layers and incubated at 30°C for 48 h. After 24 and 48 h the cellophane disc with the LABs was carefully removed and a total of 100 µL of *T. rubrum* spore suspension (10^6 spore/mL) was spread plated onto the agar surface with a sterile glass spreader and incubated at 30°C for 72 h. After 24, 48 and 72 h incubation, two agar plugs (18 mm diameter) were randomly removed with a sterile spatula, placed on a glass slide and stained with lactophenol cotton blue, and a coverslip placed over the surface. A compound microscope was used to examine the numbers of germinated spores. A total of 50 spores in each of four fields were counted in each replicate and the number recorded. Spores were considered germinated when the germ tube length was equal to or greater than the spore diameter (Magan, 1988). The experiments were all carried out with three replicates per treatment.

3.2.4 Antifungal efficacy of the four chosen LABs grown in modified MRS (mMRS) broth

L. plantarum VS1, *L. plantarum* VN5, *P. acidilactici* 84 and *P. pentosaceus* 95 cell suspensions were prepared as described in Section 2.2.2(a). Each LAB (2.0 %) and a mixture of four LABs were inoculated into 40 mL sterile mMRS broth and incubated at 30°C for 48 h in static conditions. Periodically, after 24 and 48 h, 20 mL of the culture was dispensed and the supernatant separated from the cells by centrifugation at 2000 rpm and 4°C for 10 min. This was then filter sterilized using a sterile cellulose acetate membrane filter (0.22 µm, Sartorius, Göttingen, Germany) and freeze dried aseptically. The dried cell free supernatants (CFS) were dissolved in sterile distilled water to a 20-fold concentration. The antifungal activity against *T. rubrum* was measured using the agar spot assay as described later.

3.2.5 Efficacy of single and co-cultures of LABs against *T. rubrum* growth

Different combinations of *L. plantarum* VS1, and *L. plantarum* VN5 were used to study the effect of single and co-cultures on efficacy against *T. rubrum*. Each cell suspension was prepared as describe in Section 2.2.2(a). The cell suspensions (2%) were inoculated into 40 mL mMRS broth and incubated at 30°C under static conditions. Periodically, after 24 and 48 h, a total of 20 mL of the supernatant was collected and filtered through a sterile cellulose acetate membrane filter (0.22 µm). The sterile supernatant was then freeze dried aseptically and dissolved in sterile distilled water (to a 20-fold concentration). The antifungal activity was measured using the agar spot assay as described later.

3.2.6 Optimisation of growth conditions to enhance antifungal activity of *L. plantarum* VS1

(a) Incubation time

200 µL of *L. plantarum* VS1 cell suspension (approx. 10^7 CFUs/mL) was inoculated into 200 mL of mMRS broth and incubated at 30°C in static condition for 60 h. At different time intervals 20 mL of the culture was withdrawn and centrifuged at 3,000 rpm for 10 min at 4°C to separate the supernatant from the cells. The supernatant was collected, filter sterilize and dried as described previously. The dried supernatant was then dissolved in sterile distilled water to a 15-folds concentration. The antifungal activity against *T. rubrum* was tested using the agar spot assay.

(b) Inoculum size.

Different concentration (0.5%, 1.0%, 2.0% and 3.0%) of *L. plantarum* VS1 cell suspension (approx. 10^7 CFUs/mL) was inoculated into 40 mL of mMRS and incubated at 30°C in static condition for 48 h. The supernatant was separated from the cells, sterilized, concentrated and the antifungal assays run as described above.

(c) Temperature

A total of 1200 µL of *L. plantarum* VS1 cells (approx. 10^7 cfu/mL) were inoculated into 40 mL of mMRS (3%; v/v) and incubated at different temperatures (20°C, 25°C, 30°C, 35°C and 37°C) in static condition for 48 h. The supernatant was separated from the cells, sterilized, concentrated and the antifungal assay done.

(d) pH of mMRS broth

The mMRS broth was aliquoted and the pH adjusted to 4.0, 4.5, 5.0, 5.5, 6.2 and 6.5 with 1 N HCl or 1 N NaOH. After this, *L. plantarum* VS1 cell suspensions (approx. 10^7 CFUs/mL) was inoculated into 40 mL of mMRS (3%; v/v) and incubated at 30°C in static condition for 48 h. The supernatant was separated from the cells, sterilized, concentrated and the antifungal assays carried out.

(e) Carbon sources

The ability of lactose, fructose, sucrose and glucose as sole carbon sources in mMRS broth to enhance the production of antifungal compound production by *L. plantarum* VS1 was studied. Cell suspensions of this LAB strain (approx. 10^7 CFUs/mL) was inoculated into 40 mL of mMRS (pH 6.2) (3%; v/v) containing different carbon sources and incubated at 30°C in static condition for 48 h. The same procedure described previously was used for the antifungal assay.

3.2.7 Effect of heat and pH modified supernatant from *L. plantarum* VS1 on antifungal activity

The treatment was applied to the supernatant to determine the nature of the antifungal compounds. Those treatments were: (1) maintained the original pH (3.3) of the supernatant and autoclaved at 121°C for 15 min and (2) the pH was adjusted to 6.8 and autoclaved at 121°C for 15 min. The supernatant was freeze dried and later dissolved in sterile distilled water to a 15-

fold concentration. The antifungal activity against *T. rubrum* was tested using the agar spot assay described later.

3.2.8 Growth of *T. rubrum* on keratin media

Keratin agar media mainly consists of 0.5% keratin (w/v) (Santa Cruz Biotechnology Inc, Texas, USA) as the main carbon and nitrogen sources, MgSO₄.H₂O (0.5 g/L), KH₂PO₄ (0.1g/L), FeSO₄.7H₂O (0.01g/L) and Zn SO₄.7H₂O (0.005g/L) (Wawrzekiewicz et al., 1991). The pH was adjusted to 4.0, 4.5, 5.0 and 5.5 using 1N HCl or 1N NaOH before agar (Sigma Aldrich) was added (1.5%) and autoclaved at 121°C for 15 min. Sterile keratin agar media was poured aseptically into Petri plates (Ø 53 mm) and after cooling to room temperature 5 µL of spore suspension (10⁶ spores/mL) was inoculated in the middle of the agar media and incubated at 30°C, 35°C and 37°C for 10 days. The colony diameter was measured every two days, in two perpendicular directions at right angles to each other. The radial growth rate was calculated from the linear regression slope of the growth curve (colony radius vs incubation days).

3.2.9 Efficacy of *L. plantarum* VS1 supernatant against *T. rubrum* growth

Different concentration of supernatant was tested to determine the MIC, IC₅₀ and minimum fungicide concentration (MFC) against *T. rubrum* growth using the agar dilution method (EUCAST, 2000). *L. plantarum* VS1 was inoculated and incubated under optimum condition for antifungal activity which was 3% inoculum, glucose as a carbon source, pH 6.2, incubation time and temperature of 48 h and 35°C, respectively. After 48 h, the supernatant was separated from the cells as described in Section 3.2.6(a) and filter sterilised into sterile pre-weighed 50 mL tubes. These were freeze dried and the final weight of the dried supernatant noted. The dried supernatant was then dissolved in sterile distilled water and mixed with keratin agar (the final concentration VS1 was 0 - 30 mg/mL) and poured into Petri plates (Ø = 53mm). After cooling to room temperature 5 µL of spore suspension (10⁶ spores/mL) was inoculated in the centre of the agar plates and incubated at 30°C for 12 days. The diameter of the fungal colony was measured in two perpendicular direction after 12 days and the colony area calculated as described in Section 3.2.19.

3.2.10 Quantification of lactic acid and acetic acid by HPLC-UV/VIS

Lactic acid and acetic acid produced by *L. plantarum* VS1 were quantified using HPLC. The supernatant containing organic acids were diluted using 5 mN sulphuric acid (H₂SO₄) and filtered using nylon syringe filters (0.22 µm pore size, Fisher) into HPLC vials. The separation

and quantification of lactic and acetic acids were made using the HPLC Shimadzu UFLC XR, CTO-20A system. This consists of a UV/VIS detector (SPD-20A), column oven (CTO-20AC), degasser (DGU-20A₅), pump (LC-30AD) and autosampler (SIL-20AC_{XR}). The separation was done at 55°C using a Rezex ROA-Organic Acid H⁺ (8%) column (150 x 7.8 mm) fitted with a guard column (security guard, 4 mm x 3 mm cartridge, Phenomenex, USA). The mobile phase was 5 mN sulphuric acid. The flow rate was 0.8 mL/min and injection volume 10 µL. The detection wavelength was 210 nm. The total run time was 15 min with a retention time at 5.0 and 5.9 minutes for L-lactic acid and acetic acid, respectively. A mixture of L-lactic acid (0.5 - 2.5 mg/mL) ($R^2 = 0.9999$) and acetic acid standard (4 - 20 µg/mL) ($R^2 = 0.9999$) was prepared by dissolving a stock solution in 5 mN H₂SO₄. The limit of detection (LOD) for L-lactic acid and acetic acid were 0.03 mg/mL and 0.18 µg/mL, respectively based on a signal to noise ratio (S/N) of 3:1. A limit of quantification (LOQ) was 0.1 mg/mL for L-lactic acid and 0.62 µg/mL for acetic acid based on the S/N 10:1. mMRS broth was analysed as a control.

3.2.11 Comparison of the antifungal activity between *L. plantarum* VS1 supernatant and selected compounds identified in the supernatant against *T. rubrum*.

Sterile dried *L. plantarum* VS1 supernatant, L-lactic acid and acetic acid were dissolved in sterile distilled water. These antifungal compounds (100 µL each) were then mixed with keratin agar (approx. 52°C) and poured into Petri plates (Ø 53 mm). The final concentration of supernatant, L-lactic acid and acetic acid were 20 mg/mL, 6.6 mg/mL and 2.1 µg/mL, respectively. After the keratin agar solidified, 5 µL of spore suspension (10⁶ spores/mL) was centrally inoculated and incubated at 30°C for 21 days. The diameter of the fungal colony was measured in two perpendicular direction after 21 days and the area calculated as described in Section 3.2.19.

II: *Streptomyces* AS1

3.2.12 Effect of *Streptomyces* AS1 metabolites on the spore germination of *F. verticillioides*, *P. verrucosum* and *A. fumigatus* (Mi538)

Sterilized half strength nutrient agar (1/2 NA) was poured into Petri plates (90 mm Ø) and after the agar was solidified an overlay of sterile cellophane (90 mm Ø) was placed on the agar surface. A single colony of *Streptomyces* AS1 was inoculated on the 1/2 NA previously overlaid with a sterile cellophane sheet and incubated at 25°C for 10 days. At different time

intervals (day 2, 5 and 10) the cellophane layer with the *Streptomyces* AS1 biomass was removed and a total of 100 µL of spore suspension (10^6 spores/mL) of the test pathogens were spread plated onto the surface with a sterile glass spreader. These Petri plates were then incubated for 48 h. *P. verrucosum* and *F. verticillioides* were incubated at 25°C and *A. fumigatus* (Mi538) treatments at 30°C. After 24 h and 48 h two agar plugs (18 mm Ø) were taken randomly and placed on a glass slide. They were stained with lactophenol cotton blue covered with a coverslip prior to microscopic examination. The number of spores that germinated were counted as described in Section 3.2.3. The experiments were all carried out with three replicates per treatment

3.2.13 Extraction of *Streptomyces* AS1 bioactive metabolites using ethyl acetate

The *Streptomyces* AS1 spore suspension (200 µL) was inoculated into 200 mL ½ strength sterile nutrient broth (NB) and incubated at 30°C at 200 rpm for 4 days. After 4 day the supernatant was separated from the mycelium by filtration using Whatman filter paper no 4.

Bioactive metabolites from AS1 supernatant were extracted three times with acetyl acetate (Sultan and Magan, 2011). Basically 900 mL of cell free supernatant was mixed with 300 mL of ethyl acetate in a separating funnel and after shaking for a few seconds the mixture was left to separate into two layers. The ethyl acetate layer (upper layer) containing bioactive metabolites was collected and the extraction was repeated three times. The ethyl acetate layers containing the bioactive metabolites were combined and the solvent was removed using a rotary evaporator at 38°C. The dried film was dissolved in dimethyl sulfoxide (DMSO) under sterile conditions for performing the assays

3.2.14 Efficacy of *Streptomyces* AS1 ethyl acetate extract against *P. verrucosum*, *F. verticillioides* and *A. fumigatus* using the BioScreen C turbidimetric assay

(a) Preparation of culture medium

The culture medium used to inoculate the fungal pathogen was prepared as described by Medina et al. (2012). Water was used to prepare the medium and this was adjusted with glycerol to 0.99 a_w and 0.95 a_w . The culture media used were semi-solid YES (yeast extract sucrose) for *P. verrucosum* and *A. fumigatus* and PD (potato dextrose) for *F. verticillioides*. Each culture medium contained:

- 1) YES - yeast extract 20g/L, sucrose 150g/L, magnesium sulphate 0.5g/L and 0.05% agar

2) PD – potato extract 4g/L, dextrose 20g/L and 0.03% agar

Both culture mediums were sterilised at 121°C for 15 min.

(b) Culture medium containing bioactive metabolites and spore suspensions

A mixture of 9800 µL culture medium, 100 µL of spore suspensions (final spore count 10^5 spores/mL) and 100 µL of ethyl acetate extract (final concentration 5 µg/mL, 10 µg/mL, 20 µg/mL, 30 µg/mL and 40 µg/mL; w/v) were prepared in sterile 25 ml Universal bottles. A total of 300 µL of the mixture were loaded into 100 well microtiter plates and the density measured automatically at 600 nm every 30 minutes at 25°C for seven days for *P. verrucosum* and *F. verticillioides* and at 37°C for *A. fumigatus* (Mi538). This method was based on that developed for screening efficacy against filamentous fungi using the BioScreen assay (Medina et al., 2012). Each set of experiments was carried out with ten replicates. The relative times to reach an absorbance value of 0.1 (Time To Detection; TTD 0.1) was then compared as an indication of growth rates. In addition, the wells from the 10 replicates could be used for subsequent mycotoxin quantification. The raw data was analysed using Microsoft Excel to obtain the growth curve of fungi. The TTD 0.1, minimum inhibitory concentrations (MIC) and IC₅₀ were calculated using the Lambert Model (Lambert and Pearson, 2000).

3.2.15 Agar media containing ethyl acetate (EA) extract and inoculation of fungal spores.

Approximately 100 µL of the different concentration of EA extract (0 - 40 µg/mL) was added aseptically into 10 mL sterile YES (for *P. verrucosum* and *A. fumigatus* (Mi538)). and PD (for *F. verticillioides*) agar ($\pm$ 52°C) with different a_w levels (0.99 and 0.95) and inverted several times before pouring into Petri plates (53 mm, Ø). After the agar media had solidified, 100 µL of spore suspension (10^7 spores/mL) was spread plated using a surface-sterilised spreader and incubated at 25°C for *P. verrucosum* and *F. verticillioides* and 37°C for *A. fumigatus* (Mi538) for seven days.

3.2.16 Extraction and quantification of ochratoxin A, gliotoxin and fumonisins

(a) Ochratoxin A (OTA)

A total of five agar plugs of *P. verrucosum* after seven days grown on YES medium were collected using a sterile cork borer (number 5) and transferred into pre-weighed 2 mL tubes and re-weighed to obtain the weight of the agar plug. The extraction of OTA from agar plug

was carried out using one millilitre of methanol. The samples were shaken at 200 rpm and 30°C in the dark for 1 h. Samples were then centrifuged at 15,000 rpm for 5 min and the methanol containing OTA was filtered (nylon syringe filter, 0.22 µm pore size, Fisher) into amber and silanized HPLC vials (Agilent, UK) for analysis.

The separation and quantification of OTA was done using an Agilent 1200 Series HPLC system (Agilent, UK). The system consists of an auto sampler (ALS G1329A), fluorescence detector (FLD 1321A), pump (G 1312A), oven (FC/ALS-therm G1330A) and degasser (G1379B). The separation was done at 25°C using a Poroshell 120 EC-C18 (4.6 mm x 100 mm, 2.7 µm) column fitted with a guard column (security guard, 4 mm x 3 mm cartridge, Phenomenex, USA). The mobile phase was water:acetonitrile:acetic acid (41:57:2, v/v/v). The flow rate and injection volume were 1 mL/min and 20 µL, respectively. The detection wavelength was 333 nm for excitation and 460 nm for emission. The total run time was 10 min with the retention time for OTA was 2.52 minutes. Different concentrations of OTA standard (0 - 400 ng/mL) was prepared by dissolving OTA (Sigma) stock (5 mg/mL) in methanol ($R^2 = 0.9994$). The limit of detection (LOD) and limit of quantification (LOQ) was 12.3 ng/mL and 41.1 ng/mL, respectively. Three replicates were used at all times. Standards were used in all quantification batches of samples.

(b) Gliotoxin

Gliotoxin is a toxin produced by *A. fumigatus*. The preparation of agar plugs was as described above. The extraction was carried out using chloroform (1 mL) for 1 h at 30°C and shaken at 200 rpm. After this 800 µL chloroform containing gliotoxin was transferred into a new 2 mL tube and dried overnight in a fume cupboard. The dried extract was dissolved in 700 µL of mobile phase solution (1% acetic acid:acetonitrile (75:25, v/v) and filtered (nylon syringe filter, 0.22 µm pore size, Fisher) into amber and silanized HPLC vials (Fisher, UK) for analysis.

The separation and quantification of gliotoxin was done according to Alonso et al. (2016) with slight modification. The gliotoxin concentration was measured using HPLC (Agilent, UK) with a diode array detector (DAD). The separation was done at 25°C using a Zorbax Eclipse XDB-C18 (4.6 mm x 150 mm, 5.0 µm, Agilent, USA) column fitted with a guard column (security guard, 4 mm x 3 mm cartridge, Phenomenex, USA). The mobile phase was acetic acid:water (1:99, v/v) (eluent A) and acetonitrile (eluent B). The flow rate, injection volume and detection wavelength were 1.3 mL/min, 50 µL and 268 nm respectively. The gradient program was 25%

B for 10 min followed by rapid increased to 100% in 1 min and this was held for 8 min before decreasing this to 25% in 2 min. The retention time was at 5.1 min. Working gliotoxin standard solution with a concentration range from 0 - 3000 ng/mL was prepared by diluting gliotoxin (Sigma) stock (5 mg/5 mL acetonitrile) in mobile phase ($R^2 = 0.9999$). The LOD and LOQ was 41 ng/mL and 138 ng/mL, respectively.

(c). Fumonisin

Fumonisin B₁ and B₂ are the main mycotoxins in the suite produced by *F. verticillioides*. The agar plugs were extracted by adding 1 mL of acetonitrile:water (50:50, v/v) and shaking the 2 mL tube at 200 rpm for 1 h at 30°C. Acetonitrile:water (50:50,v/v) containing FB1 and FB2 was then filtered (nylon syringe filter, 0.22 µm pore size, Fisher) into silanized HPLC vials for analysis.

The separation and quantification of FBs was run using the HPLC-FLD system (Agilent, UK). The separation was done using a Zorbax Eclipse Plus C18 (4.6 mm x 150 mm, 3.5 µm) column joined with a guard column (security guard, 4 mm x 3 mm cartridge, Phenomenex, USA) at 30°C. The detection was 335 nm for the excitation wavelength (λ_{ex}) and 440 nm for the emission wavelength (λ_{em}). The mobile phase was 0.05 M sodium dihydrogen phosphate (pH 4.01): methanol (50:50, v/v) (eluent A) and acetonitrile:water (80:20) (eluent B). The injection volume was 15 µL with a flow rate of 1 mL/min. The total run time was 20 min with the retention time of FB₁ and FB₂ at 10.9 and 16.2 minutes, respectively. The gradient programme was 0% B for 5 min., increasing B to 50% in 1 min. and held for 7.10 min before slowly increasing to 80% in 6.90 min. Before injecting the standards or samples, 10 µL of standards or samples were mixed with 5 µL of derivatization solution (OPA). The derivatization solution consists of 1 mL ortho-phthaldialdehyde (Sigma-Aldrich, USA) (40 mg of OPA in 1 mL absolute methanol), 5 mL of 0.1 M disodium tetraborate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) and 50 µL of 2-mercaptoethanol (Sigma-Aldrich, USA). These solutions were prepared separately and filtered using a nylon syringe filter (0.22 µm) for OPA and cellulose acetate syringe filter (0.22 µm) for 0.1 M disodium tetraborate. These solutions were kept at -20°C and mixed on the day the analysis was carried out. The derivation was carried out at the HPLC system with the auto-derivatization programmed. The programme was:

- 1) Draw 10 µL from sample, max. speed
- 2) Needle wash in vial 100, one time
- 3) Draw 5 µL OPA from vial 99, max. speed

- 4) Mix 15 μL in air, max. speed, 10 times
- 5) Wait 0.2 min
- 6) Inject
- 7) Wait 0.1 min
- 8) Valve bypass

Different concentrations of FB standards (0-5 $\mu\text{g/mL}$) was prepared by diluting 5 mg/mL FB standard solution (Sigma-Aldrich, USA) in acetonitrile:water (50:50, v/v) ($R^2 = 0.9972$ for FB1 and 0.9959 for FB2). The LOD and LOQ was 0.37 $\mu\text{g/mL}$ and 1.21 $\mu\text{g/mL}$ for FB₁ and 0.44 $\mu\text{g/mL}$ and 1.48 $\mu\text{g/mL}$ for FB₂, respectively.

3.2.17 Identification of bioactive compounds from *Streptomyces* AS1 EA extract

Sample preparation, detection and quantification were performed as described by Malachová et al. (2014). Briefly, the extraction solvent (acetonitrile/water/acetic acid; 79/20/1) was added to dried ethyl acetate extract and after shaking and centrifugation the extract was injected into liquid chromatography-tandem mass spectrometry (LC-MS/MS) equipped with a TurboV electrospray ionization (ESI) source. The Phenomenex C18-column (150 x 4.6mm, 5 μm) fitted with a C18 security guard cartridge (4 x 3mm) was used to separate the compounds. The mobile phases consisted of methanol/water/acetic acid with the ratio of 10/89/1 (v/v/v) for eluent A and 92/2/1 (v/v/v) for eluent B. Both eluents contain 5 mM ammonium acetate. These assays were kindly made by Dr. M. Sulyok, at IFA-Tulln, Austria.

3.2.18 Comparison of antifungal activity between *Streptomyces* AS1 EA extract and selected compounds identified in EA extract against *P. verrucosum*

Two major antifungal compounds (cyclo(L-Pro-L-Tyr) and valinomycin) in the EA extract was chosen for the comparative study. These compounds and the EA extract were dissolved in DMSO (100 μL each) and mixed with 9900 μL of 0.95 a_w YES agar (approx. 52°C) and poured into Petri plates (53 mm $\varnothing$). The final concentration of AS1 ethyl acetate extract, valinomycin and cyclo(L-Pro-L-Tyr) was 5 $\mu\text{g/mL}$, 22 ng/mL and 4 ng/mL, respectively. After the YES agar was solidified, 5 μL of spore suspension (10^6 spores/mL) was centrally inoculated and incubated at 25°C for 10 days. The colony diameter was measured every two days in two perpendicular directions and averaged. The radial growth rate was obtained from the linear regression slope of the growth curve (colony radius vs incubation days). All treatments were

done in triplicate. After 10 days five agar plugs were collected and the OTA was extracted and quantified as described in Section 3.2.16a

3.2.19 Agar spot assay

The agar medium (approximate 52°C) was mixed with antifungal extract and poured into Petri plates (90 mm or 53 mm Ø). After the agar media were solidified 5 µL of spore suspension (10⁶ spores/mL) was dropped onto the middle of the agar medium. The spore suspension was left to dry before being incubation at the respective temperatures. The fungal colony diameters were measured in two directions at right angles to each other. The area of the fungal colony was calculated.

Calculation of area:

$$A = \pi r^2$$

3.2.20 Statistical analysis

Normal distribution of data was checked by Shapiro-Wilk W Test. For data with a normal distribution, the general influence of antifungal extract on fungal growth and mycotoxin production were checked using one way analysis of variance (ANOVA) and Kruskal Wallis Tests (Rank Sums) for non-normally distributed data. Student's t test was further applied to compare the means for each treatment for normally distributed data and Wilcoxon method (non-parametric comparison) for non-normal distribution dataset. $p < 0.05$ was accepted as significant difference. The JMP Pro (SAS Institute Inc., Cary, North Carolina, USA) was used in the analyses. All experiments were carried out with at least three replicates per treatment and some repeated.

3.3 RESULTS

I: Lactic acid bacteria

3.3.1 Effect of lactic acid bacteria metabolites on spore germination of *T. rubrum*

The efficacy of metabolites from the *L. plantarum* VS1 on the spore germination of *T. rubrum* is shown in Figure 3.1. The germination of *T. rubrum* spores in all experimental conditions was completely inhibited by the LAB metabolites over a 72 h incubation period.

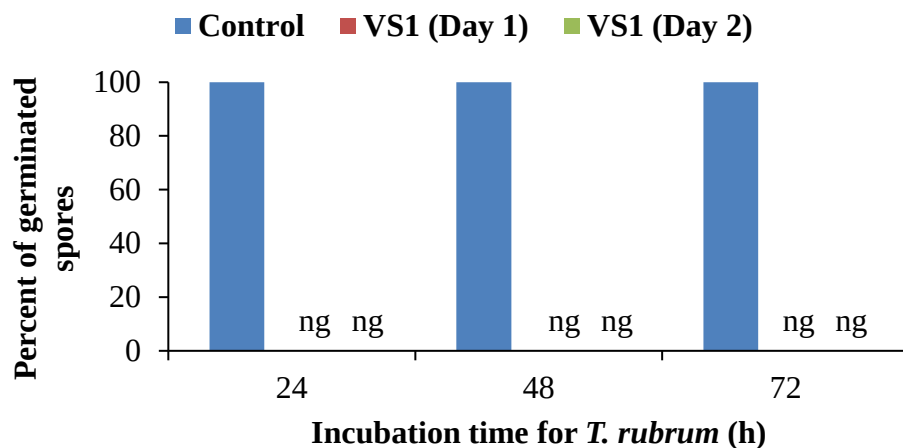


Figure 3.1 Mean percentage germination of *T. rubrum* spores on MRS agar containing a *L. plantarum* VS1 metabolites. Data are means of three replicates. In all cases the standard deviation (SD) was smaller than the data symbols for the controls. The data analysis was carried out on actual number of germinated spores. These were back transformed to percentages for presentation purposes. Key: ng – no germination

3.3.2 Effect of four LAB strains on *T. rubrum* growth

Figure 3.2. shows the effect of four LAB strains on colony area after 24 and 48 h incubation at 30°C. Overall, the *T. rubrum* growth was influenced by the LAB strains and the incubation time. The colony area of *T. rubrum* was significantly reduced ($p < 0.05$) after 24 and 48 h when compared to the control and between the strains. The highest reduction was by *L. plantarum* VS1 followed by *L. plantarum* VN5 and a mixture of four LAB strains, *P. pentosaceus* 95 and *P. acidilactici* 84. The inhibition was more pronounced when the incubation time was prolonged to 48 h as no fungal growth was detected with *L. plantarum* VS1, *L. plantarum* VN5 and a mixture of the four LAB strains.

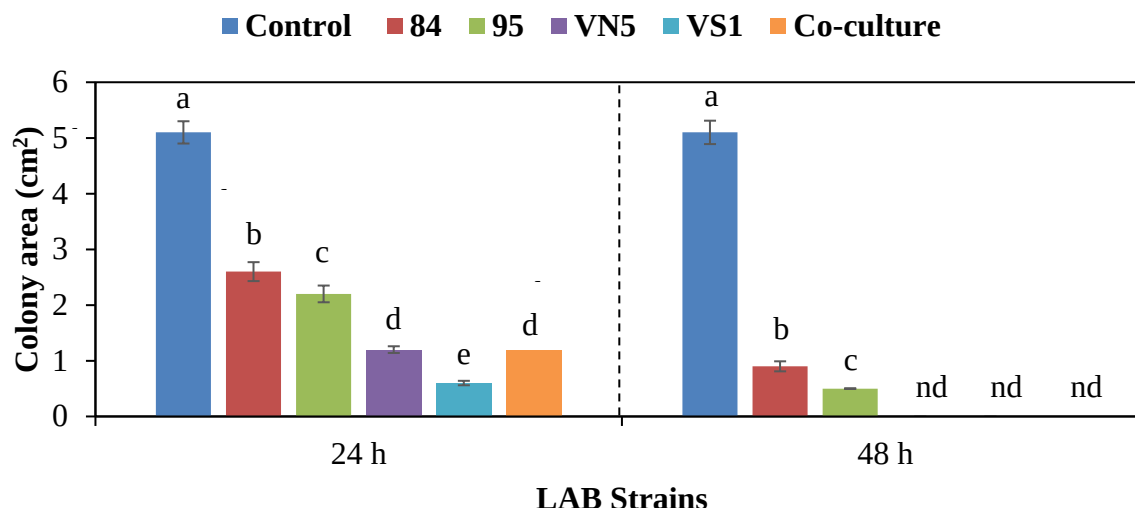


Figure 3.2 Effect of LABs grown on modified MRS on antifungal activity against *T. rubrum* using the agar spot assay. Data are means of triplicate with bars indicating standard deviation (SD). Different letters indicate significant differences ($p < 0.05$) using the Student's *t* test. Key to LB strains: 84; *P. acidilactici*, 95; *P. pentosaceus*, VS1; *L. plantarum*, VN5; *L. plantarum*.

3.3.3 Efficacy of *L. plantarum* VS1 and *L. plantarum* VN5 and a combination of the two strains against *T. rubrum*.

Figure 3.3. compares the efficacy of *L. plantarum* VS1 and *L. plantarum* VN5 and a mixture of both strains on *T. rubrum* growth. There was a significant reduction ($p < 0.05$) of the *T. rubrum* colony area when compared to the control, irrespective of the incubation time and a combination of two LAB strains. However, the antifungal activity of LABs and all combinations of LAB strains were not significant from each other after 24 h and 48 h although the *T. rubrum* colony area treated with VS1 strain was the smallest after 48 h.

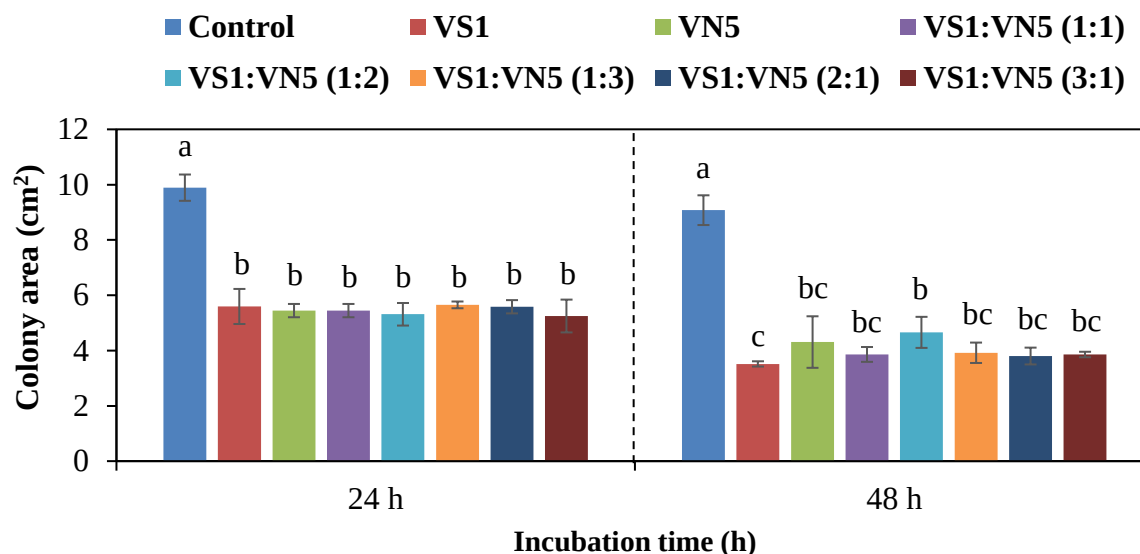


Figure 3.3 Effect of single LAB and combinations of the two LABs on antifungal activity against *T. rubrum* using the agar spot assay. Data are mean of triplicates with bars indicating standard deviation. Different letter indicates significant different ($p < 0.05$) using the Student's t test. Key to strains: VS1: *L. plantarum*, VN5: *L. plantarum*.

3.3.4 Effect of incubation time, *L. plantarum* VS1 concentration, temperature, pH of growth medium and carbon sources on efficacy against *T. rubrum* growth

Figure 3.4. shows the changes in growth of *L. plantarum* VS1, pH of supernatant and effects on the colony area of *T. rubrum* over 60 h incubation. The growth of the LAB strain *L. plantarum* VS1 was rapid for 24 h. This was followed by a plateau and then a slight decreased after 60 h incubation. The growth was significantly increased ($p < 0.05$) after four hours incubation. The colony forming units (CFUs) increased from $0.7 \log_{10}$ CFUs/mL after four hours to $3.7 \log_{10}$ CFUs/mL after 48 h. The pH of the supernatant was significantly reduced ($p < 0.05$) during the 60 h period. The reduction was rapid within 12 h followed by a gradual reduction until the end of the incubation period. A significant reduction of *T. rubrum* colony area ($p < 0.05$) was caused by the cell free supernatant of the *L. plantarum* VS1 strain after 8 h. A significant reduction occurred after 48 h until the end of the experiment.

Figure 3.5. shows the changes in lactic and acetic acid production over 60 h. The production of lactic acid was increased slowly in the first 8 h and increase significantly ($p < 0.05$) after 12 h. It was then maintained for 24 h and increased again significantly after 36 h. At the end of

incubation period the lactic acid was increased from 0.01 mg/mL to 2.00 mg/mL. Acetic acid was produced later than lactic acid. Acetic acid was first detected after 8 h and significantly increase until 24 h. A second significant period of biosynthesis occurred after 36 h.

The efficacy of the LAB strain *L. plantarum* VS1 against *T. rubrum* growth was further enhanced by examination of the effect of inoculum size (%), temperature, pH and carbon sources on antifungal activity of the *L. plantarum* VS1 supernatant. Figure 3.6a shows the effect of inoculum size on *T. rubrum* growth. Growth of *T. rubrum* was significantly inhibited by all *L. plantarum* VS1 concentrations examined when compared to the control. The highest inhibition was found at 3.0% with an inhibition of >70%. Concentrations of 0.5%, 1.0% and 2.0% gave similar levels of inhibition.

Figure 3.6b illustrates the effect of temperature on efficacy against *T. rubrum* growth. The efficacy of *L. plantarum* VS1 supernatant was enhanced as the temperature was increased from 20°C to 35°C. When compared with the control, there was a significant reduction ($p < 0.05$) of fungal colony area of between 38 and 70%. However, at high temperature (37°C) the efficacy of *L. plantarum* VS1 was significantly reduced.

The influence of mMRS broth pH from 4.0 to 6.5 on the antifungal activity of *L. plantarum* VS1 supernatant was also examined (Figure 3.6c). The fungal colony area was significantly reduced at all pH values tested when compared to the control. pH levels of between 4.0 to 5.5 gave similar significant inhibition of *T. rubrum* growth. The best inhibition was achieved at pH values >6.0.

Other than physical parameters, chemical factors such as carbon sources were also important for *L. plantarum* VS1 growth and production of antimicrobial metabolites. Mono (glucose and fructose) and disaccharide (lactose and sucrose) sugars were examined. Generally, glucose was the best carbon source as it showed total inhibition of growth (Figure 3.6d). This was followed by sucrose, fructose and lactose.

Figure 3.7 compares the *L. plantarum* VS1 cell dry weight and the effectiveness of *L. plantarum* VS1 supernatant to inhibit *T. rubrum* growth under optimized and non-optimized fermentation conditions. There was no significant difference in *L. plantarum* VS1 cell dry weight under both conditions although the cell dry weight from optimized fermentation conditions was high. Under optimized conditions the fungal colony area was significantly reduced ($p < 0.05$) by 30 times when compared with the non-optimized treatment.

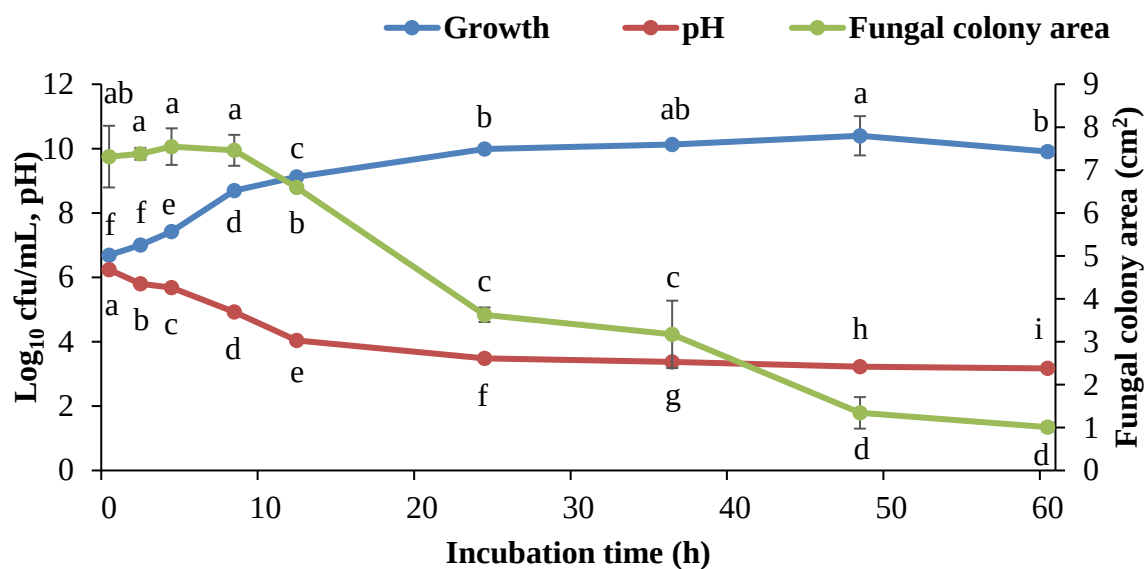


Figure 3.4 Growth of *L. plantarum* VS1, pH of supernatant and effect on *T. rubrum* colony area during 60 hrs incubation at 30°C. Data are means of triplicates $\pm$ SD. Different letters indicate significant differences ($p < 0.05$) using the Student's t test.

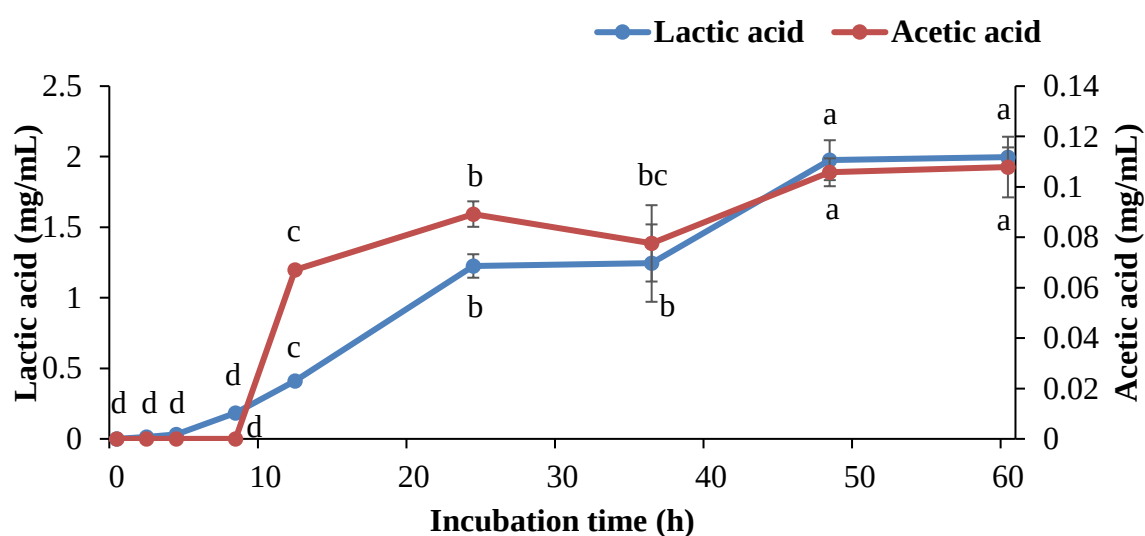


Figure 3.5 Lactic acid and acetic acid production by *L. plantarum* VS1 during 60 h incubation at 30°C. Data are means of triplicates $\pm$ SD. Different letters indicate significant differences ($p < 0.05$) using the Student's t test.

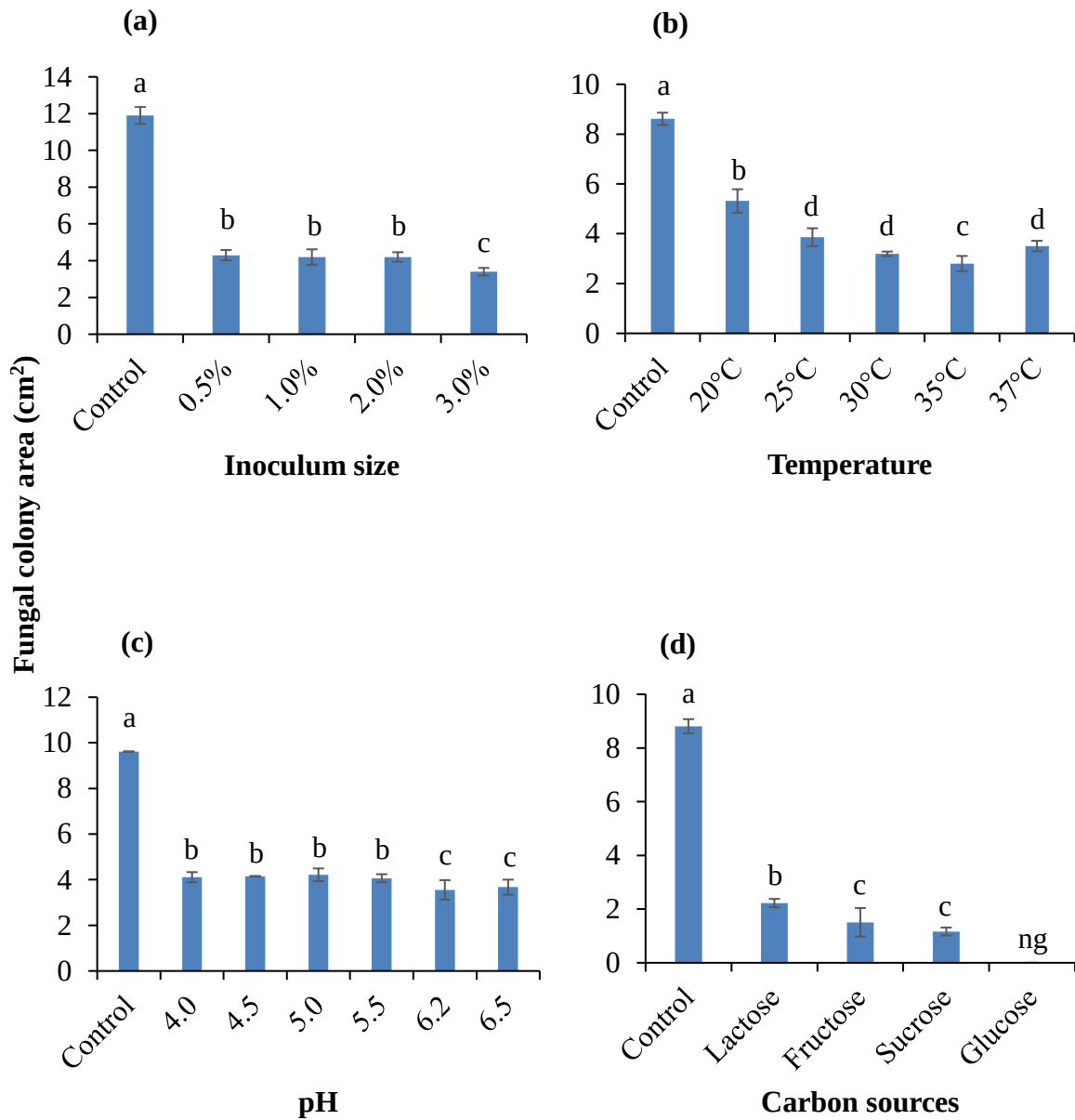


Figure 3.6 Effects of (a) inoculum size, (b) temperature, (c) pH and (d) carbon sources and efficacy against *T. rubrum* growth. Data are means of triplicates with bars indicating standard deviations. Different letters indicate significant difference ($p < 0.05$) using the Wilcoxon method (non parametric comparison) (a, b) and Student's t test (c, d). ng: no growth

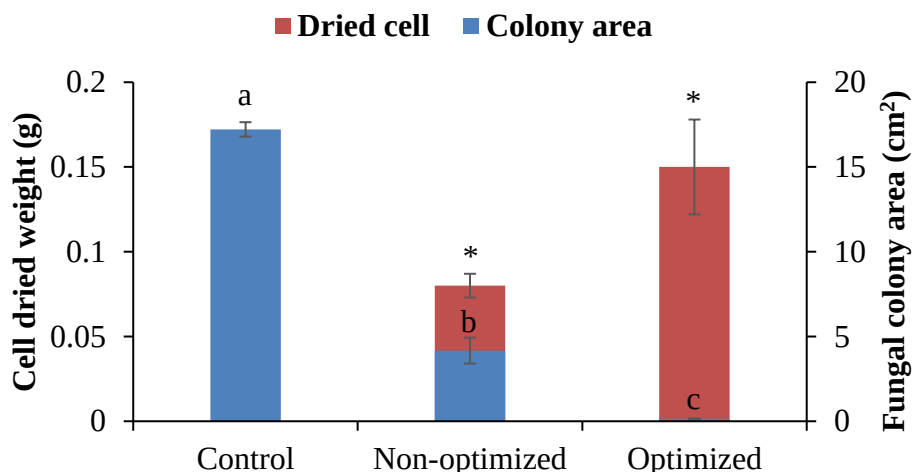


Figure 3.7 Comparison of optimized and non-optimized *L. plantarum* VS1 growth condition on *L. plantarum* VS1 cell dry weight and efficacy against *T. rubrum* growth. Data are means of triplicate with bars indicating standard deviation. Different letters indicate significant differences ($p < 0.05$); different number of asterisks indicate significant differences ($p < 0.05$) using the Student's t test.

3.3.5 Effect of heat and pH modified supernatant from *L. plantarum* VS1 on efficacy against *T. rubrum* growth.

The effect of heat treatment and pH modified *L. plantarum* VS1 supernatant on the efficacy against *T. rubrum* growth was examined (Figure 3.8). There was a significant reduction ($p < 0.05$) in *T. rubrum* colony area as the supernatant was heated at 121°C for 15 min when compared to control and untreated supernatant. However, as the pH was modified to 6.8 and treated with the same heat treatment, the fungal colony area was significantly increased ($p < 0.05$) when compared to untreated supernatant but significantly reduced ($p < 0.05$) when compared to the control.

3.3.6 Antifungal compounds isolated from VS1 cell free supernatant

A total of six compounds secreted by *L. plantarum* VS1 were isolated and identified from the cell free supernatant (Table 3.1). Organic acids were the major antifungal compounds produced by *L. plantarum* VS1. Two organic acids were identified as L-lactic acid and acetic acid with concentrations of 19,113 mg/g and 2.2 mg/g dried cells, respectively. Four minor compounds were identified as cyclo(L-Pro-L-Val), tryptophol, culmorin and phenopyrozin.

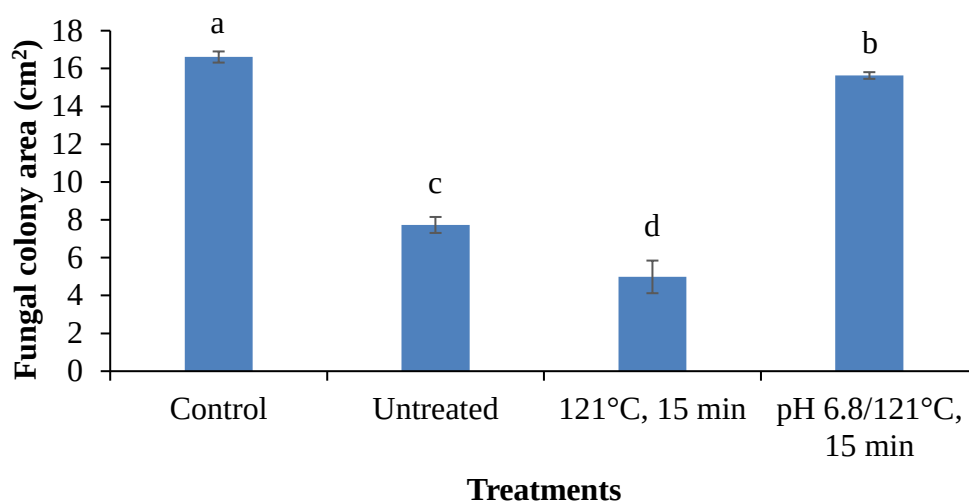


Figure 3.8 Antifungal activity of *L. plantarum* VS1 supernatant after heat treatment with/without modification to pH 6.8. The unmodified pH of supernatant was 3.3. Data are means of three replicates with bars indicating standard deviation. Different letters indicate significant differences ($p < 0.05$) using the Student's *t* test.

Table 3.1 Concentration of organic acids and other metabolites in cell free supernatants of *L. plantarum* VS1 grown in mMRS broth for 48 h at 30°C.

Antifungal metabolites	Concentration (mg/g cell dry weight)
Organic acid	
L-lactic acid	19,113.0 $\pm$ 66.2
Acetic acid	2.2 $\pm$ 0.11
Other metabolites	
Cyclo(L-Pro-L-Val)	0.0551
Tryptophol	0.0005
Culmorin	0.0003
Phenopyrrozin	0.0001

*Values were substrate with uninoculated medium.

3.3.7 Growth of *T. rubrum* on keratin agar

Figure 3.9. shows the effect of pH and temperature on the growth rate of *T. rubrum*. Generally, the growth rate was significantly decreased ($p < 0.05$) as the temperature was increased from 30°C to 37°C regardless of pH. The highest growth rate was at 30°C. The statistical analysis of the effect of temperature is shown in Table 3.2. The effect of pH on growth rate was observed at 30°C with the highest growth at pH 4.5 which was significantly different ($p < 0.05$) from the other three pH levels examined. In contrast, the growth rate at all pH values was the same at 35°C and 37°C.

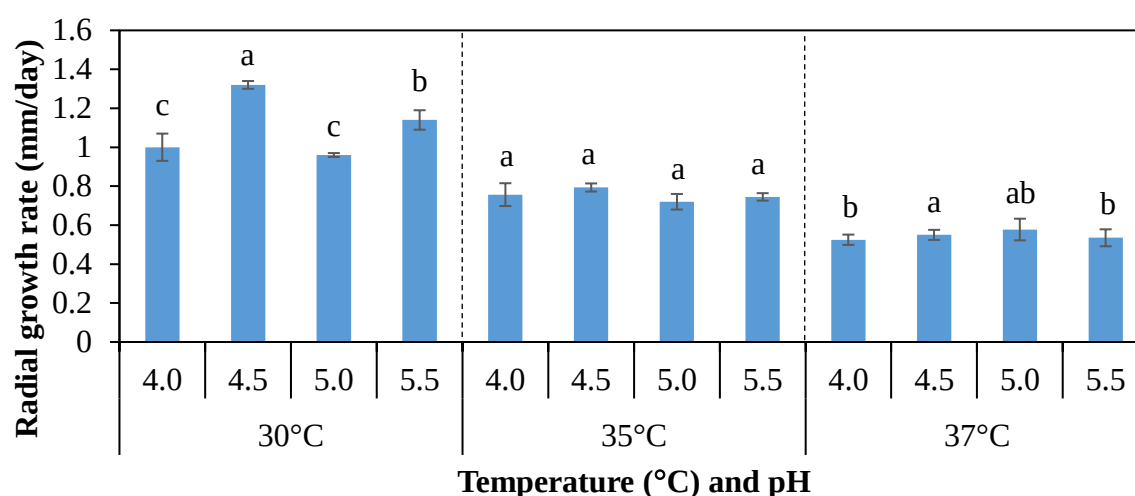


Figure 3.9 Effect of pH of keratin media incubated at different temperature on the growth of *T. rubrum*. Data are means of triplicate with bars indicating standard deviation. Different letters indicate significant differences ($p < 0.05$) within treatments under the same conditions using the Student's t test.

Table 3.2 Statistical analysis of the effect of temperature on *T. rubrum* growth on keratin agar with different pH levels using the Student's t test.

Temperature		
Level	Level	p-value
30	37	<0.0001*
30	35	<0.0001*
35	37	<0.0001*

3.3.8 Efficacy of *L. plantarum* VS1 supernatant against *T. rubrum* growth on keratin media

Figure 3.10 shows the effect of *L. plantarum* VS1 supernatant on fungal colony area on keratin-based medium. The fungal area was continuously reduced as the *L. plantarum* VS1 concentration increased and at 20 mg/mL no fungal growth was observed. Table 3.3. shows the minimum inhibitory concentration (MIC), concentration inhibit 50% fungal growth (IC_{50}) and minimum fungicidal concentration (MFC) of VS1 supernatant on keratin agar. The MIC, IC_{50} and MFC were 20 mg/mL, 14 mg/mL and 30 mg/mL, respectively.

The efficacy of the *L. plantarum* VS1 supernatant and major individual compounds (L-lactic acid and acetic acid) identified in VS1 supernatant against *T. rubrum* growth was compared (Figure 3.11). The concentration and the pH of the L-lactic acid and acetic acid used in this study was the same with the concentration calculated as 20 mg/mL and the pH of *L. plantarum* VS1 supernatant. There was no *T. rubrum* growth when VS1 supernatant was used as the antifungal. L-lactic acid alone and in combination with acetic acid significantly reduced ($p < 0.05$) the *T. rubrum* colony area when compared to the control and acetic acid only. However, there was no significant inhibition when acetic acid was used as the sole antifungal inhibitor when compared to the control.

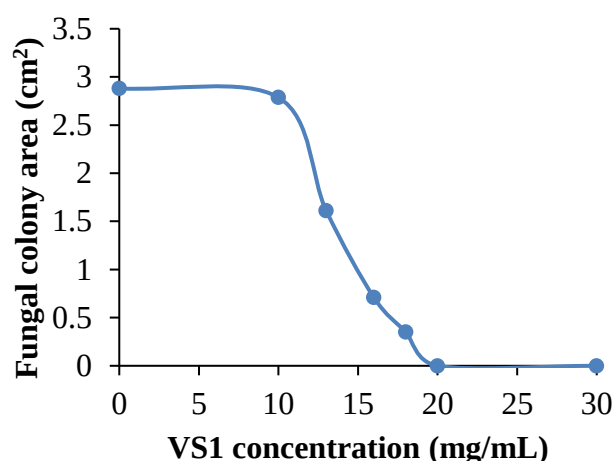


Figure 3.10 Effect of *L. plantarum* VS1 supernatant on *T. rubrum* growth on keratin agar

Table 3.3 Minimum inhibitory concentration (MIC), IC₅₀ and minimum fungicidal concentration (MFC) of *L. plantarum* VS1 supernatant against *T. rubrum* growth on keratin agar.

Antifungal	MIC (mg/mL)	IC ₅₀ (mg/mL)	MFC (mg/mL)
VS1 Supernatant	20	14	30

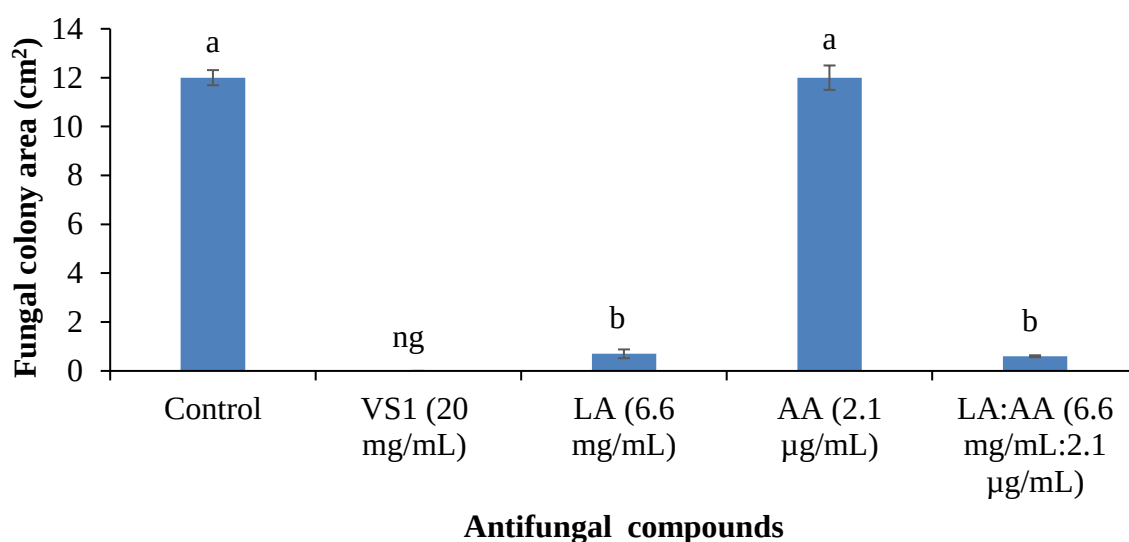


Figure 3.11 Efficacy of *L. plantarum* VS1 supernatant, acetic acid and lactic acid for control growth of *T. rubrum*. Data are mean of triplicates with bars indicating standard deviation. Different letter indicates significant differences ($p < 0.05$) using the Student's t test. Key: VS1 - natural mixture of *L. plantarum* compounds, LA - L-Lactic acid, AA - Acetic acid, ng -no growth

II *Streptomyces* AS1

3.3.9 Efficacy of *Streptomyces* AS1 metabolites against spore germination of *P. verrucosum*, *F. verticillioides* and *A. fumigatus* (Mi538)

Figure 3.12 shows the efficacy of metabolites secreted by *Streptomyces* AS1 after 10 days growth, on the germination of conidia of *P. verrucosum*, *A. fumigatus* (Mi538) and *F. verticillioides*. It was found that none of the *P. verrucosum* spores germinated in all the

treatment conditions tested. However, 85% of *A. fumigatus* (Mi538) spores germinated after 48 h incubation. For *F. verticillioides* there was very little efficacy in controlling spore germination.

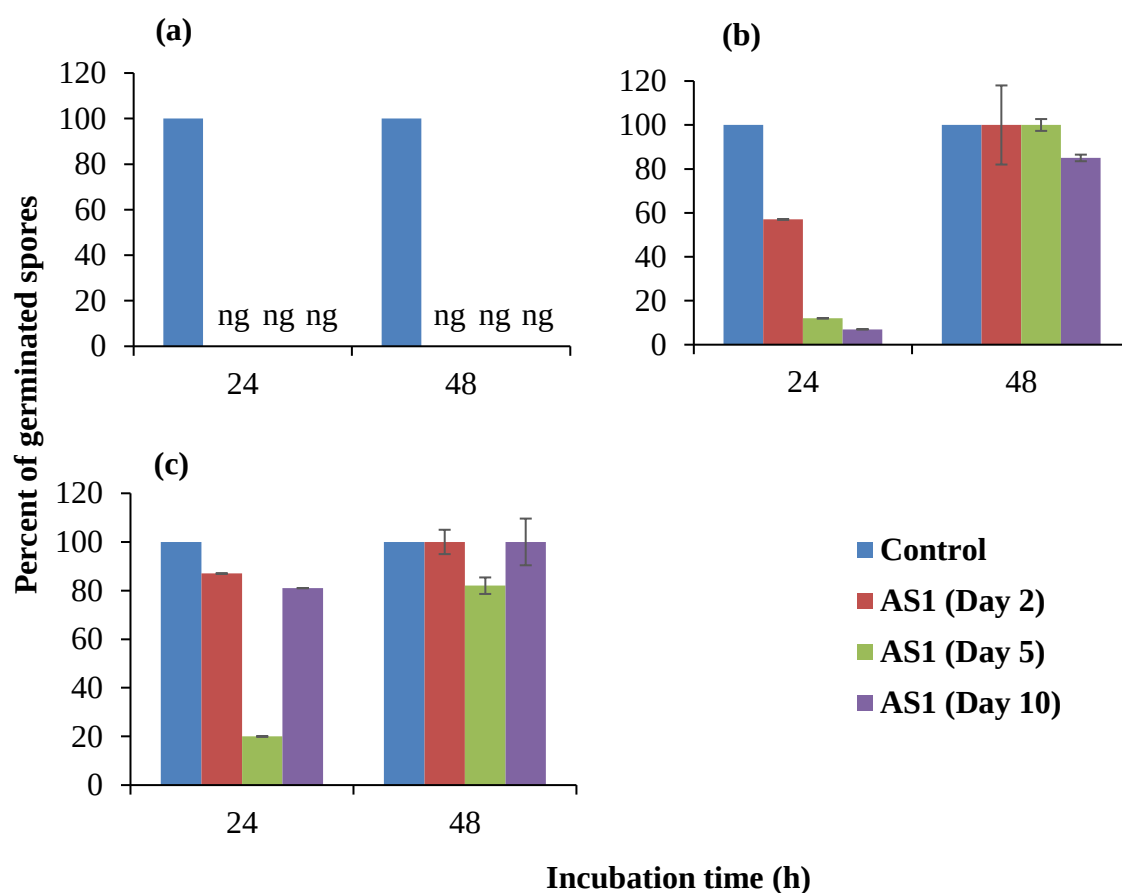


Figure 3.12 Mean percentage germination of (a) *P. verrucosum*, (b) *A. fumigatus* (Mi538) and (c) *F. verticillioides* spores on $\frac{1}{2}$ nutrient agar containing *Streptomyces* AS1 metabolites. Data are means of three replicates with bars indicating standard deviation. The data analysis was carried out on the actual data. The percentages were plotted for presentation purposes only. Key: ng - no germination

3.3.10 Efficacy of *Streptomyces* AS1 ethyl acetate extract on time to detection (TTD) of *P. verrucosum*, *A. fumigatus* (Mi538) and *F. verticillioides* and mycotoxin production using the BioScreen C assay method

Figure 3.13 shows an example of the average growth curve of *P. verrucosum* obtained after inoculation with different concentrations of *Streptomyces* AS1 ethyl acetate extract at two a_w levels (0.95 and 0.99 a_w) over seven days.

Figure 3.14 shows the effect of different concentration of ethyl acetate extract at two a_w levels (0.95 and 0.99 a_w) on the time to detection (TTD) at O.D. equal to 0.1 for *P. verrucosum*, *A. fumigatus* (Mi538) and *F. verticillioides*. For *P. verrucosum*, AS1 ethyl acetate extract at 5 $\mu\text{g/mL}$ resulted in a drastic and significant increase ($p < 0.05$) of TTD at both a_w levels. However, at 0.95 a_w an increase in the AS1 concentration did not show any significant increase in the TTD. In contrast, the TTD was continuously increased significantly ($p < 0.05$) at 0.99 a_w until 30 $\mu\text{g/mL}$ after which there was no significant increase. For *A. fumigatus* (Mi538) at 0.95 a_w the addition of AS1 extract up to 20 $\mu\text{g/mL}$ significantly increased ($p < 0.05$) the TTD. Meanwhile, at 0.99 a_w the TTD was significantly increased until 30 $\mu\text{g/mL}$ and after that there was no change. For *F. verticillioides*, the TTD was significantly increased ($p < 0.05$) until 20 $\mu\text{g/mL}$ of AS1 extract at both a_w levels.

The effect of AS1 ethyl acetate extract (Table 3.4) on toxin production was examined. The production of gliotoxin (*A. fumigatus*) and fumonisins (B_1 and B_2 ; *F. verticillioides*) was high at 0.99 a_w and for ochratoxin A (OTA; *P. verrucosum*) this was at 0.95 a_w . The OTA production was totally inhibited by the ethyl acetate extract at all doses and a_w treatment levels. There was a significant reduction ($p < 0.05$) in FB_1 and FB_2 at both a_w levels when compared with the control. AS1 concentration of 5 - 40 $\mu\text{g/mL}$ gave similar inhibition of FB_1 at both a_w levels and FB_2 at 0.95 a_w . For $\text{FB}_2/0.99 a_w$, >20 $\mu\text{g/mL}$ AS1 was needed to significantly ($p < 0.05$) inhibit production. However, there was no significant effect on gliotoxin production. In fact, gliotoxin production was stimulated significantly ($p < 0.05$) at 0.95 a_w when treated with low AS1 extract concentrations (5 $\mu\text{g/mL}$ and 10 $\mu\text{g/mL}$).

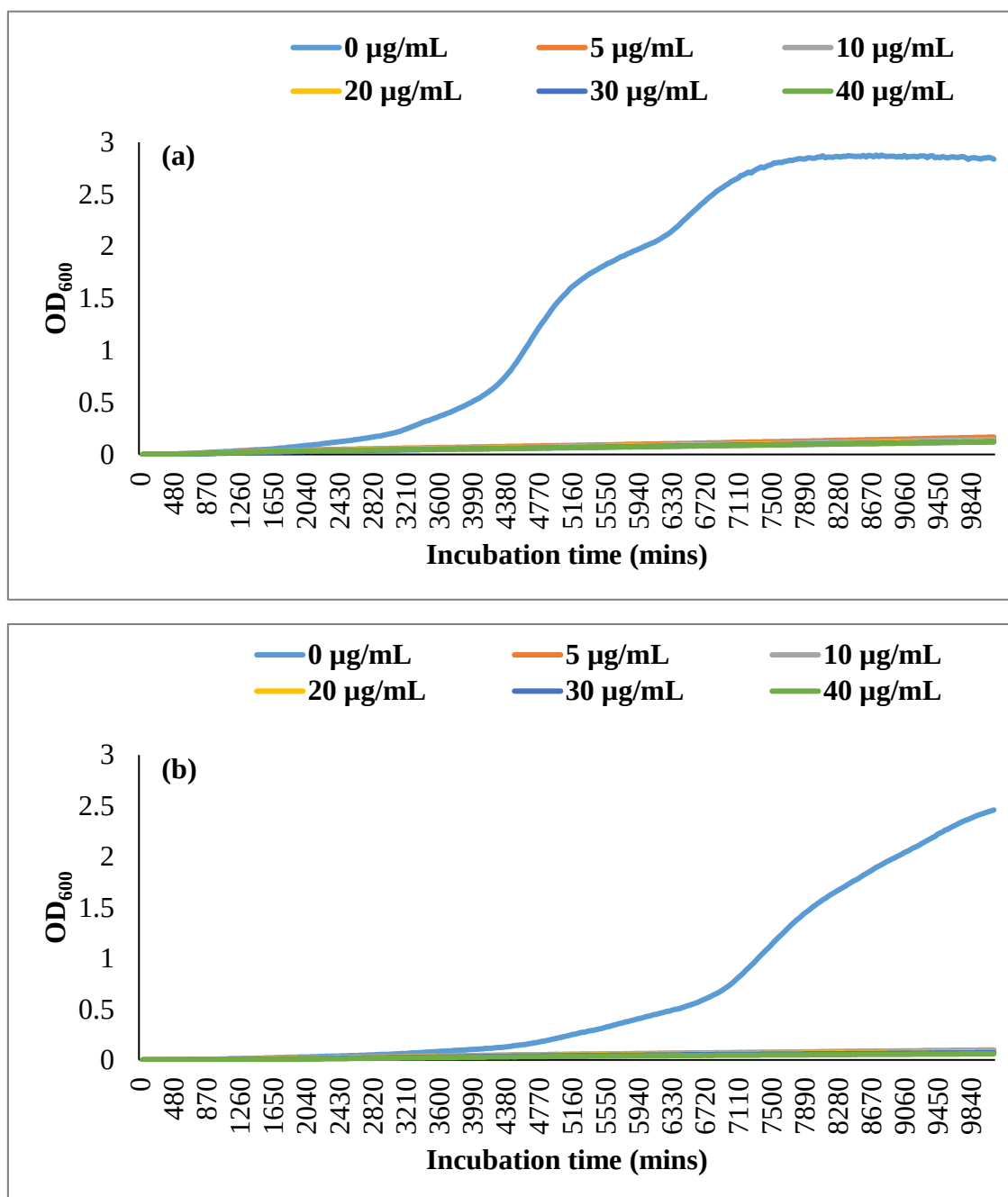


Figure 3.13 Growth curves of *P. verrucosum* at a_w (a) 0.99 and (b) 0.95 in semi-solid YES media containing different concentrations (0 - 40 µg/mL) of *Streptomyces* AS1 ethyl acetate extract over seven days incubation at 25°C using the Bioscreen C. Each curve represents the mean of ten individual replicates.

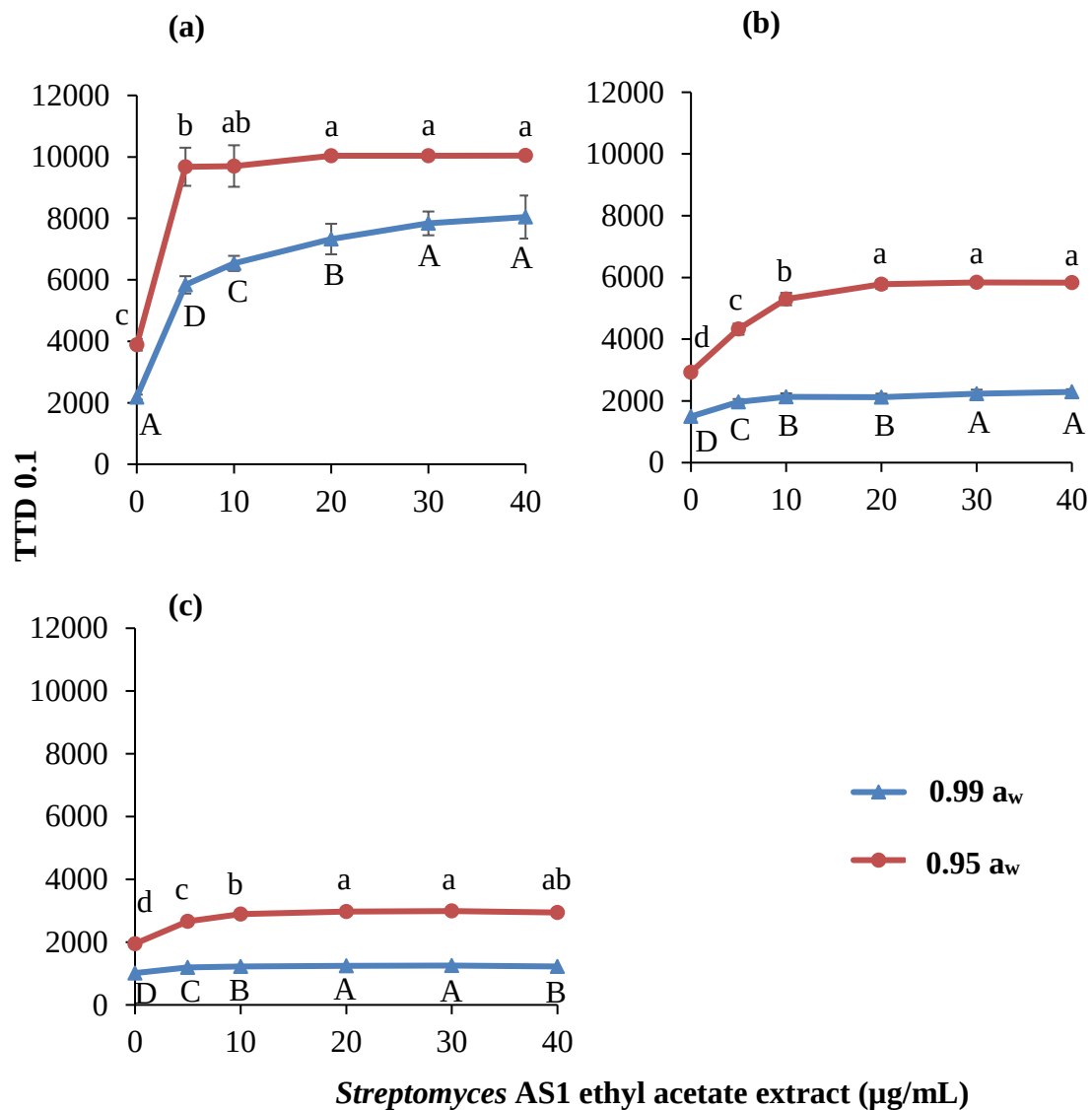


Figure 3.14 Effect of *Streptomyces* AS1 ethyl acetate extract on the time to detection (TTD) of (a) *P. verrucosum*, (b) *A. fumigatus* (Mi538) and (c) *F. verticillioides*. TTD 0.1; time to detection for an O.D. of 0.1. Data are mean of ten replicates with bars indicating standard deviation. Different letters indicate significant differences ($p < 0.05$) within treatments by the Student's *t* test for *P. verrucosum* and *A. fumigatus* at 0.99 a_w and *F. verticillioides* at 0.95 and 0.99 a_w and the Wilcoxon method (non parametric comparison) for *P. verrucosum* and *A. fumigatus* (Mi538) at 0.95 a_w .

Table 3.4 Efficacy of *Streptomyces* AS1 ethyl acetate extract for control of OTA, gliotoxin and fumonisins production by *P. verrucosum*, *A. fumigatus* (Mi538) and *F. verticillioides* grown on solid media, respectively. Data are means with SD; ND, none detected.

Concentration (µg/mL)	a _w	OTA (µg/g agar)	Gliotoxin (µg/g agar)	Fumonisin (µg/g agar)	
				FB ₁	FB ₂
0	0.99	10.5 ± 4.9	4.1 ± 0.56 ^A	3.73±0.07 ^A	4.57±0.49 ^A
	0.95	13.2 ± 3.1	2.2 ± 0.44 ^{cd}	0.84±0.01 ^a	0.96±0.15 ^a
5	0.99	ND	6.9 ± 0.30 ^A	0.10±0.002 ^B	4.20±0.15 ^A
	0.95	ND	3.8 ± 0.34 ^a	0.06±0.04 ^b	0.18±0.04 ^b
10	0.99	ND	4.1 ± 0.20 ^A	0.09±0.003 ^B	4.70±0.66 ^A
	0.95	ND	3.4 ± 0.70 ^{ab}	0.03±0.01 ^b	0.09±0.02 ^{bc}
20	0.99	ND	7.0 ± 0.03 ^A	0.07±0.01 ^B	2.26±0.70 ^B
	0.95	ND	2.9 ± 0.14 ^{bc}	0.04±0.002 ^b	0.07±0.01 ^{bc}
30	0.99	ND	5.0 ± 1.1 ^A	0.06±0.003 ^B	1.42±0.23 ^B
	0.95	ND	1.9 ± 0.06 ^d	0.03±0.01 ^b	0.07±0.01 ^c
40	0.99	ND	4.1 ± 0.15 ^A	0.06±0.03 ^B	1.32±0.56 ^B
	0.95	ND	2.4 ± 0.50 ^{cd}	0.03±0.01 ^b	0.08±0.01 ^{bc}

Data are means of triplicates with bars indication standard deviation. Different letters indicate significant differences ($p < 0.05$) within treatment by the Student's t test for fumonisins (0.95, 0.99 a_w) and gliotoxin (0.95 a_w) and the Wilcoxon Method (non parametric comparison) for 0.99 a_w. Experiments were carried out with agar spot assay

The minimum inhibitory concentration (MIC) and concentrations of ethyl acetate extracts that were necessary for 50% growth inhibition (IC₅₀) were determined and are shown in Table 3.5. The MIC of *P. verrucosum*, *A. fumigatus* (Mi538) and *F. verticillioides* at both *a_w* levels was more than the highest concentration tested (> 40 µg/mL). The IC₅₀ for *P. verrucosum* was the lowest which is 0.15 µg/mL and 0.005 ng/mL at 0.99 and 0.95 *a_w*, respectively. Based on these results, subsequent studies focussed only on control of *P. verrucosum* and OTA production.

Table 3.5 Minimum inhibitory concentration (MIC) and IC₅₀ of *Streptomyces* AS1 ethyl acetate extract for controlling growth of *P. verrucosum*, *A. fumigatus* (Mi538) and *F. verticillioides*.

Pathogenic fungi	<i>a_w</i>	MIC (µg/mL)	IC ₅₀ (µg/mL)
<i>P. verrucosum</i>	0.99	> 40	0.15
	0.95	> 40	0.000005
<i>A. fumigatus</i> (Mi538)	0.99	> 40	> 40
	0.95	> 40	10.45
<i>F. verticillioides</i>	0.99	> 40	> 40
	0.95	> 40	>40

Because no OTA was detected at 5 µg/mL AS1 extract, further studies with lower concentration of AS1 extract was carried out to identify at which concentration the total OTA production was inhibited. Figure 3.15 shows that there was a significant decreased in OTA production at 0.99 and 0.95 *a_w* as the concentration of AS1 extract was increase to 1.25 µg/mL and totally inhibited at ≥2.5 µg/mL. However, the production of OTA was enhanced significantly at 0.15 µg/mL and 0.99 *a_w* when compared with the control. Figure 3.16 shows the growth rate of *P. verrucosum* at 0.99 and 0.95 *a_w*. The growth was continuously and significantly reduced (p<0.05) as the AS1 concentration was increased to 5 µg/mL. However, at 0.95 *a_w* the growth of *P. verrucosum* was the same when AS1 extract was increased from 1.25 µg/mL to 5.0 µg/mL.

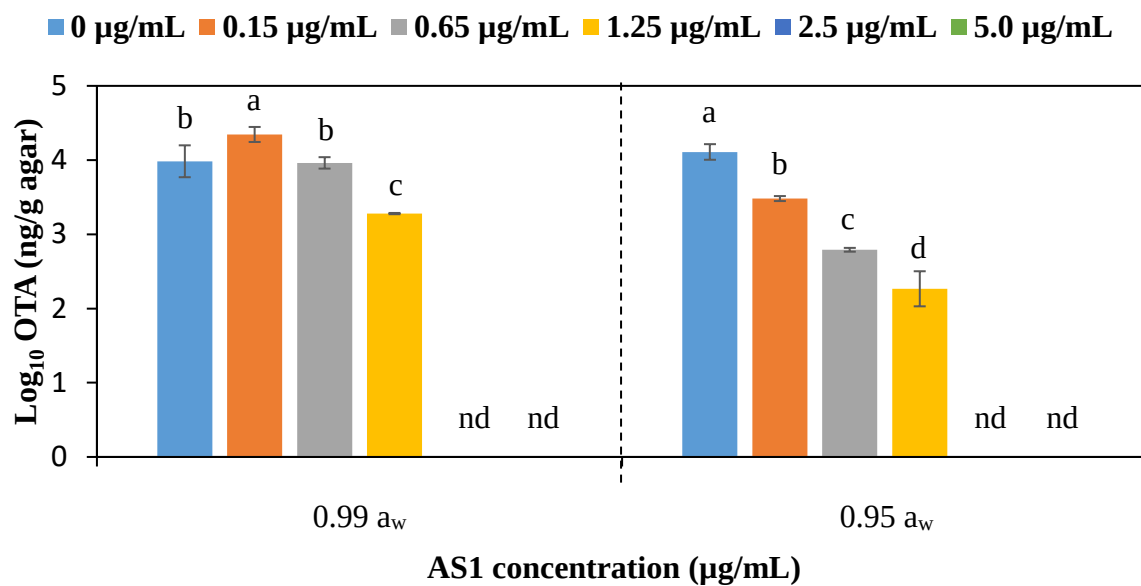


Figure 3.15 Efficacy of *Streptomyces* AS1 ethyl acetate extract for control of OTA production by *P. verrucosum* at 25°C. Data are means of triplicates with bars indicating standard errors (SE). Different letters indicate significant differences ($p < 0.05$) within treatments using the Student's *t* test. Key: n.d; none detected.

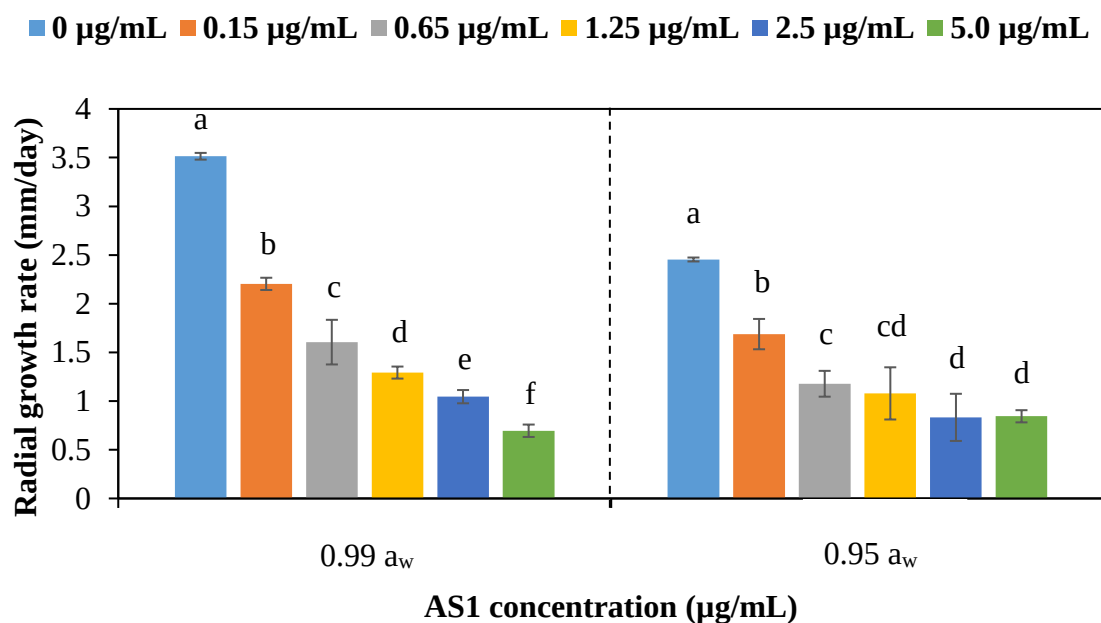


Figure 3.16 Efficacy of *Streptomyces* AS1 ethyl acetate extract for control of *P. verrucosum* growth at 25°C. Data are means of triplicates with bars indicating standard error (SE). Different letters indicate significant differences ($p < 0.05$) within treatments by the Student's *t* test.

3.3.11 Identification of bioactive compounds from *Streptomyces* AS1

Table 3.6 shows the 10 compounds isolated from *Streptomyces* AS1 ethyl acetate extract. Four major compounds were determined as valinomycin, cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Val) and brevianamide F. Another six minor compounds were identified as rugulusovin, tryptophol, chloramphenicol, monactin, dinactin and nonactin. From these four major compounds, two compounds with the highest concentration (valinomycin and cyclo(L-Pro-L-Tyr) were selected for the next experiment.

Table 3.6 Compounds isolated from ethyl acetate extract of *Streptomyces* AS1 supernatant.

Compounds	Concentration (µg/g dried biomass)
Valinomycin	149.22
Cyclo(L-Pro-L-Tyr)	21.64
Cyclo(L-Pro-L-Val)	10.07
Brevianamide F	2.68
Rugulusovin	0.72
Tryptophol	0.31
Chloramphenicol	0.23
Monactin	0.02
Dinactin	0.01
Nonactin	0.007

3.3.12 Comparison antifungal activity between *Streptomyces* AS1 ethyl acetate extract and valinomycin, cyclo(L-Pro-L-Tyr) and a mixture of these two compounds against *P. verrucosum*

The effect of AS1 ethyl acetate extract and individual antifungal compounds valinomycin and cyclo(L-Pro-L-Tyr) and a mixture of these two antifungal compounds on growth of *P. verrucosum* and OTA production is shown in Figure 3.17a and b. AS1 significantly inhibited ($p < 0.05$) growth of *P. verrucosum* when compared with the control and other

antifungal compounds and also inhibited the total production of OTA. However, similar inhibition was not observed when a single and a mixture of the two predominant antifungal compounds were used. There was no significant difference ($p>0.05$) between valinomycin, cyclo(L-Pro-L-Tyr) and a mixture of these two compounds when compared with the control in relation to growth rate and OTA production.

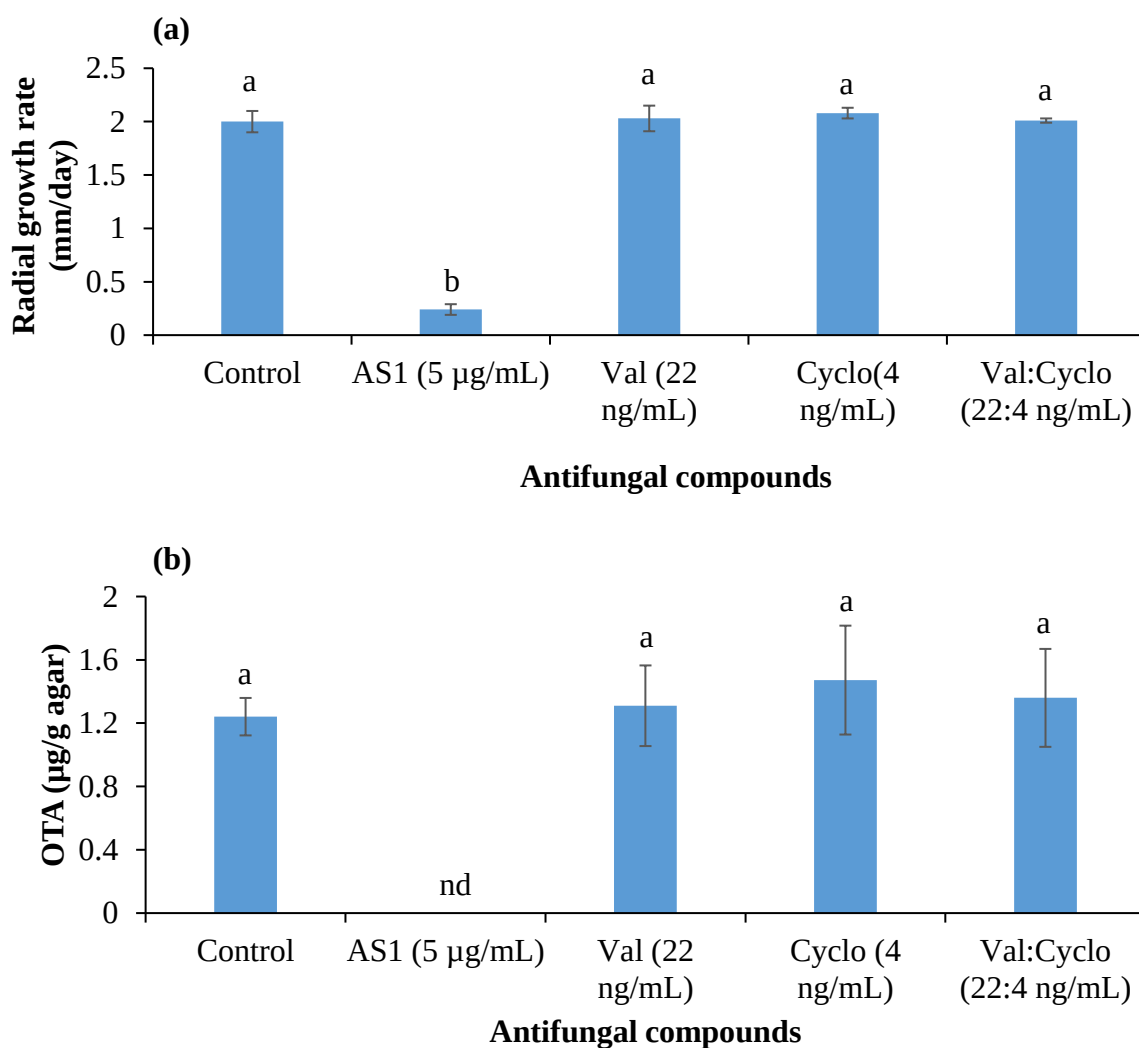


Figure 3.17 Efficacy of *Streptomyces* AS1 ethyl acetate extract, valinomycin and cyclo(L-Pro-L-Tyr) for control of growth of (a) *P. verrucosum* and (b) OTA production at 0.95 a_w . Data are means of triplicates with bars indicating standard deviation. Different letters indicate significant differences ($p<0.05$) using the Student's *t* test. Key: AS1 - natural mixture of AS1 compounds, Val - valinomycin, Cyclo - cyclo(L-Pro-L-Tyr), Val:Cyclo - mixture of these two.

3.4 DISCUSSION

3.4.1 *L. plantarum* VS1

This study aimed to determine the efficacy of selected LABs to inhibit the growth of human fungal pathogen particularly the dermatophyte fungus; *T. rubrum* and to identify the antifungal compounds involved. Four strains with high inhibition levels were identified in Chapter 2 and tested against *T. rubrum* spore germination. In this study a mixture of these four strains strongly inhibited the spore germination, completely inhibiting germinative capacity. The inhibition of spore germination is the critical first step to prevent colonisation and establishment of fungi on growth matrices. Based on these findings further studies were conducted to identify whether the inhibition was due to production of specific antagonistic substances. Therefore, the cell free supernatant from an individual and a mixture of four strains were tested. The growth inhibition by both *L. plantarum* strains were significantly stronger than the *Pediococcus* strains. This inhibition could have been due to a strong acidification of *L. plantarum* strains. For example, the final pH of *L. plantarum* VS1, *L. plantarum* VN5, *P. pentosaceus* 84 and *P. acidilactici* 95 was 3.14, 3.20, 3.33 and 3.43, respectively. In this study a mixture of four LAB strains was also tested to determine if this can enhance the antagonistic activity against *T. rubrum*. Co-cultivation of the four LABs in this study significantly enhanced ($p < 0.05$) the inhibition when compared to the individual *Pediococcus* strains. However, there was no improvement in inhibition when compared to a single *L. plantarum* strain. By contrast, *L. plantarum* VS1 alone was more effective than when co-cultivated with the other strains. Based on these findings, both *L. plantarum* strains with high inhibition were selected and the effect of different combinations of both strains on *T. rubrum* growth was studied. Again, co-cultivation did not show an advantage over a single strain. The *T. rubrum* growth inhibition by supernatants of a co-culture of *L. plantarum* VS1 and *L. plantarum* VN5 was not statistically significant ($p = 0.05$). Previous studies have suggested that co-cultivation provided some benefits for inhibiting the growth of bacterial or fungal pathogen either through bacteria-bacteria (Rojo-Bezares et al., 2007), bacteria-fungal (Esmaeilshirazifard et al., 2018; Ström et al., 2005) or fungal-fungal (Azzollini et al., 2018) co-cultivation. The advantage of this co-cultivation was suggested to be due to enhanced secretion of known antagonistic substances from each individual, as well as the induction of new compounds such as antibiotics or secondary metabolites with antifungal

and cytotoxic activity (Marmann et al., 2014). In addition, the induction of bacteriocin activity by *L. plantarum* J23 was more pronounced when the mixture is between living cells or partially heat denatured cells (Rojo-Bezares et al., 2007). However, co-cultivation did not always significantly enhance the antimicrobial activity of the LAB. Tirloni et al. (2014) found that the antibacterial activity of the *L. animalis* SB310 and *L. paracasei* subsp *paracasei* SB137 mixture varied depending on how strong the antimicrobial activity of the individual LAB strain and the ratio of the mixture was.

Since co-cultivation of *L. plantarum* VS1 and *L. plantarum* VN5 did not enhanced the fungal inhibition, the rest of the study focused on *L. plantarum* VS1, because it was shown to have the highest inhibition rate among the four LAB strains tested. Temporal effects of *L. plantarum* VS1 growth on antifungal activity against *T. rubrum* was studied. The inhibitory effect of this strain was found at the late log phase and continued during the stationary growth phase. It has been suggested that lactic and acetic acid secreted by *L. plantarum* strains may be involved in this inhibitory activity due to their high production during the log phase of growth. Various organic acids produced by LABs have been suggested to contribute to high antifungal activity. Among these are lactic acid, acetic acid, propionic acid, formic acid, succinic acid, butyric acid, phenyllactic acid, citric acid, oxalic and malonic acids (Özcelik et al., 2016; Şehirli and Saydam, 2016; Valerio et al., 2016; Crowley et al., 2013). The effect of different growth conditions and carbon sources on antifungal activity was further studied and identified. Under the optimum conditions for batch culture (incubation time: 48 h, inoculum size: 3%, pH: 6.2, temperature: 35°C and carbon source: glucose) the antifungal activity against *T. rubrum* growth was successfully increased by 30x when compared to the non-optimized.

The cell free supernatant was subjected to pH neutralization and heat treatment (121°C/15 min) to identify the nature of the antifungal compounds. The antifungal activity of the supernatant was active at low pH (3.33; original pH) and lost its activity after the neutralization process. This indicated that the acidic nature of the antifungal compounds and organic acid may be involved in the anti-dermatophyte activity. Furthermore, the application of high temperature (121°C/15 min) to the supernatant resulted in increased antifungal activity even though the pH was modified to 6.8. The application of high temperature may denature or alter the primary configuration of certain compounds (Qian

et al., 2017) secreted by the *L. plantarum* strain, forming new structures which contribute to antifungal activity or work synergistically with lactic or acetic acid against *T. rubrum*. L-lactic was found the most abundant organic acid produced by the *L. plantarum* strain which was 8,700 times higher than acetic acid. The antifungal compounds produced by the *L. plantarum* strain basically appeared to have some fungicidal efficacy with the MIC and MFC of 20 mg/mL and 30 mg/mL, respectively. To the best of my knowledge this is the first study on antifungal activity of *L. plantarum* isolated from Malaysian fermented foods against *T. rubrum*.

T. rubrum is normally isolated from human skin, nails and hairs or keratin rich matrices. To better understand the growth properties of *T. rubrum*, it was grown on a synthetic keratin medium with different pH levels and incubated at different temperatures. *T. rubrum* grew better at low pH (4.5) and 30°C which is lower than the normal human skin pH (5.5) and body temperature (37°C). Kadhim et al. (2015) reported the optimum growth conditions of clinical isolates of *T. rubrum* on SDA was at a higher pH (pH 6.0) and 30°C. Meanwhile Ferreira-Nozawa et al., (2003) found that *T. rubrum* isolate H6 grew better at pH 4.0 in non-buffered high phosphate minimal media.

The efficacy of *L. plantarum* strain supernatant and L-lactic acid, acetic acid and a mixture of these two organic acids against *T. rubrum* growth was compared. The antifungal activity of *L. plantarum* strain supernatant was more pronounced and gave better control than an individual or a mixture of individual compounds. Among these organic acids, L-lactic acid was mainly responsible for the antifungal effect observed. This contrasts with the study by Kuwaki et al. (2002) who found better control of tinea by acetic and malonic than lactic acid. Moreover, Sharma and Srivastava (2014) showed the growth of *C. albicans* was inhibited by acetic acid at concentration >5 g/L whereas no inhibition by lactic acid (>10 g/L) was observed.

3.4.2 *Streptomyces* AS1

The effect of *Streptomyces* AS1 metabolites on spore germination of *P. verrucosum*, *A. fumigatus* (Mi538) and *F. verticillioides* were examined. The efficacy of metabolites on spore germination was dependent on the temporal growth of the *Streptomyces* AS1 and thus metabolite production and on the target fungal species. The AS1 metabolites were

very effective against *P. verrucosum* as no spores germinated under all conditions tested. In contrast, it was not effective against *A. fumigatus* (Mi538) and *F. verticillioides* although the metabolites did delay germination. Previously, complete inhibition of *A. flavus* spore germination by *Streptomyces* AS1 was reported (Sultan and Magan, 2011).

The efficacy of AS1 extract was further studied using a spectrophotometric turbidimetric assay method using the Bioscreen C system. The effect of different concentrations of metabolites from the AS1 strain and water activity (a_w) on TTD of *P. verrucosum*, *A. fumigatus* (Mi538) and *F. verticillioides* and mycotoxin production were studied. TTD represents the initial growth phase of the fungi. The shorter the TTD the faster the fungal growth. In this study AS1 extract was able to control fungal growth, but with different efficacies. Overall, AS1 extract was more effective in controlling growth of *P. verrucosum*, even at low concentrations of the extract. This result was in parallel with the earlier findings on spore germination inhibition. Furthermore, AS1 extract at all concentrations successfully suppressed production of OTA and reduced production of fumonisin B₁ and B₂ at both a_w levels. However, this did not occur with gliotoxin production by *A. fumigatus* (Mi538). Since AS1 extract was very effective in controlling *P. verrucosum* growth and OTA production, it was decided to focus on control of this species. Indeed, OTA was not detected when 5 µg/mL AS1 extract was used. Thus, more detailed efficacy testing was done to determine the lowest concentrations which could be used to inhibit OTA production. The AS1 extract was more effective in inhibiting OTA production than *P. verrucosum* growth. Complete inhibition of OTA production was achieved at very low concentrations (2.5 µg/mL), while only 70% growth inhibition was achieved. Previous studies with other *Streptomyces* strains have suggested no effect on *P. verrucosum* growth, only some inhibition of *A. fumigatus*, but good efficacy against *F. verticillioides* (Paškevičius et al., 2014; Nguyen et al., 2018). However, these studies only focused on control of mycelial growth and not on mycotoxin production. Another study by Medina et al. (2007) found compounds from another *Streptomyces* strain was effective at controlling *Aspergillus carbonarius* growth and OTA production.

A total of 10 compounds were found to be present in the AS1 ethyl acetate extract. Four major antifungal compounds with >2 µg/g dry biomass were identified as valinomycin, cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Val) and brevianamide F. The production of these

compounds by other *Streptomyces* species and bacteria such as *Bacillus* sp. N strain, *Pseudomonas aurantiaca* and *Cellulosimicrobium cellulans* has been reported previously (Buedenbender et al., 2018; Wattana-Amorn et al., 2016; Park & Shim 2014; Kumar et al., 2013, Smaoui et al., 2012; Gwee Kyo et al., 2011; Yonezawa et al., 2011, and Li et al., 2006). Valinomycin is an antibiotic with a cyclic (dodeca)depsipeptide structure which contains three identical sequences that form a 36 members cyclic molecule. The unit of the sequence comprises of D- α -hydroxy-isovaleric acid, D-valine, L-lactic acid and L-valine (Kimbrell et al., 2011). This structure binds the salt ion, particularly potassium ion and transports it across the cell membrane. This causes the swelling and high respiration of the mitochondria, and hence disturbs the function of this organelle (Paananen et al., 2000; Azzi and Azzone, 1965). Cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Val) and brevianamide F are cyclic dipeptides which consist of two amino acids. The antifungal activity of cyclo(L-Pro-L-Tyr), and cyclo(L-Pro-L-Val) was reported with a different level of efficacy depending on the target fungi such as *Aspergillus*, *Fusarium*, *Penicillium*, *Rhizoctania* and *Candida* species (Park & Shim 2014; Kumar et al., 2013; Smaoui et al., 2012; Gwee Kyo et al., 2011; Yonezawa et al., 2011). However, no antifungal activity was described for brevianamide F.

A comparative study was done to of the efficacy of AS1 ethyl acetate extract and the two major individual antifungal compounds identified from the AS1 extract. The concentration of valinomycin and cyclo(L-Pro-L-Tyr) in this study was similar to that determined in the 5 μ g/mL AS1 extract. In our study, there was no synergistic effect of combinations of cyclo(L-Pro-L-Tyr) and valinomycin against growth and OTA production by *P. verrucosum*. In contrast, the natural mixture of compounds from the AS1 extract showed more effective antifungal properties, with better control of growth of *P. verrucosum* and of OTA production. The discrepancy in these results may be due to the existence of various minor compounds (Williams et al., 2012) with biological activities in the natural AS1 extract which may exhibit a synergistic effect when compared to the two main components only. Previously, several studies examined the antifungal effects of a combination of two biological active compounds (Zhao et al., 2017; Kumar et. al., 2014; Del Poeta et al., 2000) which gave variable effects. For example, a combination of cyclo(L-Pro-L-Tyr) and imipenem (an antibiotic) was successfully shown to have synergistic effects against bacterial pathogen and reduced the MICs. However,

the effect was indifferent when combined with ceftazidime (an antibiotic) (Kumar et al., 2014).

3.5 CONCLUSIONS

Four LAB strains were very effective at inhibiting *T. rubrum* spore germination and controlled mycelial growth. The best strain with the highest antifungal activity was determined as *L. plantarum* VS1. The cell free supernatant had fungicidal activity with the MFC of 30 mg/mL. Two major compounds produced by VS1 were determined as L-lactic acid and acetic acid and the antifungal effect was mainly from L-lactic acid. However, the application of cell free supernatant as antifungal compounds was more effective than a single or a mixture of L-lactic and acetic acid.

Streptomyces AS1 is more effective in suppressing spore germination and mycelial growth of *P. verrucosum* than *A. fumigatus* (Mi538) and *F. verticillioides*. A very low concentration of AS1 extract was able to inhibit mycelial growth by 50% of *P. verrucosum* although >40 µg/mL AS1 was needed for complete inhibition. AS1 successfully inhibited 100% OTA production at concentrations ≥ 2.5 µg/mL. Four major bioactive compounds from AS1 were successfully identified. This study found that the application of antifungal compounds in the naturally formed state were better than single or a two main components as a mixture.

CHAPTER 4

Impact of *L. plantarum* VS1 and *Streptomyces* AS1 metabolites on mechanism of action in relation to production of extracellular hydrolytic enzymes by the target pathogens

4.1 INTRODUCTION

There is significant interest in a better understanding of the mechanism of action of potential anti-fungal compounds against the fungi examined in this thesis. Many mechanisms of action reported have been based on observations of the morphological alteration of hyphae and conidia and ultrastructural changes in the fungal cells, especially in the permeability of the cell wall/membrane (Avanco et al., 2017; Khan and Ahmad, 2011; De Oliveira Pereira et al., 2013; Ghahfarokhi et al., 2004). In addition, often measurements have been made of the release of intracellular material, primarily nucleotides, as an indication of leakage from the fungal cells (De Oliveira Pereira et al., 2013).

The production of extracellular hydrolytic enzymes is critical for the germination, growth and colonisation of different matrices by the fungi examined in this study. Depending on the fungal species and the availability of nutrients, fungi will secrete different enzymes to hydrolyse complex nutrients such as carbohydrates and proteins into simple forms and use these as an energy source for colonisation and establishment (Alam et al., 2009; Kaufman et al., 2007; Marin et al., 2003). It has also been suggested that hydrolytic enzyme production can be a good indicator of early activity of fungi prior to microscopic and visible growth occurring (Magan, 1993; Keshri et al., 2002). In addition to growth, enzyme production may also be used as an indicator for early mycotoxin production (Magan and Aldred, 2007). This method (the measurement of hydrolytic enzyme production by fungi) has an advantage as it can be completed in 1 - 2 h, many samples can be examined relatively quickly and the enzyme activity accurately quantified (Magan, 1993). Thus, many previous studies have mainly focused on early detection of fungal growth in relation to different interacting environmental conditions. Few studies have examined the effect of anti-fungal compounds on the hydrolytic enzyme production as a mechanism of action of inhibiting the activity of the human pathogens and spoilage mycotoxigenic fungi.

The objectives of this study were to examine the following:

1. To identify the key enzymes produced by the different target fungi using a semi-quantitative screen of 19 different enzymes using the bioMérieux kit assay
2. Examine the effect of different concentrations of *L. plantarum* VS1 cell free supernatants on *T. rubrum* growth, sporulation and hydrolytic enzyme production on a keratin-based matrix.
3. To study the effect of different concentrations of *Streptomyces* AS1 ethyl acetate extracts on *P. verrucosum* growth, ochratoxin A (OTA) production and hydrolytic enzyme activity on a conducive YES agar medium modified to 0.99 and 0.95 a_w.

4.2 MATERIALS AND METHODS

4.2.1 Semi-quantitative screening for extracellular hydrolytic enzymes production by *P. verrucosum* and *T. rubrum* at 0.99 and 0.95 water activity using the API ZYM kit

Spore suspensions (approx. 10⁶ spores/ mL) were prepared as described in Section 2.2.2(b) and 20 mL of YES and SBD broth modified with glycerol to 0.99 and 0.95 a_w prepared in 100 mL conical flasks. A total of 200 µL of *P. verrucosum* spore suspension was inoculated into 20 mL YES broth and *T. rubrum* in SBD broth and incubated in an incubator shaker (Thermo Fischer Ltd) for 5 days with an agitation rate of 150 rpm. *P. verrucosum* was incubated at 25°C and *T. rubrum* at 30°C. After 5 days the culture was filtered with Whatman filter paper and the supernatant collected for extracellular enzyme assays. The supernatant was tested for enzyme activities using API ZYM kit (bioMérieux SA, Marcy-l'Etoile, France) as described by the manufacturer.

In brief, a strip with 20 cupules which contain different substrates and buffer, were placed in a tray humidified with water. Sixty-five microliters of sample were loaded into each cupule and incubated at 37°C for four hours, in the dark, under static conditions. After four hours 35 µL of ZYM A was added followed by 35 µL of ZYM B. The mixture was then incubated in daylight for 30 minutes. The developed colour was recorded and the value from 0-5 was assigned correspond to colours developed: (1) 0 negative reaction, (2) 1-2 intermediate reaction and (3) 3-5 positive reaction.

Table 4.1 List of enzymes, substrate and pH for each cupule

No	ENZYMES	SUBSTRATE	pH	RESULT	
				POSITIVE	NEGATIVE
1.	Control			Colourless or colour of the sample if it has an intense coloration	
2.	Alkaline phosphatase	2-naphthyl phosphate	8.5	Violet	Colorless or very pale yellow
3.	Esterase (C 4)	2-naphthyl butyrate	6.5	Violet	
4.	Esterase Lipase (C 8)	2-naphthyl caprylate	7.5	Violet	
5.	Lipase (C 14)	2-naphthyl myristate	7.5	Violet	
6.	Leucine arylamidase	L-leucyl-2-naphthylamide	7.5	Orange	
7.	Valine arylamidase	L-valyl-2-naphthylamide	7.5	Orange	
8.	Cystine arylamidase	L-cystyl-2-naphthylamide	7.5	Orange	
9.	Trypsin	N-benzoyl-DL-arginine-2-naphthylamide	8.5	Orange	
10	α -chymotrypsin	N-glutaryl-phenylalanine-2-naphthylamide	7.5	Orange	
11.	Acid phosphatase	2-naphthyl phosphate	5.4	Violet	
12.	Naphthol-AS-B1-phosphohydrolase	Naphthol-AS-B1-phosphate	5.4	Blue	
13.	α -galactosidase	6-Br-2-naphthyl- α D-galactopyranoside	5.4	Violet	
14.	β -galactosidase	2-naphthyl- β D-galactopyranoside	5.4	Violet	
15.	β -glucuronidase	Naphthol-AS-B1- β D-glucuronide	5.4	Blue	
16.	α -glucosidase	2-naphthyl- α D-glucopyranoside	5.4	Violet	
17.	β -glucosidase	6-Br-2-naphthyl- β D-glucopyranoside	5.4	Violet	
18.	N-acetyl- β -glucosaminidase	1-naphthyl-N-acetyl- β D-glucosaminide	5.4	Brown	
19.	α -mannosidase	6-Br-2-naphthyl- α D-mannopyranoside	5.4	Violet	
20.	α -fucosidase	2-naphthyl- α L-fucopyranoside	5.4	Violet	

4.2.2 Preparation of *L. plantarum* VS1 cell free supernatant

The modified MRS broth was prepared as described in Section 3.2.2. LAB cell suspensions were prepared as described in Section 2.2.2(a). Cell suspensions (3.0 %) were inoculated into 100 mL sterile mMRS broth and incubated at 30°C for 48 h in static conditions. After 48 h the supernatant was separated from the cells by centrifugation at 2000 rpm, 4°C for 10 min, filtered using sterile cellulose acetate membrane filters and freeze dried aseptically. The dried cell free supernatant was then dissolved in sterile distilled water.

4.2.3 Inoculation of *T. rubrum* on keratin agar

Keratin agar with pH 4.5 was prepared as described in Section 3.2.8.

Ten millilitres of keratin agar (approx. 52°C) was mixed aseptically with *L. plantarum* VS1 supernatant (final concentration 4 mg/mL and 15 mg/mL) and poured into Petri plates (Ø 53 mm). These were left to cool to room temperature before inoculation with the spore suspensions. These plates were divided into two parts:

- a) for hydrolytic enzyme and protein assay - 100 µL of spore suspension (10^4 spores/mL; final spore count was 10^3) was spread plated on the keratin agar plates
- b) for growth measurement - 5 µL of spore suspension (10^6 spores/mL) was inoculated in the centre of the keratin agar plates

These cultures were incubated at 30°C for 12 days. Every three days three replicates were destructively sampled. The keratin agar with *T. rubrum* was transferred into pre-weighed 50 mL tubes and weighed again to obtain the agar amount. This was kept at -20°C until enzyme extraction was carried out. Meanwhile, in separate replicates, the diameter of the colony were measured every two days in two perpendicular directions at right angles to each other and the means calculated. The radial growth rate was obtained from linear regression slopes of the growth curve (colony radius vs incubation days). All treatments were done with three replicates per treatment.

4.2.4 Hydrolytic enzyme assays

(a) Extraction of extracellular hydrolytic enzymes

The extraction of enzymes was done using distilled water at 30°C. Firstly, the agar sample in the 50 mL tube was left to defrost at room temperature. Then, distilled water was added (1:1,

w/v) to agar samples and the mixture shaken for one hour at 30°C and 200 rpm. The supernatant was separated from the biomass and agar by filtration using Whatman filter paper and transferred into a 15 mL tube. The supernatant was kept at -20°C until used.

(b) Measurement of hydrolytic enzyme activity

Enzyme assay

The enzyme assay consisted of a substrate, buffer and enzyme. The substrate, buffer and the concentration used to assay N-acetyl- β -glucosaminidase, acid phosphatase, alkaline phosphatase, esterase and keratinase activity are shown in Table 4.2. Each substrate with the required molar concentration was prepared in the respective buffer except p-nitrophenyl butyrate which was dissolved in acetonitrile. For the N-acetyl- β -glucosaminidase, acid phosphatase and alkaline phosphatase reaction, 50 μ L of buffer, 50 μ L of substrate and 50 μ L of enzyme extract were loaded into a 96 well plate and incubated at 37 °C for 30 min with the agitation rate of 150 rpm. Subsequently, 6.25 μ L of 1N disodium carbonate (Na_2CO_3) was added to stop the enzyme reaction and for the ionisation of p-nitrophenol to yellow p-nitrophenolate ion. After being left for 10 min at room temperature the absorbance was measured at 405 nm using a microplate reader (Tecan, Infinite M200PRO). For the control blank, the enzyme extract was added after 1N Na_2CO_3 .

For esterase, 50 μ L of 100 mM MOPS buffer (pH 7.5), 50 μ L of substrate and 50 μ L of enzyme extract were loaded in the 96 well plate and the absorbance measured at 405 nm (minute zero) followed by incubation at 37°C for 10 min. After 10 min the absorbance was measured again (minute 10). The difference in absorbance value at minute 10 and minute zero was used to calculate the esterase activity.

For keratinase activity, the keratin azure was used as a substrate. Keratin azure is a sheeps' wool keratin that has been impregnated with azure dye to give a blue colour. Keratinase breaks the keratin azure (insoluble) and releases soluble blue peptides and amino acids. For the preparation of keratin azure, it was first kept at -20°C for 24 h and transferred into a mortar. The liquid nitrogen was subsequently poured into the mortar and the keratin azure was ground to a powder using a pestle. After this, 228 mg keratin azure powder was weighed into a 50 mL tube and 28.5 mL of 0.05 M Tris buffer (pH 8.0) added (final concentration 8 mg/mL). This was thoroughly mixed before being aliquoted (250 μ L) into 2 mL tubes. Finally, 250 μ L of enzyme solution was added and incubated at 40°C for 64 h with the agitation rate of 200 rpm.

After 64 h the mixture was kept in ice to stop the reaction and centrifuged for 5 min at 4°C and 12,000 rpm to separate the insoluble keratin azure. The supernatant containing soluble azure (150 µL) was transferred into the 96 well plate and the absorbance measured at 595 nm using the microplate reader (Tecan, Infinite M200PRO). For the control blank, the enzyme was added after 64 h incubation of keratin azure, immediately mixed, kept in ice and centrifuged to separate insoluble keratin azure.

One unit of enzyme is defined as the amount of enzyme needed to hydrolyse 1 µmol of substrate permin under specific condition. The enzyme activity was calculated using the equation as shown below and the enzyme specific activity was expressed as enzyme activity (U) per mg protein.

$$\text{Enzyme activity (U/g sample)} = \frac{\Delta \text{Abs}_{405\text{nm}} \times 0.15625 \text{ mL} \times \text{wt}_{\text{agar}}}{30 \text{ min} \times 0.05 \text{ mL} \times \epsilon \times \text{volume (mL)}}$$

$$\Delta \text{Abs}_{405\text{nm}} = \text{Abs}_{405\text{nm}} \text{ sample} - \text{Abs}_{405\text{nm}} \text{ blank}$$

$$\text{wt}_{\text{agar}} = \text{weight of agar (g)}$$

$$\epsilon = \text{slope of standard curve}$$

$$\text{volume (ml)} = \text{volume of extraction enzyme}$$

$$0.15625 \text{ ml} = \text{total volume of assay}$$

$$0.05 \text{ ml} = \text{volume of enzyme/assay}$$

$$30 \text{ min} = \text{incubation time}$$

Standard curve

p-nitrophenol: Different concentrations (0-400 µmol) of p-nitrophenol (pNP) (Sigma Aldrich, UK) was prepared in 50 mM acetate buffer (pH 5.4 and 4.8) and 50 mM Tris buffer (pH 8.5). Basically, 50 µL/well of p-nitrophenol was loaded into the 96 well plate followed by 50 µL of distilled water, 50 µL of respective buffer and 6.25 µL of 1N disodium carbonate (Na₂CO₃). After resuspension the plate was left for 10 min at room temperature for the ionisation of p-nitrophenol to form the yellow colour, the absorbance was measured at 405 nm using

microplate reader (Tecan, Infinite M200PRO). The calibration curve with absorbance at 405 nm versus p-nitrophenol concentration was constructed. A slightly different protocol for preparation of the p-nitrophenol standard for esterase enzyme, which involved absorbance measurement without adding 1N sodium carbonate ($R^2 = 0.9999$).

Azure B: Different concentration (0 - 12 μmol) of azure B (Sigma Aldrich, UK) were prepared in 0.05 M Tris buffer pH 8.0. The absorbance of azure B was measured at 595 nm using the microplate reader (Tecan, Infinite M200PRO). The calibration curve with absorbance at 595 nm versus azure B concentration was constructed ($R^2 = 0.9991$).

4.2.5 Measurement of sporulation

Spores from fungal colonies were harvested after 12 days incubation according to Parra et al. (2004). Spores were harvested twice by pouring 10 mL of 0.1% sterile Tween-80/water solution and scraping the culture surface gently using a surface sterilised stainless-steel rod. The spore solution was then transferred into a 50 mL tube and centrifuged at 3000 rpm for 5 min. The supernatant was removed and a known volume of 0.1% Tween-80/water was added and mixed. The number of spores were counted using a haemocytometer. The total number of spores were divided by the colony area and expressed as spores/cm².

4.2.6 Preparation of ethyl acetate extract of *Streptomyces* AS1 supernatant

As described in Section 3.2.13.

4.2.7 Inoculation of *P. verrucosum* on YES agar

Water was adjusted with glycerol to the target a_w (0.99 and 0.95) levels and used to prepare YES agar. Sterile YES agar (approx. 52°C) was mixed with different concentration of AS1 ethyl acetate extract (final conc. 0, 0.15 and 1.25 $\mu\text{g/mL}$) in sterile 50 mL tubes and poured into small Petri plates ($\varnothing$ 53 mm). These plates were divided into 2 groups: (1) for growth rate and (2) for enzyme, protein assay and OTA quantification. After the agar was cooled, 100 μL of *P. verrucosum* spore suspension (10^7 spores/mL) was spread plated onto the agar surface and incubated at 25°C for seven days. Periodically, initially (0 h) and after 24 h, 72 h, 120 h and 168 h, five agar plugs were collected for OTA analysis and the remaining YES agar used for enzyme analyses and protein quantification. Meanwhile, in a separated plate 5 μL of spore suspension (10^8 spores/mL) was applied to the centre of YES agar plates and incubated at 25°C for seven days for growth measurement. The diameter of the colony was measured every day

in two perpendicular directions to each other. The radial growth rate was obtained from linear regression slope of the growth curve (colony radius vs incubation days). All treatments consisted of at least three replicates.

4.2.8 Ochratoxin A analysis

As described in Section 3.2.16a

4.2.9 Measurement of protein concentration

The concentration of protein was measured using the Bradford reagent (Bio Rad Laboratories, Inc., UK) which consists of Coomassie Brilliant Blue G-250 dye, methanol and phosphoric acid. Coomassie Brilliant Blue G-250 in Bradford reagent is in a cationic form (red), primarily double protonated. When it binds to protein it changed into a stable unprotonated form (blue) which was detected at 595 nm using a spectrophotometer or microplate reader. The protein concentration was determined by mixing 75 μ L of Bradford reagent and 75 μ L of sample or standard in a 96 well plate. This was left for 10 min at room temperature and then the absorbance measured at 595 nm using a microplate reader. Different concentrations of a standard (0-10 μ g/mL) was prepared using bovine serum albumin (BSA) in distilled water.

4.2.10 Statistical Analysis

Normal distribution of data was checked by Shapiro-Wilk W Test. For data with a normal distribution, the general influence of *L. plantarum* VS1 supernatant concentrations and incubation time on enzyme activities, *T. rubrum* growth and sporulation and AS1 ethyl acetate extract concentration, a_w and incubation time on enzyme activities, *P. verrucosum* growth and OTA production was checked using a one way analysis of variance (ANOVA) and Kruskal Wallis Tests (Rank Sums) for non-normally distributed data. Student's t test was further applied to compared mean of each treatment for normally distributed data and Wilcoxon method (non-parametric comparison) for non-normal distribution dataset. $p < 0.05$ was accepted as significant difference. The JMP Pro (SAS Institute Inc., Cary, North Carolina, USA) was used in the analysis.

Table 4.2 Substrate, buffer and pH for enzyme assay

Enzyme	Substrate	Buffer	pH	Chemical reaction/Hydrolysis
N-acetyl- β -glucosaminidase	4 mM p-nitrophenyl-N-acetyl- β D-glucosaminidin (Sigma-Aldrich)	50 mM acetate buffer	5.4	<p>p-nitrophenyl-N-acetyl-βD-glucosaminidin (colorless) $\longrightarrow$ p-nitrophenol (yellow) + N-acetyl-βD-glucosamine</p>
Acid phosphatase	8 mM p-nitrophenyl phosphate (Sigma-Aldrich)	50 mM acetate buffer	4.8	<p>p-nitrophenyl phosphate (colorless) + H₂O $\longrightarrow$ p-nitrophenol (yellow) + H₂O + PO₄³⁻</p>
Alkaline phosphatase	8 mM p-nitrophenyl phosphate (Sigma-Aldrich)	50 mM Tris buffer	8.5	<p>p-nitrophenyl phosphate (colorless) + H₂O $\longrightarrow$ p-nitrophenol (yellow) + H₂O + PO₄³⁻</p>
Keratinase	Keratin azure (8 mg/mL) (Sigma-Aldrich)	50 mM Tris buffer	8.0	Keratin azure + H ₂ O $\longrightarrow$ soluble peptide and amino acids (blue)
Esterase	8 mM p-nitrophenyl butyrate	100 mM MOPS	7.5	<p>p-nitrophenyl-butyrate (colorless) $\longrightarrow$ p-nitrophenol (yellow) + butyric acid</p>

4.3 RESULTS

4.3.1 Identification of extracellular hydrolytic enzymes production by *P. verrucosum* and *T. rubrum* at 0.99 and 0.95 a_w

Figure 4.1 shows an example of the API ZYM strips with colour changes due to enzymatic reactions. Figure 4.2 (a) and (b) compare the number and quantity of enzymes produced by *P. verrucosum* and *T. rubrum*, respectively. For *P. verrucosum* a total number of nine enzymes was produced at 0.99 a_w and seven at 0.95 a_w . Except for enzymes number 3 and 4, the colour intensity of the enzymes at 0.99 a_w were higher than at 0.95 a_w . Similar trends were found with *T. rubrum*. Ten enzymes were secreted at 0.99 a_w with higher colour intensity compared to five enzymes at 0.95 a_w .



Figure 4.1 API ZYM strips showing enzymatic activity (colour changes and intensity) for 19 different enzymes in the supernatant of *T. rubrum* grown on SBD broth at (a) 0.99 a_w and (b) 0.95 a_w for five days at 30°C.

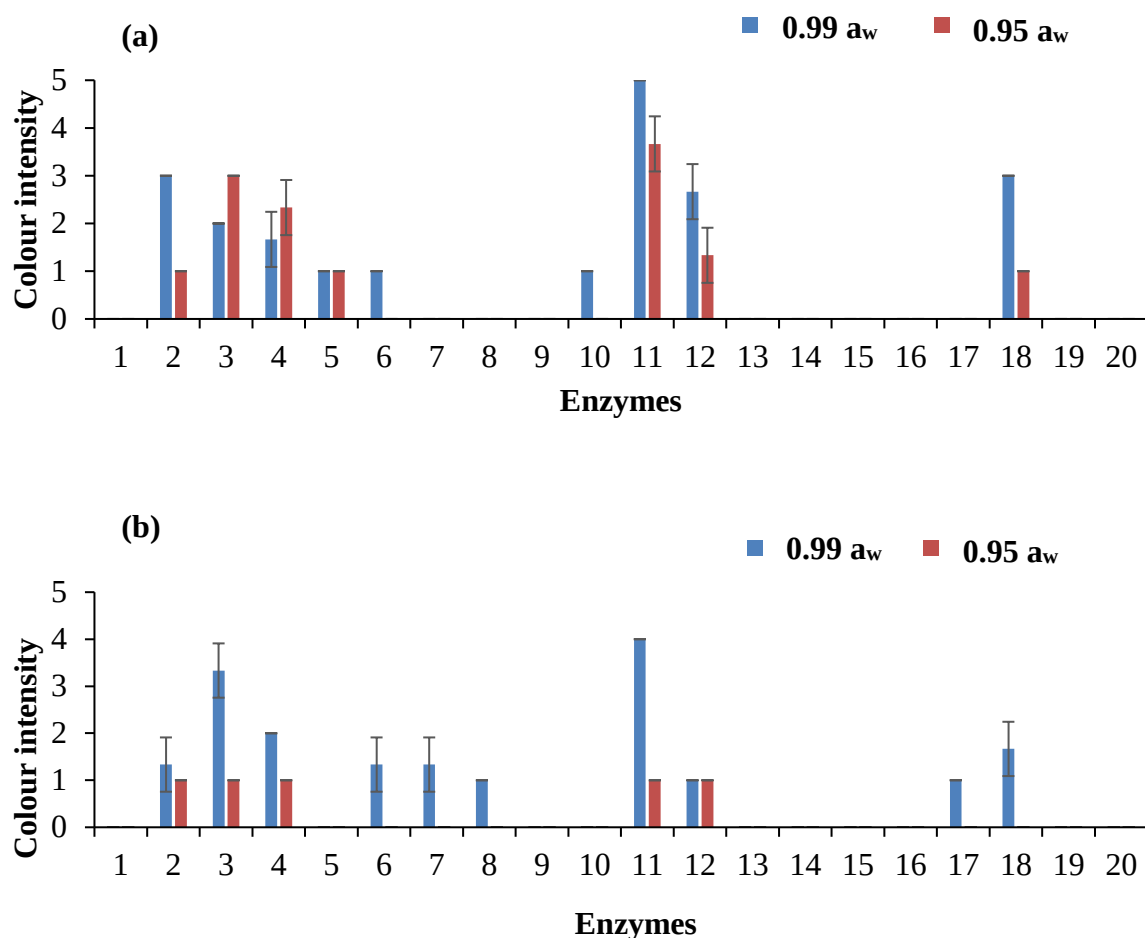


Figure 4.2 Semi-quantitative enzyme profiles of (a) *P. verrucosum* and (b) *T. rubrum* produced after 5 days incubation at 25°C and 30°C, respectively with an agitation rate of 200 rpm. Data are means of triplicates with bars indicating standard deviation. Key to enzymes – see Table 4.1. The colour intensity of 1 to 5 is equivalent to 5, 10, 20, 30 and 40 nM, respectively of the API ZYM substrate being hydrolysed.

4.3.2 Effect of *L. plantarum* VS1 supernatant concentrations and incubation time on extracellular hydrolytic enzyme activities produced by *T. rubrum* and effects on relative sporulation and growth

Figure 4.3 shows the temporal changes of total and specific activity of alkaline phosphatase, N-acetyl- β -glucosaminidase, esterase and keratinase produced by *T. rubrum* in the presence of different concentrations of the *L. plantarum* VS1 supernatant. The total activity of four enzymes were significantly increased ($p < 0.05$) over time. The highest total enzyme activities

were at day 12, except for esterase production by the control (0 µg/mL) which was on day 9. Among these enzymes, the highest total enzyme activities were alkaline phosphatase followed by N-acetyl-β-glucosaminidase, esterase and keratinase activity. These four enzyme activities were also significantly influenced ($p<0.05$) by the *L. plantarum* VS1 supernatant concentrations. The increment of the enzyme activities was delayed as the concentration of *L. plantarum* VS1 supernatant increased from 0 to 15 mg/mL. At 15 mg/mL all enzyme activities were detected after day 9. These enzymes activities were also at a lower level than the control and 4 mg/mL treatment, except for keratinase activity. This increased significantly when compared to the control and 4 mg/mL treatments at day 12. At 4 mg/mL the total enzyme activities of alkaline phosphatase, N-acetyl-β-glucosaminidase and keratinase were higher than the control throughout the study. No acid phosphatase activity was detected from *T. rubrum* at all *L. plantarum* VS1 supernatant concentrations and incubation times.

For specific enzyme activity, the changes in enzyme production mirrored those obtained for total enzyme production. The specific activity of the enzymes assayed was significantly influenced by the incubation time and *L. plantarum* VS1 supernatant concentration used. At 15 mg/mL concentration, the specific activities were suppressed, except for keratinase activity, which was highest after day 9. Table 4.3 (a) and (b) shows the statistical analyses of the effect of the VS1 supernatant concentration and incubation time on total and specific enzyme activity. Overall, the incubation time and *L. plantarum* VS1 supernatant concentration significantly influenced ($p<0.05$) the total and specific activity of the predominant four enzymes tested.

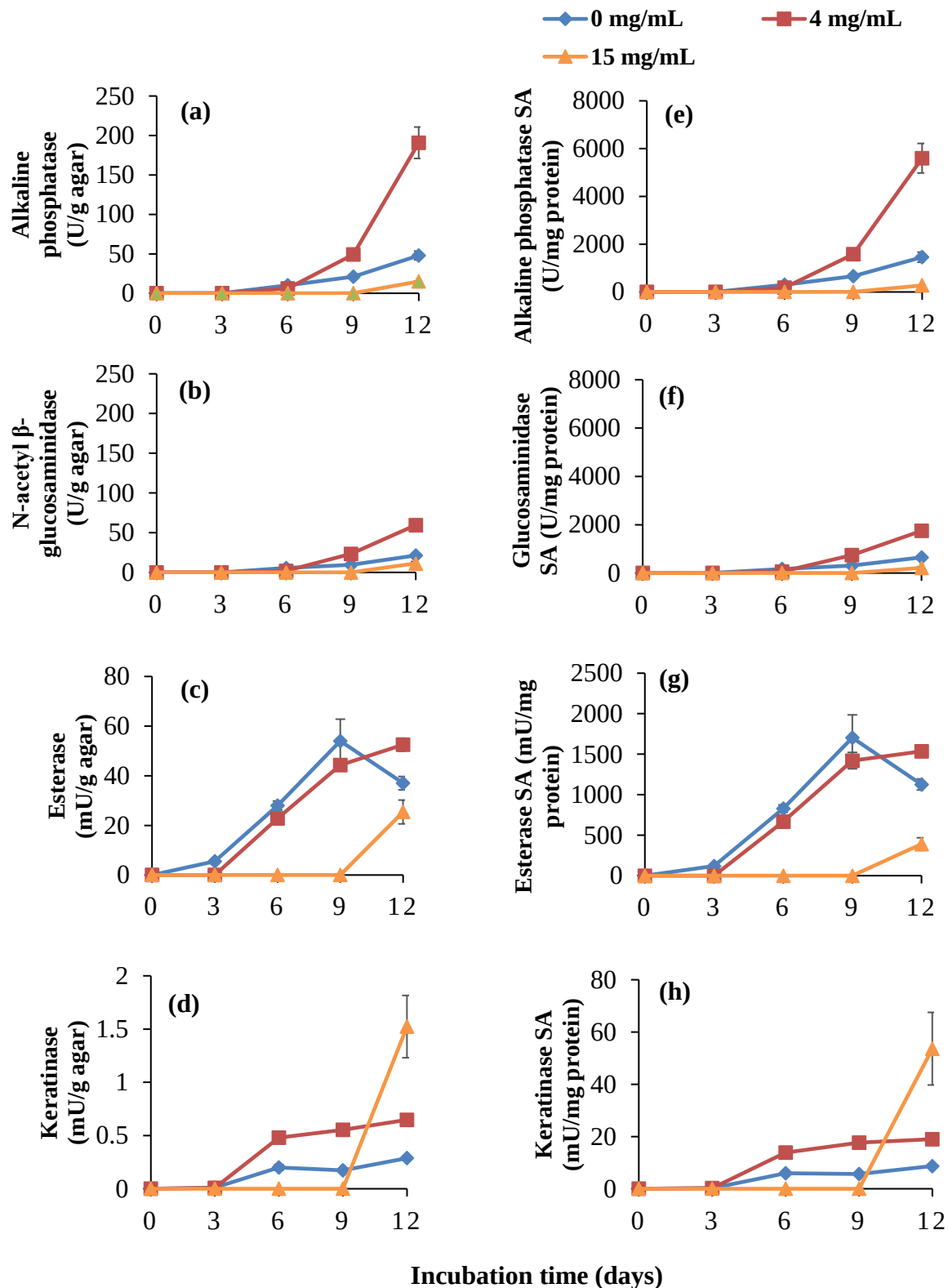


Figure 4.3 Changes in total (a, b, c, d) and specific (e, f, g, h) enzyme activities produced by *T. rubrum* treated with different concentration of *L. plantarum* VS1 supernatant over a period of 12 days. Data are means of triplicate with bars indicating standard error (SE). SA: specific activity

Table 4.3 Statistical analysis (Kruskal-Wallis Tests, Rank Sums for (a) total activity and (b) specific activity of alkaline phosphatase N-acetyl- β -glucosaminidase, esterase and keratinase as a function of incubation time and *L. plantarum* VS1 supernatant concentration.

(a)

Factor	Alkaline phosphatase			N-acetyl- β -glucosaminidase		Esterase		Keratinase	
	DF	X ²	p	X ²	P	X ²	P	X ²	p
Incubation time	4	40.3992	<0.0001*	33.6999	<0.0001*	29.5722	<0.0001*	30.4015	<0.0001*
VS1 concentration	2	10.2003	0.0061*	12.5725	0.0019*	14.9645	0.0006*	7.7429	0.0208*

(b)

Factor	Alkaline phosphatase			N-acetyl- β -glucosaminidase		Esterase		Keratinase	
	DF	X ²	p	X ²	P	X ²	P	X ²	p
Incubation time	4	39.3424	<0.0001*	32.4325	<0.0001*	27.7355	<0.0001*	29.8422	<0.0001*
VS1 concentration	2	11.0148	0.0041*	13.7266	0.0010*	17.1454	0.0002*	7.7429	0.0208*

* Indicate significant different at p<0.05

4.3.3 Effect of *L. plantarum* VS1 supernatant concentrations on *T. rubrum* growth and sporulation

Figure 4.4 shows the effect of the *L. plantarum* VS1 supernatant concentration on the growth rate of *T. rubrum* on keratin-based agar media. The growth rate of *T. rubrum* varied after treatment with different concentrations of the *L. plantarum* VS1 supernatant. Low concentrations of the supernatant significantly ($p<0.05$) increased growth, while higher concentrations inhibited growth significantly ($p<0.05$) when compared to the untreated control.

The effect of the *L. plantarum* VS1 supernatant on sporulation was also examined (Figure 4.5). A low concentration of the supernatant (4 mg/mL) significantly ($p<0.05$) increased the sporulation of *T. rubrum*. In contrast no significant ($p=0.05$) difference was observed at 15 mg/mL VS1 when compared to the control although the sporulation was generally higher.

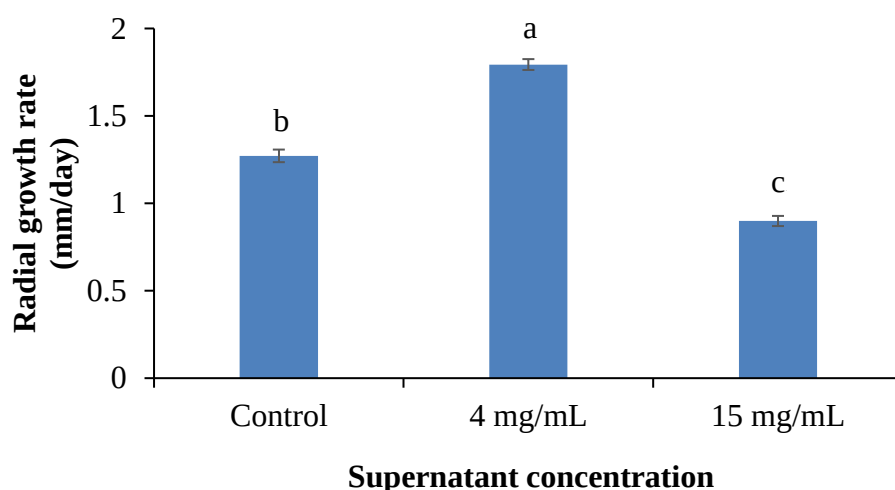


Figure 4.4 Effect of different concentrations of *L. plantarum* VS1 supernatant on growth of *T. rubrum* on keratin-based media. Data are mean of triplicate with bars indicating standard deviation. Different letter indicates significant differences at $p<0.05$ using the Student's t test.

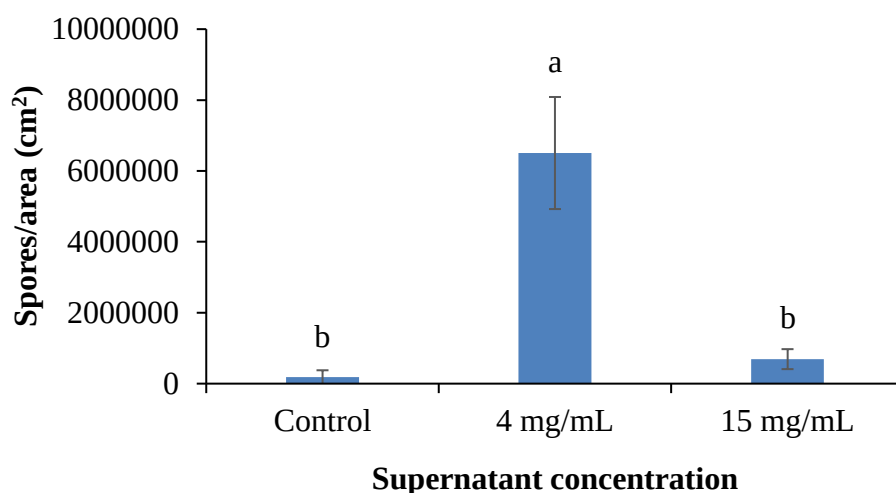


Figure 4.5 Effect of different concentration of *L. plantarum* VS1 supernatant on the sporulation of *T. rubrum* after 12 days incubation. Data are means of triplicates with bars indicating standard deviation. Different letters indicate significant differences at $p < 0.05$ using the Student's *t* test.

4.3.4 Effect of *Streptomyces* AS1 ethyl acetate extract, water activity (a_w) and incubation time on the production of extracellular hydrolytic enzymes, *P. verrucosum* growth and ochratoxin (OTA) production

Figure 4.6 shows the effect of AS1 ethyl acetate extract concentration, incubation time, and a_w on the total activity N-acetyl- β -glucosaminidase, acid phosphatase, alkaline phosphatase and esterase produced by *P. verrucosum*. Generally, the total activity of these enzymes was increased as the incubation time increased up to a certain duration. The highest total enzymes production at 0.99 a_w and 0.95 a_w was N-acetyl- β -glucosaminidase followed by acid phosphatase, alkaline phosphatase and esterase. No esterase activity was detected with 1.25 $\mu\text{g/mL}$ AS1 extract when applied at both water activities. For the other enzymes, the reduction was more pronounced for N-acetyl- β -glucosaminidase and acid phosphatase which were $>80\%$ at 0.99 a_w and $>30\%$ at 0.95 a_w . At 0.15 $\mu\text{g/mL}$ AS1 extract, the total enzyme activities of N-acetyl- β -glucosaminidase, and esterase at 0.99 a_w and acid phosphatase at 0.95 a_w were increased and higher than the control. The statistical analysis is shown in Table 4.4(a). Overall, the incubation time significantly influenced ($p < 0.05$) the total activity of these four enzymes tested. The effect of AS1 concentration was significant ($p < 0.05$) on acid phosphatase and

esterase activity. However, the total activity of these four enzymes were not significantly affected by a_w .

Figure 4.7 compares the specific activity of these four enzymes. The specific activity was increased as the incubation time increased until a specific time and then decreased. At 0.99 a_w the specific activity of all enzymes when compared to control was decreased when AS1 concentration was increased to 1.25 $\mu\text{g/mL}$. The specific activity of esterase, acid phosphatase, N-acetyl- β -glucosaminidase, and alkaline phosphatase was reduced by 100%, 84%, 76% and 54%, respectively. At 0.15 $\mu\text{g/mL}$ AS1, there were 33% and 26% reduction of alkaline and acid phosphatase specific activity, respectively. However, an increase of esterase was observed when compared to the control.

At 0.95 a_w there was 100% inhibition of esterase and 14% of alkaline phosphatase when *P. verrucosum* was treated with 1.25 $\mu\text{g/mL}$ AS1. In contrast, the specific activity of N-acetyl- β -glucosaminidase and acid phosphatase was higher than the control. At 0.15 $\mu\text{g/mL}$ AS1 there was a reduction on esterase and alkaline phosphatase specific activities by 98% and 15%, respectively and an increment in acid phosphatase when compared to the control. Table 4.4(b) shows the statistical analysis on the effect of incubation time, AS1 concentrations and a_w on the specific activity of enzymes. Overall, the incubation time significantly influenced ($p < 0.05$) the activity of all the enzymes and AS1 concentration significantly affected ($p < 0.05$) esterase activity. No significant differences were found between a_w levels examined.

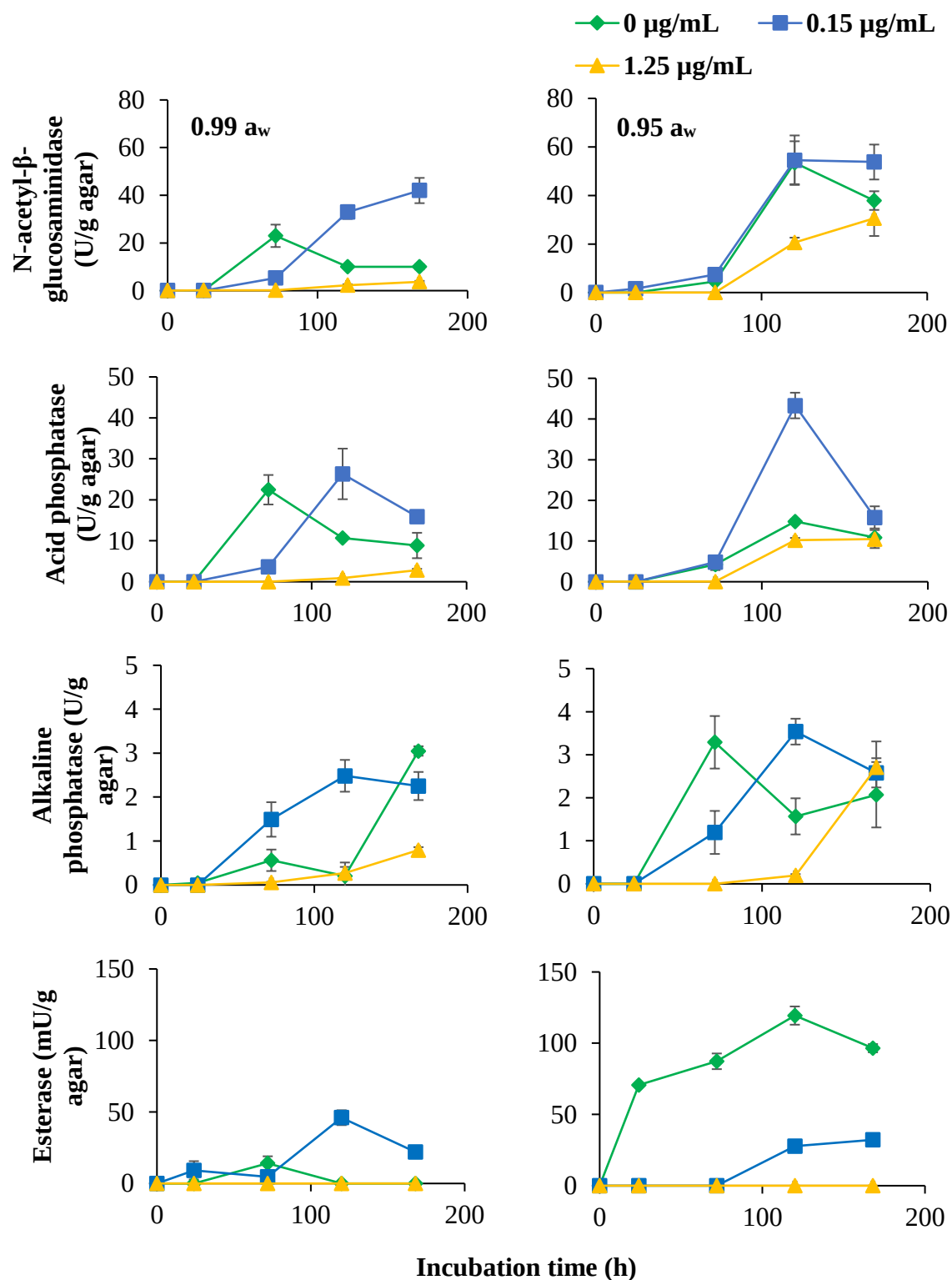


Figure 4.6 Effect of different concentrations of the ethyl acetate extract (0-1.25 µg/mL) of the *Streptomyces* AS1 on the temporal total production of four hydrolytic enzymes by *P. verrucosum* at 0.99 a_w and 0.95 a_w. Data are mean of triplicate with bars indicating standard error.

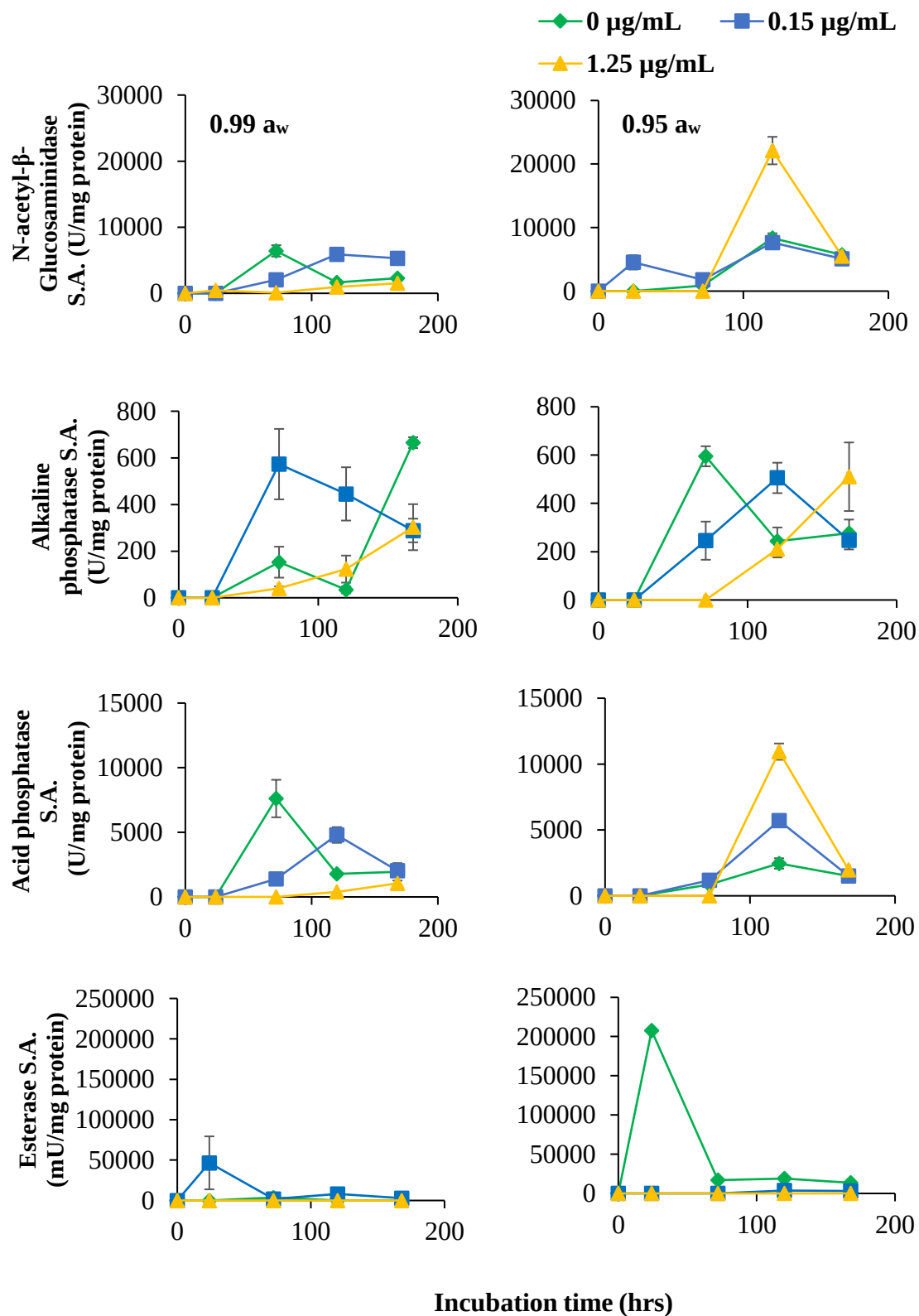


Figure 4.7 Effect of the ethyl acetate extracts (0-1.25 µg/mL) of the *Streptomyces* AS1 on the temporal specific activity of N-acetyl-β-glucosaminidase, acid phosphatase, alkaline phosphatase and esterase by *P. verrucosum* at 0.99 and 0.95 a_w. Data are means of triplicates with bars indicating standard error. S.A.: specific activity

Table 4.4 Statistical analysis (Kruskal-Wallis Tests, Rank Sums) for (a) total activity and (b) specific activity of N-acetyl- β -glucosaminidase, acid phosphatase, alkaline phosphatase and esterase as a function of incubation time, *Streptomyces* AS1 concentration and a_w .

(a)

Factor	N-acetyl β -glucosaminidase			Acid phosphatase		Alkaline phosphatase		Esterase	
	DF	X ²	p	X ²	P	X ²	p	X ²	p
Incubation time	4	64.9120	<0.0001*	59.7867	<0.0001*	61.8584	<0.0001*	14.4808	0.0059*
AS1 concentration	2	5.1274	0.0770	6.8833	0.0320*	4.7191	0.0945	22.0361	<0.0001*
a_w	1	0.7571	0.3842	0.7652	0.3817	0.0543	0.8158	0.1153	2.4796

(b)

Factor	N-acetyl β -glucosaminidase			Acid phosphatase		Alkaline phosphatase		Esterase	
	DF	X ²	p	X ²	P	X ²	p	X ²	p
Incubation time	4	58.0448	<0.0001*	59.2056	<0.0001*	59.1529	<0.0001*	12.1397	0.0163*
AS1 concentration	2	2.9928	0.2239	3.2133	0.2006	2.5580	0.2783	22.0448	<0.0001*
a_w	1	0.8547	0.3552	0.7024	0.4020	0.0380	0.8454	1.5027	0.2202

* Indicate significant difference ($p < 0.05$), DF: Degrees of freedom

4.3.5 Effect of *Streptomyces* AS1 ethyl acetate extract and water activity on growth of *P. verrucosum* and ochratoxin A (OTA) production

The effect of AS1 concentrations and a_w on the radial growth rate of *P. verrucosum* is shown in Figure 4.8. Overall, both the AS1 concentration and a_w had significant impacts on the growth rate. There was a significant reduction ($p<0.05$) of growth as the concentration of AS1 was increased irrespective of a_w . When comparisons were made between the a_w levels, a significant difference ($p<0.05$) was found between the control (0 $\mu\text{g/mL}$) and the 0.15 $\mu\text{g/mL}$ AS1 ethyl acetate extract (Table 4.5)

Figure 4.9 shows the effect of AS1 concentrations and a_w on the OTA production. Incubation time significantly affected the production of OTA. OTA was detected initially after 72 h and production was increased up to the end of the experiment at 168 h. At 0.99 a_w , the production of OTA was stimulated after 72 h at 0.15 $\mu\text{g/mL}$ AS1 ethyl acetate extract. However, OTA production was suppressed as the AS1 concentration was increased to 1.25 $\mu\text{g/mL}$. At 0.95 a_w the OTA production was continuously reduced as the AS1 concentration was increased from 0.15 $\mu\text{g/mL}$ to 1.25 $\mu\text{g/mL}$. Table 4.6 shows the statistical analysis on the effect of AS1 concentration, incubation time and a_w on OTA production. Overall, the incubation time significantly increased ($p<0.05$) the OTA production. There was a significant difference ($p<0.05$) between AS1 concentration at 0.15 $\mu\text{g/mL}$ and 1.25 $\mu\text{g/mL}$. However, no significant differences were found between the a_w levels examined.

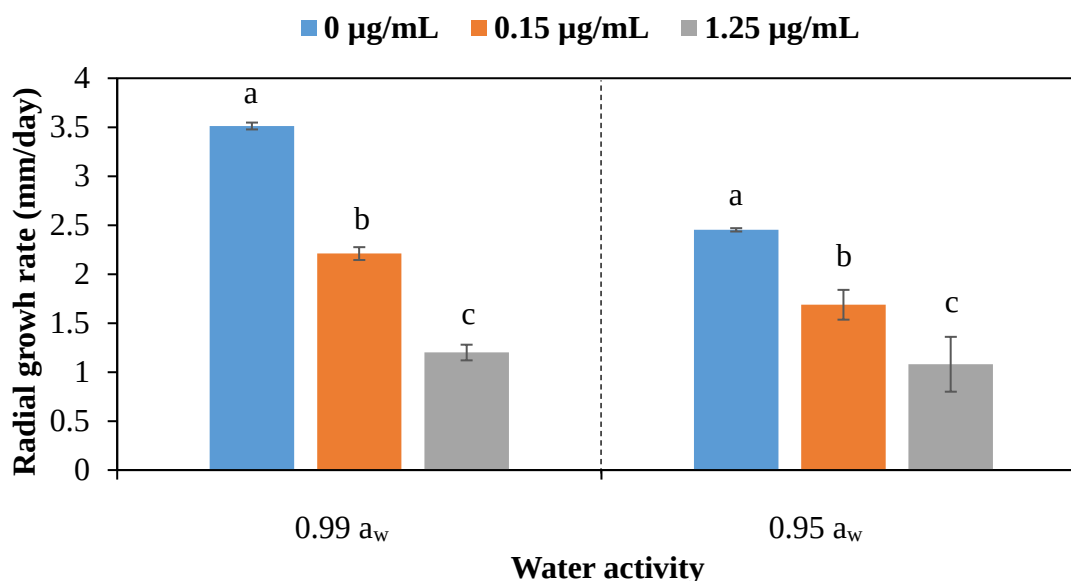


Figure 4.8 Effect of *Streptomyces* AS1 ethyl acetate extract concentrations on the growth rate of *P. verrucosum* grown on YES agar at 25°C. Data are mean of triplicate with bars indicating standard deviation. Different letter indicates significant different ($p < 0.05$) within treatment using Wilcoxon method (non parametric)

Table 4.5 Statistical analysis on the effect of *Streptomyces* AS1 ethyl acetate extract concentrations x a_w on the growth rate of *P. verrucosum* using the Dunn method for joint ranking (nonparametric comparison)

Factor	0 µg/mL			0.15 µg/mL		1.25 µg/mL	
	DF	SMD	p-value	SMD	p-value	SMD	p-value
a _w	1	3.7500	0.0304*	2.7500	0.0304*	1.7500	0.3094

SMD: Score mean difference, DF: Degree of freedom

*Significant different at $p < 0.05$

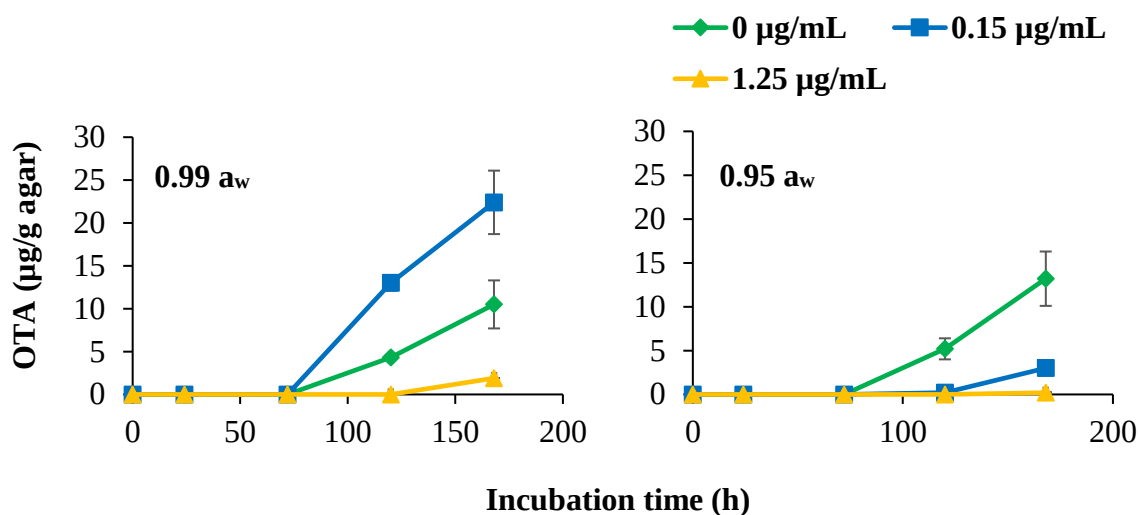


Figure 4.9 Effect of the ethyl acetate *Streptomyces* AS1 natural mixture of compounds on the temporal ochratoxin A production by *P. verrucosum* over a period of 168 h at 0.99 a_w and 0.95 a_w . Data are means of triplicates with bars indicating standard deviation.

Table 4.6 Statistical analysis of the effect of incubation time, *Streptomyces* AS1 ethyl acetate extract concentration and a_w on the ochratoxin A production using the Wilcoxon Method (non-parametric comparison).

Factors								
Incubation time (h)			AS1 concentration ($\mu\text{g/mL}$)			a_w		
Level	Level	p-value	Level	Level	p-value	Level	Level	p-value
168	0	<0.0001*	0.15	0	0.9268	0.95	0.99	0.5197
168	24	<0.0001*	1.25	0.15	0.0588			
168	120	<0.0001*	1.25	0.15	0.0313*			
120	0	<0.0002*						
120	24	<0.0002*						
120	72	<0.0001*						
168	120	0.0700						
24	0	1.000						
72	0	1.000						
72	24	1.000						

* Indicate significant difference ($p < 0.05$)

4.4 DISCUSSION

4.4.1 Effect of *L. plantarum* VS1 supernatant concentration and incubation time on the *T. rubrum* growth rate, sporulation and hydrolytic enzyme production

The effect of sub-inhibitory concentrations of *L. plantarum* VS1 supernatant on *T. rubrum* growth and enzyme production was studied on a keratin-based medium to simulate conditions relevant to where this dermatophyte pathogen. The *L. plantarum* VS1 concentration (15 mg/mL) used was slightly higher than IC₅₀ values for inhibiting growth of *T. rubrum* on keratin-based medium which was approx. 14 mg/mL. While the initial concentration of *L. plantarum* VS1 to inhibit *T. rubrum* growth on keratin media was >10 mg/mL (see Figure 3.10), the effect of *L. plantarum* VS1 concentrations at <10 mg/mL on *T. rubrum* growth was not previously known. The application of *L. plantarum* VS1 supernatant at concentration <10 mg/mL (in this case 4 mg/mL) appeared to significantly stimulate the growth of *T. rubrum*. This stimulation might be because of the presence of *L. plantarum* VS1 supernatant may response to a stress factor to try and survive. This stress also perhaps initiated a reprogramming of genomic expression which may have led to up-regulation of certain genes associated with cell wall synthesis (Parveen et al., 2004) and thus increasing fungal growth. Another proposed explanation was because of the *L. plantarum* VS1 supernatant concentration was relatively ineffective to control growth, allowing this dermatophyte pathogen to utilize the nutrients available in the supernatant as an energy source to support growth (Parra et al., 2004). The growth enhancement by antifungal compounds has also been reported previously by Mills et al. (2004). They found that the use of antifungal compounds (0.002 M, 0.02 M and 0.2 M) such as aluminium chloride amended media stimulated the mycelial growth of *Phytophthora infestans* and sodium bicarbonate enhanced mycelial growth of *Verticillium albo-atrum*. However, at high *L. plantarum* VS1 concentration (15 mg/mL) it becomes toxic to the *T. rubrum* and significantly inhibited ($p < 0.05$) the *T. rubrum* growth.

In addition to this, VS1 supernatant enhanced sporulation of *T. rubrum* regardless of whether growth was stimulated or inhibited. It was speculated that stress conditions such as the use of sub-inhibitory concentrations of antifungal agents may stimulate sporulation as a physiological mechanism for survival by producing a large number of propagules (spores). Previously, application of sub-inhibitory concentrations of amphotericin B, griseofulvin and clotrimazole enhanced the level of arthro sporulation of *T. rubrum* (Yazdanparast and Barton, 2006). Mills et al. (2004) found that although the growth of *Phytophthora erythroseptica* on phosphonic

acid amended media (0.002 M, 0.02 M and 0.2 M) was significantly reduced, the sporulation was increased when compared to the control. Other than stress conditions, nutrient composition in growth media may also contribute to the high sporulation level. For example, *Colletotrichum gloeosporioides* sporulated significantly in media containing maltose and ammonium nitrate as the main carbon and nitrogen sources (Sangeetha and Rawal, 2008). In this study the high spore production by *T. rubrum* may also be due to the nutritional or other compounds present in the *L. plantarum* VS1 supernatant which stimulated the sporulation.

The key hydrolytic enzyme produced by *T. rubrum* in Sabouraud broth was found to be acid and alkaline phosphatase, N-acetyl- β -glucosaminidase and esterase. However, subsequent studies on the keratin agar as a sole carbon and nitrogen source found no acid phosphatase activity. It was reported that the production of enzymes is dependent on the nutrient supplied in the growth medium. Brash et al. (1991) found that the number of enzymes produced by *T. rubrum* was not the same when the growth medium was supplied with different kinds of nutrient such as keratin, lipid and peptone. In this discussion, the focus is on the four main enzymes; alkaline phosphatase, N-acetyl- β -glucosaminidase, esterase and keratinase. The total enzyme activity of untreated *T. rubrum* was detected after day three and increased over time. However, after 15 mg/mL of *L. plantarum* VS1 supernatant was applied, the total and specific activity of these enzymes were detected after day 9 and was lower than the control. The highest reduction for both total and specific enzyme activities over the control was alkaline phosphatase (69% and 81%, respectively) followed by esterase (53% and 77%) and N-acetyl- β -glucosaminidase (47% and 67%). However, there was a significant increase in keratinase activity once it was produced by *T. rubrum* after day 9. Keratinase is one of the main enzymes produced by dermatophyte fungi known to grow on keratin rich matrices and utilized these as the only carbon and nitrogen sources for energy (Sharma et al., 2017). It is speculated that the keratinase activity detected after day 9 is not only used to support the growth (Malviya et al., 1992) of *T. rubrum* but also to aid the sporulation when the environment did not favour growth. Earlier results of this study showed that 15 mg/mL *L. plantarum* VS1 significantly inhibited the growth of *T. rubrum* but in the meantime increased the sporulation. It was reported that the hydrolytic enzymes normally increased significantly during sporulation. For example, the α -mannosidase, protease C and B activity was recorded increased approximately 9-, 2.5- and 9-fold, respectively during sporulation of *Saccharomyces cerevisiae* strains SK-1 (Opheim, 1979). These enzymes may active in this period to provide more precursors needed for new

synthesis. The present study is in agreement with Khan and Ahmad (2011) who showed an inhibition in keratinase activity of *T. rubrum* by essential oils.

4.4.2 Effect of *Streptomyces* AS1 ethyl acetate extract, water activity and incubation time on *P. verrucosum* growth, OTA production and extracellular hydrolytic enzymes activities

The application of AS1 ethyl acetate extract at 0.15 µg/mL and 1.25 µg/mL was able to control growth of *P. verrucosum* significantly at 0.99 a_w and 0.95 a_w levels. In the meantime, the production of OTA was successfully suppressed at both a_w levels when the AS1 concentration was increased to 1.25 µg/mL. However, at low AS1 concentration (0.15 µg/mL) the inhibition was more pronounced in increased water stress conditions (0.95 a_w). The inhibition of fungal growth does not always lead to toxin reduction. The presence of 0.15 µg/mL of AS1 extract at 0.99 a_w may have induced some chemical stress to *P. verrucosum* and triggered the synthesis of OTA as a defence reaction. Previously, Magan et al. (2002) demonstrated that the application of sub-inhibitory concentrations of propiconazole stimulated the production of deoxynivalenol although it was able to partially inhibit growth of *Fusarium culmorum*.

Initial screening to identify the key hydrolytic enzymes produced by *P. verrucosum* demonstrated that an elevated production of enzyme activities was obtained under freely available water condition (0.99 a_w). Similar results were reported by Magan and Aldred (2007b) who showed that the number, and activities of hydrolytic enzymes produced by *A. flavus* were higher at 0.96 a_w than 0.90 a_w levels. Four key hydrolytic enzymes produced by *P. verrucosum* which may play a crucial role in colonisation were identified as acid and alkaline phosphatase, N-acetyl-β-glucosaminidase and esterase and thus the effect of the AS1 extract on these enzyme activities were studied in more detail. The temporal study of the key hydrolytic enzymes showed that there was an increase in the production over time. The application of 1.25 µg/mL AS1 extract at both a_w levels resulted in a reduction of total activities of all four of these hydrolytic enzymes. The most significant reduction was esterase, where no activity was detected at both a_w levels. The other two enzymes that were inhibited significantly when compared to control, were N-acetyl-β-glucosaminidase and acid phosphatase. The specific activity of these four enzymes were also reduced when the concentration of AS1 was increased to 1.25 µg/mL although acid phosphatase and N-acetyl-β-glucosaminidase at 0.95 a_w showed an increase which was higher than the control.

The changes in production of key hydrolytic enzyme activities during fungal growth was reported previously. N-acetyl- β -D-glucosaminidase, α -D-galactosidase and β -D-glucosidase activities were significantly increased during the growth of *Penicillium chrysogenum* on bread (Keshri et al., 2002). This was later supported by Marin et al. (2003), who studied *Penicillium corylophilum*. Dorn and Rivera (1966) reported the increase in *Aspergillus nidulans* growth was paralleled with acid and alkaline phosphatase activity. Other than growth, hydrolytic enzymes may also be involved in OTA synthesis as proposed by Huff and Hamilton (1979). The author proposed the role of esterase was to de-esterify the ochratoxin C (an ochratoxin A ethylester) to ochratoxin A in the final step in the OTA biosynthesis pathway. Therefore, the reduction of these enzyme activities in the present study could be relate to the efficacy of AS1 ethyl acetate extract at 1.25 μ g/mL to inhibit growth of *P. verrucosum* and OTA production.

4.5 CONCLUSIONS

Overall, this study demonstrated that the application of 15 mg/mL *L. plantarum* VS1 supernatants significantly inhibited growth of the dermatophyte, *T. rubrum*. This inhibition resulted in the reduction in the production of keratinase, alkaline phosphatase, N-acetyl- β -glucosaminidase and esterase which are key hydrolytic enzymes for infection produced by *T. rubrum*. However, the *L. plantarum* VS1 supernatant did not affect sporulation.

The presence of *Streptomyces* AS1 ethyl acetate extract at 1.25 μ g/mL significantly inhibited the growth of *P. verrucosum* and OTA production at both a_w levels examined. In addition, the total activity of N-acetyl- β -glucosaminidase, acid phosphatase and alkaline phosphatase were reduced, and esterase were totally inhibited at both a_w levels. However, the specific activity of N-acetyl- β -glucosaminidase and acid phosphatase were higher than the control at 0.95 a_w .

CHAPTER 5

***In-situ* studies of the effects of bacterial metabolites in controlling (a) the growth of *T. rubrum* on nail matrices (from LAB) and (b) *P. verrucosum* and ochratoxin A production in stored wheat (from *Streptomyces* AS1)**

5.1 INTRODUCTION

Onychomycosis or tinea unguium is a fungal infection on the nail plate and/or the nail bed that causes destruction to the nail plate. This is not a life-threatening disease, but the prevalence was reported as high as 51% in Ethiopia (Teklebirhan and Bitew, 2015), 39% in Italy (Vena et al., 2012) and 14% in Sweden (Drakensjö and Chryssanthou, 2011). The species that is most frequently isolated from the patients is *T. rubrum*, the main causative agent of onychomycosis (Vena et al., 2012; Drakensjö and Chryssanthou, 2011; Havlickova et al., 2008). There are several treatments of onychomycosis which include oral or topical drug therapy, removal of the infected nail, pulse and laser therapy or a combination of these (Ghannoum and Isham, 2014; Gupta et al., 2013). The drugs normally used for topical therapy are amorolfine and ciclopirox and for oral therapy are terbinafine, itraconazole and fluconazole (Gupta et al., 2013). Topical therapy is limited to the efficacy of the drugs to penetrate through the nail plate and reach the infected nail bed area with the concentration above the minimum inhibitory concentration (Ghannoum and Isham, 2014; Ghannoum et al., 2008). Oral drug therapy is generally more effective than topical therapy. The patients with drug therapy are, however, prone to the risk of drug-drug interactions. This is more severe particularly to immunocompromised patients including those with diabetes, liver disease, renal dysfunctions and the elderly (Gupta et al., 2013). Therefore, the application of natural antifungal compounds which are generally less toxic or non-toxic to patients, both normal and immunocompromised would be very beneficial.

Penicillium verrucosum is one of the main contaminants of wheat in cool damp regions of Northern Europe and also in Canada. Improper harvesting, a delay in drying after harvesting, and poor storage condition increases the risk of post-harvest colonisation of stored cereals by *P. verrucosum*, and more importantly could result in ochratoxin A contamination. It was reported that stored wheat with $>0.80 a_w$ and temperatures $>20^{\circ}\text{C}$ can increase potential for *P. verrucosum* contamination and OTA production (Cairns-Fuller et al., 2005; Magan et al., 2004). Lindblat et al. (2004) suggested that the threshold limit for *P. verrucosum* populations

which would result in OTA contamination above the EU legislative limit (5 µg/kg grain) was 1000 CFUs/g of this species on a selective medium. Several strategies have been suggested to prevent *P. verrucosum* colonisation and OTA contamination including both physical and chemical approaches. Some food grade preservatives are commercially available to control the growth of fungi based on mixtures of salts of aliphatic acids including calcium propionate and potassium sorbate. However, these preservatives are fungistatic and uniform distribution is very important to significantly control the growth of such spoilage mycotoxigenic fungi (Magan and Aldred, 2007a). In addition, it has been reported that sub-inhibitory concentrations of these preservatives can stimulate OTA production (Schmidt-Hyedt et al., 2007). Research has focused on new potential antifungals with better efficacy against *P. verrucosum* and OTA production. Most of the natural antifungal compounds tested previously were plant extracts, especially essential oils and antioxidants (Hossain et al., 2014; Alexa et al., 2012; Aldred et al., 2008) with very few studies of bacteria (Sriwattanachai et al., 2018), particularly *Streptomyces* species. Earlier screening in *in-vitro* results (see Chapter 3) demonstrated that the *Streptomyces* AS1 ethyl extract was very effective in inhibiting the growth of *P. verrucosum* and OTA production on a conducive YES media.

The objectives of this study were (a) to examine the efficacy of *L. plantarum* VS1 cell free supernatant on hydrolytic enzyme activity and inhibition of *T. rubrum* growth on nail matrices (b) to study the efficacy of *Streptomyces* AS1 ethyl acetate on control of *P. verrucosum* colonisation and OTA production in stored wheat of different initial moisture contents.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of spore suspensions

Spore suspensions (10^6 spores/mL) were prepared as described in Section 2.2.2(b).

5.2.2 Preparation of *Streptomyces* AS1 ethyl acetate extract.

The extract was prepared as described in Section 3.2.13.

5.2.3 Preparation of *L. plantarum* VS1 supernatant

The modified MRS medium was prepared as described in Section 3.2.2. LAB cell suspensions were prepared as described in Section 2.2.2(a). Inoculation and cell free supernatant was prepared as described in Section 4.2.2.

5.2.4 Preparation of RPMI 1640 medium

The Roswell Park Memorial Institute medium (RPMI 1640) with glutamine, phenol red and without bicarbonate was prepared as described by the National Committee for Clinical Laboratory Standard (NCCLS, 2002). The RPMI 1640 (Difco Laboratories Ltd, UK) powder (1.04 g) was first dissolved into 80 mL of distilled water and after that 3.45 g of MOPS (3-[N-morpholino] propanesulfonic acid) was added and stirred continuously until dissolved. The pH of the medium was later adjusted to pH 4.5 with 1N HCl and the volume was brought to 100 mL with distilled water. Finally, the medium was filter sterilized through a cellulose acetate membrane filter (0.22 μ m pore size) and kept at 4 °C until used. The composition of the RPMI 1640 medium is shown in Appendix H.

5.2.5 Preparation of the nail matrices

A mixture of finger and toe nails were washed with chloroform for 1 h followed in ethanol for 30 min. After drying in a fume hood, the nails were ground using a mortar and pestle and 10 mg weighed in a 2 mL tube. The milled nails were sterilized at 121°C for 15 min before aseptically being transferred to sterile microtiter 96 well plates (approx. 10 mg/well).

5.2.6 Inoculation of *T. rubrum*

Five microliter of *T. rubrum* spore suspensions (10^5 spores/mL) was inoculated onto the surface of the ground nails and kept at 30°C for 1 h. After that 25 μ L of LAB VS1 supernatant was added followed by 470 μ L of RPMI 1640 (pH 4.5) and further incubated at 30°C for two weeks. The final concentration of VS1 supernatant was 25 mg/mL and 50 mg/mL and the final spore concentration was 10^3 spores/mL. In another treatment, the VS1 supernatant was added after the mycelia were established on the nail matrix. Briefly, after the spores were inoculated onto the surface of the ground nails and kept at 30°C for 1 h followed by addition of 470 μ L of RPMI 1640 medium and then incubated at 30°C for 6 days. After that, 25 μ L of VS1 supernatant was added and the treatments incubated again at the same temperature for two weeks. Every week the samples were withdrawn and transferred into sterile 2 mL Eppendorf tubes and centrifuged at 14,000 rpm for 5 min at 4°C to separate the supernatant with the nails and mycelium or spores. The supernatant was transferred into new 2 mL tubes and kept at -80°C until used for enzyme assays. The nails with mycelium or spores were washed with sterile RPMI 1640 pH 7.0 twice and placed on SBD agar and incubated at 30°C for 10 days to check the viability of *T. rubrum* spores/mycelia.

5.2.7 Hydrolytic enzyme assays

The assays for N-acetyl- β -glucosaminidase, alkaline phosphatase and keratinase activities were performed as described in Section 4.2.4(b)

5.2.8 Measurement of protein concentration

Protein content was measured as described in Section 4.2.9.

5.2.9 Determination of moisture adsorption curve of wheat grain to accurately modify the stored wheat grain treatments to the target a_w values

Wheat grain was gamma irradiated at 12-15 kGys (Synergy Health, Swindon, U.K.). These wheat grains were clean of any surface moulds but had retained germinative capacity. Initially, a moisture adsorption curve was made by adding different volumes of distilled water to 10 g dry wheat, mixed well, and stored at 4°C for 48 h. After 48 h the wheat was equilibrated at 25°C and the a_w measured using a water activity meter (Aqua Lab 4TE, Labcell Ltd, Washington State, U.S.A.). The wheat was then dried at 100°C overnight and left to cool down to room temperature in a desiccator. The dried wheat was weight to determine the moisture content. The moisture content was calculated as $[\text{wet wheat (g)} - \text{dried wheat (g)}] / \text{wet wheat (g)} \times 100$

5.2.10 Inoculation of wheat grain with *P. verrucosum* spore suspensions.

10 g of wheat was aseptically weighed in sterile culture vessel and 1,250 μL and 550 μL of sterile distilled water were added to modify the treatments to the target a_w values of 0.95 and 0.90, respectively. These a_w levels were chosen because it was relevant to moist grain during storage (Cairns Fuller et al., 2005). The wheat treatments and replicates were mixed thoroughly and kept at 4°C for 48 h. After this 50 μL of AS1 ethyl acetate extract was added (final concentration of 2.5, 25 and 50 $\mu\text{g/g}$ wheat grain), mixed again and kept at 4°C for 3 h. Finally, 100 μL of *P. verrucosum* spore suspension (10^6 spores/mL) was added to obtain a final concentration of 10^4 spores/g wheat, again mixed and then the treatments were stored in a storage boxes containing beakers of glycerol/water mixtures, with the same target a_w levels of the treatments (0.95, 0.90 a_w). The different a_w treatments then incubated at 15 and 25°C in constant temperature rooms for 28 days. A glycerol/water solution was replaced every week to maintain the equilibrium relative humidity (erh) the same as the treatment conditions for the stored wheat. After 28 days, 1 g of wheat was used to examine the *P. verrucosum* populations

present in each treatment and replicate. In addition, the remaining wheat was dried at 55°C for 72 h for subsequent OTA analyses. As a positive control, a mixture of the *P. verrucosum* and DMSO was used to inoculate the stored wheat treatments. The experiments were carried out with three replicates per treatment and repeated once.

5.2.11 Populations of *P. verrucosum* in stored wheat treatments

P. verrucosum populations was quantified by measuring the colony forming units (CFUs/g dried wheat grain) by serial dilution. Thus, 1 g of wheat was soaked in 9 mL sterile water + 0.1% Tween-80 in a sterile Stomacher bag for 2 h at room temperature. After 2 h, the wheat was homogenized using the Stomacher for 2 min. After this, serial dilution was performed between 10^{-1} to 10^{-4} and 100 μ L of the diluent was spread plated on ME agar using a sterile glass spreader. The ME agar plates were incubated at 25°C for 72 h before the colonies were counted.

5.2.12 Quantification of OTA in stored wheat grain treatments

The OTA in the wheat treatment were performed using the method used previously by Aldred et al. (2008) with slight modifications. Initially, a mixture of 10 mL of methanol and 4 g of dried milled wheat was shaken at 170 rpm for 12 h at 25°C in the dark. After 12 h, methanol fraction was transferred into glass vials and 10 mL of fresh methanol was added and the same procedure was repeated for another 12 h. After 24 h both methanol extracts were mixed and filtered through qualitative filter paper (QL 125, Fisher Scientific UK Ltd) containing 2-4 g of Celite 545 (Fisher Scientific UK Ltd) to purify the sample. Finally, the methanol extract was centrifuge at 2,000 rpm for 10 min at 10°C for final purification. Two millilitres of the extract were transferred into a glass vial and left to dry overnight in a fume cupboard. The dried extract was then dissolved in 1 mL of methanol and filtered into amber silanised HPLV vials. The recovery rate of OTA extracted from 5 g wheat spiked with OTA (final concentration 40 ng/g) was 100%.

5.2.13 Quantification of OTA using HPLC-FLD

Quantification of OTA concentration was done as described in Section 3.2.16(a)

5.2.14 Statistical Analysis

Normal distribution of data was checked by using the Shapiro-Wilk W Test. For data with a normal distribution, the general influence of *L. plantarum* VS1 supernatant concentrations and

incubation time on enzyme activities and *Streptomyces* AS1 ethyl acetate extract concentration, a_w and temperature on *P. verrucosum* growth and OTA production was checked using a one way analysis of variance (ANOVA) and Kruskal Wallis Tests (Rank Sums) for non-normally distributed data. Student's t test was further applied to compared mean of each treatment for normally distributed data and Wilcoxon method (non-parametric comparison) for non-normal distribution dataset. $p < 0.05$ was accepted as significant difference. The JMP Pro (SAS Institute Inc., Cary, North Carolina, USA) was used in the analysis.

5.3 RESULTS

5.3.1 Effect of *L. plantarum* VS1 supernatant concentrations and incubation time on hydrolytic enzyme activities of *T. rubrum* grown on nail matrices

Figure 5.1 shows the total and specific activities of N-acetyl- β -glucosaminidase, alkaline phosphatase and keratinase produced by *T. rubrum* growing on the nail medium with different concentrations of *L. plantarum* VS1 supernatant for 14 days. For the control, the total and specific activities of N-acetyl- β -glucosaminidase and alkaline phosphatase were significantly increased ($p < 0.05$) by day 14 when compared to day 7. No significant differences were however observed on keratinase activities. At 25 mg/mL and 50 mg/mL *L. plantarum* VS1 treatment, complete inhibition of the three enzymes occurred.

Figure 5.2 illustrates the changes in hydrolytic enzyme activities after *L. plantarum* VS1 supernatant was added to the nail medium with established mycelia of *T. rubrum* (6 days old). The total and specific activities of N-acetyl- β -glucosaminidase, alkaline phosphatase and keratinase were significantly increased ($p < 0.05$) when the incubation time was increased from 7 to 14 days. However, when 25 mg/mL and 50 mg/mL of *L. plantarum* VS1 supernatant were added the activity of these three enzymes were totally inhibited.

Figure 5.3 and 5.4 shows the viability of the spores and mycelia, respectively after treated with 25 mg/mL and 50 mg/mL of *L. plantarum* VS1 supernatant. The germination of the spores was totally inhibited by *L. plantarum* VS1 supernatant at both concentrations. The mycelial colonisation was completely inhibited with 50 mg/mL *L. plantarum* VS1 supernatant. However, in the presence of 25 mg/mL *L. plantarum* VS1 supernatant after 7 days some growth was observed on the SBD agar. However, this growth was subsequently completely inhibited after 14 days.

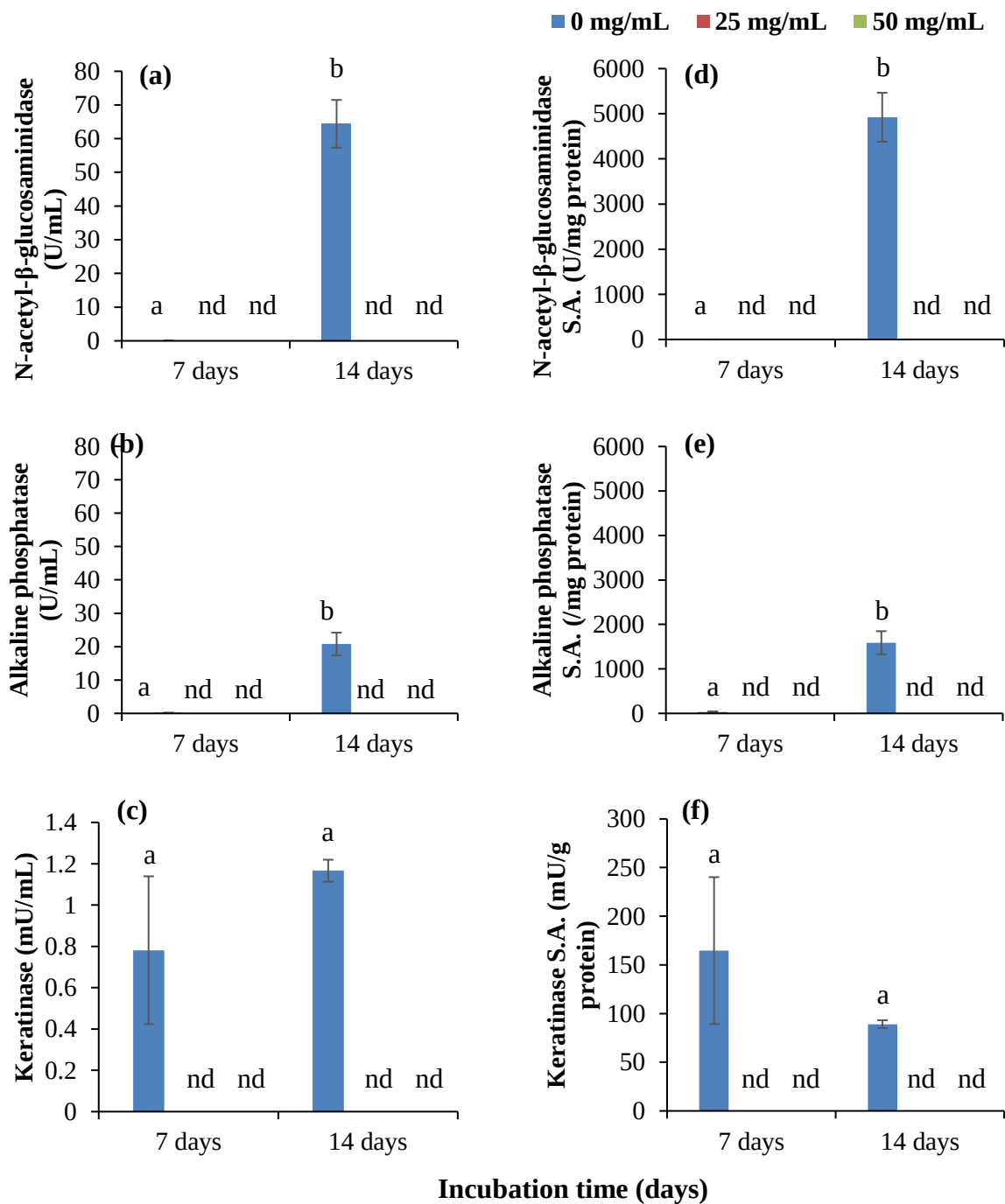


Figure 5.1 Changes in total (a, b, c) and specific (d, e, f) enzyme activities produced by *T. rubrum* treated with different concentration of *L. plantarum* VS1 supernatant over a period of 14 days. Data are means of triplicate with bars indicating standard error. Different letters indicate significant difference (p<0.05) using the T-test. SA: specific activity, nd: not detected.

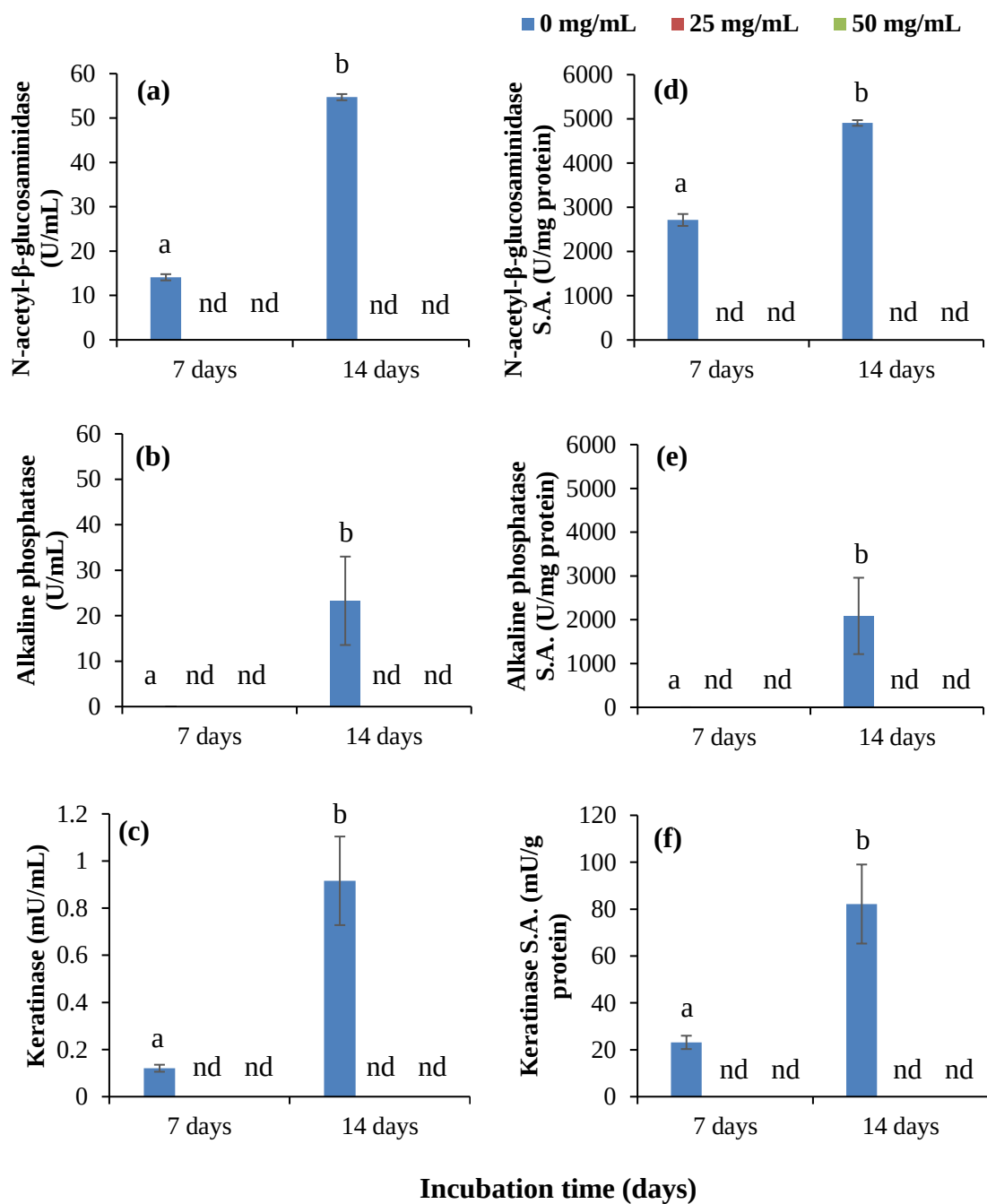


Figure 5.2 Changes in total (a, b, c) and specific (d, e, f) enzyme activities produced by *T. rubrum* mycelia treated with different concentration of *L. plantarum* VS1 supernatant over a period of 14 days. The enzyme activities presented here were after deducting the enzyme activities obtained after spores were inoculated for 6 days. Data are means of triplicates with bars indicating the standard error. Different letters indicate significant differences ($p < 0.05$) using the T-test. SA: specific activity, nd: not detected

Example of *T. rubrum* grown on nails after exposure to the VS1 supernatant for 7 days

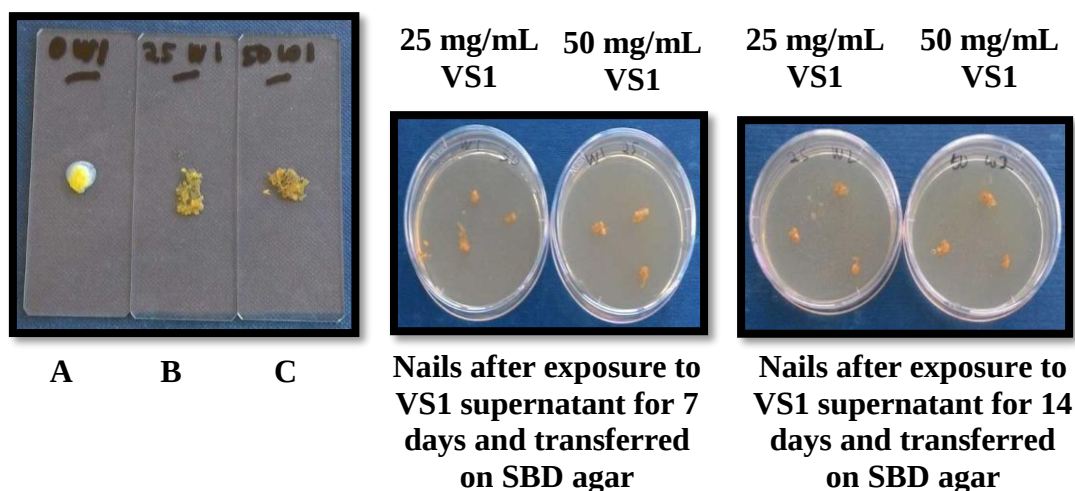


Figure 5.3 Viability of *T. rubrum* spores on nail-based medium after exposed to 25 mg/mL and 50 mg/mL *L. plantarum* VS1 supernatant for 14 days. After 7 and 14 days the nails were separated from the supernatant by centrifugation and washed twice with RPMI 1640 solution before being placed on SBD agar and incubated at 30°C for 10 days. Key: A- Control, B - 25 mg/mL VS1, C - 50 mg/mL *L. plantarum* VS1 supernatant.

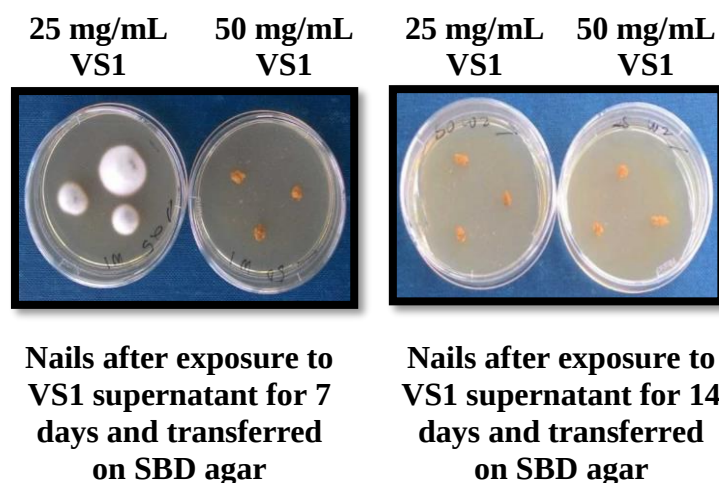


Figure 5.4 Viability of *T. rubrum* mycelia on nail-based medium after exposure to 25 mg/mL and 50 mg/mL *L. plantarum* VS1 supernatant for 14 days. After 7 and 14 days the nails were separated from the supernatant by centrifugation and washed twice with RPMI 1640 solution before being placed on SBD agar and incubated at 30°C for 10 days.

5.3.2 *In-situ* study on influence of *Streptomyces* AS1 compounds on populations of *P. verrucosum* and ochratoxin A (OTA) production in stored wheat

Table 5.1 shows the volume of water needed to adjust the water activity (a_w) level of 10 g wheat grain sub-samples to 0.95 and 0.90 a_w was 1.4 mL and 0.7 mL, respectively. Figure 5.5 shows the changes in viable populations of *P. verrucosum* on stored wheat treated with the AS1 ethyl acetate extracts at two different temperatures and a_w levels. At 25°C the populations of *P. verrucosum* treated with 2.5 µg/mL, 25 µg/mL and 50 µg/mL AS1 extract was significantly increased ($p < 0.05$) after 28 days at both a_w levels when compared to day 0. In addition, in the 28 day/0.90 a_w treatment there was a significant reduction ($p < 0.05$) in the populations of *P. verrucosum* CFUs/g wheat with the AS1 extract when compared to the control (0 µg/mL). The populations of CFUs of *P. verrucosum* also increased significantly at 15°C after 28 day storage at both a_w levels except in the 2.5 µg/mL and 0.95 a_w treatments where there was no significant difference when compared to day 0. For the 28 day/0.90 a_w treatment the populations of *P. verrucosum* isolated from the AS1 extract treatments were significantly reduced when compared to the control (0 µg/mL).

The effect of different concentrations of AS1 extract, temperature and a_w levels on OTA production by *P. verrucosum* on the stored wheat treatments are shown in Figure 5.6. Overall, there was no significant ($p > 0.05$) reduction in OTA production at all AS1 concentrations, temperatures and a_w levels tested.

Table 5.1 The volume of water required to adjust the water activity of gamma irradiated wheat to 0.90 a_w and 0.95 a_w

Gamma irradiated wheat (g)	Volume added (mL)	water a_w	Moisture content (%)
10.05 ± 0.02	0.7	0.90 ± 0.005	19.5 ± 0.07
10.04 ± 0.02	1.4	0.95 ± 0.005	24.5 ± 0.29

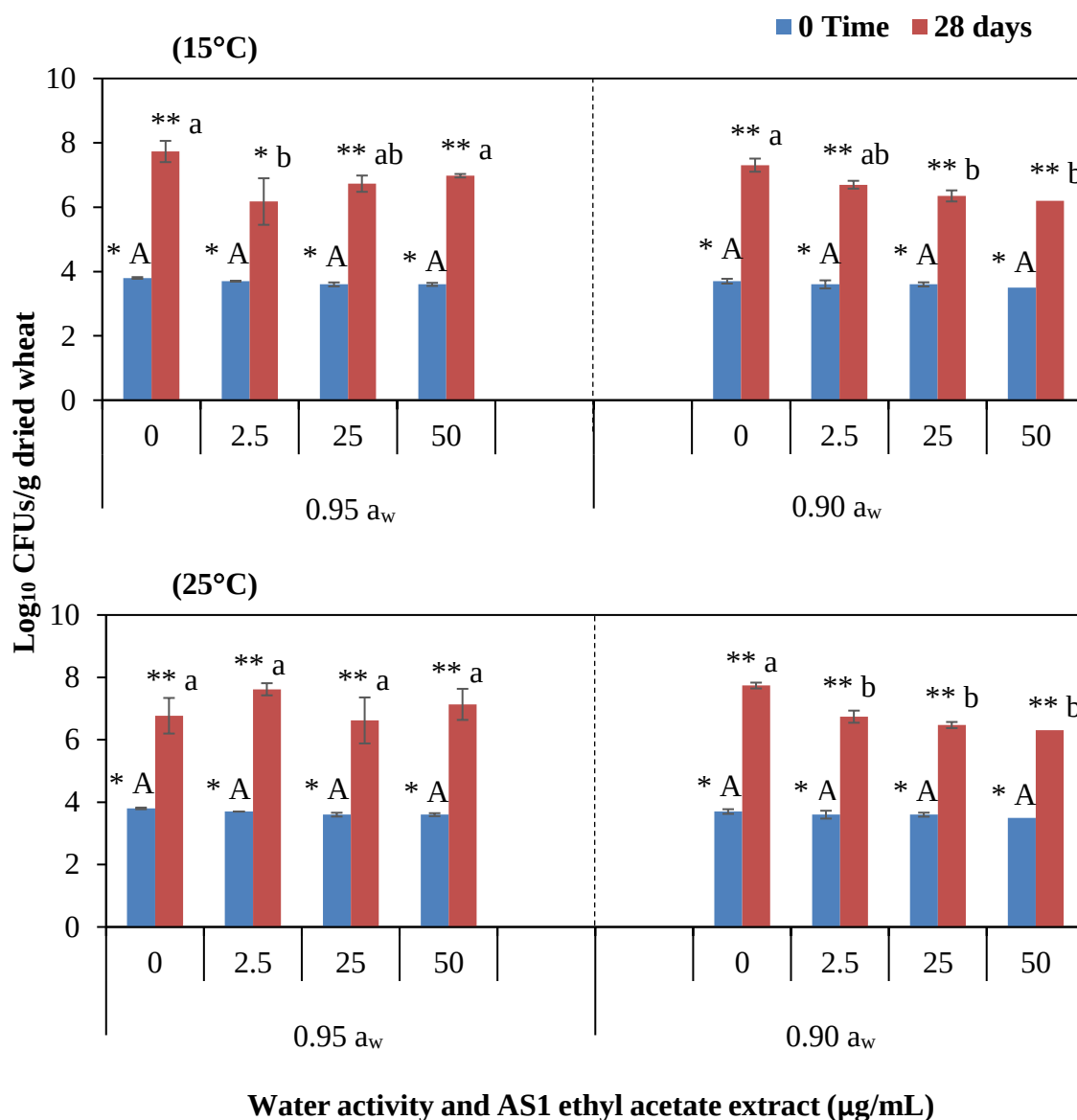


Figure 5.5 Efficacy of *Streptomyces* AS1 ethyl acetate extract on the populations of *P. verrucosum* isolated from stored wheat after storage at 25 and 15°C for 28 days. Data are means of three replicates with bars indicating standard error. Different numbers of asterisks indicate significant difference ($p < 0.05$) at the same AS1 concentration, different letters indicate significant difference ($p < 0.05$) between concentration on the same storage day. Comparison between time=0 and t=28 days using the T-test. Comparison between AS1 concentrations was done using the Wilcoxon method (non parametric comparisons).

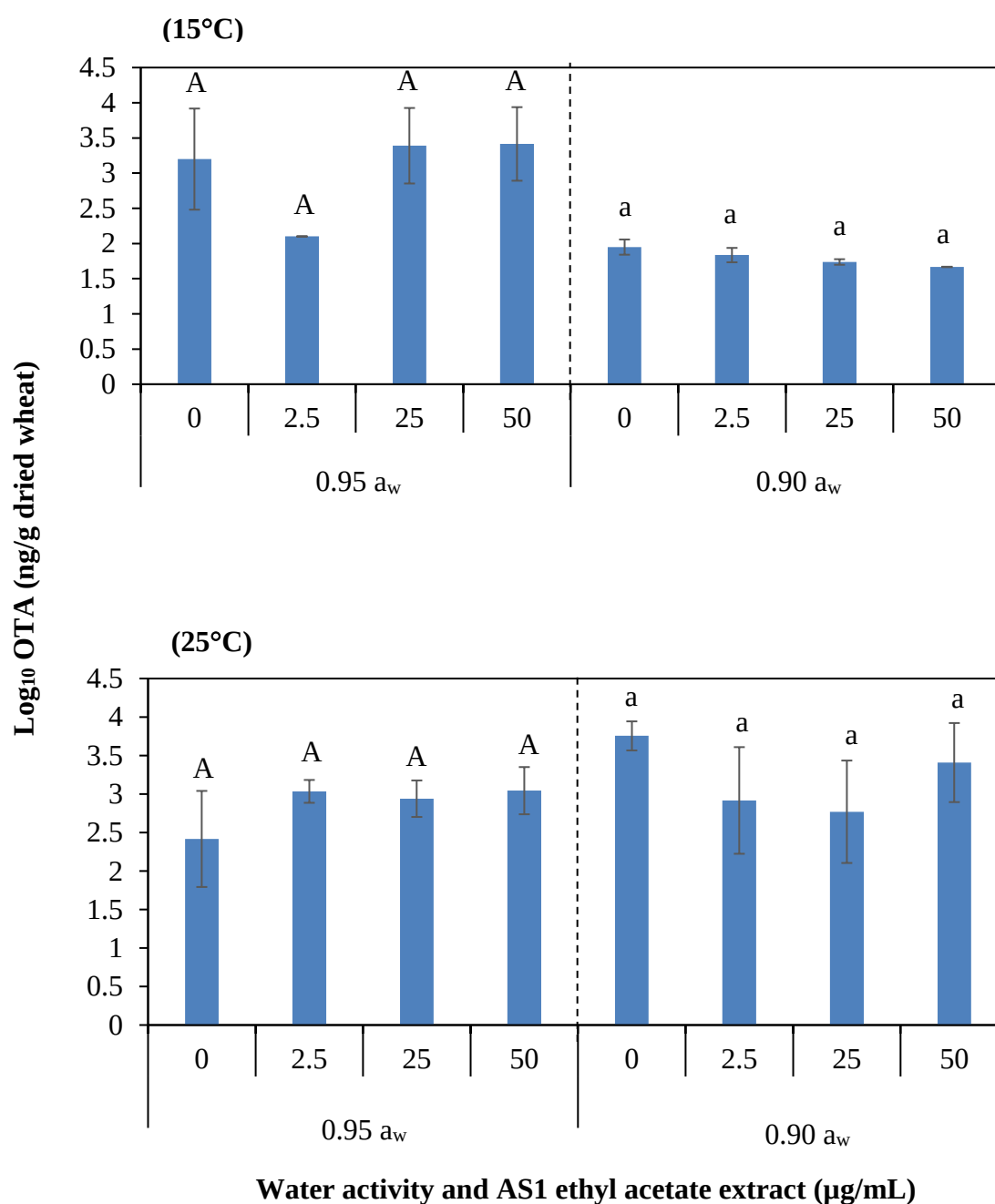


Figure 5.6 Effect of *Streptomyces* AS1 ethyl acetate extract on the OTA production by *P. verrucosum* grown on wheat at 25°C and 15°C for 28 days. Data are mean of three replicates with bars indicating standard error. Different letters indicate significant difference ($p < 0.05$) within treatments under the same conditions using Wilcoxon method (non parametric comparison).

5.4 DISCUSSION

5.4.1 Control of *T. rubrum* colonisation on nail-based media using *L. plantarum* VS1 supernatant

In the current study *L. plantarum* VS1 supernatant was mixed with *T. rubrum* spores and mycelia on nails to determine the susceptibility of *T. rubrum* to the *L. plantarum* VS1 supernatant. This was determined by measuring the hydrolytic enzymes activities after 7 and 14 days. The growth medium consisted of nails and RPMI 1640 to simulate the natural environment in which fungal infection of nails occurs. The application of *L. plantarum* VS1 supernatant to the spores and mycelium at both concentrations totally inhibited the activity of N-acetyl- β -glucosaminidase, alkaline phosphatase and keratinase. Furthermore, the *L. plantarum* VS1 extract was able to eliminate the germination of spores of *T. rubrum* on nails after 7 days. In contrast, inhibition of mycelial growth required a much longer period (14 days), especially with 25 mg/mL *L. plantarum* VS1 supernatant. This anti-dermatophyte activity of *L. plantarum* VS1 supernatant in this study was mainly from L-lactic acid and minor metabolites identified in the supernatant (see Chapter 3 sub-Section 3.3.6). The role of organic acids as anti-dermatophytes was reported previously and thus used as a main ingredient in the skin and nail treatment formulations but none of them used L-lactic acid. Shokri and Asadi (2011) reported a cream containing a mixture of citric (2.5%) and tartaric acids (2.5%) were able to inhibit *T. mentagrophyte* infection on guinea pig skin. Moreover, several commercial products such as Nonyx gel and Varisi solution use acetic acid and ascorbic acid, respectively, as the main ingredients to treat nail infections. In my study the use of *L. plantarum* VS1 supernatant to inhibit *T. rubrum* growth was more effective than the organic acid (L-lactic acid) alone (see Chapter 3 sub Section 3.3.8). Although the application of L-lactic acid as an anti-dermatophyte is perhaps much easier or might be cheaper than *L. plantarum* VS1 supernatant, it is less efficient than *L. plantarum* VS1 supernatant. Furthermore, further studies are needed to compare with the commercial nail treatment products which use organic acid to determine which one is more effective and cost-effective and be of benefit to the patient.

The nail treatment normally takes place after mycelial establishment. Complete drug therapy normally takes a long time, from a few months to a year with a relapse rate of

between 40-100% (Thomas et al., 2010). In human clinical therapy the fungicides normally used to treat dermatophytic fungi are triazoles (itraconazole, fluconazole) and others such as terbinafine, amorolfine and ciclopirox (Gupta et al., 2013). Because of resistance concerns, studies have focused on alternative treatments using natural products with better efficacy and less side effects. Previously, Lopes et al. (2017) reported the efficacy of chitosan with different molecular weight in preventing hair damage by *T. rubrum* and *Microsporum canis* over periods of three weeks. Also, nail fragments treated with hydroxypropyl chitosan were shown to be protected from infection by *T. rubrum* and *T. mentagrophytes* over seven-day periods (Bulgheroni et al., 2015). Li et al. (2015) found that ellagic acid (8.0 mg/mL) from *Euphorbia humifusa* Willd inhibited *T. rubrum* growth by 89% in the guinea pig dermatophytosis model. Guo et al. (2012) identified *Lactobacillus reuteri* ee1p and the antifungal compounds (reuterin) with activity against dermatophytes such as *Microsporum canis*, *Microsporum gypseum* and *Epidermophyton floccosum*. Koroishi et al. (2008) reported hydroalcoholic extracts of *Piper regnillii* at >1.2 mg/mL was able to inhibit *T. rubrum* growth on nail-based media for 14 days. In the present study the efficacy of *L. plantarum* VS1 supernatant was able to eliminate the colonisation of *T. rubrum* on nails which could be a potentially alternative approach to treating such nail infections.

5.4.2 Control of *P. verrucosum* colonisation and ochratoxin A (OTA) production in stored wheat grain by *Streptomyces* AS1 ethyl acetate extract

This study has shown that the AS1 ethyl acetate extract was more effective in controlling the colonisation of *P. verrucosum* at the intermediate a_w level (0.90) than with more available water (0.95 a_w) at both 15 and 25°C. However, regardless of the concentration used, the AS1 extract was unable to control OTA contamination, regardless of the a_w x temperature conditions. Initial studies on a conducive YES medium particularly at 0.95 a_w /25°C showed inhibition of >90% of *P. verrucosum* growth with 5 µg/mL, with 2.5 µg/mL completely inhibiting OTA production. This could potentially be due to the AS1 extract compounds binding to some of the wheat components reducing the efficacy of the compounds *in-situ*. Perhaps, much higher concentrations (>50 µg/mL) of AS1 may be needed in the natural substrate (cereal grain) to utilise this type of treatment effectively. Previously, Sultan and Magan (2011) reported that AS1ethyl acetate extract (50 ppm)

was very effective at inhibiting the total production of aflatoxins by *A. flavus* under water stress on YES media after 10 days incubation. However, a much higher concentration was needed to significantly reduce aflatoxin contamination of stored peanuts. In addition, the use of naturally contaminated wheat grains may give a better idea of the potential efficacy of AS1 extract in controlling *P. verrucosum* colonisation and OTA contamination than on the relatively “clean” grain used in the present study. This is because the competition between the mycobiota in naturally contaminated wheat in the stored grain niche may affect the colonisation by *P. verrucosum* and thus influence the lack of efficacy in OTA control observed. The competition between mycobiota and the effect on fungal growth and mycotoxin production has indeed been reported previously. Magan et al. (2010) showed that the presence of different fungal species were able to inhibit or stimulate OTA production by *Aspergillus carbonarius*, and DON production by *Fusarium culmorum*. These effects were influenced by the storage a_w and temperature conditions. Vankudoth et al. (2016) reported the interaction between *P. verrucosum* and various fungal species from different genera (*Alternaria*, *Aspergillus*, *Cladosporium*, *Chetomium*, *Dreschlera*, *Fusarium*, *Penicillium*, *Rhizopus* and *Trichoderma*) resulting in inhibition of *P. verrucosum* growth and OTA production by 40-90% and 60-100%, respectively. Furthermore, Barberis et al. (2014) reported the presence of different non-toxicogenic fungal strains were able to inhibit the OTA contamination by *A. carbonarius* by different relative amounts.

Previously, some studies have screened different antifungal compounds for efficacy against growth of *P. verrucosum* on cereal grains and on toxin production. Alexa et al. (2012) demonstrated that the ethanolic extract of grape pomace and grape seeds at 500-2500 ppm significantly inhibited *P. verrucosum* populations and OTA production in wheat. Aldred et al. (2008) reported that the antioxidant resveratrol (500 $\mu\text{g/g}$) was able to inhibit >90% of *P. verrucosum* growth and totally inhibited OTA production in stored wheat at 15°C/ 0.90 a_w and 0.95 a_w levels. Hossain et al., (2014) found that a combination of 2% basil essential oil and 2 kGy of radiation totally inhibited *P. chrysogenum* population in stored rice. However, in many cases the economics of use of these compounds have not been addressed. For example, the use of resveratrol was found to be too expensive to use when compared with the existing commercial fungistats available.

Thus, a cost-benefit analyses needs to be done on AS1 extract and compared with other existing compounds to evaluate whether it would be feasible to use or not.

5.5 CONCLUSIONS

In summary the present study has shown that *L. plantarum* VS1 supernatant at 25 and 50 mg/mL totally inhibited the growth of *T. rubrum*, based on the inhibition of the ability to produce N-acetyl- β -glucosaminidase, alkaline phosphatase and keratinase. Furthermore, it also completely inhibited spore germination and mycelial growth of *T. rubrum* on nail-based media over 14 days exposure periods. *Streptomyces* AS1 ethyl acetate extract at 2.5 μ g/mL - 50 μ g/mL significantly inhibited the colonisation of *P. verrucosum* of stored wheat grain at 0.90 a_w and both 15 and 25°C. However, this extract was unable to inhibit OTA production. This study suggests that the concentration of AS1 extract to inhibit *P. verrucosum* colonisation and control OTA contamination of stored wheat needs to be much higher than that used in the present study to control OTA contamination.

CHAPTER 6

Overall discussion, final conclusions and future work

6.1 OVERALL DISCUSSION

The potential of bacteria as antifungal agents has been reported widely for food applications (Deepthi et al., 2016). Thus, the screening of LABs isolated from Malaysian fermented foods, and metabolites produced by a *Streptomyces* AS1 against human and food fungal pathogens is an area of growing importance. Metabolites from *Streptomyces* have previously been demonstrated to have a wide spectrum of antifungal activities (Singh et al., 2013) when compared to the application of LABs. In this study, of 66 LABs, only two strains each of *Lactobacillus* and *Pediococcus* had antifungal activity, and then predominantly only against the dermatophyte *T. rubrum*. No efficacy against the other important human pathogens such as *A. fumigatus* and *C. albicans* or against mycotoxigenic species was observed.

The present study has reported, for the first time, the efficacy of these four LAB strains isolated from Malaysian fermented foods, for controlling the dermatophyte species, both *in-vitro* and *in-situ*. For LABs, it was interesting to note that the efficacy of *L. plantarum* VS1 alone was better than when co-cultured with the others. This certainly suggests that depending on the LAB strains, a single effective LAB strain may be better than co-cultures of several ones. From biotechnological point of view, the use of a single strain may be better as the relative production costs and downstream processing cost would probably be more economic. The efficacy of bacteria as antifungal agents generally depends on the type of metabolites produced. The metabolites can be fungistatic or fungicidal depending on the targeted fungal pathogens (Park et al., 2008). In this study the metabolites produced by *L. plantarum* VS1 were shown to have fungicidal properties for controlling both spore germination and mycelial growth of *T. rubrum*. This is important as there are indications that there is some build-up of resistance against the existing recommended fungicidal drugs being used.

In contrast, the *Streptomyces* AS1 metabolites were fungistatic against spore germination and growth of *P. verrucosum*, although the IC₅₀ concentrations were <0.20 µg/mL and resulted in complete inhibition of OTA mycotoxin production at both a_w levels tested.

This study has identified the key metabolites secreted by the *Streptomyces* AS1. The metabolites produced were in different concentrations and may act synergistically, enhancing the overall antifungal activity (Cain et al., 2003). This was supported when comparing the natural mixture of antifungal metabolites produced by the *Streptomyces* AS1 strain with the predominant individual compounds alone. The latter did not significantly inhibit fungal growth or OTA production on a conducive medium. This suggests that a mixture of compounds naturally produced by this particular strain has better efficacy than a specific individual one alone. This has not been shown previously.

Inhibition of components of the life cycle of the two targeted pathogens (*T. rubrum*, *P. verrucosum*) in relation to the tested compounds was examined to evaluate whether inhibition of extracellular enzyme production was a key factor in their control. To this end the key hydrolytic enzymes which may play a role in fungal colonisation/establishment were identified using the API ZYM kit. Generally, fungi rely on the ability to produce hydrolytic enzymes which breakdown complex nutrients into simple sugars and utilise these for colonisation of different matrices (Kaufman et al., 2007). Therefore, the inhibition of key hydrolytic enzyme activities could be related to the inhibition of fungal growth and/or mycotoxin production (Reynoso et al., 2002). This study demonstrated that the activity of hydrolytic enzymes was inhibited in the presence of *L. plantarum* VS1 supernatant (15 mg/mL) and *Streptomyces* AS1 extract (1.25 µg/mL at both a_w levels) and this paralleled the reduction in fungal growth. For *P. verrucosum*, the enzyme inhibition may also be related to the reduction in OTA production, in the *in-vitro* studies, particularly of esterase activity. However, when lower concentrations of antifungal mixture of compounds was applied, a stimulation in some enzyme activities occurred which paralleled effects on growth, sporulation and OTA production. This could possibly be a stress-related response to the presence of very low concentrations of antifungal compounds. Previous studies have suggested that under water stress, for some mycotoxigenic fungi, there is a stimulation in mycotoxin production which was confirmed by analyses of key genes involved in the biosynthesis of the toxin (Schmidt-Heydt et al., 2008).

The efficacy of antifungal compounds against *T. rubrum* and *P. verrucosum* colonisation was further studied on nail-based matrices and in stored wheat, respectively. For *T.*

rubrum it is important to examine whether protection can be offered by the compounds produced by the LAB strain, especially related to inhibition of spore germination and mycelial extension, the main method of skin colonisation by this dermatophyte. This would provide information on the potential treatment of such infections with these naturally produced compounds. The production of key hydrolytic enzymes was used as an indicator of the efficacy against *T. rubrum*. The supernatant of *L. plantarum* VS1 gave significant inhibition of enzyme activity, as no activity was detected, either from spore or mycelial-based inoculums. Furthermore, the spores were not viable, even 7 days after treatment, while mycelial inhibition took about 14 days when using 25 mg/mL of the LAB supernatant. Previously, such enzyme activity has been found to be a very good early indicator of fungal activity *in-vitro* and *in-situ* (Alam et al. 2009; Keshri et al., 2002). Overall, this is the first time LABs from Malaysian fermented foods have been shown to have potential control of medically important fungal pathogens, such as *T. rubrum*, *in-vitro* and *in-situ*.

For *P. verrucosum*, the concentration of AS1 extract used in this study (50 µg/mL) was not sufficient to control fungal populations and OTA production *in-situ*. However, some decrease in isolation of populations of *P. verrucosum* was observed at 0.90 a_w at both temperatures. Perhaps, some components of the mixture become bound to the nutritional components of the wheat, especially proteins. Much higher concentrations may need *in-situ* to obtain effective control of OTA using this mixture of compounds from the *Streptomyces* AS1. Recently, Sriwattanachai et al. (2018) found a significant inhibition of *Penicillium* populations in stored rice with application of ≥MIC of a mixture of *L. plantarum* supernatant and thyme essential oil. In addition, if higher concentrations are necessary then the economics of production of the natural mixture of compounds needs to be examined. Previously, Aldred et al. (2008) demonstrated that resveratrol was very effective at controlling OTA production by *P. verrucosum* in stored wheat grain. However, the costs were prohibitive when compared to existing fungistatic compounds based on salts of aliphatic acids.

6.2 FINAL CONCLUSIONS

The efficacy of LABs isolated from Malaysian fermented foods showed that only a few LAB strains produced natural inhibitory compound against a number of human and plant

fungus pathogens. These were more effective against human fungus pathogens than plant pathogens. The natural mixture of compounds produced by the *Streptomyces* strain AS1 appeared to be very effective against some of these fungi, especially the mycotoxigenic *P. verrucosum*. This allowed more detailed *in-vitro* and *in-situ* studies to be carried out. The metabolites that were responsible for the antifungal activity were determined. The major conclusions of the finding are listed below according to the different Chapters of the thesis.

CHAPTER 2: Screening of lactic acid bacteria (LABs) from Malaysian fermented foods and a *Streptomyces* strain (AS1) for efficacy against human and plant fungus pathogens

- (a) Two *Lactobacillus* and two *Pediococcus* strains had the highest activity against the human fungus dermatophyte pathogen *T. rubrum*.
- (b) *Streptomyces* AS1 metabolites had the broad spectrum of antifungal activity against the plant and human fungus pathogens tested.

CHAPTER 3: *In-vitro* efficacy of the best lactic acid bacteria (LABs) and the *Streptomyces* AS1 metabolites for control of growth and mycotoxin production of selected pathogenic fungi

Lactic acid bacteria

- (a) Spore germination of *T. rubrum* was totally inhibited by the mixture of *L. plantarum* VN5, *L. plantarum* VS1, *P. acidilactici* 84 and *P. pentosaceus* 95
- (b) The antifungal activity of *L. plantarum* VS1 was significantly higher than the other individual LAB strains and co-cultures of all four LAB strains
- (c) The efficacy of *L. plantarum* VS1 against *T. rubrum* growth was successfully increased by 30-fold after optimization of the growth conditions.
- (d) The MIC, IC₅₀ and MFC of *L. plantarum* VS1 supernatant, which were approximately 20 mg/mL, 14 mg/mL and 30 mg/mL, respectively.
- (e) Six compounds which may have antifungal activities were determined, with the major compounds produced being L-lactic acid and acetic acid.

- (f) Natural antifungal compounds (cell free supernatants) were more effective than a single compound (L-lactic and acetic acid) or a mixture of the two against *T. rubrum* growth
- (g) The optimum growth conditions for *T. rubrum* on keratin-based media was at pH 4.5 and 30°C

***Streptomyces* strain AS1**

- (a) *Streptomyces* strain AS1 metabolites were more effective at inhibiting the germination of spores of *P. verrucosum* than *A. fumigatus* (Mi538) and *F. verticillioides*
- (b) This was supported by the TTD results using the rapid screening approach using the Bioscreen assay.
- (c) The MIC for control of *P. verrucosum*, *A. fumigatus* (Mi538) and *F. verticillioides* was >40 µg/mL AS1 extract; with the IC₅₀ concentration for *P. verrucosum* growth the lowest at both a_w levels (0.99 and 0.95) tested.
- (d) Production of OTA by *P. verrucosum* was completely inhibited at ≥2.5 µg/mL AS1 extract at both a_w levels on a conducive medium.
- (e) Ten bioactive compounds from the AS1 extract which may be related to the antifungal activity were determined. The two major compounds identified were valinomycin and cyclo(L-Pro-L-Tyr)
- (f) The antifungal activity of the AS1 natural extract containing a mixture of compounds was more effective than the single major compounds (valinomycin and cyclo(L-Pro-L-Tyr)) alone or as a mixture of the two compounds against *P. verrucosum* growth and OTA production.

CHAPTER 4: Impact of LAB (*L. plantarum* VS1) and *Streptomyces* AS1 metabolites on mechanism of action in relation to production of extracellular hydrolytic enzymes by the target pathogens

Lactic acid bacteria

- (a) The number and quantity of hydrolytic enzymes secreted by *T. rubrum* was higher at 0.99 than 0.95 a_w.

- (b) *L. plantarum* VS1 supernatant at 15 mg/mL delayed the production and decreased the activity of alkaline phosphatase, N-acetyl- β -glucosaminidase, esterase and keratinase. However, keratinase activity was higher than the control after day 9.
- (c) *L. plantarum* VS1 supernatant at 15 mg/mL significantly decreased the *T. rubrum* growth but not the sporulation when compared to the control
- (d) *L. plantarum* VS1 supernatant at 4 mg/mL increased the *T. rubrum* growth, sporulation and activities of the four enzymes when compared to the control

***Streptomyces* strain AS1**

- (a) The number and quantity of hydrolytic enzymes secreted by *P. verrucosum* was higher at 0.99 than 0.95 a_w
- (b) AS1 extract at 1.25 $\mu\text{g/mL}$ completely inhibited esterase activity and reduced the activity of acid phosphatase, N-acetyl- β -glucosaminidase and alkaline phosphatase and reduced *P. verrucosum* growth and OTA production at both a_w levels.
- (c) At low concentrations of the AS1 mixture of metabolites (0.15 $\mu\text{g/mL}$) some enzyme activities increased at both a_w levels, with a stimulation of OTA production at 0.99 a_w .

CHAPTER 5: *In-situ* studies of the effects of bacterial metabolites in controlling (a) the growth of *T. rubrum* on nail matrices (from LAB) and (b) *P. verrucosum* and ochratoxin A production in stored wheat (from *Streptomyces* AS1).

Lactic acid bacteria

- (a) *L. plantarum* VS1 supernatant at 25 and 50 mg/mL totally inhibited the activity of N-acetyl- β -glucosaminidase, alkaline phosphatase and keratinase production by *T. rubrum* spores and mycelia grown on nail matrices
- (b) The viability of spores and mycelia was totally inhibited after 7 and 14 days, respectively.

***Streptomyces* strain AS1**

- (a) The antifungal activity of AS1 extract for the control of populations of *P. verrucosum* in stored wheat was more effective at 0.90 than 0.95 a_w at both temperatures examined (15 and 25°C).

- (b) AS1 extract (2.5 - 50 µg/mL) was unable to inhibit OTA production by *P. verrucosum* in stored wheat under all the conditions tested.

6.3 FUTURE WORK

- (a) *In-situ* studies in store cereal grain with higher concentrations of the mixtures of the natural compounds produced by the *Streptomyces* AS1 strain
- (b) Use of molecular markers in the toxin biosynthesis pathway (e.g. OTApks) for monitoring effects of metabolites on toxin control
- (c) The potential for pilot scale production of the mixture of compounds to identify the conditions for high titre and the economics of such production
- (d) Study the stability of metabolites from *L. plantarum* VS1 and *Streptomyces* AS1 such as at different temperatures and storage times
- (e) Potential for medical use of the *L. plantarum* VS1 supernatant by immobilising in gel systems which can be applied to the infected area and provide control of dermatophyte infections
- (f) Potential for using low cost by products for the production of these metabolites by both the LAB and *Streptomyces* strain and to examine the economics of the production and use.

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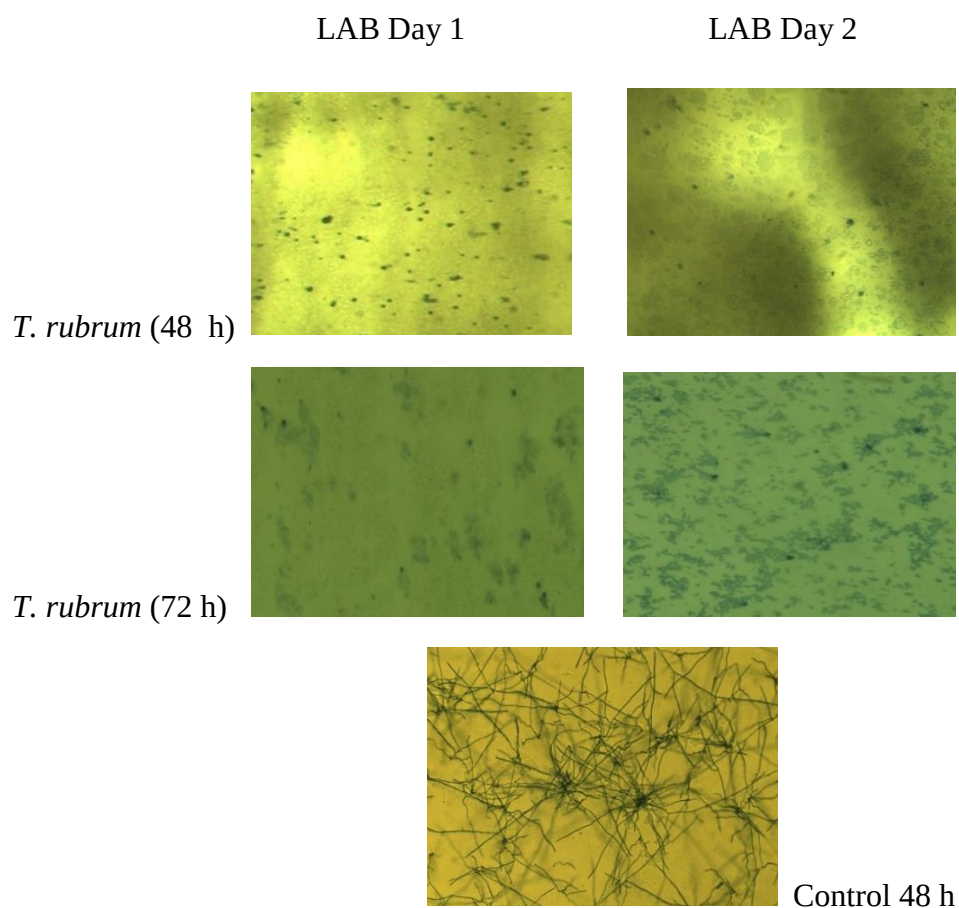
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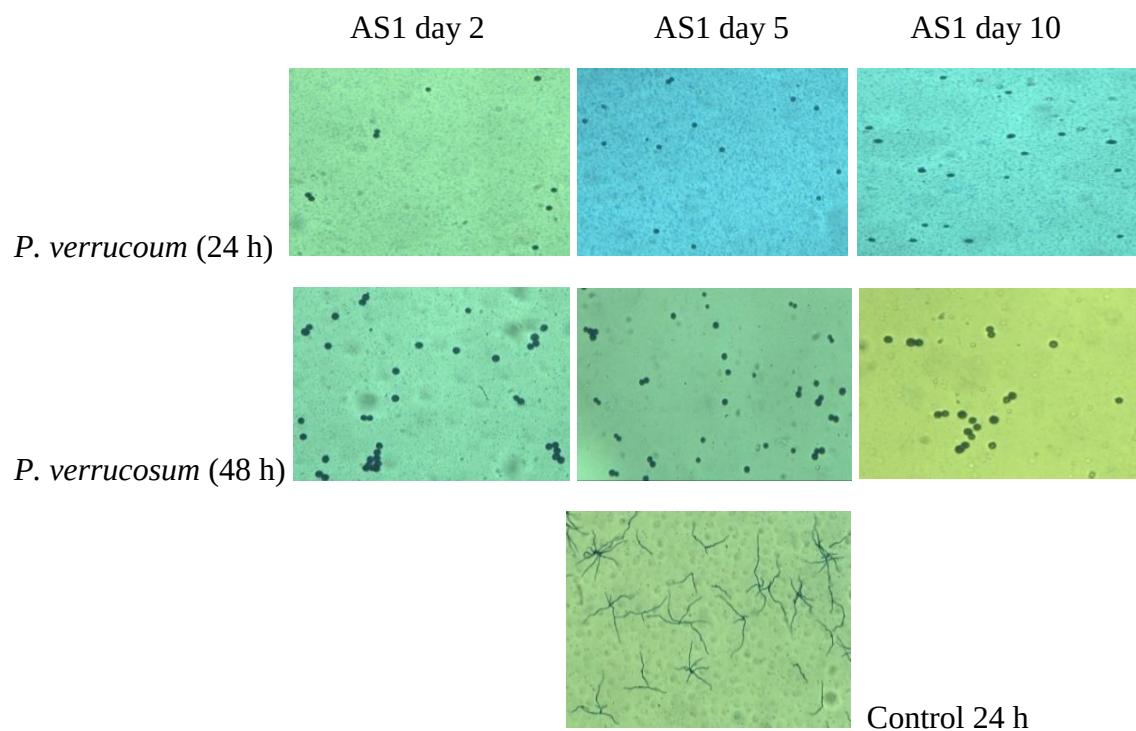
APPENDICES

Appendix A Spore germination of *T. rubrum* after grown on MRSA containing lactic acid bacteria (LAB) metabolites

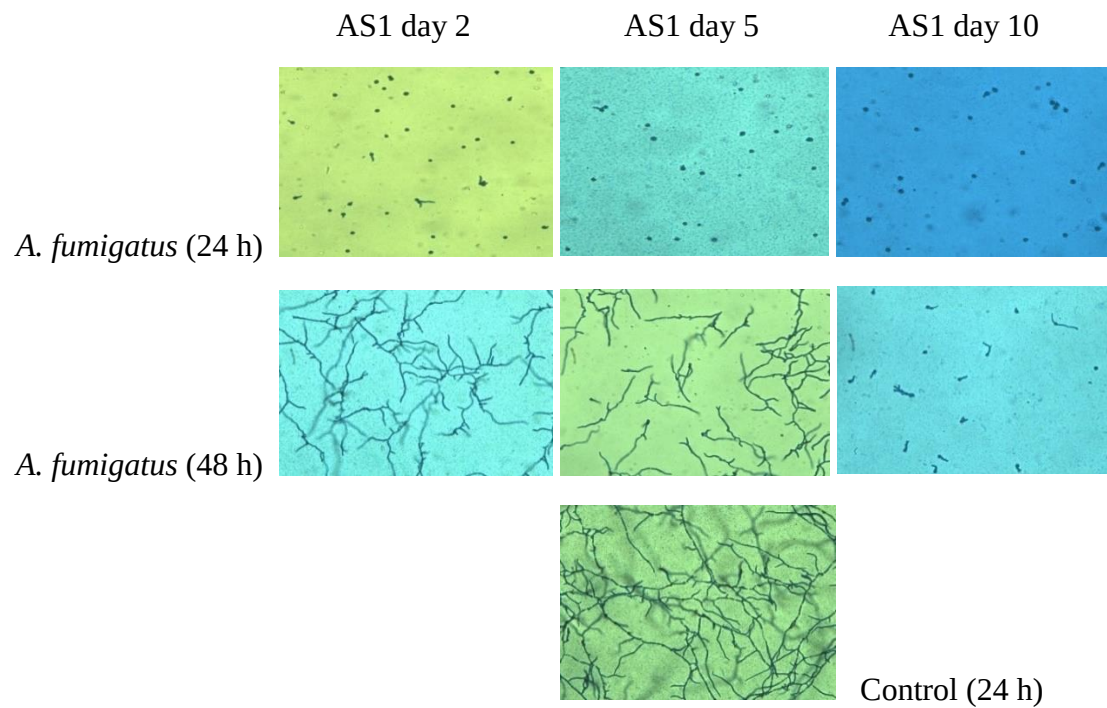


Appendix B Spore germination of (a) *P. verrucosum*, (b) *A. fumigatus* and (c) *F. verticillioides* after grown on ½ NA containing *Streptomyces* AS1 metabolites

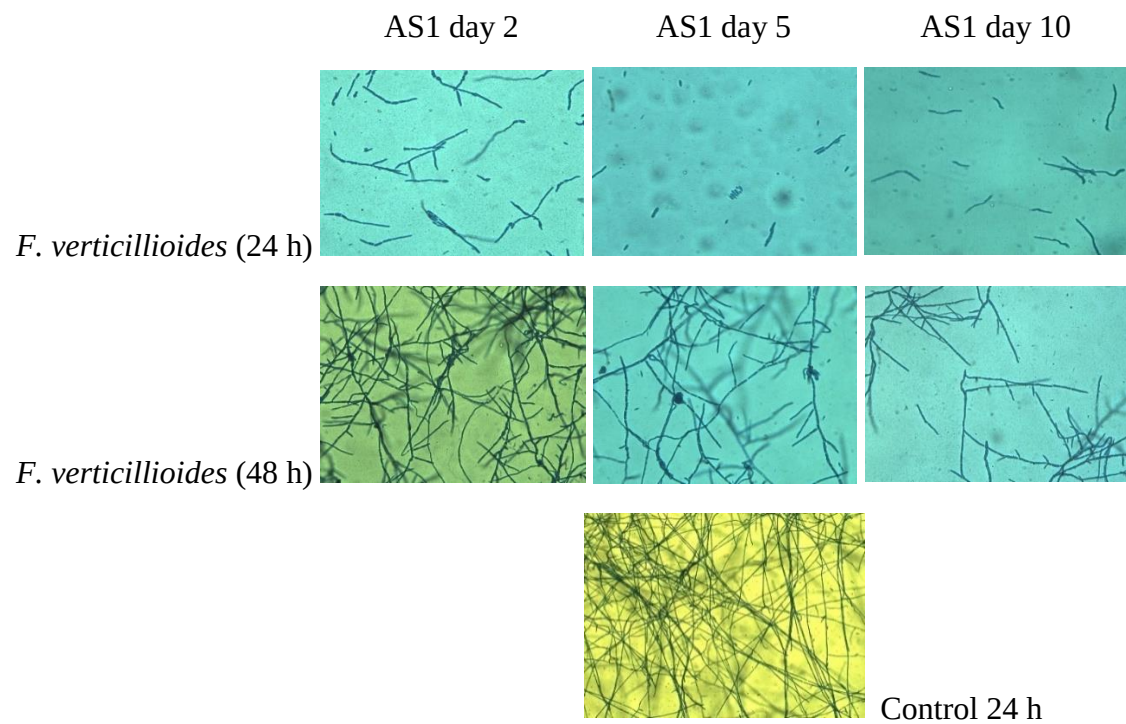
(a) *P. verrucosum*



(b) *A. fumigatus*

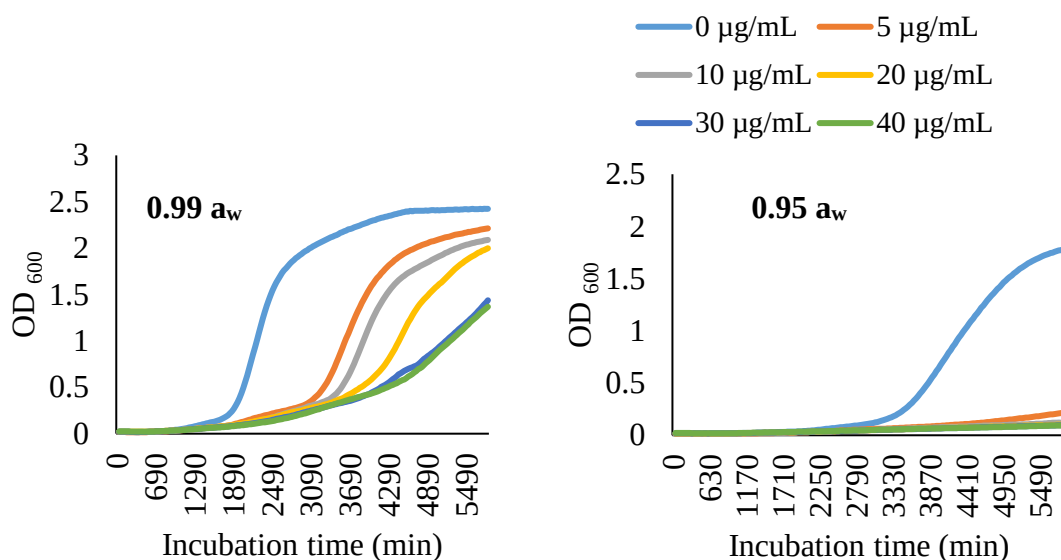


(C) *F. verticillioides*

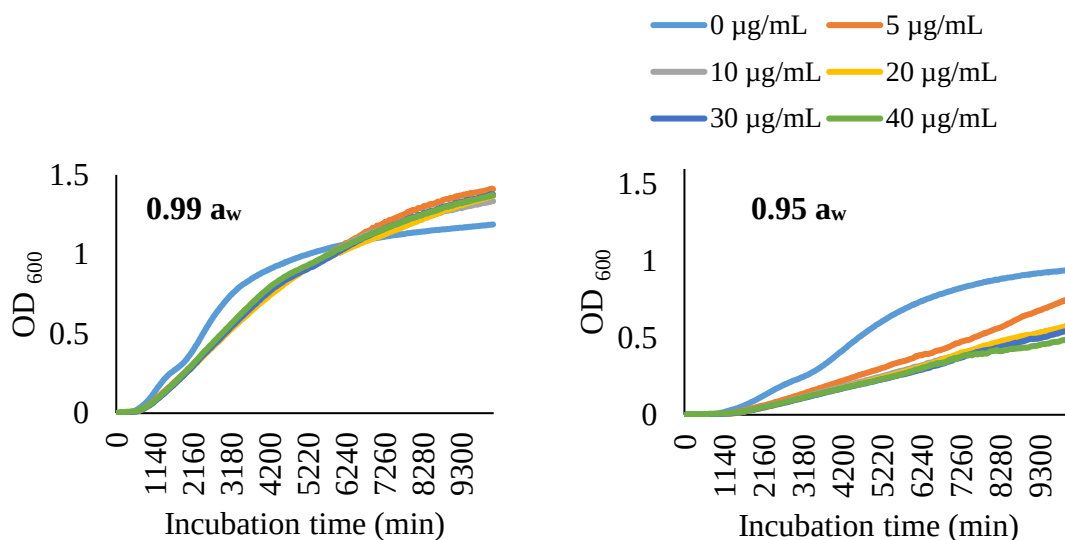


Appendix C Growth curve of *A. fumigatus* and *F. verticillioides* at 0.99 and 0.95 a_w semi solid YES media containing different concentration (0 - 40 $\mu\text{g/mL}$) of *Streptomyces* AS1 extract

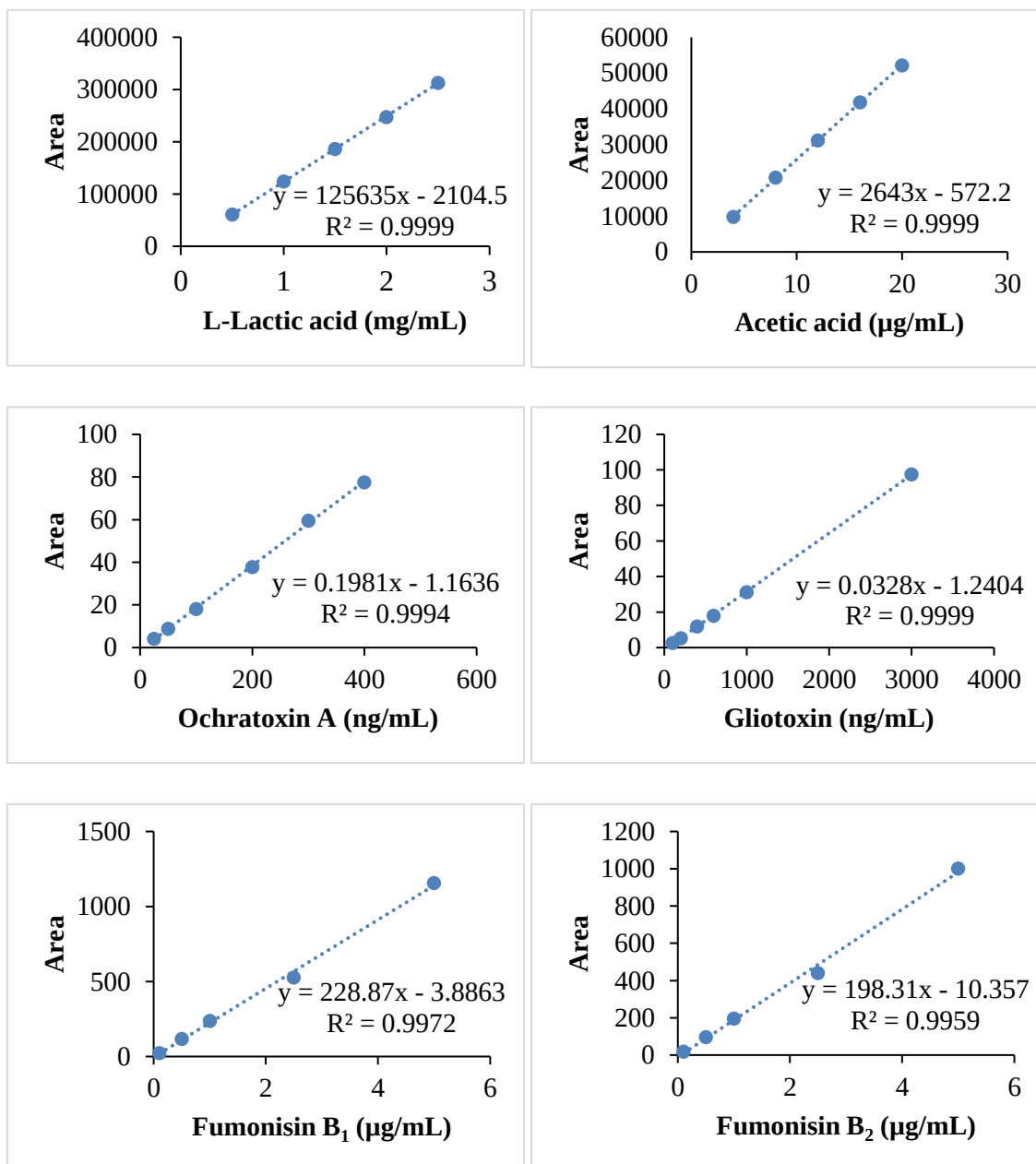
A. fumigatus



F. verticillioides

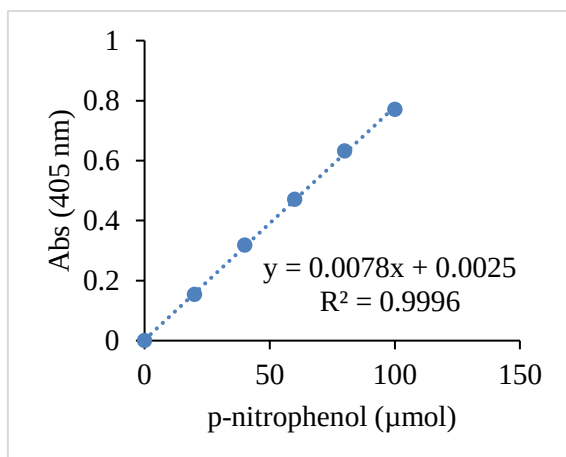


Appendix D Standard curve for L-lactic acid, acetic acid, ochratoxin A, gliotoxin, fumonisin B₁ and fumonisin B₂

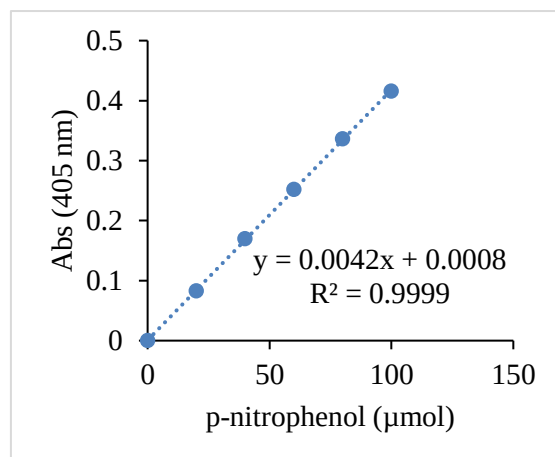


Appendix E Standard curve for (A) acid phosphatase, (B) alkaline phosphatase, (C) N-acetyl- β -glucosaminidase, (D) keratinase and (E) protein

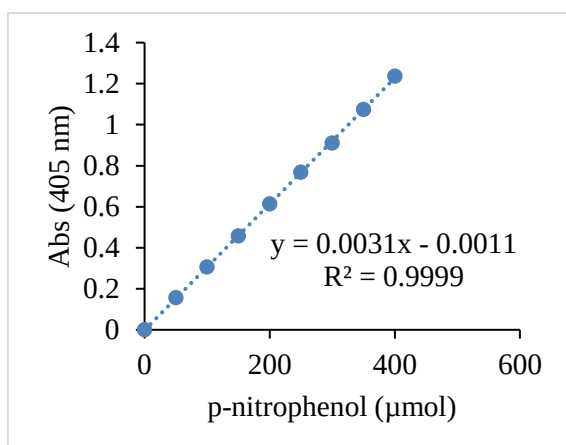
(A)



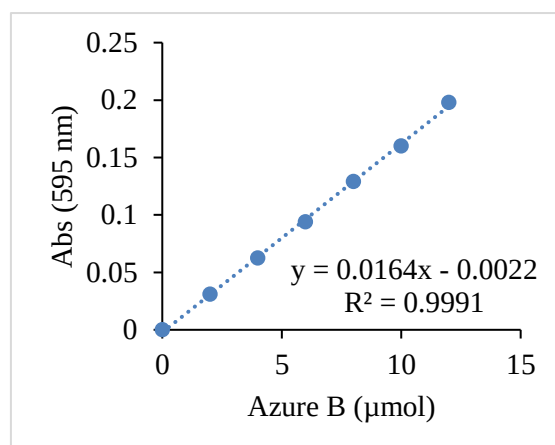
(B)



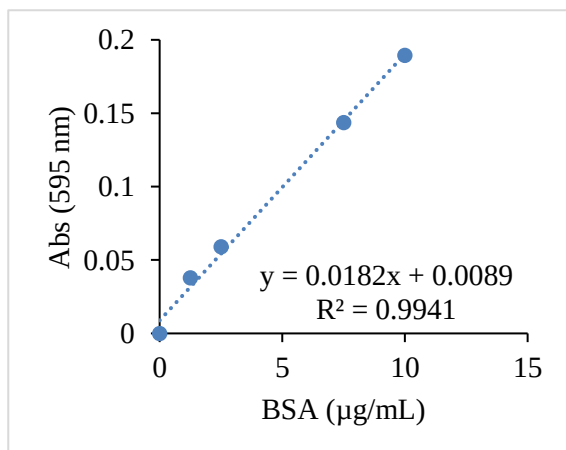
(C)



(D)



(E)



Appendix F Statistical analysis of the effect of incubation time, *Streptomyces* AS1 concentration and a_w on the hydrolytic enzyme activities

Statistical analysis of the N-acetyl β -glucosaminidase activity and N-acetyl β -glucosaminidase specific activity using Wilcoxon Method (nonparametric comparison).

N-acetyl β -Glucosaminidase activity (U/mL)			N-acetyl β -Glucosaminidase specific activity (U/mg protein)		
Incubation time (h)	AS1 concentration ($\mu\text{g/mL}$)	a_w	Incubation time (h)	AS1 concentration ($\mu\text{g/mL}$)	a_w
Level - p-value level	Level - p-value level	Level - p-value level	Level - p-value level	Level - p-value level	Level - p-value level
120 - 0 <0.0001*	0.15 - 0 0.3428	0.95 - 0.3867 0.99	120 - 0 <0.0001*	0.15 - 0 0.3428	0.95 - 0.3576 0.99
168 - 0 <0.0001*	1.25 - 0 0.1757		168 - 0 <0.0001*	1.25 - 0 0.4015	
120 - 24 <0.0001*	1.25 - 0.0260* 0.15		120 - 24 <0.0001*	1.25 - 0.0912 0.15	
168 - 24 <0.0001*			168 - 24 <0.0001*		
72 - 0 <0.0001*			72 - 0 <0.0001*		
168 - 72 0.0003*			120 - 72 0.0013*		
120 - 72 0.0009*			168 - 72 0.0052*		
72 - 24 0.0003*			72 - 24 0.0035*		
24 - 0 0.0286*			24 - 0 0.0286*		
168 - 120 0.0789			168 - 120 0.0956		

Statistical analysis of the acid phosphatase activity and acid phosphatase specific activity using Wilcoxon Method (nonparametric comparison).

Acid phosphatase activity (U/mL)			Acid phosphatase specific activity (U/mg protein)		
Incubation time (h)	AS1 concentration (µg/mL)	a _w	Incubation time (h)	AS1 concentration (µg/mL)	a _w
Level - p-value level	Level - p-value level	Level - p-value level	Level - p-value level	Level - p-value level	Level - p-value level
168 - 0 <0.0001*	0.15 - 0 0.5531	0.95 - 0.3842 0.99	168 - 0 <0.0001*	0.15 - 0 0.9268	0.95 - 0.4046 0.99
120 - 0 <0.0001*	1.25 - 0 0.0396*		120 - 0 <0.0001*	1.25 - 0 0.2169	
168 - 24 <0.0001*	1.25 - 0.0168* 0.15		168 - 24 <0.0001*	1.25 - 0.0653 0.15	
120 - 24 <0.0001*			120 - 24 <0.0001*		
72 - 0 <0.0001*			72 - 0 <0.0001*		
72 - 24 0.0006*			72 - 24 0.0028*		
120 - 72 0.0081*			120 - 72 0.0052*		
168 - 72 0.0159*			168 - 72 0.00174*		
24 - 0 0.0023*			24 - 0 0.0023*		
168 - 120 0.7595			168 - 120 0.0351*		

* Indicate significant difference (p<0.05)

Statistical analysis of the alkaline phosphatase activity and alkaline phosphatase specific activity using Wilcoxon Method (nonparametric comparison).

Alkaline phosphatase activity (U/mL)			Alkaline phosphatase specific activity (U/mg protein)		
Incubation time (h)	AS1 concentration (µg/mL)	a _w	Incubation time (h)	AS1 concentration (µg/mL)	a _w
Level - p-value level	Level - p-value level	Level - p-value level	Level - p-value level	Level - p-value level	Level - p-value level
120 - 0 <0.0001*	0.15 - 0 0.8829	0.95 - 0.8195 0.99	120 - 0 <0.0001*	0.15 - 0 0.8693	0.95 - 0.8492 0.99
120 - 24 <0.0001*	1.25 - 0 0.0616		168 - 0 <0.0001*	1.25 - 0 0.1873	
168 - 0 <0.0001*	1.25 - 0.0588 0.15		120 - 24 <0.0001*	1.25 - 0.1514 0.15	
168 - 24 <0.0001*			168 - 24 <0.0001*		
72 - 0 <0.0001*			72 - 0 <0.0001*		
72 - 24 <0.0001*			72 - 24 <0.0001*		
168 - 72 0.0037*			168 - 72 0.0417*		
168 - 120 0.0662			168 - 120 0.1446		
120 - 72 0.2902			120 - 72 0.4499		
24 - 0 0.1512			24 - 0 0.1512		

* Indicate significant difference (p<0.05)

Statistical analysis of the esterase activity and esterase specific activity using Wilcoxon Method (nonparametric comparison).

Esterase activity (mU/mL)			Esterase specific activity (mU/mg protein)		
Incubation time (h)	AS1 concentration (µg/mL)	a _w	Incubation time (h)	AS1 concentration (µg/mL)	a _w
Level - p-value level	Level - p-value level	Level - p-value level	Level - p-value level	Level - p-value level	Level - p-value level
168 - 0 0.0008*	0.15 - 0 0.4649	0.99 - 0.1165 0.95	168 - 0 0.0008*	0.15 - 0 0.4543	0.99 - 0.2222 0.95
72 - 0 0.0013*	1.25 - 0 <0.0001*		72 - 0 0.0013*	1.25 - 0 <0.0001*	
120 - 0 0.0013*	1.25 - 0.15 <0.0001*		120 - 0 0.0013*	1.25 - 0.15 <0.0001*	
168 - 24 0.1100			24 - 0 0.0286*		
120 - 24 0.1378			168 - 24 0.4186		
24 - 0 0.0286*			72 - 24 0.4903		
72 - 24 0.2023			120 - 24 0.4903		
168 - 72 0.5113			120 - 72 0.6952		
120 - 72 0.5381			168 - 72 1.000		
168 - 120 0.6962			168 - 120 0.5944		

* Indicate significant difference (p<0.05)

Appendix G Statistical analysis of the effect of incubation day and *L. plantarum* VS1 concentration on hydrolytic enzyme activity

Statistical analysis of the alkaline phosphatase activity using Wilcoxon method (nonparametric comparison)

Incubation day		VS1 concentration (mg/mL)			VS1 concentration		Incubation day				
		0	4	15	(mg/mL)		0	3	6	9	12
Level	Level	p-value	p-value	p-value	Level	Level	p-value	p-value	p-value	p-value	p-value
9	6	0.0304*	0.0304*	0.0319*	4	0	1.0000	0.0552	0.0304*	0.0304*	0.0304*
12	0	0.0436*	0.0436*	0.1066	4	15	1.0000	1.0000	0.0211*	0.0518	0.1052
6	0	0.0436*	0.0436*	1.0000	15	0	1.0000	0.0552	0.0211*	0.0518	0.1052
9	0	0.0436*	0.0436*	0.0636							
6	3	0.1052	0.0211*	1.0000							
9	3	0.1052	0.0211*	0.0319*							
3	0	0.1066	1.0000	1.0000							
3	12	0.1052	0.0211*	0.0552							
6	12	0.0304*	0.0304*	0.0552							
9	12	0.0304*	0.0304*	0.1489							

* Indicate significant different at $p < 0.05$

Statistical analysis of the alkaline phosphatase specific activity using Wilcoxon method (nonparametric comparison)

Incubation day		VS1 concentration (mg/mL)			VS1 concentration (mg/mL)	Incubation day				
		0	4	15		0	3	6	9	12
Level	Level	p-value	p-value	p-value	Level	Level	p-value	p-value	p-value	p-value
9	6	0.0304*	0.0304*	0.0319*	4	0	1.0000	0.0552	0.0304*	0.0304*
12	0	0.0436*	0.0436*	0.1066	4	15	1.0000	1.0000	0.0211*	0.0518
6	0	0.0436*	0.0436*	1.0000	15	0	1.0000	0.0552	0.0211*	0.0518
9	0	0.0436*	0.0436*	0.0636						
6	3	0.1052	0.0211*	1.0000						
9	3	0.1052	0.0211*	0.0319*						
3	0	0.1066	1.0000	1.0000						
3	12	0.1052	0.0211*	0.0552						
6	12	0.0304*	0.0304*	0.0552						
9	12	0.0304*	0.0304*	0.1489						

* Indicate significant different at $p < 0.05$

Statistical analysis of the N-acetyl- β -glucosaminidase activity using Wilcoxon method (nonparametric comparison)

Incubation day		VS1 concentration (mg/mL)			VS1 concentration (mg/mL)	Incubation day				
		0	4	15		0	3	6	9	12
Level	Level	p-value	p-value	p-value	Level	Level	p-value	p-value	p-value	p-value
6	3	0.0304*	0.1052	0.0552	4	0	1.0000	0.4875	0.0304*	0.0304*
9	3	0.0304*	0.1052	1.0000	4	15	1.0000	0.0552	0.1052	0.0211*
9	6	0.0304*	0.0304*	0.0552	15	0	1.0000	0.0211*	0.1052	0.0211*
12	0	0.0436*	0.0436*	0.0636						
3	0	0.0436*	0.1066	1.0000						
6	0	0.0436*	0.0436*	0.1066						
9	0	0.0436*	0.0436*	1.0000						
3	12	0.0304*	0.1052	0.0319*						
6	12	0.0304*	0.0304*	0.1489						
9	12	0.0304*	0.0304*	0.0319*						

* Indicate significant different at $p < 0.05$

Statistical analysis of the N-acetyl- β -glucosaminidase specific activity using Wilcoxon method (nonparametric comparison)

Incubation day		VS1 concentration (mg/mL)			VS1 concentration (mg/mL)	Incubation day				
		0	4	15		0	3	6	9	12
Level	Level	p-value	p-value	p-value	Level	Level	p-value	p-value	p-value	p-value
6	3	0.0304*	0.1052	0.0552	4	0	1.0000	0.4875	0.0304*	0.0304*
9	3	0.0304*	0.1052	1.0000	4	15	1.0000	0.0552	0.1052	0.0211*
9	6	0.03048	0.0304*	0.0552	15	0	1.0000	0.0211*	0.1052	0.0211*
12	0	0.0436*	0.0436*	0.0636						
3	0	0.0436*	0.1066	1.0000						
6	0	0.0436*	0.0436*	0.1066						
9	0	0.0436*	0.0436*	1.0000						
3	12	0.0304*	0.1052	0.0319*						
6	12	0.0304*	0.0304*	0.1489						
9	12	0.0304*	0.0304*	0.0319*						

* Indicate significant different at $p < 0.05$

Statistical analysis of the esterase activity using Wilcoxon method (nonparametric comparison)

Incubation day		VS1 concentration (mg/mL)			VS1 concentration (mg/mL)		Incubation day				
		0	4	15			0	3	6	9	12
Level	Level	p-value	p-value	p-value	Level	Level	p-value	p-value	p-value	p-value	p-value
9	6	0.0304*	0.0304*	1.0000	4	0	1.0000	0.0552	0.0606	1.0000	0.0304*
12	0	0.0536	0.0436*	0.0636	4	15	1.0000	1.0000	0.0211*	0.0211*	0.0518
6	0	0.0436*	0.0436*	1.0000	15	0	1.0000	0.0552	0.0211*	0.0211*	0.1116
9	0	0.0436*	0.0436*	1.0000							
6	3	0.1052	0.0211*	0.0319*							
9	3	0.1052	0.0211*	1.0000							
3	0	0.1066	1.0000	1.0000							
9	12	0.3123	0.0304*	1.0000							
3	12	0.1052	0.0211*	0.0319*							
6	12	0.0606	0.0304*	0.0319*							

* Indicate significant different at $p < 0.05$

Statistical analysis of the esterase specific activity using Wilcoxon method (nonparametric comparison)

Incubation day		VS1 concentration (mg/mL)			VS1 concentration (mg/mL)		Incubation day				
		0	4	15			0	3	6	9	12
Level	Level	p-value	p-value	p-value	Level	Level	p-value	p-value	p-value	p-value	p-value
9	6	0.0304*	0.0304*	1.0000	4	0	1.0000	0.0552	0.1939	0.8852	0.0304*
12	0	0.0436*	0.0436*	0.0636	4	15	1.0000	1.0000	0.0211*	0.0211*	0.0518
6	0	0.0436*	0.0436*	1.0000	15	0	1.0000	0.0552	0.0211*	0.0211*	0.0518
9	0	0.0436*	0.0436*	1.0000							
9	12	0.1124	0.3123	0.0319*							
6	3	0.1052	0.0211*	1.0000							
9	3	0.1052	0.0211*	1.0000							
3	0	0.1066	1.0000	1.0000							
3	12	0.1052	0.0211*	0.0319*							
6	12	0.0304*	0.0304*	0.0319*							

* Indicate significant different at $p < 0.05$

Statistical analysis of the keratinase activity using Wilcoxon method (nonparametric comparison)

Incubation day		VS1 concentration (mg/mL)			VS1 concentration (mg/mL)		Incubation day				
		0	4	15			0	3	6	9	12
Level	Level	p-value	p-value	p-value	Level	Level	p-value	p-value	p-value	p-value	p-value
12	0	0.0436*	0.0436*	0.0636	4	0	1.0000	0.0552	0.0518	0.0518	0.0304*
6	0	0.0636	0.0436*	1.0000	15	0	1.0000	0.0552	0.0319*	0.0319*	0.0518
9	0	0.0636	0.0436*	1.0000	4	15	1.0000	1.0000	0.0211*	0.0211*	0.0518
3	0	0.1066	0.1066	1.0000							
6	3	0.1489	0.0211*	1.0000							
9	3	0.1489	0.0211*	1.0000							
9	6	1.0000	0.3123	1.0000							
3	12	0.1052	0.0211*	0.0319*							
6	12	0.1116	0.0606	0.0319*							
9	12	0.0518	0.0304*	0.0319*							

* Indicate significant different at $p < 0.05$

Statistical analysis of the keratinase specific activity using Wilcoxon method (nonparametric comparison)

Incubation day		VS1 concentration (mg/mL)			VS1 concentration (mg/mL)		Incubation day				
		0	4	15			0	3	6	9	12
Level	Level	p-value	p-value	p-value	Level	Level	p-value	p-value	p-value	p-value	p-value
12	0	0.0436*	0.0436*	0.0636	4	0	1.0000	0.0552	0.0518	0.0518	0.0304*
6	0	0.0636	0.0436*	1.0000	15	0	1.0000	0.0552	0.0319*	0.0319*	0.0518
9	0	0.0636	0.0436*	1.0000	4	15	1.0000	1.0000	0.0211*	0.0211*	0.0518
3	0	0.1066	1.0000	1.0000							
6	3	0.1489	0.0211*	1.0000							
9	3	0.1489	0.0211*	1.0000							
9	6	1.0000	0.1124	1.0000							
3	12	0.1052	0.0211*	0.0319*							
6	12	0.1116	0.0304*	0.0319*							
9	12	0.0518	0.4705	0.0319*							

* Indicate significant different at $p < 0.05$

Appendix H Composition of RPMI 1640

Constituent	g/L water	Constituent	g/L water
L-arginine (free base)	0.200	Biotin	0.0002
L-asparagine (anhydrous)	0.050	D-pantothenic	0.00025
L-aspartic acid	0.020	Choline chloride	0.003
L-cystine.2HCl	0.0652	Folic acid	0.001
L-glutamic acid	0.020	Myo-inositol	0.035
L-glutamine	0.300	Niacinamide	0.001
Glycine	0.010	PABA	0.001
L-histidine (free base)	0.015	Pyridoxine HCl	0.001
L-hydroxyproline	0.020	Riboflavin	0.0002
L-isoleucine	0.050	Thiamine HCl	0.001
L-leucine	0.050	Vitamine B ₁₂	0.000005
L-lysine.HCl	0.040	Calcium nitrate.H ₂ O	0.100
L-methionine	0.015	Potassium chloride	0.400
L-phenylalanine	0.015	Magnesium sulfate (anhydrous)	0.04884
L-proline	0.020	Sodium chloride	6.000
L-serine	0.030	Sodium phosphate, dibasic (anhydrous)	0.800
L-threonine	0.020	D-glucose	2.000
L-tryptophan	0.005	Glutathione, reduced	0.001
L-tyrosine.2Na	0.02883	Phenol red, Na	0.0053
L-valine	0.02		

Appendix I Poster Presentation

Abstract submitted and poster presented at the Mycotoxin Forum – 10th Conference, Amsterdam, Netherlands (March 12 to 14, 2018)



Antifungal compounds from *Streptomyces* AS1: efficacy against *P. verrucosum* growth and ochratoxin A (OTA) production

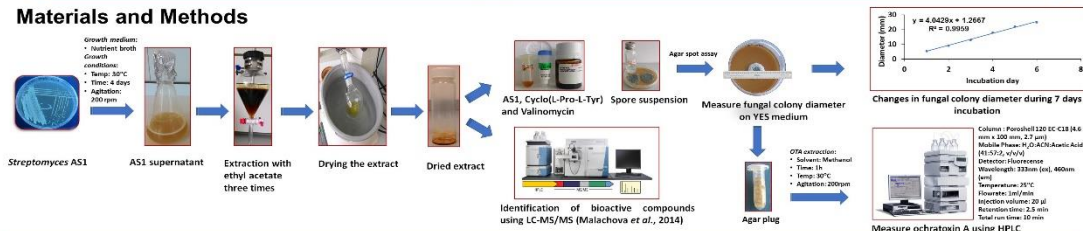
Azlina M. Danial, Angel Medina and Naresh Magan

Applied Mycology Group, Environment and AgriFood Theme, Cranfield University, Cranfield, Bedford, MK43 0AL, U.K

Introduction

- * The mycotoxigenic fungal spoilage species *P. verrucosum* contaminates cereals and cereal-based food products which can have impacts during downstream processing.
- * This has resulted in the development of strict legislative limits in many regions world-wide. For example the EU Commission Regulation has maximum permissible levels for ochratoxin A (OTA) in unprocessed (5.0 µg/kg) and processed cereal products (3.0 µg/kg).
- * There is significant interest in the use of natural metabolites from microorganisms to control *P. verrucosum* and ochratoxin A (OTA) in staple food chains.
- * However, most previous studies only examined the effect of metabolites on growth of *P. verrucosum* and not on OTA production.
- * **The objective of this study was to examine the efficacy of *Streptomyces* AS1 metabolites against *P. verrucosum* on (a) growth and (b) OTA production on yeast extract sucrose (YES) agar.**

Materials and Methods



Results

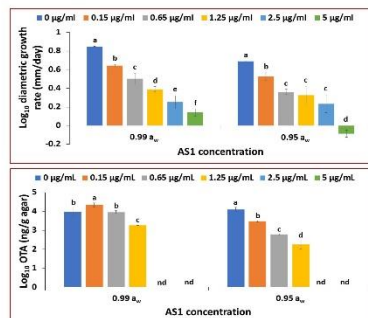


Figure 1. Efficacy of *Streptomyces* AS1 ethyl acetate extract for control of *P. verrucosum* growth and OTA production at 25°C after 7 days. Data are mean of triplicate with bars indicating standard errors. Different letter indicates significant difference ($p < 0.05$) within treatments by Tukey-Hramer HSD, nd: none detected.

Table 1. Compounds isolated from ethyl acetate extract of *Streptomyces* AS1 strain and concentration in 2.5 µg/ml AS1 extract which totally inhibited production of OTA

Bioactive compounds	Concentration (µg/g dried biomass)	Concentration in 2.5 µg/ml AS1 extract (ng/ml)
Valinomycin	149.22	11.25
Cyclo(L-Pro-L-Tyr)	21.64	1.63
Cyclo(L-Pro-L-Val)	10.07	0.76
Brevianamide F	2.68	0.202
Rugulussavin	0.72	0.054
Tryptophol	0.31	0.023
Chloramphenicol	0.23	0.017
Monactin	0.02	0.002
Dinactin	0.01	0.001
Nonactin	0.007	0.0005

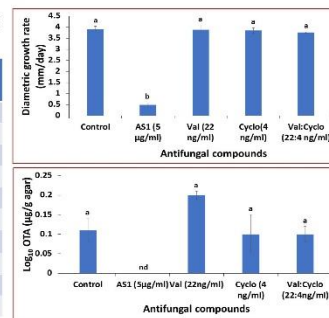


Figure 2. Efficacy of *Streptomyces* AS1 ethyl acetate extract, valinomycin and cyclo(L-Pro-L-Tyr) for control of *P. verrucosum* growth and OTA production at 25°C. Data are means of triplicate with bars indicating standard errors. Different letters indicate a significant difference ($p < 0.05$) compared to the control using the Wilcoxon Method (nonparametric comparison), nd: none detected.

Conclusions

- > The use of naturally produced combination of antifungal compounds was very effective in controlling growth and OTA production of *P. verrucosum*.
- > Naturally produced AS1 compounds in an ethyl acetate extract inhibited the growth of *P. verrucosum* by 88% when compared to control and totally inhibited production of OTA at concentration >2.5 µg/ml
- > Interestingly, no inhibition of growth and OTA production was observed in chemically derived individual compounds alone (cyclo(L-Pro-L-Val), 4 ng/ml; valinomycin, 22 ng/ml) or combination of the two compounds (4:22 ng/ml).
- > The better efficacy of naturally produced antifungal compounds present in the EA extract from the *Streptomyces* AS1 was possibly due to the synergistic effect of major and/or minor compounds together.
- > Studies are in progress to treat stored cereals under different environmental conditions to examine *in situ* efficacy.

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This work was supported by Malaysian Agricultural Research and Development Institute and Ministry of Agriculture

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2. Sulyok, M. and Magan, N. (2015). Impact of *Streptomyces* AS1 strain and its metabolite on control of *Aspergillus flavus* and aflatoxin B1 contamination in vitro and in stored product. *Biocatalysis and Biotechnology*, 21: 12–22/27–32.

Abstract submitted and poster presented at the First Mycokey International Conference in Ghent Belgium (September 11 to 14, 2017)



Efficacy of metabolites from *Streptomyces* AS1 to control growth and ochratoxin A (OTA) and gliotoxin production by the mycotoxigenic spoilage species *P. verrucosum* and *A. fumigatus*

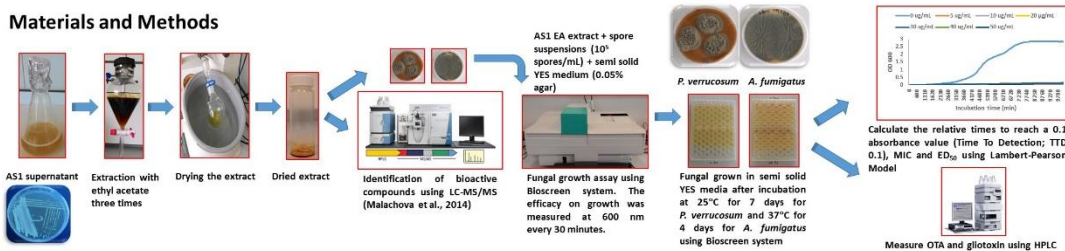
Azlina M. Danial, Angel Medina and Naresh Magan

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Introduction

- The mycotoxigenic fungal spoilage species *P. verrucosum* contaminates cereals and cereal-based food products which can have impacts during downstream processing. Similar impacts have been reported for animal feed contaminated with *A. fumigatus* which is a health risk not only to livestock but also to humans.
- This has resulted in the development of strict legislative limits in many regions world-wide. For example the EU Commission Regulation has maximum permissible levels for ochratoxin A (OTA) in unprocessed (5.0 µg/kg) and processed cereal products (3.0 µg/kg)
- There is significant interest in the use of natural metabolites from microorganism to control mycotoxigenic spoilage fungi in staple food chains
- The objective of this study was to examine the efficacy of *Streptomyces* AS1 metabolites against *P. verrucosum* and *A. fumigatus* on (a) growth and (b) OTA and gliotoxin production by *P. verrucosum* and *A. fumigatus* respectively.**

Materials and Methods



Results

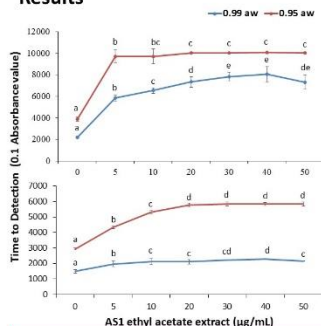


Figure 1. Effect of *Streptomyces* AS1 ethyl acetate extract and water activity (0.99 and 0.95 a_w) on time to detection of (A) *P. verrucosum* and (B) *A. fumigatus*. Means of 10 replicates. Bars indicate SE. Experiments carried out with the Bioscreen assay system. Different letters indicate significant difference ($p < 0.05$) using Wilcoxon method (nonparametric comparisons; Lambert et al., 2000; 2012).

Table 1. Minimum inhibitory concentration (MIC) and ED_{50} of *Streptomyces* AS1 ethyl acetate extract for controlling growth of *P. verrucosum* and *A. fumigatus*

Pathogenic fungi	a_w	MIC (µg/mL)	ED_{50} (µg/mL)
<i>P. verrucosum</i>	0.99	> 50	0.15
	0.95	> 50	0.000005
<i>A. fumigatus</i>	0.99	> 50	> 50
	0.95	> 50	10.45

Table 2. Compounds isolated from *Streptomyces* AS1 cell free supernatant (Malachova et al., 2014).

Bioactive compounds	Concentration (ng/mL)
Cyclo(L-Pro-L-Tyr)	3253
Valinomycin	2510
Cyclo(L-Pro-L-Val)	1537
Brevianamide F	367.4
Rugulosovin	22.76
Chloramphenicol	15.5
Monactin	0.4
Nonactin	0.1

Table 3. Efficacy of *Streptomyces* AS1 ethyl acetate extract for control of ochratoxin A (OTA) and gliotoxin production by *P. verrucosum* and *A. fumigatus*. Data are means with SE; LOD, limit of detection; ND, none detected

Concentration (µg/mL)	a_w	OTA (ng/mL)	Gliotoxin (ng/mL)
0	0.99	3021.4 ± 278.1	613 ± 38.3
	0.95	24.1 ± 0.5	ND
5	0.99	ND	ND
	0.95	ND	ND
10	0.99	ND	ND
	0.95	ND	ND
20	0.99	ND	ND
	0.95	ND	ND
30	0.99	ND	ND
	0.95	ND	ND
40	0.99	ND	ND
	0.95	ND	ND
50	0.99	ND	ND
	0.95	ND	ND

Conclusions

- Ethyl acetate extract of *Streptomyces* strain AS1 as low as 5 µg/mL significantly inhibited the growth of *P. verrucosum* at 0.99 and 0.95 a_w and for *A. fumigatus* at 0.95 a_w as well as production of OTA and gliotoxin.
- The MIC for *P. verrucosum* and *A. fumigatus* at both a_w levels were >50 µg/mL
- For ED_{50} , except for *A. fumigatus* at 0.99 a_w the ED_{50} values were ≤10 µg/mL
- Four major potential compounds which suppress the fungal growth and mycotoxin production were identified as cyclo(L-Pro-L-Tyr), valinomycin, cyclo(L-Pro-L-Val) and brevianamide F
- These findings suggest that the natural mixture of metabolites produced by *Streptomyces* AS1 in the ethyl acetate extract, especially the four major compounds, may be effective fungistatic antibiotic compounds to control *P. verrucosum* and *A. fumigatus* and toxin production in food/feed matrices.
- Work is in progress to study the effect of single and mixture of these four compounds on fungal growth and toxin production *in vitro* and *in situ*.

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Investigating mycological complexity

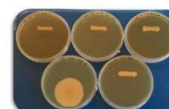
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- Medina, A., Lambert, R.J.W. and Magan, N. (2012). Rapid throughput analysis of filamentous fungi growth using turbidimetric measurements with the Bioscreen C as a tool for screening antifungal compounds. *Fungal Biology*, 115, p. 185-195

This work was supported by Malaysian Agricultural Research and Development Institute and Ministry of Agriculture



Efficacy of *Streptomyces* strain (AS1) and Lactic Acid Bacteria (LABs) against medically and food-related fungal pathogens



INTRODUCTION

- Malaysian fermented foods are considered healthy as they contain antimicrobial compounds such as lactic acid and bacteriocins which are naturally produced by the fermentative lactic acid bacteria (LABs).
- There is interest in examining these useful LAB strains isolated from Malaysian fermented food for potentially useful metabolites for the control of medically and food-related fungal pathogens.
- A *Streptomyces* (strain AS1) was isolated from peanuts and found to produce anti-fungal compounds which had efficacy against growth of *Aspergillus flavus* and aflatoxin B₁ production (Sultan & Magan, 2011).
- This *Streptomyces* strain and its metabolites have not previously been screened for efficacy against other pathogenic moulds.
- Objectives:** To screen and examine the efficacy of selected LAB strains from Malaysian fermented food and the *Streptomyces* strain (AS1) against *Penicillium*, *Fusarium*, *Aspergillus* and *Trichophyton* species.

MATERIAL AND METHOD: **Bacteria strains:** Lactic acid bacteria, *Streptomyces* strain (AS1). **Fungal pathogens:** Species of *Penicillium*, *Fusarium*, *Aspergillus*, *Trichophyton*. **Inhibition assay:** diffusion assay, dual culture assay, turbidimetric assays were used. **Turbidimetric assay:** The assay was done using the Bioscreen C Microbiological Growth Analyser. A mixture of spore suspension (final count 10⁵ spore/ml), semi-solid YES media (YES broth + 0.05% agar) with different water activity levels (0.99, 0.95, 0.90 a_w) and ethyl acetate extract of AS1 strain were used. The absorbance was measured every 30 min at 600 nm for 7 days at 25°C. Data were analysed using Easy Bioscreen Experiment software and Excel (Medina et al., 2012). **Bioactive compound extraction:** Bioactive compounds from strain AS1 was extracted with ethyl acetate prior to evaporation using rotary evaporator to remove the extractant solvent (Sultan & Magan 2011)

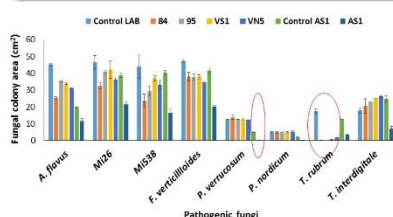


Figure 1. Effect of interaction between LABs and *Streptomyces* (AS1) strain and pathogenic fungi based on fungal colony area. M26 and M538: *A. fumigatus*

Table 1. Effect of interaction between LABs and *Streptomyces* (AS1) strains and different pathogenic fungi on the interaction scores and total Index of Dominance (I_D).

Bacteri	Interaction scores								I _D
Strains	<i>T. rubrum</i>	<i>T. interdigitale</i>	<i>A. flavus</i>	<i>A. fumigatus</i> M26	<i>A. fumigatus</i> M538	<i>P. nordii</i>	<i>P. verrucosum</i>	<i>F. vert.</i>	
B4	5/0	1/1	1/1	1/1	1/1	n.g.	n.g.	1/1	10/5
95	5/0	1/1	1/1	1/1	1/1	n.g.	n.g.	1/1	10/5
V51	5/0	1/1	2/2	1/1	1/1	n.g.	n.g.	1/1	11/6
VNS	5/0	1/1	1/1	1/1	1/1	n.g.	n.g.	1/1	10/5
AS1	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	40/0

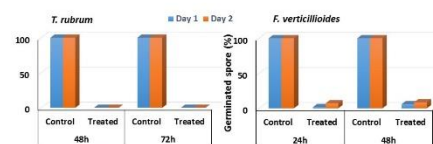


Figure 3. Effect of LABs metabolites on spore germination of fungal pathogens on MRSA media. The mixture of four LAB strains was grown on MRSA overlaid with sterile cellophane disc for two days. Colonies were removed prior to fungal spore assays.

RESULTS

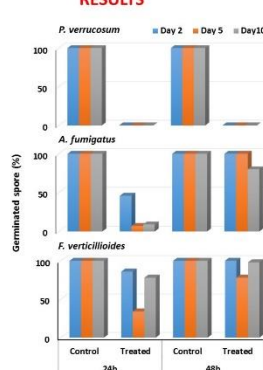


Figure 2. Effect of *Streptomyces* AS1 metabolites on spore germination of fungal pathogens on 1/2 NA media. The *Streptomyces* AS1 was grown on 1/2 NA overlaid with sterile cellophane disc for 10 days. The colonies and cellophane were removed prior to fungal spore germination assays

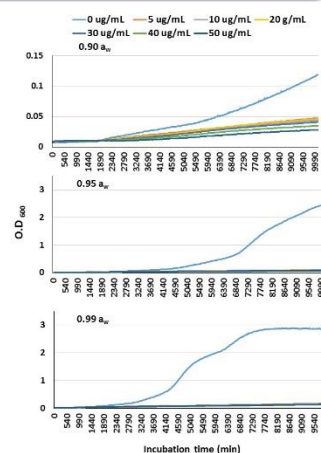


Figure 4. Growth curve of *P. verrucosum* in semi-solid YES media with different a_w and *Streptomyces* AS1 ethyl acetate extract concentration.

CONCLUSIONS

- Streptomyces* AS1 showed high efficacy against all pathogenic fungi tested and inhibited all spores of *P. verrucosum* from germinating (100%).
- Ethyl acetate metabolite extract of *Streptomyces* strain AS1 as low as 5µg/ml was very effective at inhibiting growth of *P. verrucosum* at 0.99 and 0.95 a_w.
- Four LAB strains (*L. plantarum* strain MCC 2156, *L. plantarum* strain HT-W104-B1, *P. acidilactici* 1498 and *P. pentosaceus* 1426) showed high efficacy against *T. rubrum* and completely inhibited spore germination.
- Studies are in progress to examine the relative concentrations of the metabolites to inhibit growth of *A. fumigatus*, *F. verticillioides* and *T. rubrum* and potential for control of mycotoxin production (ochratoxin A, gliotoxin and fumonisin B₁).

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Acknowledgement: This work was supported by Ministry of Agriculture and Malaysian Agricultural Research and Development Institute



References

Medina, A., Lambert, R. J. W. & Magan, N. (2012). Rapid throughput analysis of filamentous fungal growth using turbidimetric measurement with the Bioscreen C: a tool for screening bioactive compounds. *Fungal Biology*, 116, 161-169

Sultan, Y. & Magan, N. (2011). Impact of *Streptomyces* AS1 strain and its metabolite on control of *Aspergillus flavus* and aflatoxin B₁ contamination in-vitro and in stored peanut. *Biocontrol Science and Technology*, 21, 12, 1437-1455

Abstract submitted and poster presented at the International Commission for Food Mycology (Food Mycology workshop, Freising, Germany, June 13-15, 2016)

Screening of lactic acid bacteria (LABs) and *Streptomyces* strain (AS1) for efficacy against food spoilage fungi

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INTRODUCTION

- Malaysian fermented foods are considered healthy as they consist of antimicrobial compounds such as lactic acid and bacteriocins which are naturally produced by lactic acid bacteria (LABs).
- There is interest to study LAB strains isolated from Malaysian fermented food as no previous studies have screened these strains for inhibition of growth and mycotoxin production by spoilage fungi.
- Streptomyces* AS1 has been isolated from peanuts and was found to produce anti-fungal compounds which had efficacy against growth of *Aspergillus flavus* and aflatoxin B₁ production (Sultan & Magan, 2013).
- However this *Streptomyces* strain and its metabolites have not previously been screened against other spoilage moulds.

OBJECTIVE: To screen 66 lactic acid bacteria (LABs) from Malaysian fermented foods and a *Streptomyces* strain (AS1) isolated from peanuts for efficacy against *F. verticillioides*, *P. nordicum*, *P. verrucosum* and *A. flavus*.

MATERIALS AND METHOD



RESULTS

Table 1. Antifungal activity of LAB strains against fungal pathogens using agar well diffusion method

LAB strains	<i>A. flavus</i>	<i>F. verticillioides</i>	<i>P. verrucosum</i>	<i>P. nordicum</i>
Lb (34 strains)	-	+(3/34)	-	-
Lc (7 strains)	-	-	-	-
Ln (4 strains)	-	-	-	-
Pd (21 strains)	-	+(2/21)	-	-

-- no inhibition zone, + : 1-4 mm inhibition zone, ++ : 5-8 mm inhibition zone and +++ : >8 mm inhibition zone.
Key to strains: Lb: *Lactobacillus*; Lc: *Lactococcus*; Ln: *Leuconostoc*; Pd: *Pediococcus*;

Table 2. Antifungal effect of LABs against *F. verticillioides* using agar overlay method

LAB strains	-	+	++	+++
<i>Lactobacillus</i>	1	8	23	2
<i>Lactococcus</i>	0	3	4	0
<i>Leuconostoc</i>	1	0	3	0
<i>Pediococcus</i>	1	2	16	2

Table 3. Inhibition of selected LABs against *A. flavus* and *P. verrucosum* using agar overlay method

Lactic acid bacteria	<i>A. flavus</i>	<i>P. verrucosum</i>
<i>L. plantarum</i> strain MCC 2156 (VN5)	+	+
<i>L. plantarum</i> strain HT-W104-B1 (VS1)	+	+
<i>P. acidilactici</i> 1498 (B4)	+	+
<i>P. pentosaceus</i> 1426 (P5)	+	+

-- no suppression, + = no fungal growth on 0.1-10% of the plate area per bacterial streak, ++ = no fungal growth on 11-20% of the plate area per bacterial streak, +++ = no fungal growth on >20% of the plate area per bacterial streak.

CONCLUSIONS

- Some LAB strains and metabolites of the *Streptomyces* AS1 strain had high efficacy against some mycotoxigenic fungi.
- Studies are in progress to examine the relative concentrations (LD₅₀/LD₉₀) of the metabolites necessary to inhibit germination, growth and mycotoxin production (aflatoxin B₁, ochratoxin A, fumonisin B₁).

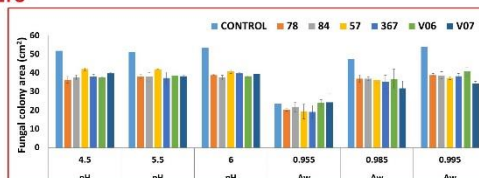


Figure 1. Effect of different LAB strains at different water activity (a_w) and pH on *A. flavus* colony area

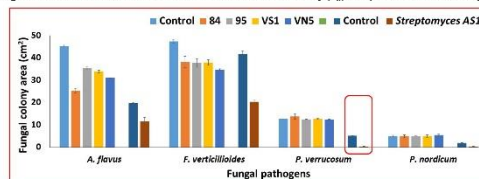


Figure 2. Effect of LABs and *Streptomyces* in dual-culture on inhibition of fungal colony area

Table 4. Effect of interaction between LABs, *Streptomyces* and fungal pathogen on interaction scores and I_0

Bacteria	Interaction scores				Index of Dominance (I_0)
	<i>A. flavus</i>	<i>F. verticillioides</i>	<i>P. verrucosum</i>	<i>P. nordicum</i>	
<i>L. plantarum</i> strain MCC 2156	1/1	1/1	1/1	1/1	4/4
<i>L. plantarum</i> strain HT-W104-B1	1/1	1/1	1/1	1/1	4/4
<i>P. acidilactici</i> 1498	1/1	1/1	1/1	1/1	4/4
<i>P. pentosaceus</i> 1426	1/1	1/1	1/1	1/1	4/4
<i>Streptomyces</i> AS1	5/0	5/0	5/0	5/0	20/5

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This work was supported by Malaysian Agricultural Research and Development Institute and Ministry of Agriculture