Detoxification of aflatoxin B_1 and T-2 by probiotic yoghurt bacteria

---

A thesis

submitted in partial fulfilment

of the requirements for the Degree of

Master of Applied Science (Food Science)

at

Lincoln University

by

Walter Odhiambo Ondiek

Lincoln University

2017

Abstract of a thesis submitted in partial fulfilment of the
requirements for the Degree of Master of Applied Science (Food Science)

Detoxification of aflatoxin B_1 and T-2 by probiotic yoghurt bacteria

by

Walter Odhiambo Ondiek

Certain strains of lactic acid bacteria (LAB) have been shown to be able to detoxify some mycotoxins. Different LAB species such as *Lactobacillus casei* and *L. acidophilus* are used as probiotics in several products including yoghurt, cheese, buttermilk, and frozen desserts. Limited work has been done to isolate LAB from foods such as probiotic-enriched yoghurts or fermented dairy foods to assess their ability to detoxify aflatoxin B_1 (AFB_1) and trichothecene-2 toxin (T-2). The objectives of this project were to (1) isolate *L. casei shirota* from a fermented milk drink (FMD) and *L. acidophilus, L. bulgaricus and Streptococcus thermophilus* from a conventional yoghurt (CY), (2) determine the viability of these LAB species when incubated with AFB_1 (5, 10, 20, 50 and 100 µg/L) and T-2 (20, 50, 100, 200 and 250 µg/L), (3) determine the capacity of these LAB species (10^9 cells/mL) to detoxify AFB_1 and T-2, and (4) compare the detoxification rate between live (non-heat-treated) and denatured (heat-treated) cells of *L. casei shirota*. A pure commercial *L. acidophilus* AS1.3342 culture was used as a positive control. *L. acidophilus* AS1.3342 and the food-derived *L. casei shirota* were used singly whereas *L. acidophilus, L. bulgaricus and Streptococcus thermophilus* isolated from CY were used as a mixture. All bacteria were able to detoxify AFB_1 and T-2 but there were no significant differences between the bacteria in their capacity to detoxify the toxins. The toxin removed [both in absolute quantity (µg/L) and as a percentage] by live cells significantly increased as the toxin concentration increased in spite of the bacterial cell viability declining with exposure to increase in toxin concentrations. Live cells of *L. casei shirota* detoxified 11–43% of AFB_1, 19–38% of T-2; *L. acidophilus* AS1.3342, 10–46% AFB_1 and 15–45% T-2; and the mixture of live CY strains, 14–43% AFB_1 and 15–45% T-2. The absolute quantity of toxin removed by denatured cells also significantly increased with increase in toxin concentration. Expressed as a percentage, denatured cells detoxified more of the toxins (*L. casei shirota* detoxified 48–62% AFB_1

and 42–53% T-2) compared with exposure to live bacterial cells, lending support to the hypothesis that denatured bacterial cells are able to remove more toxins than live cells possibly due to the higher binding of toxins to the cell membrane of non-viable cells. These findings show that bacteria in probiotic milk foods, irrespective of whether live or denatured, can bind significant amounts of AFB_1 and T-2, especially when exposed to higher toxin concentrations, and thereby markedly reduce exposure of consumers to these mycotoxins.

Keywords: Aflatoxin B_1, Trichothecene-2 toxin, *Lactobacillus casei shirota*, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus, Streptococcus thermophilus*, probiotics, fermented milk drink, conventional yoghurt, liquid chromatography-tandem mass spectrometry

Abbreviations:

AFB_1	Aflatoxin B_1
Cfu	Colony-forming unit
°C	Degrees Celsius
CY	Conventional yoghurt
FMD	Fermented milk drink
GAP	Good Agricultural Practice
GMP	Good Manufacturing Practice
GSH	Glutathione
LAB	Lactic acid bacteria
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MRS	De Man-Rogosa Sharpe
PBS	Phosphate-buffer saline
T-2	Trichothecene-2 toxin
ZEN	Zearalenone

Acknowledgements

I would like to take this opportunity to express my sincere gratitude to all those who contributed towards the success of this thesis. First and foremost, I would like to acknowledge the financial and the general support that I received from my sponsor; the New Zealand Agency for International Development (NZAID). I am really grateful for the agency for supporting my Masters studies. Through the NZAID scholarship, I have been able to acquire new knowledge, useful networks and new perspectives, which will definitely help me make a positive contribution to the economic development of my country and to a larger extent make a contribution towards realisation of the broader objectives of the scholarship. Special appreciation to Sue Bowie, representative for NZAID at Lincoln University, for the overall support during my stay in New Zealand and for supporting my request to undertake this research in China. Thanks so much, Sue.

From the onset to the end of my thesis, I have received unwavering support from Prof. Ravi Gooneratne. My sincere gratitude goes out to him for investing time in developing my research skills, presentation skills, writing skills and so much more. It is worth mentioning the effort Ravi put in working out the logistics of this research in China and being there in person to ensure that I got all the support I needed to succeed in data collection. Thanks so much and may your effort get rewarded sooner. My gratitude also goes to my associate supervisors Assoc. Prof. Steven On and Dr Haotian Zheng for offering insighful perspective to my thesis at the proposal stage and thesis writing stage. Thanks so much for critically analysing and offering constructive suggestions.

I would also like to pass my gratitude to Prof. Lijun Sun and Prof. Yaling Wang (College of Food Science & Technology, Guangdong Ocean University (GDOU), China) for offering guidance during data collection. I would also like to pass my appreciation to their students who supported me in the laboratory and especially to the PhD students Deng Qi and Rundong Wang who visited Ravi's laboratory at Lincoln University for 6 weeks in 2016. My sincere appreciation for the material support and for allowing this research to be carried out in their laboratories. I am delighted to have been the pioneer student under the research collaboration between the Department of Wine, Food & Molecular Biosciences of Lincoln University, New Zealand and GDOU (College of Food Science & Technology, College of Agriculture and Overseas student support office at GDOU, Zhanjiang, China). My appreciation also goes to Prof. Jinjun Chen (Dean, College of Agriculture, GDOU, China) for his humour and helping me network and integrate with the commmunity. This was very useful in helping me overcome some of the hurdles including the language barrier.

Thanks to the Lincoln University library team for the useful tips on literature review, proposal writing, data analysis and thesis writing. It is not possible to list everyone who contributed towards the success of this thesis but whatever role you played, please note that your input is highly appreciated.

Finally, I would like to pass my gratitude to my wife Rispah Akinyi and my twin daughters Gaby and Natalin for supporting and cheering me throughout my study. Sometimes the going proved tough but through your words and encouragement, we conquered.

Table of Contents

List of Tables

List of Figures

Chapter 1

Introduction

Mycotoxins are secondary metabolites of fungi and are commonly found in agricultural produce. They can cause teratogenicity, carcinogenicity, and oestrogenic or immune-suppressive effects in animals and humans, and overall economic loss (Bhat, Rai, & Karim, 2010; Lahtinen, Haskard, Ouwehand, Salminen, & Ahokas, 2004; Woloshuk & Shim, 2013). Major mycotoxins include aflatoxins, deoxynivalenol, fumonisins, zearalenone, T-2, ochratoxin and certain ergot alkaloids (Richard, 2007). Aflatoxins are produced by *Aspergillus flavus* and *A. parasiticus* while T-2 is produced by *Fusarium graminearum* and related species. These toxigenic fungi are ubiquitous in nature and can occur at pre-harvest, at harvest or can grow saprophytically on plants and stored agricultural products (Binder, Tan, Chin, Handl, & Richard, 2007; Jouany, 2007). The most susceptible products include cereals, peanuts, figs, peas, sunflower seeds, sesame seeds, pistachios, and almonds (Logrieco, Bottalico, Mulé, Moretti, & Perrone, 2003). Aflatoxin contamination tends to predominate in the tropical and subtropical countries (Pitt, 2000), but can also grow in the temperate regions of America, Europe and Asia, with *Fusarium* fungi identified as the most prevalent toxin-producing fungi commonly found on cereals such as oats (Creppy, 2002). Both aflatoxin and T-2 can directly or indirectly lead to human toxicity. Direct intoxication occurs when the contaminated foods are ingested by humans, while indirect intoxication can arise from mycotoxin residues in animal products as a result of usage of contaminated livestock feed (Zain, 2011).

Several types of aflatoxins have been identified. However, the main naturally produced aflatoxins with economic and health impacts include aflatoxin B_1, B_2, G_1, and G_2 (Yang et al., 2014). Among the group, AFB_1 is listed as a Group 1 carcinogen and is the most important due to significantly increased risk of hepatocellular carcinoma in humans exposed to AFB_1 (International Agency for Research on Cancer, 2002). T-2 causes a variety of toxic effects in experimental animals and livestock and is also known to cause toxic effects in human. The major effects of T-2 include inhibition of protein synthesis in several cellular systems, followed by secondary disruption to DNA and RNA synthesis (Chen et al., 2008; Zou et al., 2012). T-2 has been shown to inhibit synthesis of DNA (at exposure to 750 µg/kg body weight single or multiple doses) and RNA (at doses > 100-1000 µg/mL) in both in vivo and in vitro tests (EU, 2001).

According to Codex Alimentarius (2014), strategies for the reduction of mycotoxins in cereals consist of two approaches, namely, recommended practices based on Good Agricultural Practice (GAP) and Good Manufacturing Practice (GMP) while Hazard Analysis Critical Control Point principles are applied

as a complementary management system. In addition to Codex recommendations, institutionalisation of mycotoxin regulations has been adopted to manage mycotoxins in the food chain. By the end of 2003, around 100 countries (covering approximately 85% of the world's inhabitants) had specific regulations or detailed guidelines for limiting mycotoxins in food (van Egmond, Schothorst, & Jonker, 2007). Despite the Codex recommendations and institutionalisation of mycotoxin regulations, it is still not possible to totally preclude mycotoxin contamination. This is because the toxigenic fungi are ubiquitous in nature and most critical factors for mycotoxin production are extrinsic in nature and as such there are continued reports of incidences of mycotoxin contamination in various food commodities, particularly in the tropics and subtropics where high ambient humidity makes the control of commodity moisture difficult (Codex Alimentarius, 2014; Matumba, Van Poucke, Ediage, & De Saeger, 2015). Additionally, institutionalisation of mycotoxin regulation has not been successful due to the high prevalence of informal markets, especially in developing countries. According to ICRISAT, Ananth, and Farid (2015), aflatoxin poisoning in humans has been reported in several countries including India, China, and several African countries. For example, in a case study involving 3180 human immunodeficiency virus-negative specimens to quantify aflatoxin exposure across Kenya, serum AFB_1-lysine was detected in 78% of the samples (Yard et al., 2013). So, it appears that aflatoxin exposure is a public health problem throughout Kenya and this is probably true for many African countries. In China, a case study by Wang et al. (2001) on hepatocellular carcinoma and aflatoxin exposure, in Zhuqing Village of Fusui County, detected a considerable amount of AFB_1-albumin adducts in the serum, and in 88.9% of the subjects, AFB_1 metabolites were observed in the urine. According to the EU (2003), T-2 is a common contaminant in cereal samples from EU member states, and therefore EU member populations were at risk of dietary exposure to *Fusarium* toxins including T-2, and the most frequently contaminated cereal samples were corn (28 %), wheat (21 %) and oat (21 %). In its 2013 review, the EU Commission, through the scientific panel on contaminants in the food chain of the European Food Safety Authority (EFSA), indicated that estimates of chronic human dietary exposure to the sum of T-2 and HT-2 (a metabolite of T-2) were below the tolerable daily intake for populations of all age groups, and thus not an immediate health concern. However, the Commission acknowledged that there was large year-to-year variation in occurrence of T-2 and HT-2 in cereals (EU, 2013).

In order to decontaminate mycotoxin-contaminated food or feed, and minimise exposure to mycotoxins and reduce health risks, physical, chemical and biological methods have been used. Physical methods such as sorting, trimming, cleaning, milling, cooking, canning, flaking, and extrusion have been established to reduce mycotoxin concentrations but these methods do not eliminate them completely and hence can lead to a potential mycotoxin contamination of processed foods (Bullerman & Bianchini, 2007). Chemical means include use of alkaline compounds such as ammonia to detoxify the mycotoxins. Chemical treatments reduce mycotoxin concentration significantly but many of these

chemicals are toxic and also cause a loss of some nutrients (Bata & Lasztity, 1999). Owing to these limitations, biological methods have been pursued as the most promising safe option for decontamination of mycotoxin-contaminated food and feed (Bata & Lasztity, 1999; Kabak, Dobson, & Var, 2006). It was reported that binding of mycotoxins by LAB from fermented foods and by LAB present in the gastrointestinal tract could contribute to a decrease in toxin bioavailability (Niderkorn, Morgavi, Aboab, Lemaire, & Boudra, 2009).

Despite the great potential benefits that LAB may hold towards reducing human exposure to mycotoxins, only a handful of work has been done on isolation of LAB from probiotic foods to assess their ability to detoxify various concentrations of AFB_1 and T-2.

1.1 Aims and objectives

The major aim of this project was to determine if bacteria isolated from probiotic-enriched yoghurt (*L. casei shirota* from fermented milk drink (FMD) and *L. acidophilus, L. bulgaricus* and *Streptococcus thermophilus* from yoghurt (CY)) are capable of detoxifying AFB_1 and T-2. A secondary aim was to provide data from this experiment as a reference for use of probiotic-enriched foods, such as yoghurt, in future clinical trials to remove mycotoxins from the human digestive system.

The objectives of this project were to:

(1) Isolate *L. casei shirota* from FMD yoghurt and *L. acidophilus, L. bulgaricus and Streptococcus thermophilus* from CY yoghurt

(2) Determine the capacity of these LAB species (10^9 cells/mL) to detoxify various concentrations of AFB_1 (5, 10, 20, 50 and 100 µg/L) and T-2 (20, 50, 100, 200 and 250 µg/L)

(3) Compare the detoxification rate between live and denatured cells of *L. casei shirota*

(4) Determine the viability of LAB species when incubated with AFB_1 (5, 10, 20, 50 and 100 µg/L) and T-2 (20, 50, 100, 200 and 250 µg/L).

1.2 Research hypotheses

i. LAB (*L. casei shirota, L. acidophilus, L. bulgaricus* and *Streptococcus thermophilus*) isolated from probiotic-enriched yoghurts will detoxify AFB_1
ii. LAB (*L. casei shirota, L. acidophilus, L. bulgaricus* and *Streptococcus thermophilus*) isolated from probiotic-enriched yoghurts will detoxify T-2.

1.3 Study approach

This research was conducted at Guangdong Ocean University (GDOU), China; courtesy of research collaboration between the College of Food Science & Technology, Guangdong Ocean University, China, and the Department of Wine, Food & Molecular Biosciences of Lincoln University, New Zealand. The research objectives, aims, hypothesis, and methodology were developed in accordance with the house rules for the study of Masters at Lincoln University (Lincoln University, 2016). The input of the external advisers (College of Food Science & Technology, Guangdong Ocean University, China) was also taken into consideration. Prior to commencement of the study, the proposal was presented as a seminar to the Faculty of Agricultural and Life Sciences, Lincoln University, and also the College of Food Sciences, GDOU, attended by both staff and postgraduate students. Suggestions that came out of these two seminars and the discussions with the supervisors were taken into consideration before the final draft was written and submitted for approval by the Research Committee of the Faculty of Agriculture and Life Sciences, Lincoln University. Potential health and safety hazards that may arise during experimentation were identified and appropriate control measures were taken to manage the hazards prior to the commencement of the research (Table A1). Thesis writing was done in accordance with the house rules for the study of Masters at Lincoln University (Lincoln University, 2016).

The research involved three crucial phases: sampling and isolation of the bacteria, toxin extraction, and toxin assay. The first two phases (LAB isolation and toxin extraction) were conducted in the laboratories of the College of Food Science & Technology, Guangdong Ocean University, China. Isolation of LAB was done in the Food Microbiology laboratory while toxin extraction was performed in the Toxicology laboratory at GDOU University (Figs C1–C11). The last phase (toxin assay) was conducted at the National Marine Products Quality Supervision & Inspection Centre, Zhanjiang, China.

1.4 Chapter outline

This thesis has six chapters: Introduction, Literature Review, Materials and Methods, Results, Discussion and Conclusion.

Chapter 1 gives an introduction to the project, which covers an overview of the thesis and problem statement, aims and objectives, research hypothesis, and study approach and study area context.

In Chapter 2, a detailed literature review is presented to provide the reader with an in-depth understanding of the subject. The literature review covered a variety of topics including definitions, ecology and major groups of mycotoxins, aflatoxins and trichothecene toxins including T-2, aflatoxin toxicoses, T-2 toxicoses, aflatoxin and T-2 metabolism, and prevention and control of aflatoxin and T-

2 contamination. It also covered LAB, their characteristics and usage in food, recent studies on the use of LAB to detoxify mycotoxins and mode of mycotoxin removal by the bacteria.

Chapter 3 describes materials and methods. Materials, chemicals and all reagents that were used in this research such as samples, media, chemicals and toxins are identified. It also covers methodology such as isolation and identification of LAB, preparation of LAB working cultures, preparation of AFB_1 and T-2 standard solutions, toxin extraction and assay of the toxins. This chapter also describes the protocol for viability testing. Finally, the chapter highlights the methodology for data analysis, the software used to conduct statistical analysis, and the form in which the analytical results are presented.

Chapter 4 presents the results. The quantities of AFB_1 and T-2 detoxified by the bacteria are presented as absolute amount and percentages in a graphical format and also summarised in text. The bacterial viability results are presented in a table format and summarised in text.

In Chapter 5, the results are discussed, interpreted and compared with previous findings. The significance of the data and the new knowledge generated from this study is presented here.

Chapter 6 presents the conclusion. This chapter summarises key points including the performance of food-isolated probiotic bacteria in detoxifying AFB_1 and T-2. Finally, a suggestion is made on how the research findings could be used in the future.

Chapter 2

Literature Review

2.1 Definition, major groups of mycotoxins and ecology

The term mycotoxin has been defined in the literature in a variety of ways. For example, FAO (2016, p. 1) defines mycotoxins as ''toxic secondary metabolites of fungi belonging, essentially, to the *Aspergillus*, *Penicillium* and *Fusarium* genera''. Diaz, Whitlow, and Hagler (2013) define a mycotoxin as a fungal metabolite that causes an undesirable effect when animals or humans are exposed through consumption of contaminated feedstuffs/foods or through airborne exposure. The US Food and Drug Administration Office of Regulatory Affairs (2013) defines mycotoxins as natural poisons produced by fungi as secondary metabolites, which can occur in food due to mould growth during harvest or storage. Mycotoxins have also been defined as natural products of fungi, which cause toxic effects to higher vertebrates and other animals in low concentration when introduced through natural routes (Chelkowski, 2014). Despite the variation in definition, in principle, they all refer to mycotoxins as secondary metabolites produced by fungi and that these secondary metabolites have potential to cause toxic effects to humans and animals to a certain degree when consumed (Taevernier, Wynendaele, De Vreese, Burvenich, & De Spiegeleer, 2016).

The predominant toxigenic fungal flora include the following genera: *Aspergillus*, *Fusarium*, and *Penicillium* species (Kabak et al., 2006). These toxigenic fungi are ubiquitous in nature and can occur at pre-harvest, during harvesting or can grow saprophytically on plants and stored agricultural products such as cereals, nuts, coffee, cocoa, oilseeds, grapes, wines and vine fruits, barley, dried peas and beans (Jouany, 2007; Logrieco et al., 2003). Growth of toxigenic fungi in agricultural produce can result in production loss and hence economic losses, as well as production of mycotoxins, with possible development of mostly chronic effects in animals and humans when consumed. Chronic effects may include teratogenic, carcinogenic, oestrogenic or immune-suppressive effects and sometimes death (Binder et al., 2007). Mycotoxin contamination can lead to food poisoning by direct ingestion of contaminated food by humans or indirectly by ingestion of mycotoxin residues in animal products as a result of usage of contaminated livestock feed (Zain, 2011). *Aspergillus*, *Fusarium* and *Penicillium* are responsible for the production of five major kinds of mycotoxins: aflatoxins, fumonisins, ochratoxin, zearalenone (ZEN) and trichothecenes including T-2 and deoxynivalenol (DON) (Murphy, Hendrich, Landgren, & Bryant, 2006; Richard, 2007). Aflatoxins are produced by *Aspergillus flavus* and *A. parasiticus,* while trichothecenes are produced by *Fusarium graminearum* and related species (Pitt, 2000). However, aflatoxin contamination tends to predominate in tropical and subtropical countries –

areas with a hot and humid climate that are favourable for the growth of moulds – than in temperate regions (Pitt, 2000). In the temperate regions of America, Europe and Asia, *Fusarium* has been identified as the most prevalent toxin-producing fungi because it can tolerate lower temperatures than *Aspergillus* fungi (Creppy, 2002) and are commonly found on cereals.

Toxigenic fungi are known to grow over a wide range of pH (between 4 and 8), temperature (between 10 and 40°C), and water activity (a_w; usually at levels above 0.70) (Bhat et al., 2010). However, interactions of several factors influence the nature and quantity of mycotoxins produced by fungi. These factors include nutritional composition of substrate, moisture content, temperature, humidity in the surrounding environment, maturity of the fungal colony, co-occurrence with other fungi, competition from other microorganisms, stress factors, physical damage of the substrate due to insect activity, and other associated factors (Abbas, Valez, & Dobson, 2009; Bhat et al., 2010). According to FAO (2008), some of these factors, particularly those related to climatic change such as increase in global temperature and humidity, could have a major impact on fungal ecology and growth, persistence and patterns of occurrence and changes in the incidence and intensity of plant and pest infestations in the near future, and all these factors could impact on mycotoxin production. Aflatoxin and trichothecene contamination of agricultural staples such as wheat, barley and maize during fungal colonisation is an increasingly common problem possibly because of expanded use of 'no-till farming' and changing climatic patterns. Since food and feed contamination by aflatoxins and trichothecenes has been associated with human and animal toxicoses, serious questions remain to be answered regarding assessment of potential risks from ingesting-food borne aflatoxins and trichothecenes, and how these should be regulated and degraded or detoxified.

2.2 Aflatoxins

Several types of aflatoxins have been identified. However, the main naturally produced aflatoxins of economic and health impact include aflatoxin B_1, B_2, G_1, and G_2 (Yang et al., 2014). Aflatoxins B_1 (AFB_1) and B_2 are produced by *Aspergillus flavus* and *A. parasiticus* while aflatoxins G_1 and G_2 are produced by *A. parasiticus* (Gnonlonfin et al., 2013). 'B' and 'G' refer to the blue and green fluorescent colours produced by these compounds under UV light on thin layer chromatography plates, whereas the subscript numbers 1 and 2 indicate major and minor compounds (Bennett & Klich, 2003; Sweeney & Dobson, 1998). Fig. 1 shows the molecular structure of AFB_1.

Toxicity caused by consumption of aflatoxin-contaminated food is called aflatoxicosis, which can occur as acute and/or chronic toxicity in both humans and animals (Li, Yoshizawa, Kawamura, Luo, & Li, 2001). Aflatoxin-induced acute toxicity is often characterised by exposure to high doses over a short period and has a rapid onset and toxic effect including sudden death (Nyikal et al., 2004). Chronic

toxicity is more common and is characterised by low-dose exposure over a long period, which can generally result in long-term irreversible effects including cancer (Bennett & Klich, 2003). According to the International Agency for Research on Cancer (2002) , evaluation of previous studies of carcinogenicity of aflatoxins in experimental animals showed that there was strong evidence for AFB_1-induced carcinogenicity in animals. Oral administration of aflatoxin mixtures and AFB_1 to several strains of rats, hamsters, salmon, trout, ducks, tree shrews and monkeys was found to induce benign and malignant hepatocellular tumours. Specifically, Wogan, Edwards, and Newberne (1971) showed that AFB_1 dosed by stomach tube to rats at 1.5 mg/rat, given 5 days a week for 8 weeks, induced hepatocellular carcinoma. Again, based on statistically and significantly increased risks for hepatocellular carcinoma in humans exposed to aflatoxins, AFB_1 is now listed as a Group 1 carcinogen by the International Agency for Research on Cancer. In addition, the risk for hepatocellular carcinoma is elevated in areas where hepatitis B virus infection is endemic such as in the African continent (Lewis et al., 2005).

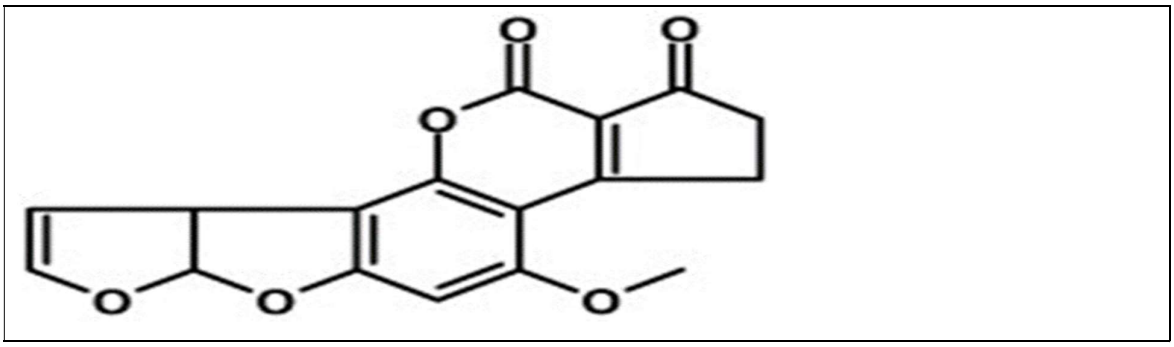

Fig. 1: Structure of aflatoxin B_1. Adapted from Richard (2007).

2.3 T-2

As many as 160 trichothecenes have been identified and are classified into four main groups according to their chemical structure, namely type A, B, C, and D (Bhat et al., 2010). One common feature among trichothecenes is the presence of a tetracyclic sesquiterpenoid 12,13-epoxytrichothec-9-ene ring (Fig. 2), with the 12,13-epoxy ring being responsible for its toxicological activity (Li et al., 2011). Type A trichothecenes have a functional group other than a ketone at position C-8, Type B a ketone at position C-8, Type C a second epoxy group at C-7, 8 or C-9, 10, and Type D a macrocyclic ring between C-4 and C-5 with two ester linkages (Sweeney & Dobson, 1998). Major toxins under Type A trichothecenes are T-2 and HT-2 but T-2 is the most important because it is considered to be the most toxic among the trichothecenes (Zou et al., 2012). Fig. 2 shows the chemical structures of Type A trichothecene and several other trichothecenes (Types B, C & D).

T-2 and HT-2 are commonly found in cereal grains such as wheat, oats and maize as well as in animal feed and human foods produced from contaminated grains (He, Zhou, Young, Boland, & Scott, 2010) and these create a food safety risk. T-2 causes a variety of toxic effects in experimental animals, livestock and also humans. The major effects of T-2 toxin include inhibition of protein syntheses in several cellular systems, followed by secondary disruption of DNA and RNA synthesis (Chen et al., 2008; Zou et al., 2012). Specifically, in both in vivo and in vitro tests, T-2 toxin has been shown to inhibit synthesis of DNA (at exposure to 750 µg/kg body weight single or multiple doses) and RNA (at exposure doses > 100–1000 µg/mL) (EU, 2001). Other toxic effects include vomiting, diarrhoea, lethargy, weight loss, anorexia, haemorrhage, immune-suppression, induction of apoptosis and necrosis of the epithelium of stomach and intestine, bone marrow, spleen, testis and ovary (EU, 2003; Li et al., 2011).

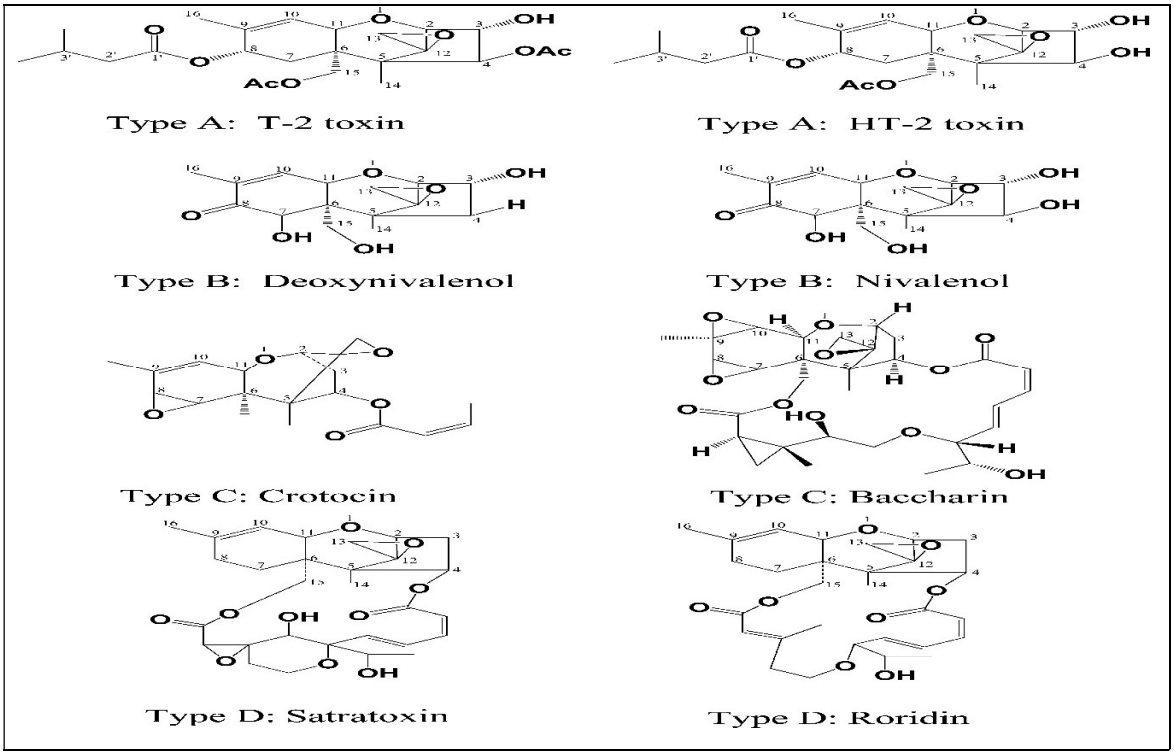

Fig. 2: Structures of type A trichothecenes (T-2 and HT-2 toxins) and other trichothecenes (types B, C, and D). Adapted from Li et al. (2011).

2.4 Aflatoxin B_1 toxicoses

AFB_1 contamination has been reported in many countries, especially in tropical and subtropical regions where conditions of temperature and humidity are conducive for maximal fungal growth and subsequent toxin production. According to ICRISAT et al. (2015), aflatoxin poisoning in human has been reported in several countries including India, China, and several African countries. For example, in April 2004, an acute outbreak of aflatoxin contamination of locally grown maize occurred in Eastern and

Central regions of Kenya, which resulted in 317 reported cases and 125 deaths (Nyikal et al., 2004). Following the outbreak, a cross-sectional survey was conducted by Lewis et al. (2005) to assess the extent of market maize contamination and to evaluate the relationship between market maize aflatoxin and the aflatoxicosis outbreak. It was established that 55% of maize products had aflatoxin levels greater than the Kenyan regulatory limit of 20 µg/kg, 35% had levels > 100 µg/kg, and 7% had levels > 1,000 µg/kg. In another case study to quantify aflatoxin exposure across Kenya, among 3180 immunodeficiency virus-negative subjects, AFB_1-lysine was detected in 78% of 600 serum specimen (Yard et al., 2013). The study showed that aflatoxin exposure was a public health problem throughout Kenya, which could impact human health and this is probably true for many African countries. In China, a case study by Wang et al. (2001) on hepatocellular carcinoma and aflatoxin exposure in Zhuqing Village, Fusui County, a considerable amount of AFB_1-albumin adducts in the serum and AFB_1 metabolites were observed in the urine of 88.9% of the subjects. Similarly, in Guangxi Province of China, a comparative study on the natural occurrence of aflatoxins and *Fusarium* toxins was conducted with corn samples in high- and low-incidence areas of human primary hepatocellular carcinoma (Li et al., 2001). Results showed that AFB_1 was the predominant toxin detected in terms of quantity and frequency. The study also reported that 13 samples (76%) exceeded the Chinese regulation of 20 µg/kg for AFB_1 in corn and corn-based products intended for human consumption. In India, Reddy, Reddy, and Muralidharan (2009) detected *Aspergillus* spp. and AFB_1 in rice from 20 states and reported that out of 1200 samples, 2% showed AFB_1 contamination above the Indian permissible limit of 30 µg/kg.

2.5 T-2 toxicoses

Contamination of cereal by T-2 and HT-2 is widely reported in the scientific literature. A study by Kassim et al. (2011) on T-2 and HT-2 in cereals sold in traditional markets in Gyeongnam Province of South Korea revealed that out of 75 samples analysed, 13 and 25 samples were found to be contaminated with T-2 (35.2–431.0 µg/kg) and HT-2 toxins (21.1–442.7 µg/kg) respectively and four samples were found to be contaminated with both toxins. In a survey by Pettersson et al. (2011) on T-2 and HT-2 in oats and oat products from European oat mills (UK, Finland, Ireland, Poland and Germany), the mean values of T-2 and HT-2 were 94, 17, 11 and 293 µg/kg in oats, oat flakes, oat meal and oat by-products respectively. However, it was established that during processing, the toxins were reduced by 82–88%. However, in the same survey, a significant increment of T-2 was observed in oat by-products. Hussein, Franich, Baxter, and Andrew (1989) investigating naturally occurring *Fusarium* toxins in New Zealand maize concluded that T-2, DON, diacetoxyscirpenol, and ZEN mycotoxins were prevalent in healthy standing crops as well as in stored maize. Some of these readings were significantly above the maximum tolerated limit for T-2 toxin (100 µg/kg) that has been set by a majority of countries (FAO, 2004).

According to the EU (2003), T-2 is a common contaminant in cereal samples from EU member states, and therefore EU member populations were at risk of dietary exposure, and the most frequently contaminated cereal samples were corn (28%), wheat (21%) and oats (21%). In its 2013 review, the EU Commission, through the scientific panel on contaminants in the food chain of the European Food Safety Authority (EFSA), indicated that estimates of chronic human dietary exposure of the EU community to the sum of T-2 and HT-2 was below the tolerable daily intake for populations of all age groups, and thus was not regarded as an immediate health concern. However, the EU Commission acknowledged that there was a large year-to-year variation in cereal T-2 and HT-2 concentrations (EU, 2013).

2.6 Metabolism of AFB_1 and T-2

The AFB_1 molecule is transported across the plasma membrane and is bio-transformed by microsomal mixed function monooxygenases to form highly reactive AFB_1-8-9- epoxide (Kirby et al., 1993). Bio-activated epoxide binds to nuclear DNA, causing nuclear damage, hepatotoxicity and carcinogenicity (Daniels, Liu, Stewart, & Massey, 1990). In vivo detoxification of AFB_1 can take place through conjugation of the reactive epoxide to glutathione (GSH). When Degen and Neumann (1978) injected AFB_1 into female Wistar rats, half of the dose was eliminated in bile as polar non-extractable metabolites. The main component of the metabolite was a GSH conjugate; identified as 2,3-dihydro-2-(*S*-glutathionyl)-3-hydroxy aflatoxin B_1 (AFB_1-GSH-conjugate). These findings clearly show that in vivo detoxification of AFB_1 involves conjugation of the reactive epoxide by GSH, which is excreted primarily through the bile. However, the amount of GSH in the liver is limited and also conjugate has the potential to be hydrolysed by the intestine microflora to release AFB_1 for reabsorption.

Another means of AFB_1 detoxification is hydroxylation, through monooxygenase-mediated biotransformation pathways to hydroxylated metabolites such as aflatoxin M_1 and aflatoxin Q_1 (Daniels et al., 1990). When AFB_1 is ingested by humans or animals, a small proportion is hydroxylated via phase I biotransformation into aflatoxins M_1 albeit with lower toxicity than the parent molecules (El-Nezami, Nicoletti, Neal, Donohue, & Ahokas, 1995).

Several studies, both in vitro and in vivo, have improved our understanding of T-2 biotransformation. Ohta, Ishii, and Ueno (1977) conducted a study to elucidate the active form of T-2 during tissue metabolism. It was apparent that T-2 was selectively hydrolysed by the microsomal esterase at C-4, giving rise to HT-2 as the only metabolite. The authors concluded that toxicity of T-2 administered to animals is also partly due to the metabolite HT-2. This was presumed because the toxicity of HT-2 is comparable to that of T-2 and that the microsomal fraction of liver possesses the capability to bio-transform the total T-2 dose to HT-2 within a few minutes. Additionally, an in vivo study by Visconti

and Mirocha (1985) on identification of various T-2 metabolites in chicken excreta and tissues noted that the majority of the T-2 metabolites were detected in the excreta, although the amount detected in the liver was also adequate to cause tissue toxicity especially to the liver. T-2 was found in trace amounts in the lungs but not in the heart or kidney. They detected several metabolites of T-2, namely HT-2, 15 acetoxy T-2 tetraol, and T-2 tetraol, 3'-hydroxy HT-2, 3'-hydroxy T-2, 4-acetoxy T-2 tetraol, trace amounts of 8-acetoxy T-2 tetraol, 3-acetoxy-3'hydroxy HT-2, and T-2 triol. Metabolism and elimination of T-2 is generally rapid after ingestion, with major metabolic reactions being hydrolysis, hydroxylation, de-epoxidation, and conjugation (Li et al., 2011).

2.7 Prevention and control of aflatoxins and T-2

According to Codex Alimentarius (2014), strategies for the reduction of mycotoxins in cereals consist of two approaches, namely, recommended practices based on GAP and GMP while Hazard Analysis Critical Control Point principles are applied as a complementary management system. GAP represents the first line of defence against contamination of food commodities with mycotoxins in the field and includes pre-harvest selection of hybrids, choice of time of planting, plant density and insect control (Codex Alimentarius, 2014). This is supplemented by the implementation of GMP during the handling, storage, and distribution of the food commodities. GMP includes minimising time between harvesting and drying, effective cleaning of cereal prior to storage, efficient drying of wet cereals for medium- and long-term storage, effective hygiene and management in store, absence of pests in store (which can provide metabolic water and initiate heating), clear specifications and traceability from field to store (Magan & Aldred, 2007). In addition to Codex recommendations, institutionalisation of mycotoxin regulations has been adopted as a means of managing mycotoxins in the food chain. By the end of 2003, about 100 countries (with approximately 85% of the world's inhabitants) had specific regulations or detailed guidelines for mycotoxins in food (van Egmond et al., 2007).

Despite all these regulations and preventive measures on the farm, it is still not possible to totally preclude mycotoxin contamination of food and feed because the toxicogenic fungi are ubiquitous in nature and most critical factors for mycotoxin production are extrinsic in nature (Codex Alimentarius, 2014; Matumba et al., 2015). Additionally, institutionalisation of mycotoxin regulation has not been successful, more so in developing countries, due to high prevalence of informal markets.

In order to prevent mycotoxin-contamination of food and feed and thereby minimise exposure to mycotoxins and reduce health risks, physical, chemical and biological methods have been tried but with little success (Bata & Lasztity, 1999). According to Bullerman and Bianchini (2007), physical methods include sorting, trimming, cleaning, milling, cooking, canning, flaking, and extrusion. These processes lower mycotoxin concentrations but do not reduce them significantly. Chemical means include the use of alkaline compounds such as ammonia to detoxify. Chemical treatments reduce mycotoxin concentration significantly but these chemicals are toxic and also degrade some nutrients (Bata & Lasztity, 1999). Owing to these limitations, biological methods have been pursued as the most promising option for decontamination of mycotoxin-contaminated food and feed (Bata & Lasztity, 1999; Kabak et al., 2006). It has been claimed that binding of mycotoxins by LAB from fermented foods and by LAB present in the gastrointestinal tract could contribute to a reduction in the toxin bioavailability (Niderkorn et al., 2009).

2.8 Lactic acid bacteria, characteristics and their use in food

Lactic acid bacteria are a group of Gram-positive, acid-tolerant, non-sporing, non-motile, catalase negative bacteria, and are either rod- or cocci-shaped. These are strictly fermentative bacteria which convert carbohydrates to lactic acid as the major end product (Teuber, 1993). Besides lactic acid, some heterofermentative strains produce acetic acid, ethyl alcohol and carbon dioxide (König & Fröhlich, 2009). Several genera of LAB have been identified and include species of the genera *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* (Stiles & Holzapfel, 1997). The cell wall of LAB has a cytoplasmic lipid membrane with polysaccharides and lipoteichoic acids (teichoic acids and lipoids), and protein subunits including surface layer proteins, commonly known as S-layer proteins, and is sheathed by a thick multi-layered peptidoglycan (Delcour, Ferain, Deghorain, Palumbo, & Hols, 1999; Nishiyama, Sugiyama, & Mukai, 2016). Thus, peptidoglycan is the main component of the LAB wall. It consists of glycan chains that are made of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid that are linked via β-1,4 bonds (Chapot-Chartier & Kulakauskas, 2014). The peptidoglycan layer helps the bacteria to maintain cell shape and integrity during growth and division and it also acts as the interface between the bacterium and its environment (Chapot-Chartier & Kulakauskas, 2014). According to Neuhaus and Baddiley (2003), teichoic acids are polymers of glycerol-phosphate or ribitol-phosphate attached to glycosyl and d-alanyl ester residues. Teichoic acids together with peptidoglycan make up a polyanionic matrix that provides cation homeostasis, regulation of autolysins, trafficking of

ions, nutrients, proteins, antibiotics, and presentation of envelope proteins (Neuhaus & Baddiley, 2003).

The bacterial S-layer is made up of either proteins or glycoproteins, with the protein subunits forming the porous lattice, which completely covers the cell (Beveridge et al., 1997). The S-layer is thought to provide several functions to bacteria. However, some of the documented functions include cell adhesion, protection from feeding by protozoa or phagocytes, virulence factor, antigenicity, anchoring sites for hydrolytic exo-enzymes, receptors for phages and porin function (Beveridge et al., 1997). Fig. 3 shows the structure of the lactic acid bacteria (Gram-positive) cell wall.

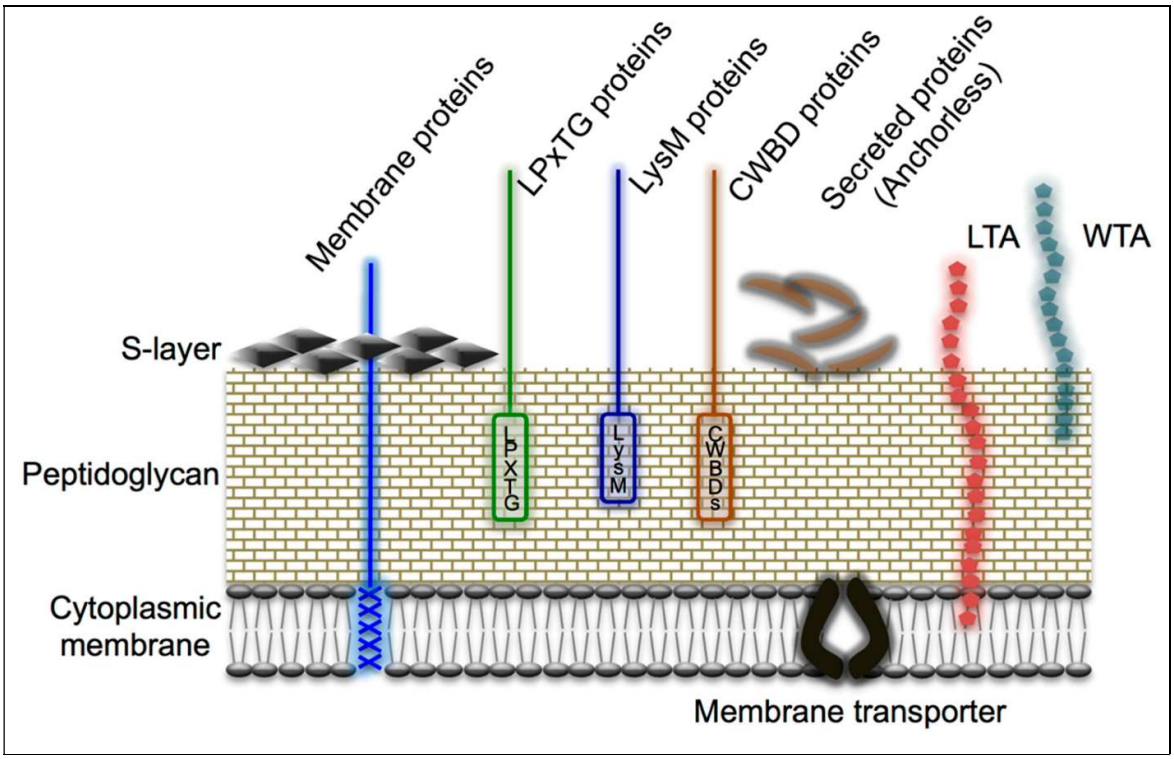

Fig. 3: Cell wall structure of Gram-positive lactic acid bacteria. The cell wall consists of a thick, multilayered peptidoglycan layer decorated with lipoteichoic acid, teichoic acid, and proteins including S-layer proteins. Adapted from Nishiyama, Sugiyama, & Mukai (2016).

Some of the LAB are of major economic importance to the food industry because they are widely used in food fermentation as natural preservatives and flavour enhancers in fermented foods such as milk, meats, vegetables and cereal products (Stiles & Holzapfel, 1997). Additionally, species of *Bifidobacteria* and *Lactobacilli* have been used as probiotics in various foods and are also found as a part of gastrointestinal microflora.

According to FAO/WHO (2001, p. 2), probiotic refers to 'live microorganisms which when administered in adequate amounts confer a health benefit to the host'. However, for microorganisms to be used as

a probiotic, they must meet the following criteria. They should (i) be generally recognised as safe (GRAS), (ii) be resistant to acid and bile salts in the gut and intestine, (iii) be capable of surviving passage through the digestive tract, and (iv) have the capability to adhere and proliferate in the gut (Lin, Hwang, Chen, & Tsen, 2006). Based on these characteristics, different species of *Lactobacillus,* such as *L. casei* and *L. acidophilus*, have been employed in the dairy industry as probiotics and incorporated in several products such as yoghurt, cheese, fermented milk drinks including buttermilk, and frozen desserts (Granato, Branco, Cruz, Faria, & Shah, 2010). Specifically, the *L. casei shirota* strain is used as a probiotic in a fermented milk drink (FMD) (Shah, 2007) while *L. acidophilus* has been used in conventional yoghurts. Recent studies, as elaborated on in sections 2.9.1 and 2.9.2, have demonstrated that a wide range of LAB strains can detoxify mycotoxins.

2.9 Recent studies on the use of lactic acid bacteria to detoxify mycotoxins

2.9.1 Aflatoxins

A wide range of LAB strains have been shown to possess the ability to remove aflatoxins specially from milk based media. El-Nezami et al. (1998), in their research on the ability of dairy strains of LAB to bind common food carcinogens including AFB_1, reported that binding was a rapid process because the percentage of AFB_1 residue in a few minutes was not significantly different from that at 72 h. At 0 h, viable cells (10^{10} cfu/ml) of *L. rhamnosus* strain GG and *L. rhamnosus* strain LC-705 removed up to 80% of AFB_1 and *L. acidophilus* was able to remove up to 68% of AFB_1 within 72 h. In the same study, the *L. casei shirota* strain bound up to 34% at 0 h and 58% at 72 h. In another case study by Hernandez-Mendoza, Garcia, and Steele (2009) on screening *L. casei* strains for their ability to bind AFB_1 in aqueous solutions, 14% to 49% of the available aflatoxin was bound. The authors noted that the amount of AFB_1 bound by *L. casei* was strain specific with the *L. casei* L30 strain significantly binding more than the several other strains examined. Separately, Peltonen, El-Nezami, Salminen, and Ahokas (2000) examined the ability of five strains of *Lactobacilli* and one strain of *Bifidobacterium* probiotic bacteria to bind AFB_1. The aflatoxin binding capacity of the two bacterial species was strain specific and the quantity bound ranged from 5.8% to 31.3%. Equally, the work by Hernandez-Mendoza, Guzman-De-Peña, González-Córdova, Vallejo-Córdoba, and Garcia (2010) on In vivo assessment of the potential protective effect of *L. casei shirota* against AFB_1, using 15 male Wistar rats, showed that AFB_1-Lys adducts quantified from blood samples were at significantly lower levels in animals receiving AFB_1 plus bacteria than in those receiving only AFB_1. The finding indicated that the presence of *L. casei shirota* had the ability to reduce aflatoxin absorption at the intestinal level even after a long period of toxin exposure.

2.9.2 Trichothecenes

Only limited studies have shown the abilities of various LAB strains to remove type A trichothecenes including T-2 toxin. This is in contrast to the enormous effort towards AFB_1 studies because of its prevalence and more serious toxic effects compared to T-2. Zou et al. (2012) reported some success on in vitro removal of DON and T-2 from a mixture of toxins by *Lactococcus lactis*, *L. brevis*, *L. casei*, and *L. plantarum.* The authors noted that all the bacterial strains removed the toxins to varying degrees but the ability of the *L. plantarum* strain was more than other three strains. The strain of *L. plantarum* LP102 was the strongest among the five tested strains at 24 h of fermentation with removal of 19.90 ± 1.70% of T-2 from a de Man-Rogosa Sharpe (MRS) broth. Thus authors clearly showed that T-2 removal was strain specific.

2.10 Mode of removal of mycotoxins by LAB

Following revelation that LAB were capable of removing mycotoxins from food and feed as well as reducing mycotoxin bioavailability, considerable effort has been directed at establishing the mode of mycotoxin removal by *Lactobacillus*. The effects of viable and heat-inactivated, acid- or alkaline-treated cells on mycotoxin removal have been investigated. El-Nezami, Polychronaki, Salminen, and Mykkanen (2002) cultured *L. rhamnosus* GG and *L. rhamnosus* LC705 with ZEN and its derivative ά-zearalenol and at 72 h of incubation no degradation products were observed in the HPLC chromatograms. This was an indicator that the strains used in the study were unable to metabolise either ZEN or its derivative ά-zearalenol. In the same experiment, both heat-killed and acid-treated bacteria were tested on their ability to remove ZEN and its derivative ά-zearalenol. The authors reported that both the heat-treated and acid-treated bacteria were capable of removing the toxins and binding increased significantly following the treatments. All these indicated that binding and not metabolism was the mechanism by which these mycotoxins were removed from the media.

Previously, Lahtinen et al. (2004) tried to establish the components of the cell envelope that are involved in the AFB_1 binding process. This was achieved by extracting exopolysaccharides and a cell wall isolate containing peptidoglycan from *L. rhamnosus* strain GG and testing for its AFB_1 binding properties. The *L. rhamnosus* strain GG was also subjected to various enzymatic and chemical treatments and their effects on the binding of AFB_1 by the bacteria were examined. The authors reported that there was no evidence for exopolysaccharides, cell wall proteins, Ca^{2+} or Mg^{2+} being involved in AFB_1 binding. AFB_1 binding was to the cell wall isolate indicating that AFB_1 was bound to the cell wall peptidoglycan of *L. rhamnosus* or compounds tightly associated with the peptidoglycan. It was also hypothesized that noncovalent binding was a possible mechanism of mycotoxin removal by LAB.The ability of LAB and *Saccharomyces cerevisiae* to remove AFB_1 from liquid medium was also

tested by Bueno, Casale, Pizzolitto, Salvano, and Oliver (2007). They noted that AFB_1 binding to microorganisms was rapid (no more than 1 min), and the binding involved formation of a reversible complex between the toxin and microorganism surface, without chemical modification of the toxin, and that both viable and heat-treated bacteria produced similar results. Based on these observations, the authors concluded that a physical binding (adsorption) and release (desorption) of AFB_1 to and from the site on the surface of the microorganism would most probably have taken place.

Despite the great potential benefits that LAB may hold towards reducing human exposure to mycotoxins, limited research has been published on isolation of LAB, from probiotic-enriched foods such as FMD and conventional yoghurt (CY), to assess their ability to detoxify various concentrations of AFB_1 and T-2.

Chapter 3

Materials and Methods

3.1 Materials and reagents

3.1.1 Samples

Reference culture of *L. acidophilus* AS1.3342 was purchased from a microbial culture collection center located in Southern China; Guangdong Microbial Culture Collection Center (GIMCC), Guangdong Institute of Microbiology, and stored at −20°C until use. Ten bottles of FMD and ten bottles of CY were purchased from a local supermarket, Zhanjiang, China, and transferred to the laboratory under refrigeration and stored at 4°C and used before the expiry date.

3.1.2 Media

For bacterial isolation and culturing, MRS agar and broth, M17 agar and MRS-sorbital agar were used; purchased from QingDao Hopebio-Technology Co., Qingdao, China.

3.1.3 Chemicals and toxins

AFB_1 and T-2 toxins were purchased from Sigma-Aldrich, China, and stored at −20°C until their use. Acetonitrile (99% purity), ethyl acetate and methanol were purchased from Guangfu Si-Tech Co. Tianjin, China.

3.2 Methods

3.2.1 Isolation and characterization of lactic acid bacteria

Only one bacterium (*L. casei Shirota)* was isolated from FMD and three (*L. acidophilus*, *L. bulgaricus* and *Streptococcus thermophilus)* from CY. From both FMD and CY, three bottles each of 100 ml were aseptically mixed, and 1 ml mixed thoroughly with 9 ml of sterilised phosphate-buffer saline (PBS, pH 7.2). Serial 10-fold dilutions with PBS were made and 0.1 ml of each dilution spread onto respective media. MRS agar was used to isolate the bacterial strain from FMD while MRS (pH 5.2), M17 agar and MRS-sorbitol agar were used to isolate the CY strains (the three strains). The agar plates were incubated anaerobically at 37°C for 48 h. Colonies were selected and characterised based on the criteria shown in Table 1 and maintained as a frozen stock (−20°C) in 50/50 glycerol: MRS broth.

Table 1: Isolation and characterization of lactic acid bacteria

Bacteria	Source	Selective medium	Morphological characteristics
L. bulgaricus	Isolated from conventional yoghurt (CY)	MRS agar, PH adjusted to 5.2	The colony size about 3–4 mm, circular, translucent and white in colour. Gram-positive and rod-shaped cells
Streptococcus thermophilus	Isolated from CY	M17 agar	Colony size about 1–2 mm, circular, smooth surface and white in colour. Gram-positive and spherical-shaped cells that occur in chains resembling a string of beads
L. casei shirota	Isolated from fermented milk drink (FMD)	MRS agar	Colony size about 2–2.5 mm, white, circular, smooth & moist surface. Gram-positive, rod-shaped bacteria
L. acidophilus	Isolated from CY	MRS-sorbital agar	Colony size 0.5–1 mm, brown, rough irregular colonies. Gram-positive and rod-shaped cells. Cells occur singly, in pairs or in short chains

3.2.2 Preparation of LAB working cultures

The reference strain and isolated cultures were grown in MRS broth at 37°C in anaerobic conditions to a concentration of 10^9 cfu/mL. Bacterial concentration was quantified by absorbance at 600 nm ($_{OD}600$) and compared using the standard plate count method. Ten millilitres of broth of each strain were centrifuged (7000 × g, 5 min, 4°C), supernatant fluid removed and bacterial pellets collected. The pellets were washed twice with 10 mL of PBS and suspended in 10 mL sterile PBS. The FMD strain and reference strains were treated as independent cultures while the three strains from CY were mixed to form a single culture.

3.2.3 Preparation of AFB_1 and T-2 standard solutions

Stock solutions of 7.703 mg/L of AFB_1 and 19.601 mg/L of T-2 were prepared by dissolving in acetonitrile. Then 0.5 mL of working stock solutions of AFB_1 (5000, 2500, 1000, 500 and 250 µg/L) and T-2 (12,500, 10,000, 5000, 2500, and 1000 µg/L) were prepared by diluting stock solutions with acetonitrile. Working stock solutions were stored at −20°C but warmed to room temperature before use.

3.2.4 Bacterial toxin treatment and toxin extraction

From each working stock solution of AFB_1 and T-2, 20 µL was mixed with 980 µL of each bacterial culture and incubated at 37°C for 72 h. The final toxin concentrations were as follows: AFB_1 (5, 10, 20, 50 and 100 µg/L) and T-2 (20, 50, 100, 200 and 250 µg/L). For each bacterial culture, a bacterial control (bacteria suspended in PBS) and AFB_1 and T-2 controls (20 µL of various working stock concentrations of AFB_1 and T-2 in 980 µL of PBS) were also incubated. All assays were conducted in duplicate. Following incubation, the bacterial toxin mixture and controls were centrifuged (7000 x g, 5 min, 4°C), 0.5mL of the supernatant fluid mixed with ethyl acetate, vortexed for 5 min, cleaned by ultrasonic vibration for 10 min, centrifuged (7000 x g, 5 min, 4°C) and supernatant collected. The same procedure was repeated three times and resulting supernatants merged, dried by N_2 gas at 50°C, re-dissolved with 1 mL of 30% methanol and filtered through a 0.22-µm micro-membrane filter. The filtered supernatants were assayed for toxin concentrations.

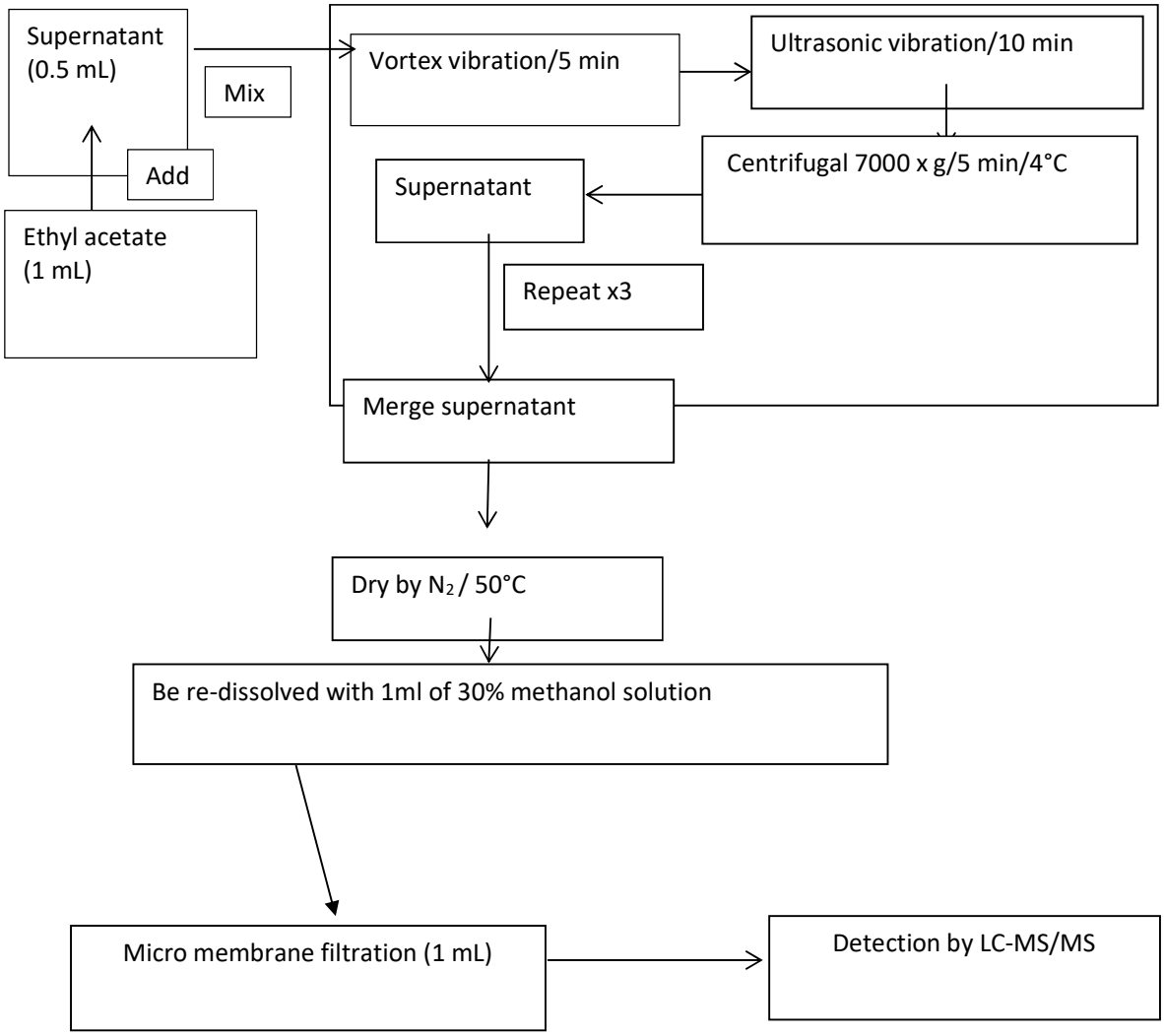

Fig. 4: Experimental design for extraction of AFB_1 and T-2 toxins.

3.2.5 LC-MS/MS assay

The assays of AFB_1 and T-2 concentrations were conducted at the National Marine Products Quality Supervision & Inspection Centre, Zhanjiang, China. For the T-2 assay, a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method by Lu et al. (2016) was used. This method was slightly modified to optimise the assay for measurement of both AFB_1 and T-2. The LC-MS/MS comprised: a Surveyor MS Pump plus, an online degasser, a Surveyor Auto Sampler Plus coupled with a Thermo TSQ Quantum Access tandem mass spectrometer equipped with an electrospray ionisation (ESI) source (Massachusetts, USA), and an analytical Hypersil GOLD column (5 µm, 100 mm × 2.1 mm) from Thermo Scientific. Methanol was used for mobile phase A, 5 mM ammonium acetate and 0.1% formic acid for mobile phase B. Elution was set as follows: 20% A at

0 min, increased to 90% A from 0 min to 4 min, held at 90% A from 4 min to 7 min, decreased to 20% A from 7 min to 7.1 min, and then held at 20% A for 10 min. Flow rate was set at 250 µL/min and a maximal retention time of 6.0 min. The samples and standards were infused directly into the LC-MS/MS, a 10-µL aliquot injected into the Hypersil GOLD column and operated in the positive ion mode using electrospray ionisation. IN the same manner, LC-MS/MS was used to measure AFB_1 concentration. The liquid chromatography conditions were similar to those used for T-2 assessment. Maximal retention time was set at 6.0 min. The most intense transition ion products (m/z) of T-2 and AFB_1 used for quantification on the selected reaction-monitoring (SRM) mode of a mass spectrometer were 304.95 m/z and 285.10 m/z.

3.2.6 Detoxification of T-2 and AFB_1 by heat-killed and live cells

To assess and compare the ability of heat-killed and live cells of LAB to detoxify the two mycotoxins, an FMD strain was used. The isolated strain was grown in MRS broth at 37°C in anaerobic conditions to a concentration of 10^9 cfu/mL. Then, 20 ml of broth was centrifuged (7000 × g, 5 min, 4°C), supernatant fluid removed and bacterial pellets collected. The pellets were washed twice with 20 mL of PBS and then suspended in 20 mL of sterile PBS. The cleaned cells were divided into two portions of 10 mL each and one portion autoclaved at 121°C for 15 min. From each working stock solution of AFB_1 and T-2, 20 µL was mixed with 980 µL of the cleaned cells and incubated anaerobically at 37°C for 24 h. A bacterial control and AFB_1 and T-2 controls were also incubated. The assays were conducted in duplicate. After incubation, the bacterial-toxin mixtures and controls were centrifuged, 0.5 ml of the supernatant fluid pipetted and mixed with ethyl acetate, vortexed under vibration for 5 min, cleaned by ultrasonic vibration for 10 min, centrifuged (7000 x g, 5 min, 4°C) and the supernatant collected. The same procedure was repeated three times and resulting supernatants merged, dried by N_2 gas at 50°C then re-dissolved with 1 mL of 30% methanol solution and finally filtered through a 0.22-µm micro membrane filter. Assay of toxin detoxification rate was carried out as described in procedure 3.2.5.

3.2.7 Viability of live bacterial cells exposed to T-2 and AFB_1

The reference strain, *L. casei shirota* and *L. bulgaricus* were grown overnight in MRS broth at 37°C in anaerobic conditions to a concentration of 10^9 cfu/mL. Then, 10 mL broth of each strain were centrifuged (7000 × g, 5 min, 4°C), supernatant fluid removed and bacterial pellets collected. The pellets were washed twice with 10 mL of PBS and then suspended in 10 mL of sterile PBS. Next, 980 µL of each bacterial culture was mixed with 20 µL of various toxin concentrations (refer to procedure 2.2.3). The bacterial-toxin mixtures were incubated anaerobically at 37°C for 72 h. For each strain, a bacterial control (bacteria suspended in PBS) was also incubated. After incubation, serial 10-fold dilutions with PBS were made and 0.1 mL of each dilution spread onto MRS agar. The agar plates were

incubated anaerobically at 37°C for 48 h. The viable cells were treated as colonies and reported as cfu/mL.

3.3 Statistical analysis

One-way analysis of variance (ANOVA) was used to determine whether the detoxification rate was significantly different between the strains and whether the rate was significantly different between toxin concentration levels for individual LAB strains. In the same way, one-way ANOVA was used to test for significant differences among means of bacterial viability in toxins. All the assay in this study were carried out in duplicate due to the wide range of toxin concentrations, three sets of bacteria and time constraint. Minitab 17 statistical software, owned by Minitab Inc., was provided by Lincoln University, New Zealand. The software was used to conduct the ANOVA test. The Tukey pairwise test was used to compare means. Results were regarded as significant if the p value was < 0.05.

The results were presented by quantity (µg/L) and percentage (%). The percentage of toxin detoxified by the bacteria was calculated using the following formula:

$$\textbf{\% Toxin detoxified} = 100 \times \left[\left[1 - \left(\frac{Concentration\ of\ Sample}{Concentration\ of\ Standard}\right)\right]\right].$$

Chapter 4

Results

4.1 Extracted ion chromatograms (T-2 and AFB_1)

The extracted ion chromatograms of samples were captured in a Full Scan-Q1MS scanning mode (Figs 5 & 6). The retention times of the AFB1 (Fig. 5) and T-2 (Fig. 6) were 2.83min and 3.32min respectively.

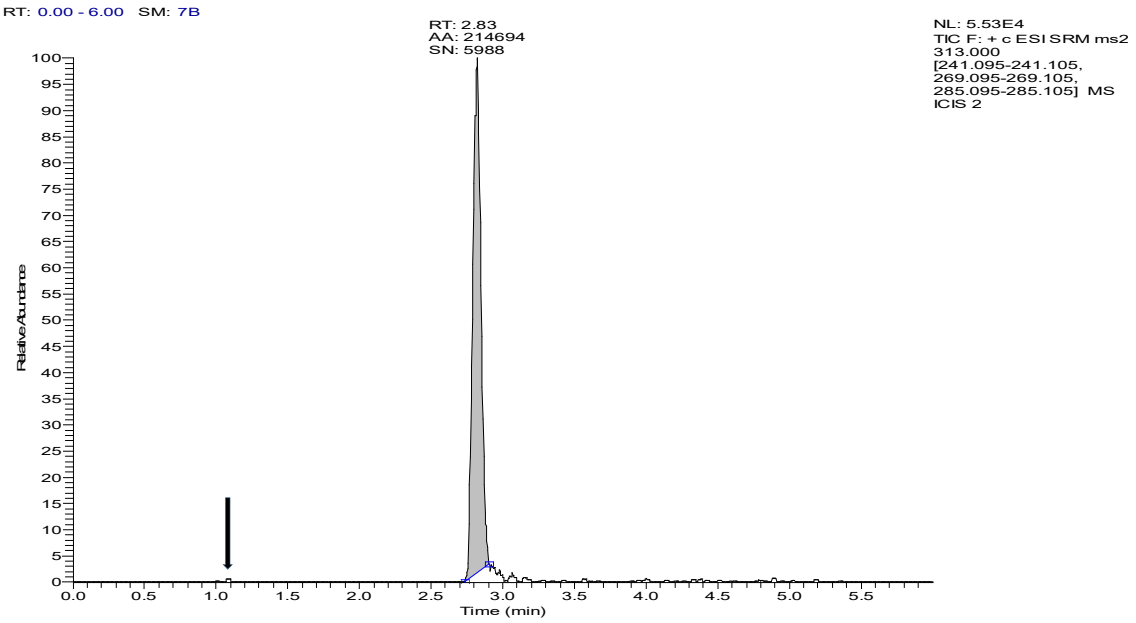

Fig. 5: Chromatogram for AFB_1 by LC-MS/MS; toxin extracted after 72 h incubation with *L. casei shirota*; retention time 2.83 min. The arrow indicates the retention time of the AFB_1 metabolite but there appears to be only an extremely low concentration of the metabolite. Groopman et al., (1985) have shown that the AFB1 metabolite is eluted around the retention time as shown above (arrow).

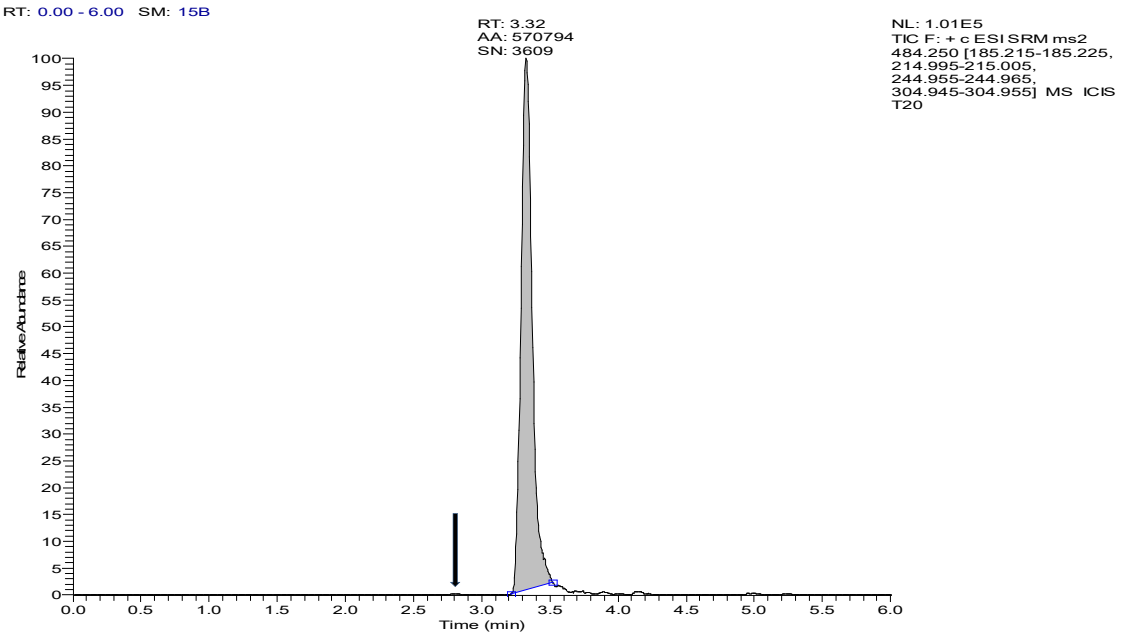

Fig. 6: Chromatogram for T-2 by LC-MS/MS; extracted after 72 h incubation with *L. casei shirota*; retention time 3.32 min. The arrow indicates the retention time of the T-2 metabolite but there appears to be only an extremely low concentration of the metabolite. Lu et al., (2016) have shown that the AFB1 metabolite is eluted around the retention time as shown above (arrow).

4.2 Quantity of AFB_1 and T-2 detoxified by live bacteria: FMD strain, Reference strain and CY strains

4.2.1 Quantity of AFB_1 detoxified by live bacteria (FMD strain, Reference strain and CY strains)

L. casei shirota and L. acidophilus AS1.3342 detoxified AFB_1 by 0.6 -42.9 µg/L (Fig. 7) and 0.5-45.7 µg/L (Fig. 8) respectively. The mixture of CY strains detoxified 0.7-42.6 µg/L AFB_1 (Fig. 9). The quantity of AFB_1 detoxified by the live cells of *L. casei shirota* (r^2 = 0.780), *L. acidophilus* AS1.3342 (r^2 = 0.805) and CY strains (r^2 = 0.829) increased as the toxin concentration increased (Figs 7–9). For individual strains, the quantities detoxified between the toxin concentrations were significantly different. However, within each level of toxin concentration, there was no significant difference among the means of the toxin detoxified by the three bacteria (Fig. 10).

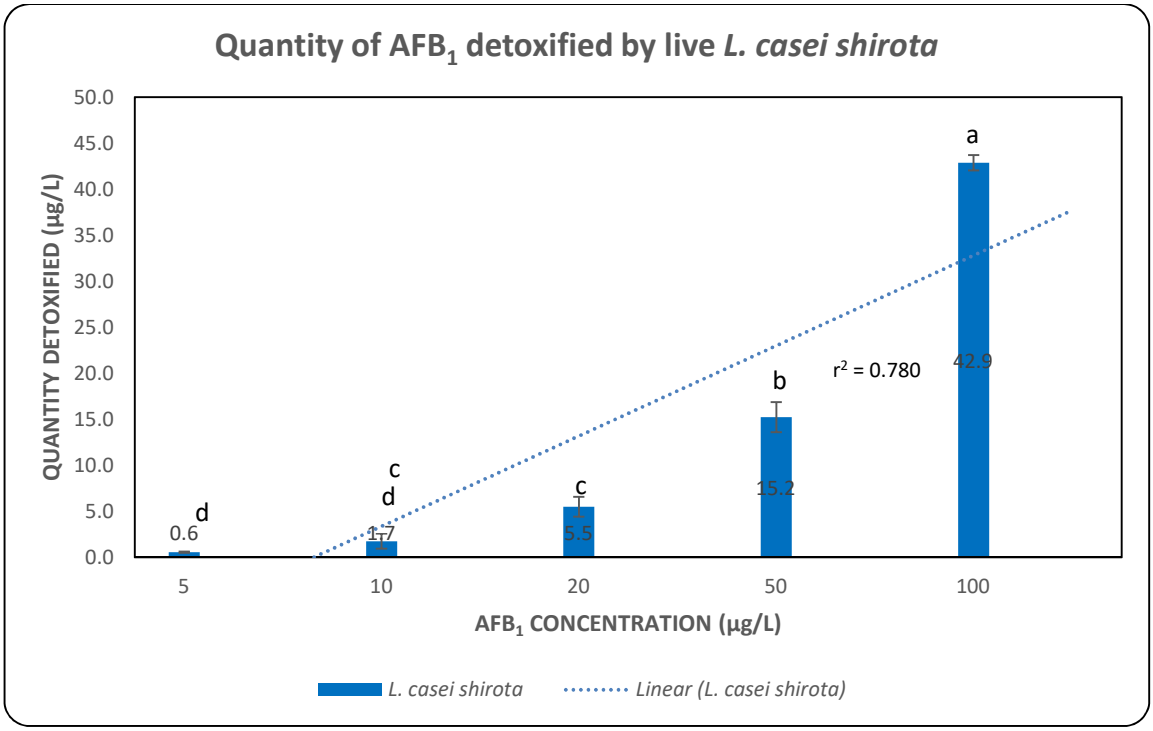

Fig. 7: Quantity of AFB_1 detoxified by live FMD isolated strain (*L. casei shirota*). Various toxin concentrations and bacteria (10^9 cfu/mL) were incubated at 37°C for 72 h. The results are shown as mean ± standard deviation. Columns with different letters are statistically different ($p < 0.05$).

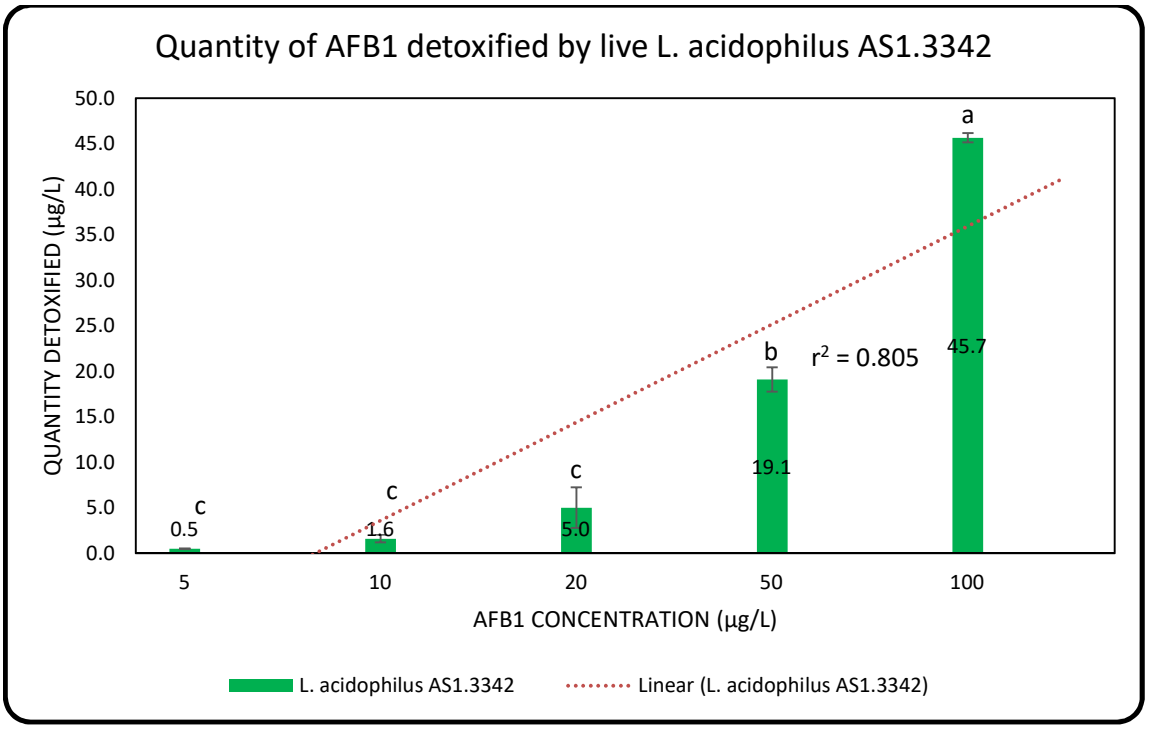

Fig. 8: Quantity of AFB_1 detoxified by live reference strain *L. acidophilus* AS1.3342. Various toxin concentrations and bacteria (10^9cfu/mL) were incubated at 37°C for 72 h. The results are shown as mean ± standard deviation. Columns with different letters are statistically different ($p < 0.05$).

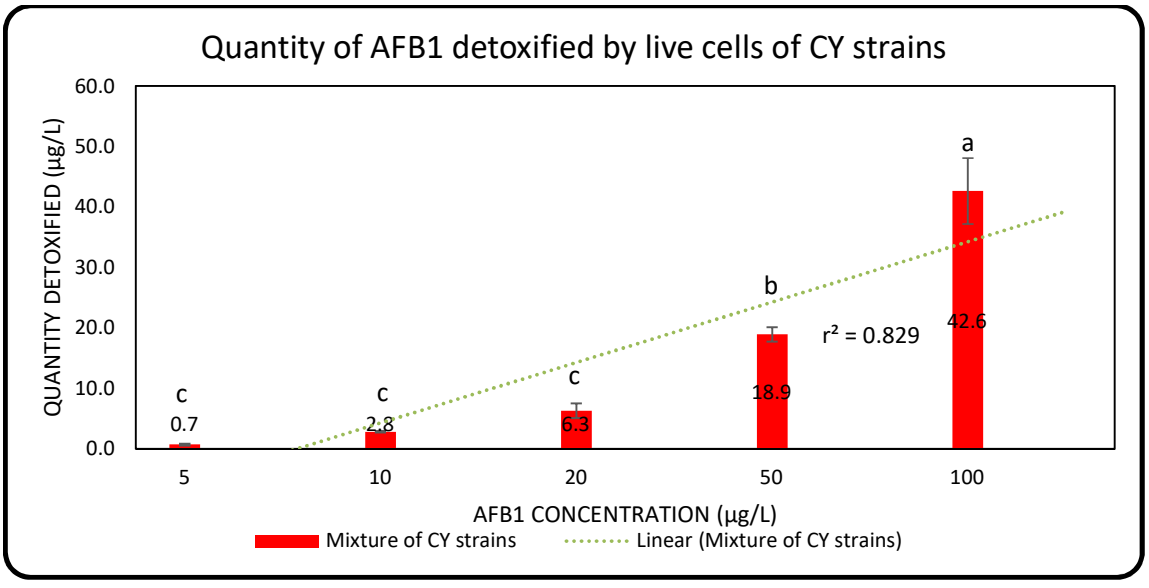

Fig. 9: Quantity of AFB_1 detoxified by a mixture of live CY strains *(L. bulgaricus, L. acidophilus and Streptococcus thermophilus)*. Various toxin concentrations and bacterial mixture (10^9 cfu/mL) were incubated at 37°C for 72 h. The results are shown as mean ± standard deviation. Columns with different letters are statistically different ($p < 0.05$).

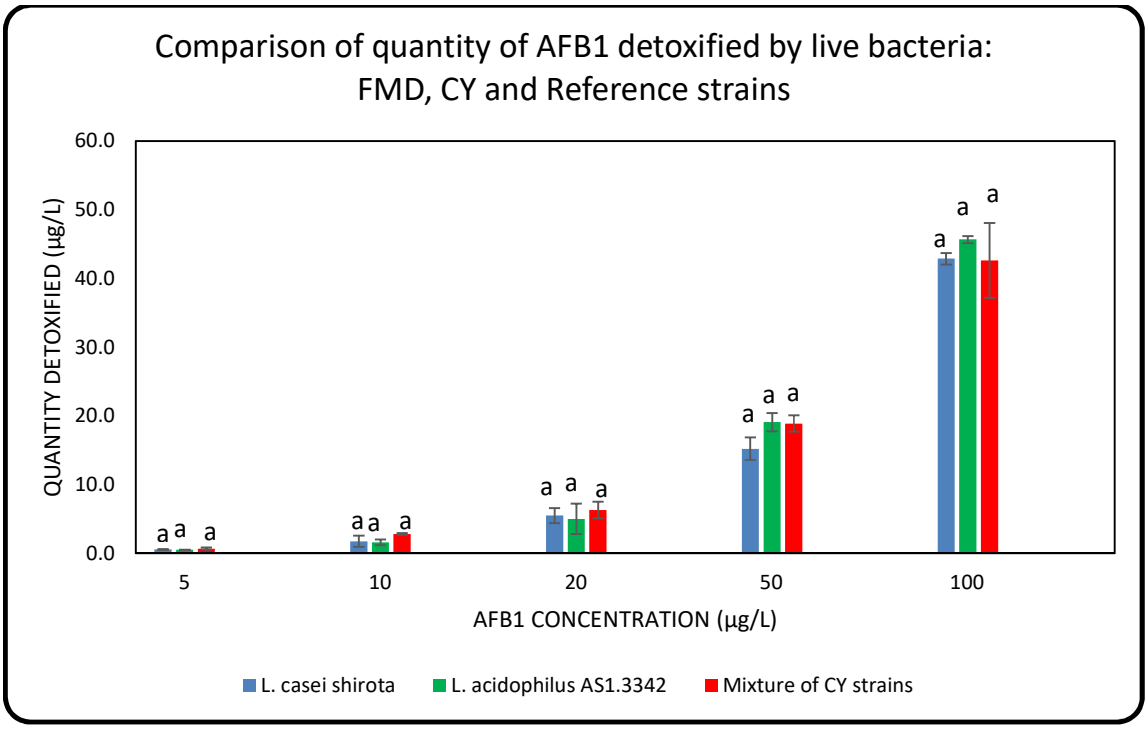

Fig. 10: Comparison of quantity of AFB_1 detoxified by the live bacterial strains in FMD (*L. casei shirota*), Reference (*L. acidophilus* AS1.3342*)* and CY (*L. bulgaricus, L. acidophilus* and *Streptococcus thermophilus*). Various toxin concentrations and bacteria (10^9 cfu/mL) were incubated at 37°C for 72 h. One-way ANOVA was used to test significant differences among means at a given concentration. There was no significant difference in detoxification by the bacterial strains at each toxin concentration.

4.2.2 Quantity of T-2 detoxified by live bacteria (FMD strain, Reference strain and CY strains)

L. casei shirota detoxified T-2 by 3.7–95.6 µg/L (Fig. 11); *L. acidophilus* AS1.3342 detoxified 2.9–110.8 µg/L (Fig. 12) and the mixture of CY strains detoxified 3.0–112.0 µg/L (Fig. 13). The quantity of T-2 detoxified by the live cells of *L. casei shirota* (r^2 = 0.954)*, L. acidophilus* AS1.3342 (r^2 = 0.941) and CY (r^2 = 0.932) strains increased as the toxin concentration increased (Figs 11–13). The quantities detoxified by individual strains between the toxin concentrations were significantly different (Figs 11–13). However, for CY strains, concentration levels were significantly different beyond 100 µg/L. Except for concentration level 200 µg/L, there was no significant difference among the means of the toxin detoxified by the three bacteria (Fig. 14).

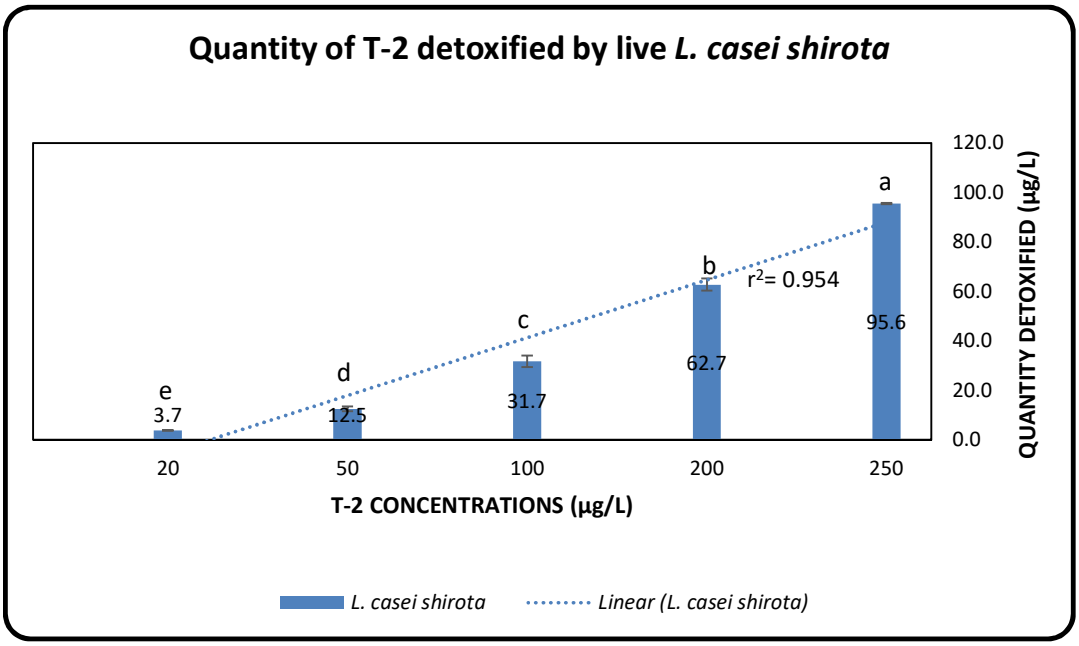

Fig. 11: Quantity of T-2 detoxified by the live FMD isolated strain (*L. casei shirota*). Various toxin concentrations and bacteria (10^9 cfu/mL) were incubated at 37°C for 72 h. The results are shown as mean ± standard deviation. Columns with different letters are statistically different ($p < 0.05$).

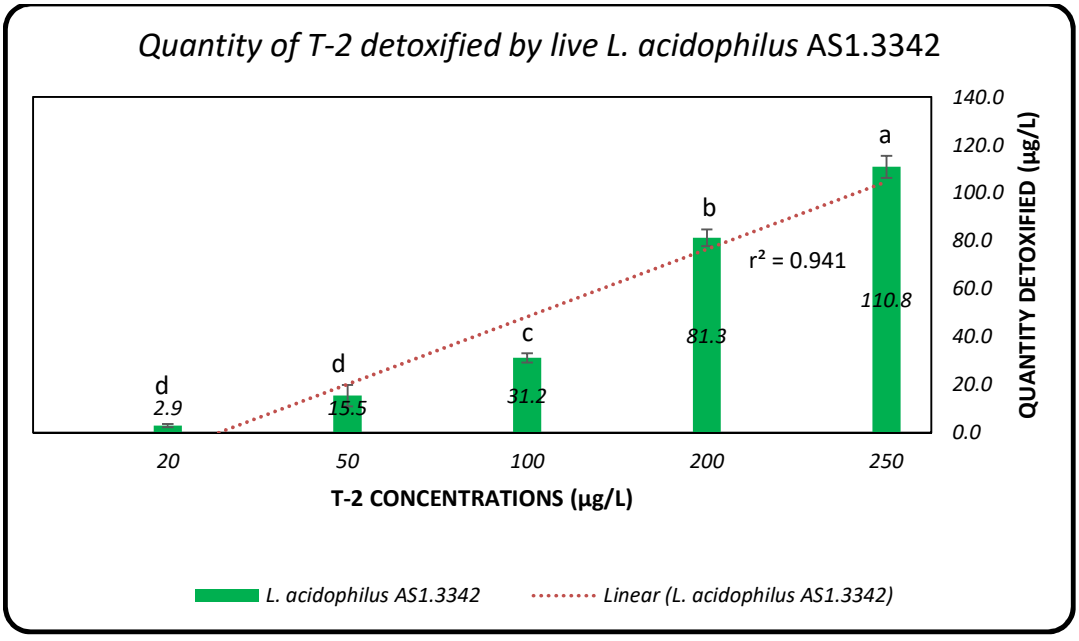

Fig. 12: Quantity of T-2 detoxified by the reference strain *(L. acidophilus* AS1.3342*).* Various toxin concentrations and bacteria (10^9 cfu/mL) were incubated at 37°C for 72 h. The results are shown as mean ± standard deviation. Columns with different letters are statistically different ($p < 0.05$).

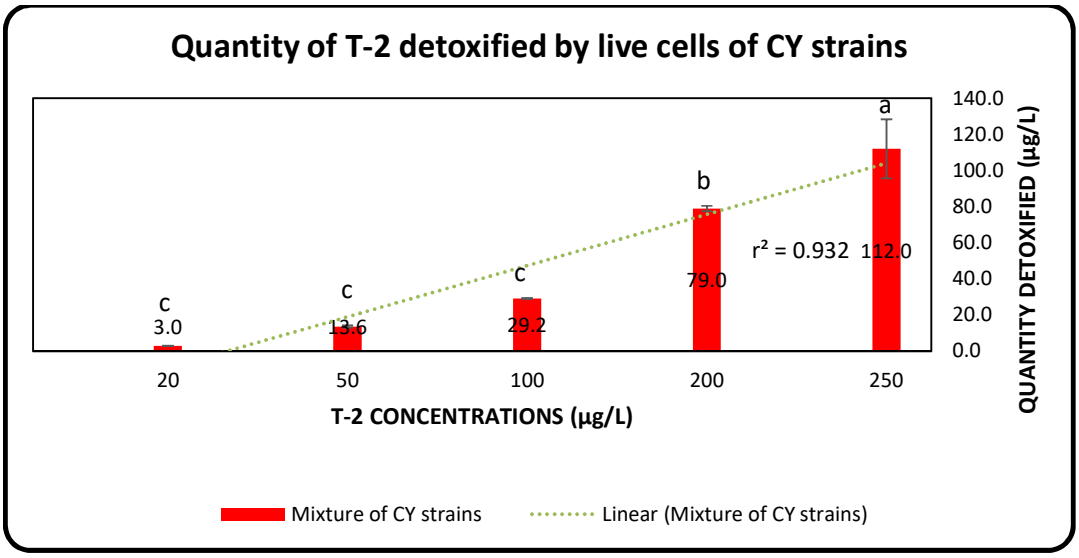

Fig. 13: Quantity of T-2 detoxified by a mixture of live CY strains (*L. bulgaricus, L. acidophilus and Streptococcus thermophilus*). Various toxin concentrations and bacteria (10^9 cfu/mL) were incubated at 37°C for 72 h. The results are shown as mean ± standard deviation. Columns with different letters are statistically different ($p < 0.05$).

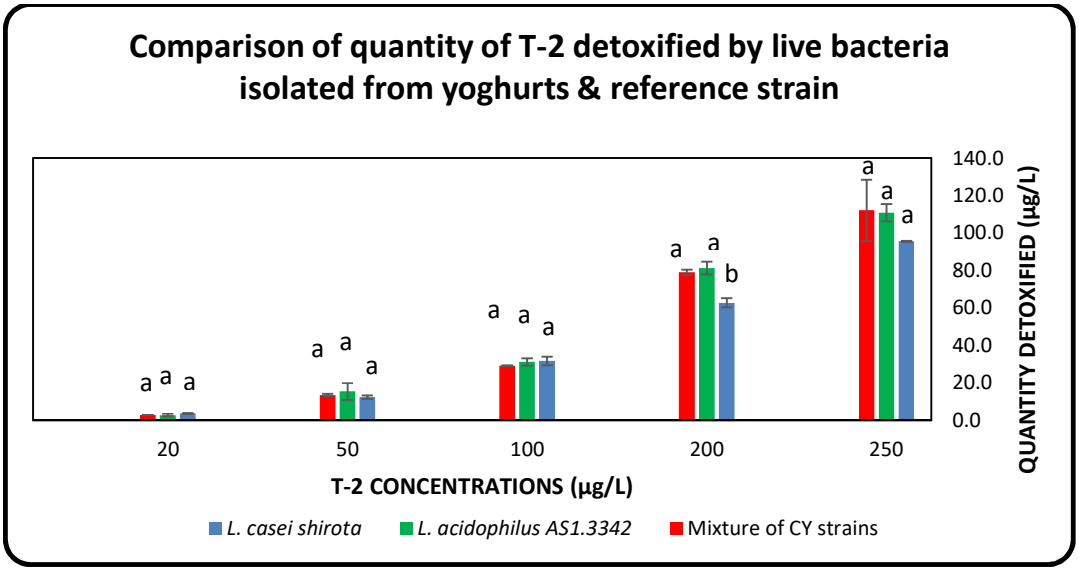

Fig. 14: Comparison of quantity of T-2 detoxified by the live FMD (*L. casei shirota*), Reference (*L. acidophilus* AS1.3342*)* and CY mixture (*L. bulgaricus, L. acidophilus and Streptococcus thermophilus*). Various toxin concentrations and live bacteria (10^9cfu/mL) were incubated at 37°C for 72 h. One-way ANOVA was used to test significant differences among means within individual concentration level. Bacteria with different letters in individual column groups are statistically different ($p < 0.05$).

4.3 Percentages of AFB_1 and T-2 detoxified by live bacteria: FMD strain, Reference strain and CY strains

4.3.1 Percentages of AFB_1 detoxified by live bacteria (FMD strain, Reference strain and CY strains)

L. casei shirota and L. acidophilus AS1.3342 *detoxified* 11–43% AFB_1 (Fig. 15) and 10–46% AFB_1 (Fig. 16) respectively while the mixture of CY strains detoxified 14–43% AFB_1 (Fig. 17). The percentage of AFB_1 detoxified by the live cells of *L. casei shirota* (r^2 = 0.976), *L. acidophilus* AS1.3342 (r^2 = 0.986) and CY strains (r^2 = 0.936) increased as the toxin concentration increased (Figs 15–17). The percentages detoxified between the toxin concentrations were significantly different (Figs 15–17). Within each toxin concentration level, there was no significant difference among the means of the toxin detoxified by the three bacteria (Fig. 18).

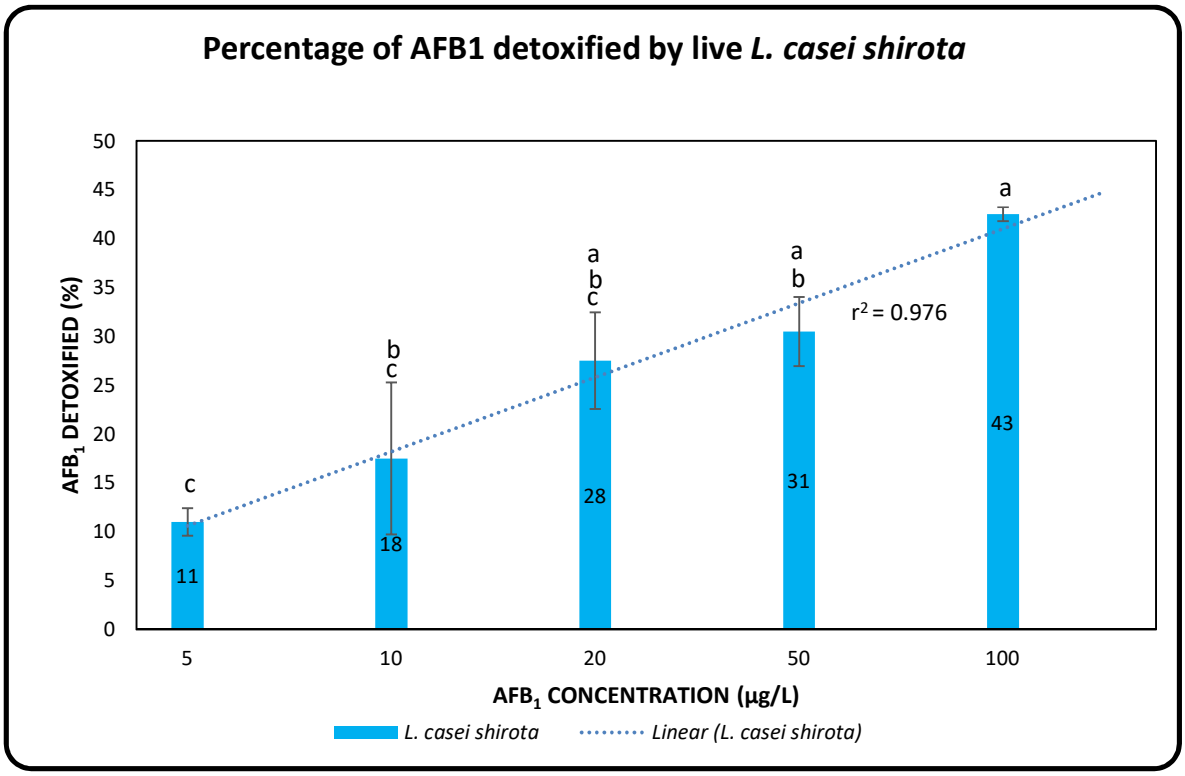

Fig. 15: Percentage of AFB_1 detoxified by live FMD isolated strain (*L. casei shirota*). Various toxin concentrations and bacteria (10^9 cfu/mL) were incubated at 37°C for 72 h. The results are shown as mean ± standard deviation. Columns with different letters are statistically different ($p < 0.05$).

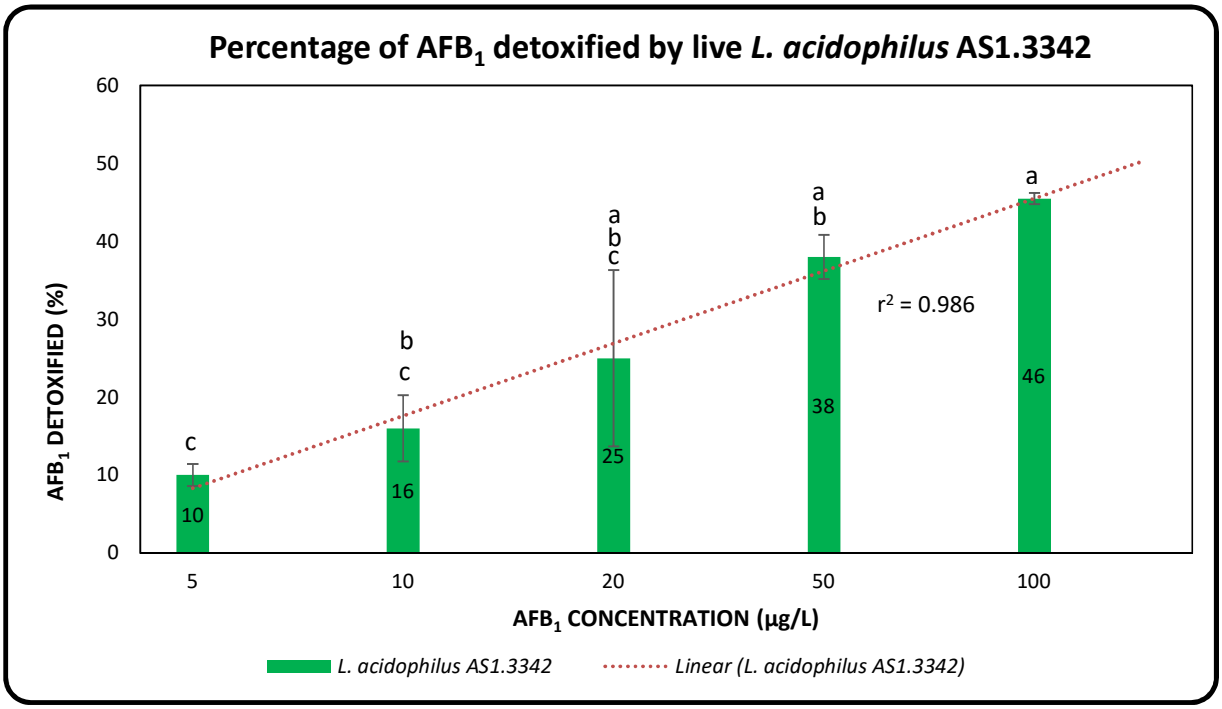

Fig. 16: Percentage of AFB_1 detoxified by live reference strain (*L. acidophilus* AS1.3342). Various toxin concentrations and bacteria (10^9 cfu/mL) were incubated at 37°C for 72 h. The results are shown as mean ± standard deviation. Columns with different letters are statistically different ($p < 0.05$).

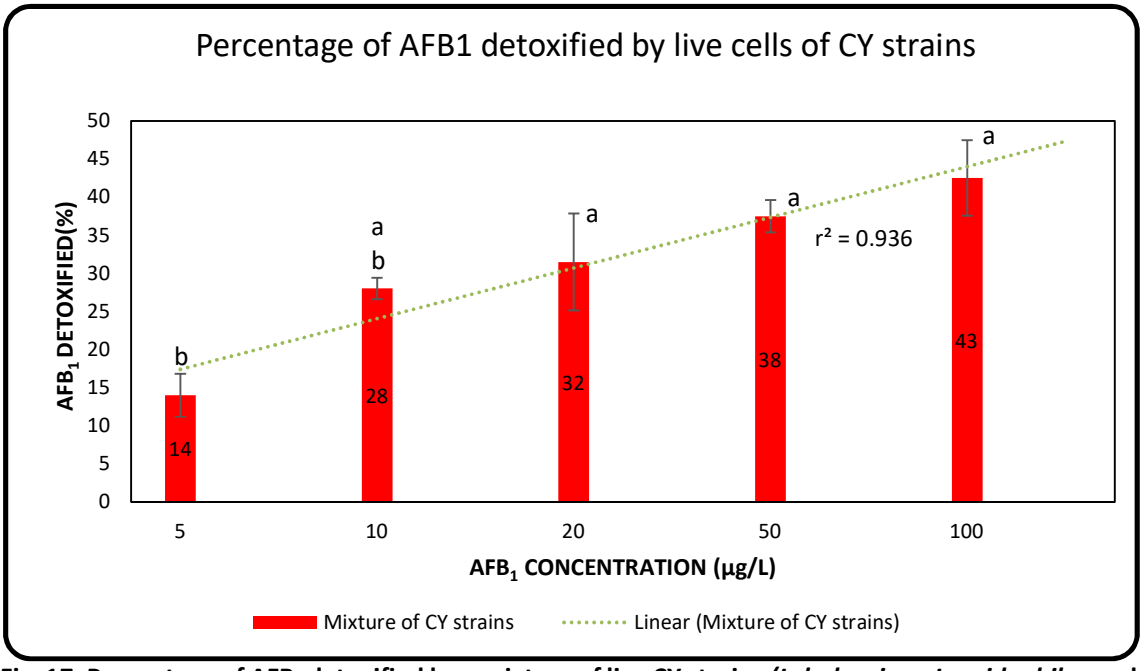

Fig. 17: Percentage of AFB_1 detoxified by a mixture of live CY strains *(L. bulgaricus, L. acidophilus* and *Streptococcus thermophilus)*. Various toxin concentrations and bacterial mixture (10^9 cfu/mL) were incubated at 37°C for 72 h. The results are shown as mean ± standard deviation. Columns with different letters are statistically different ($p < 0.05$).

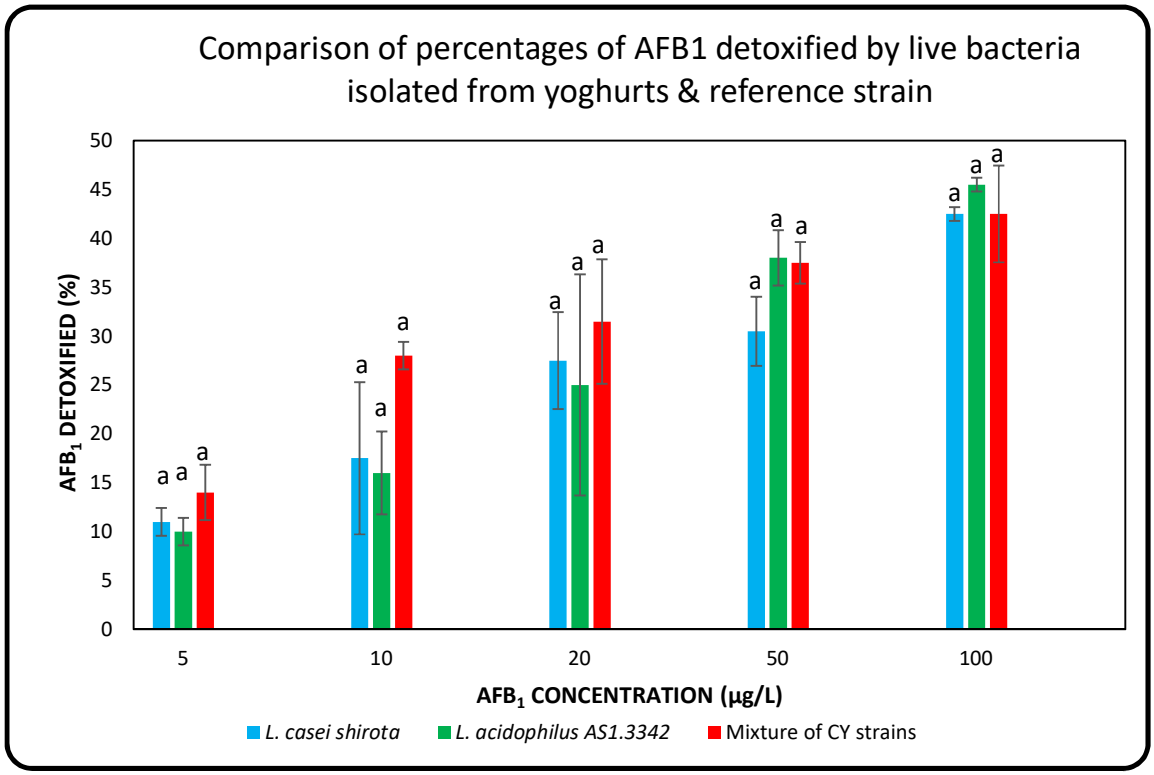

Fig. 18: Comparison of percentage of AFB_1 detoxified by the live bacterial strains in FMD (*L. casei shirota*), Reference (*L. acidophilus* AS1.3342*)* and CY mixture (*L. bulgaricus, L. acidophilus* and *Streptococcus thermophilus*). Various toxin concentrations and bacteria (10^9 cfu/mL) were incubated at 37°C for 72 h. One-way ANOVA was used to test for significant differences among means at individual concentration levels. There was no significant difference in detoxification by the bacterial strains at each toxin concentration.

4.3.2 Percentages of T-2 detoxified by live bacteria (FMD strain, Reference strain and CY strains)

L. casei shirota and L. acidophilus AS1.3342 detoxified 19–38% (Fig. 19) and 15–45% (Fig. 20) respectively. The mixture of CY strains detoxified 15–45% (Fig. 21). T-2 detoxified by the live cells of *L. casei Shirota* (r^2 = 0.983), *L. acidophilus* AS1.3342 (r^2 = 0.900), and CY strains (r^2 = 0.964) increased as the toxin concentration increased (Figs 19–21). The percentages detoxified by the various strains between the toxin concentrations were significantly different (Figs 19–21). There was no significant difference among the means of the toxin detoxified by the three bacteria at each toxin concentration (Fig. 22).

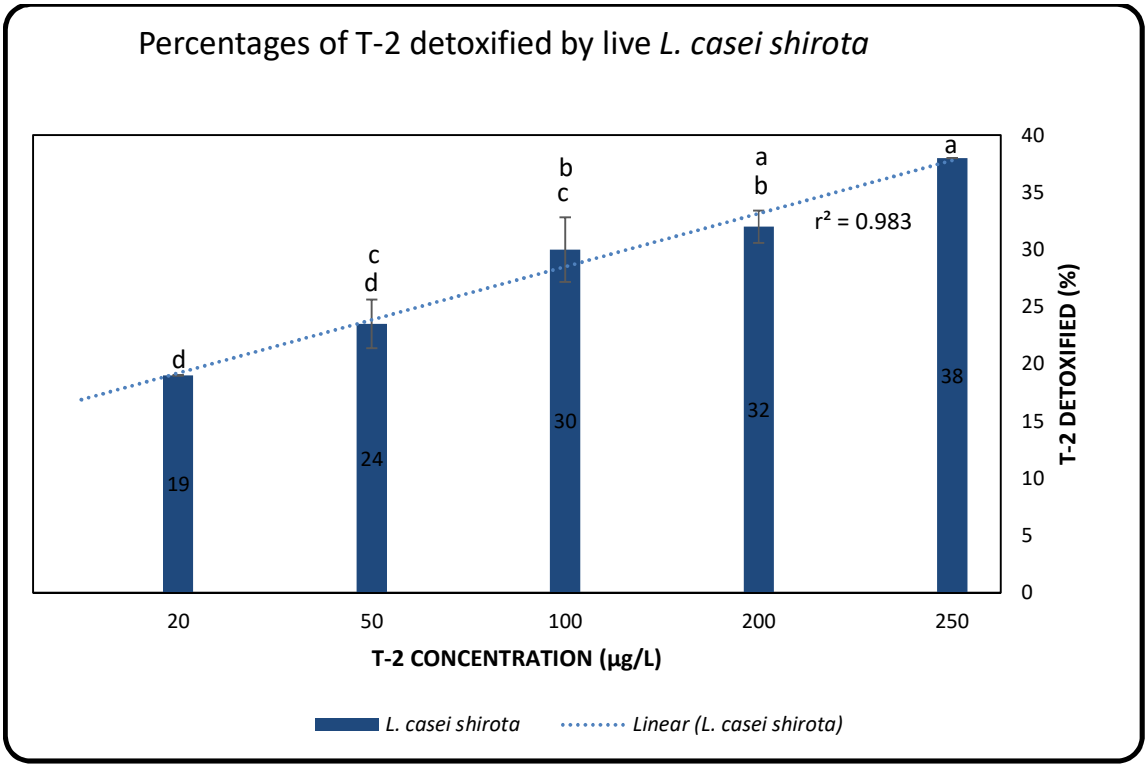

Fig. 19: Percentage of T-2 detoxified by the live FMD-isolated strain (*L. casei shirota*). Various toxin concentrations and bacteria (10^9 cfu/mL) were incubated at 37°C for 72 h. The results are shown as mean ± standard deviation. Columns with different letters are statistically different ($p < 0.05$).

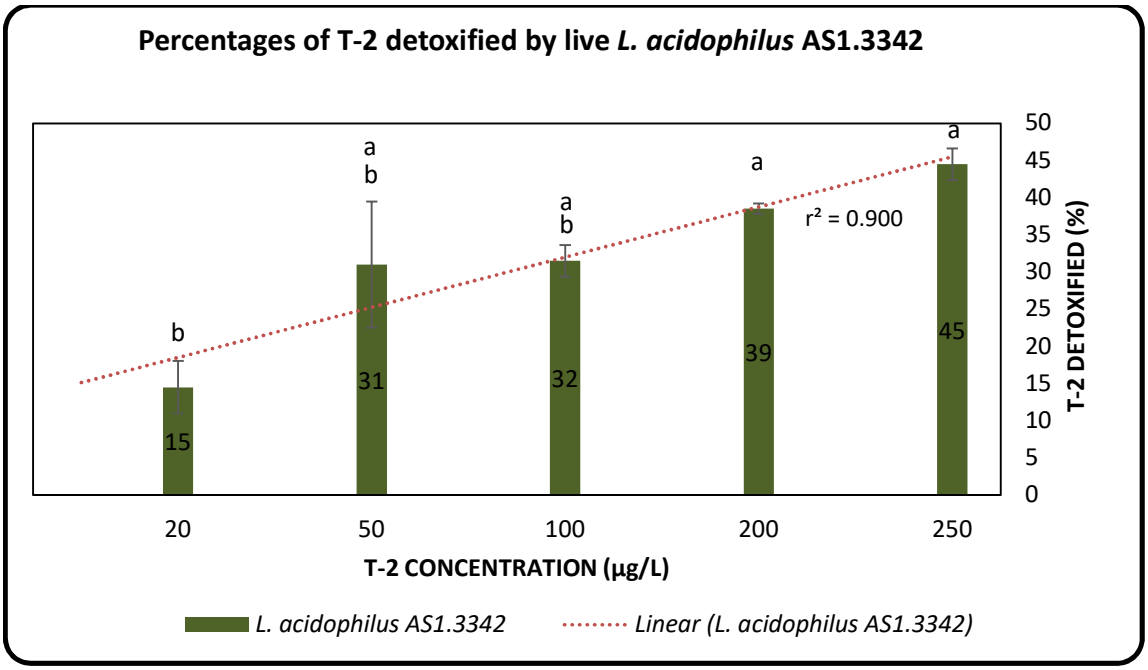

Fig. 20: Percentage of T-2 detoxified by the Reference strain *(L. acidophilus* AS1.3342*).* Various toxin concentrations and bacteria (10^9cfu/mL) were incubated at 37°C for 72 h. The results are shown as mean ± standard deviation. Columns with different letters are statistically different ($p < 0.05$).

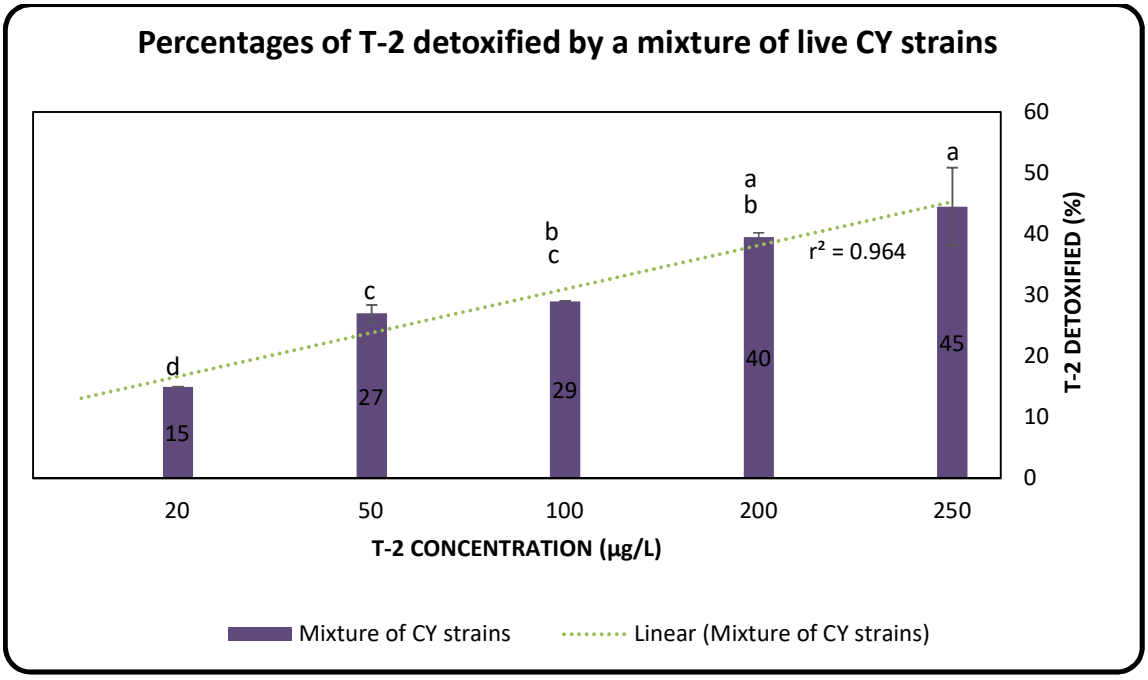

Fig. 21: Percentage of T-2 detoxified by a mixture of live CY strains (*L. bulgaricus, L. acidophilus* and *Streptococcus thermophilus*). Various toxin concentrations and bacteria (10^9 cfu/mL) were incubated at 37°C for 72 h. The results are shown as mean ± standard deviation. Columns with different letters are statistically different ($p < 0.05$).

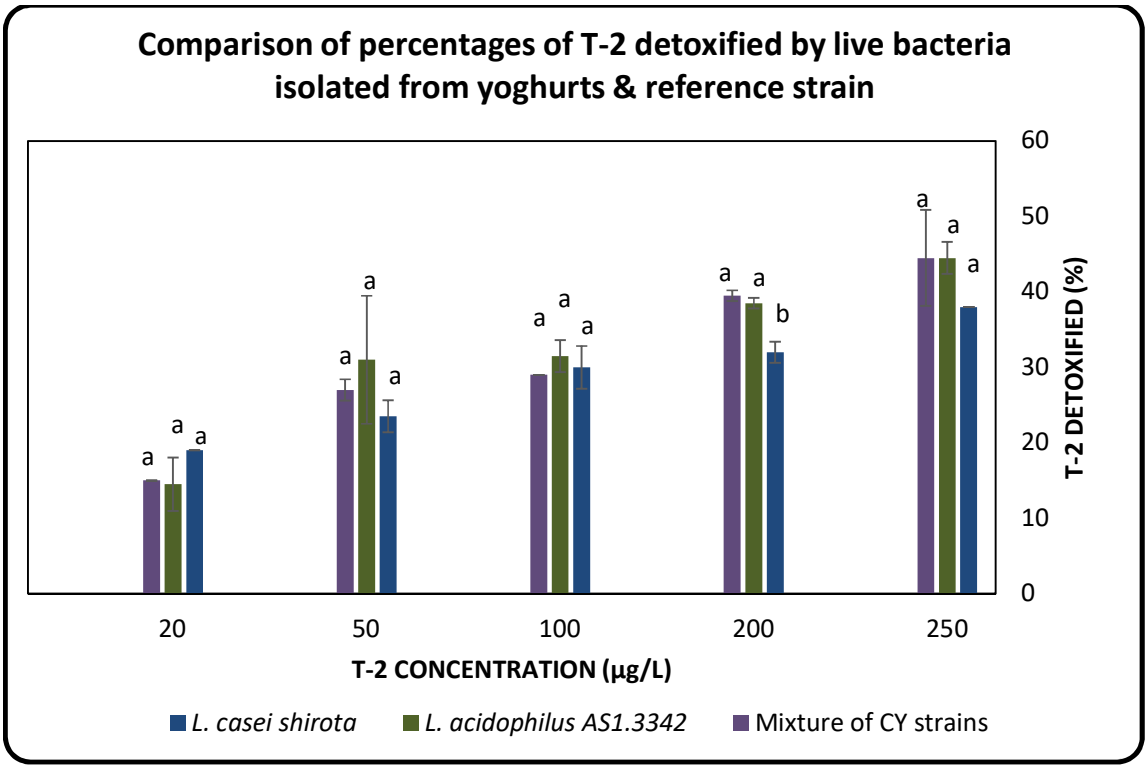

Fig. 22: Comparison of percentages of T-2 detoxified by the live FMD (*L. casei shirota*), Reference (*L. acidophilus* AS1.3342*)* and CY (*L. bulgaricus, L. acidophilus and Streptococcus thermophilus*) strains at each toxin concentration. Various toxin concentrations and live bacteria (10^9 cfu/mL) were incubated at 37°C for 72 h. One-way ANOVA was used to test for significant differences among means at a given concentration. Bacteria with different letters within individual column groups are statistically different ($p < 0.05$).

4.4 Quantity of AFB_1 and T-2 detoxified by live and denatured *L. casei shirota*

4.4.1 Quantity of AFB_1 detoxified by live and denatured *L. casei shirota*

The absolute quantity of AFB_1 detoxified by both live and denatured cells of *L. casei shirota* increased as the toxin concentration increased (Figs 23 & 24). Live cells detoxified 0.6–46.6 µg/L AFB_1 (Fig. 23) compared to 3.4–47.5 µg/L detoxified by denatured bacteria (Fig. 24). Thus denatured *L. casei shirota* cells (r^2 = 0.867) detoxified a greater quantity of AFB_1 toxin than the live cells (r^2 = 0.837) (Fig. 25). The quantity detoxified by the live cells or denatured cells were significantly different between the concentration levels (Figs 23 & 24).

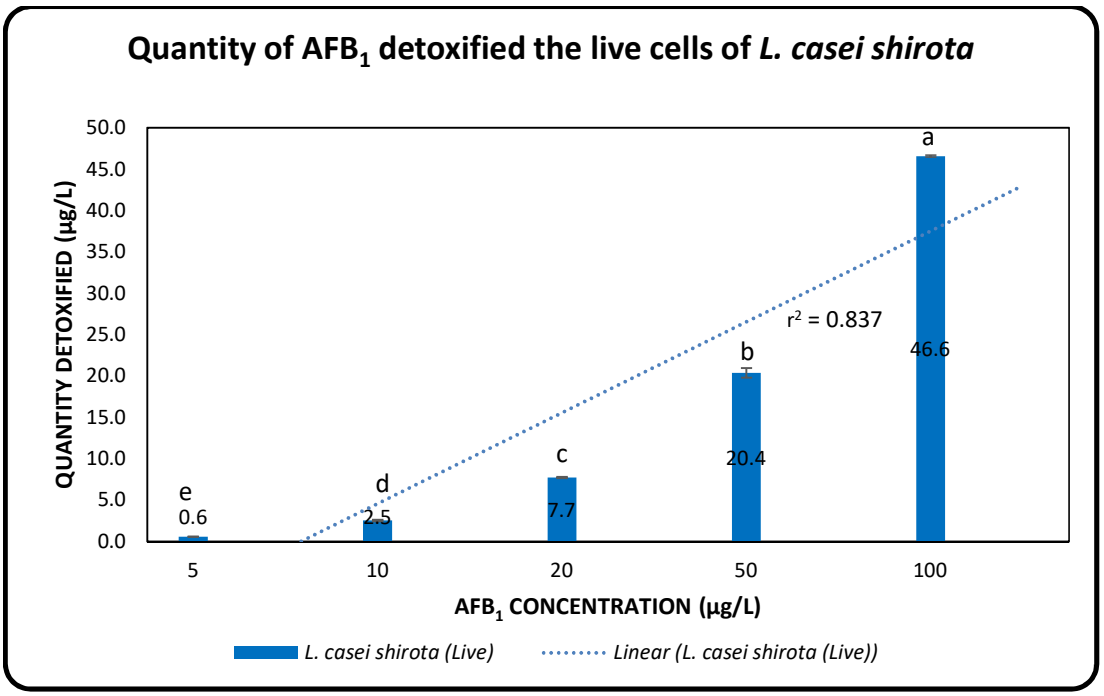

Fig. 23: Quantity of AFB_1 detoxified by live cells of *L. casei shirota* at various toxin concentrations. Various toxin concentrations and bacteria (10^9 cfu/mL) were incubated at 37°C for 24 h. The results are shown as mean ± standard deviation. Columns with different letters are statistically different ($p < 0.05$).

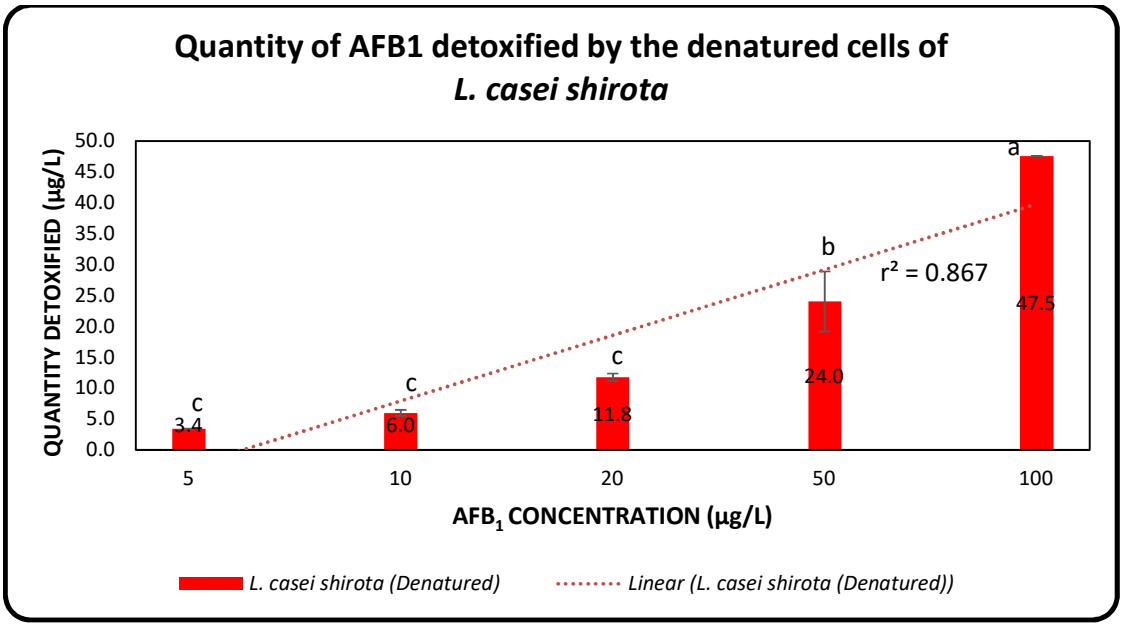

Fig. 24: Quantity of AFB_1 detoxified by denatured cells of *L. casei shirota* at various toxin concentrations. Various toxin concentrations and bacteria (10^9 cfu/mL) were incubated at 37°C for 24 h. The results are shown as mean ± standard deviation. Columns with different letters are statistically different ($p < 0.05$).

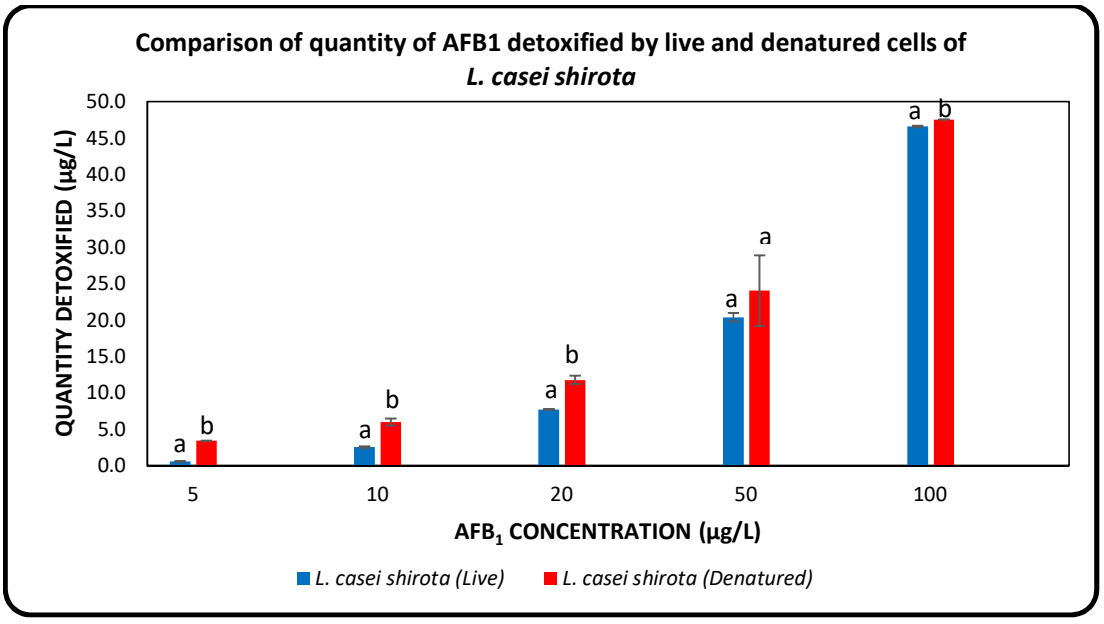

Fig. 25: Comparison of quantity of AFB_1 detoxified by live and denatured cells of *L. casei shirota* at individual toxin concentration level. Various toxin concentrations and bacteria (10^9 cfu/mL) were incubated at 37°C for 24 h. One-way ANOVA was used to test for significant differences between means at a given concentration. The results are shown as mean ± standard deviation. Treatments with different letters in individual column groups are statistically different ($p < 0.05$).

4.4.2 Quantity of T-2 detoxified by live and denatured *L. casei shirota*

The quantity of T-2 detoxified by both denatured and live cells of *L. casei shirota* increased as the toxin concentration increased (Figs 26 & 27). Live cells detoxified 5.6–94.8 µg/L T-2 (Fig. 26). Denatured cells detoxified 10.4–101.7 µg/L (Fig. 27). Thus, denatured *L. casei shirota* cells (r^2 = 0.938) detoxified a greater quantity of AFB_1 toxin than the live cells (r^2 = 0.978) (Fig. 28). The quantities detoxified by the live cells or denatured cells were significantly different between the concentration levels (Figs 26 & 27). There was a significant difference in the amount detoxified between the live and denatured cells (Fig. 28). However, the difference was only significant at 20, 50 and 100 µg/L but insignificant at 200 and 250 µg/L (Fig. 20).

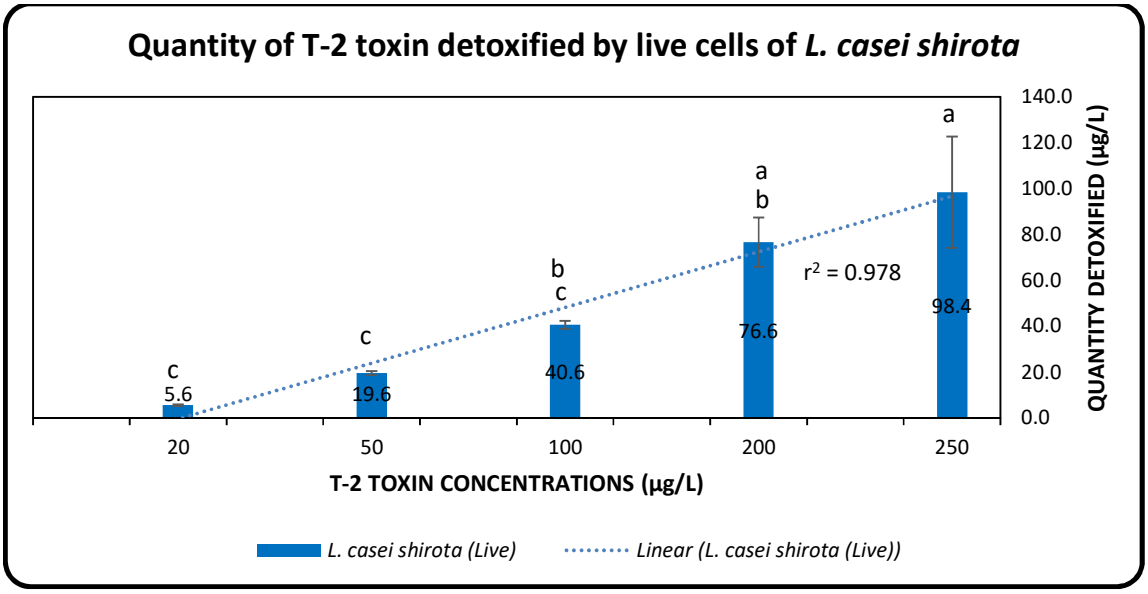

Fig. 26: Quantity of T-2 detoxified by live cells of *L. casei shirota* at various toxin concentrations. Various toxin concentrations and bacteria (10^9 cfu/mL) were incubated at 37°C for 24 h. The results are shown as mean ± standard deviation. Columns with different letters are statistically different ($p < 0.05$).

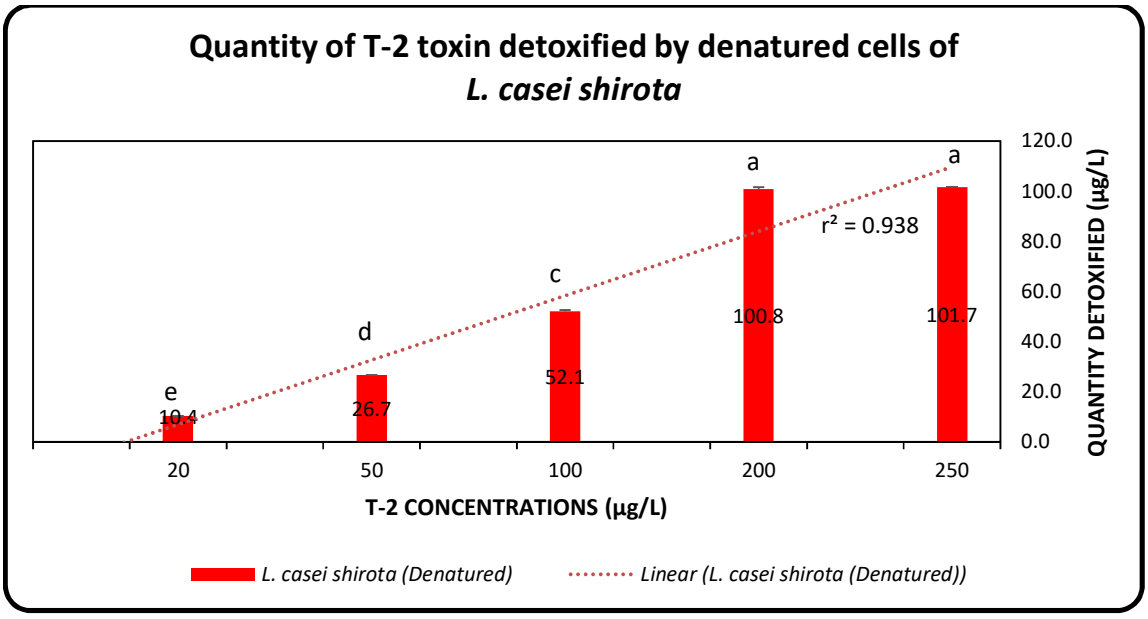

Fig. 27: Quantity of T-2 detoxified by denatured cells of *L. casei shirota* at various toxin concentrations. Various toxin concentrations and bacteria (10^9cfu/mL) were incubated at 37°C for 24 h. The results are shown as mean ± standard deviation. Columns with different letters are statistically different ($p < 0.05$).

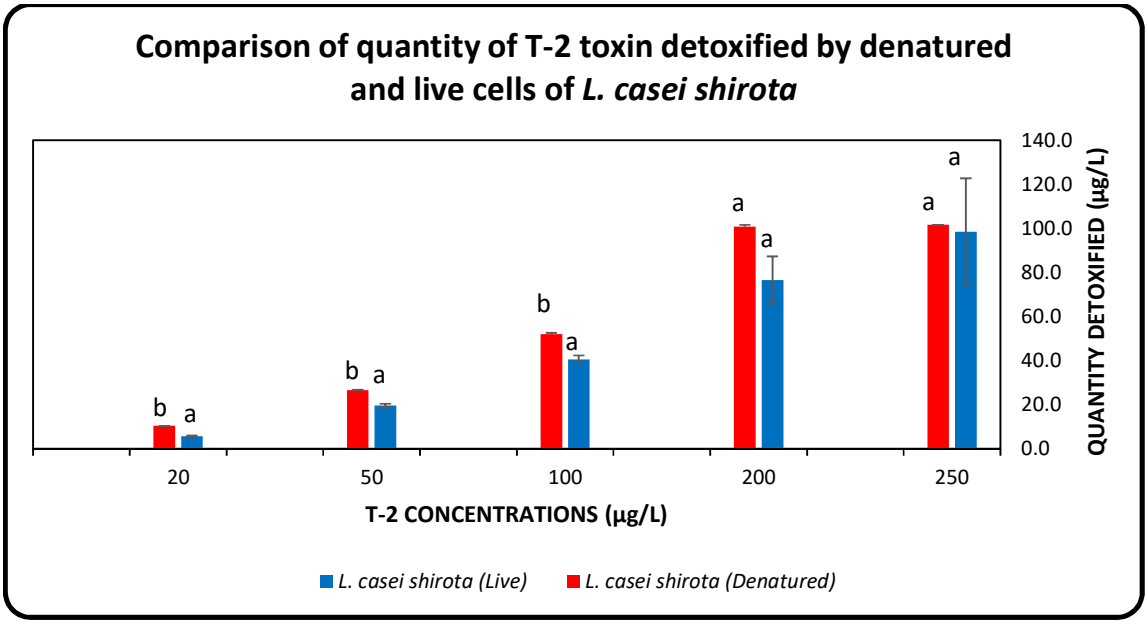

Fig. 28: Comparison of quantity of T-2 detoxified by both denatured and live cells of *L. casei shirota* at various toxin concentrations. Various toxin concentrations and bacteria (10^9 cfu/mL) were incubated at 37°C for 24 h. One-way ANOVA was used to test for significant differences between means at a given concentration. The results are shown as mean ± standard deviation. Treatments with different letters in Individual column groups are statistically different ($p < 0.05$).

4.5 Percentages of AFB_1 and T-2 detoxified by live and denatured *L. casei shirota*

4.5.1 Percentage of AFB_1 detoxified by live and denatured *L. casei shirota*

The percentage detoxified by denatured cells decreased (not significantly) as the toxin concentration increased (Fig. 29) while the percentage detoxified by live cells increased, significantly, as toxin concentration increased (Fig. 30). Denatured cells detoxified between 48% and 62% of AFB_1 (Fig. 29) while live cells detoxified between 12% and 47% (Fig. 30). There was a significant difference in the percentage of AFB_1 detoxified between the live and denatured cells (Fig. 31).

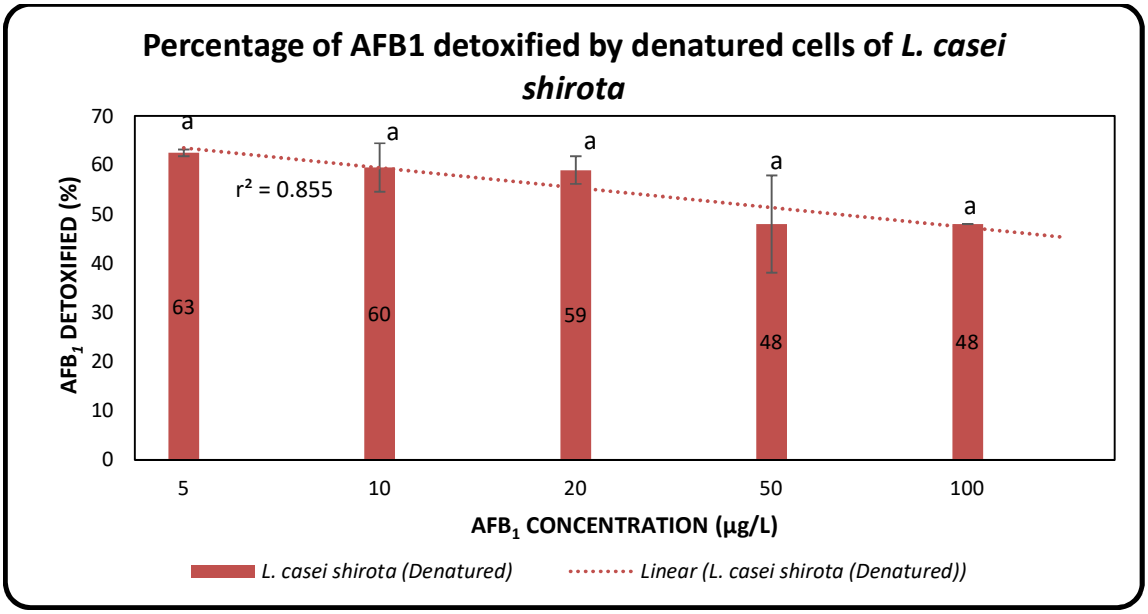

Fig. 29: Percentage of AFB_1 detoxified by denatured cells of *L. casei shirota* at various toxin concentrations. Various toxin concentrations and bacteria (10^9 cfu/mL) were incubated at 37°C for 24 h. The results are shown as mean ± standard deviation. Columns with different letters are statistically different ($p < 0.05$).

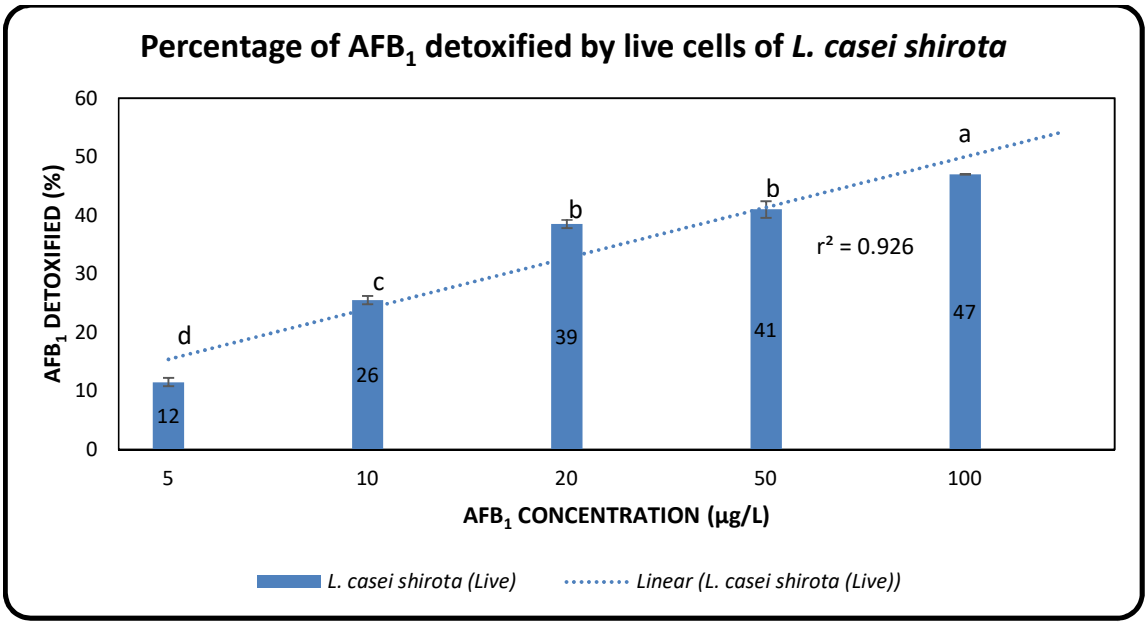

Fig. 30: Percentage of AFB_1 detoxified by the live cells of *L. casei shirota* at various toxin concentrations. Various toxin concentrations and bacteria (10^9 cfu/mL) were incubated at 37°C for 24 h. The results are shown as mean ± standard deviation. Columns with different letters are statistically different ($p < 0.05$).

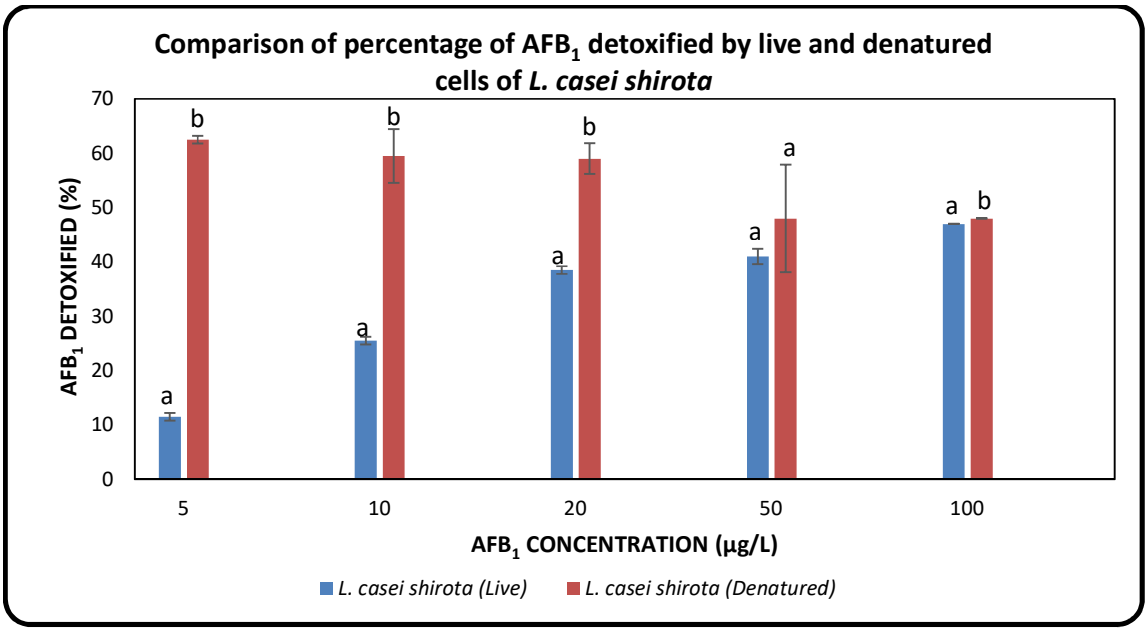

Fig. 31: Comparison of percentages of AFB_1 detoxified by live and denatured cells of *L. casei shirota* at individual toxin concentration level. Various toxin concentrations and bacteria (10^9 cfu/mL) were incubated at 37°C for 24 h. One-way ANOVA was used to test for significant differences between means at a given concentration. The results are shown as mean ± standard deviation. Treatments with different letters in individual columns are statistically different ($p < 0.05$).

4.5.1 Percentage of T-2 detoxified by live and denatured *L. casei shirota*

The percentage of T-2 detoxified by live cells increased as the toxin concentration increased (Fig. 32). The percentage detoxified by denatured cells decreased (not significantly) as the toxin concentration increased (Fig. 33). Live cells detoxified between 28% and 40% of AFB_1 (Fig. 32) while denatured cells detoxified between 42% and 53% (Fig. 33). There was a significant difference in the percentage of T-2 detoxified between the live and denatured cells at 20, 50 and 100 µg/L (Fig. 34). However, at 200 and 250 µg/L T-2 concentrations, the differences between the live and the denatured cells were insignificant (Fig. 34).

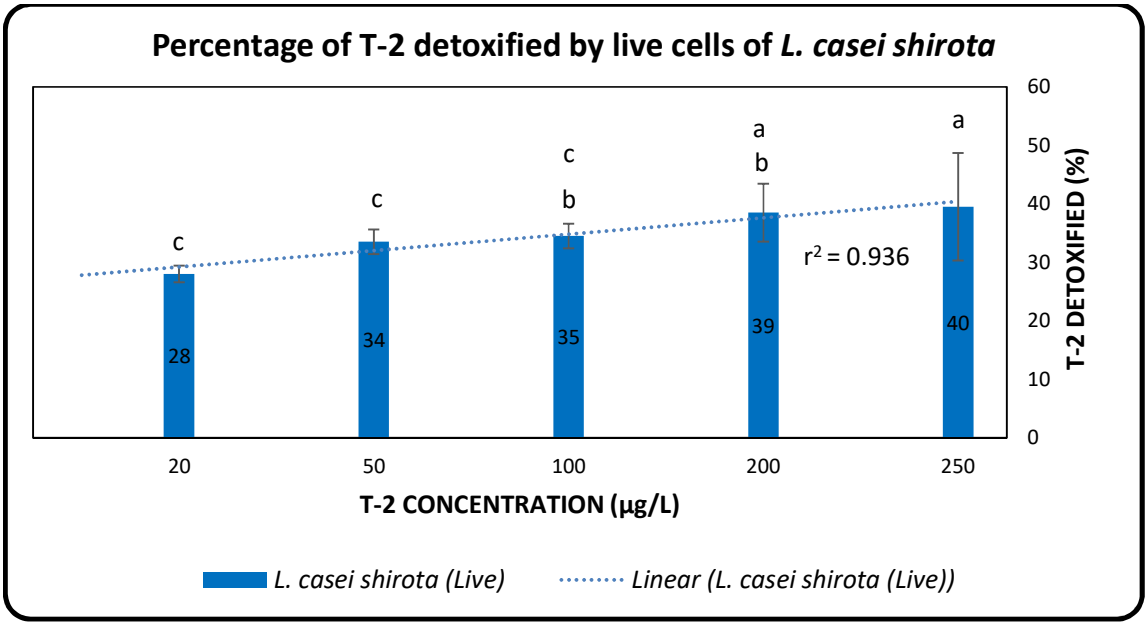

Fig. 32: Percentage of T-2 detoxified by the live cells of *L. casei shirota* at various toxin concentrations. Various toxin concentrations and bacteria (10^9 cfu/mL) were incubated at 37°C for 72 h. The results are shown as mean ± standard deviation. Columns with different letters are statistically different ($p < 0.05$).

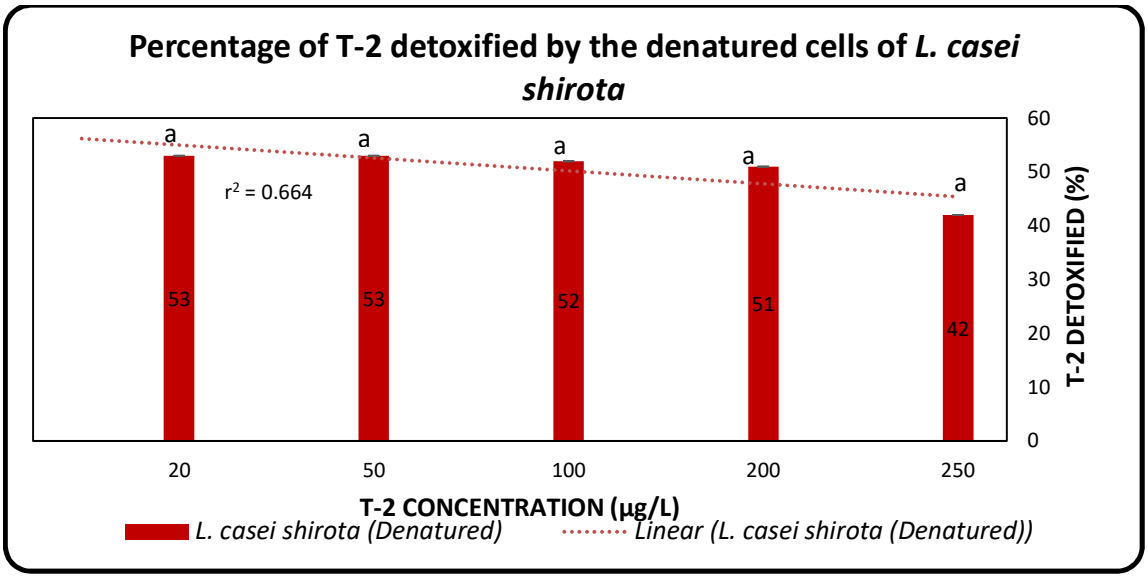

Fig. 33: Percentage of T-2 detoxified by the denatured cells of *L. casei shirota* at various toxin concentrations. Various toxin concentrations and bacteria (10^9 cfu/mL) were incubated at 37°C for 72 h. The results are shown as mean ± standard deviation. Columns with different letters are statistically different ($p < 0.05$).

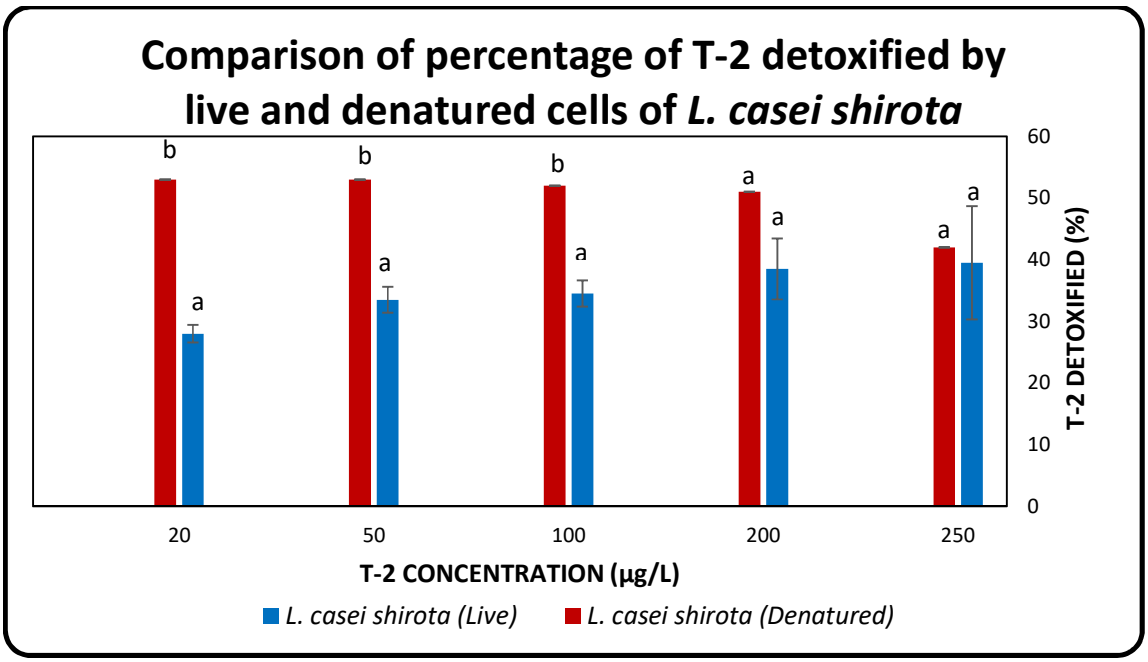

Fig. 34: Comparison of percentages of T-2 detoxified by live and denatured cells of *L. casei shirota* at individual toxin concentration level. Various toxin concentrations and bacteria (10^9 cfu/L) were incubated at 37°C for 24 h. One-way ANOVA was used to test for significant differences between means at a given concentration. The results are shown as mean ± standard deviation. Treatments with different letters in individual column groups are statistically different ($p < 0.05$).

4.6 Cell viability (cells treated with AFB_1 and T-2)

The viability of bacterial cells declined with increase in toxin concentration (Tables 2 & 3) and time (Appendix Tables A5–A10). After incubation for 72 h, reduction of AFB_1 by *L. casei shirota* was in the range of 0.2% to 18.3%, by *L. acidophilus* AS1.3342 between 0.3% to 14.5% and *L. bulgaricus* between 4.2% to 8.2% (Table 2). Similarly, reduction of *L. casei shirota* in T-2 was in the range 0.2% to 13.6%, *L. acidophilus* AS1.3342 1.0% to 19.0% and *L. bulgaricus* 4.7% to 6.1% (Table 3).

Table 2: Viability of cells treated in AFB_1 toxin; 72 h incubation (*L. casei shirota, L. acidophilus* AS1.3342 and *L. bulgaricus)*

	Bacterial viability (%) after 72 h incubation		
AFB_1 concentrations	***L. casei shirota* (%)**	***L. acidophilus* AS1.3342 (%)**	***L. bulgaricus* (%)**
5 µg/L	18.3 a (±0.8)	14.5 a (±1.9)	8.2 a (±0.6)
10 µg/L	4.4 b (±0.1)	12.5 (±0.9)	7.8 ab (±0.6)
20 µg/L	1.7 c (±0.2)	1.4 (±0.2)	5.9 bc (±0.3)
50 µg/L	0.6 cd (±0.2)	1.2 (±0.0)	4.5 c (±0.6)
100 µg/L	0.2 d (±0.0)	0.3 (±0.1)	4.2 c (±0.4)

The percentage of cell viability for each strain was calculated by dividing the number of live cells in the toxin by the number of live cells in the respective controls. Values in bracket are the standard deviation. One-way ANOVA was used to test for significant differences among means within an individual concentration level. Values in individual columns with different letters are statistically different ($p < 0.05$).

Table 3: Viability of cells treated in T-2 toxin; 72 h incubation (*L. casei shirota, L. acidophilus* AS1.3342 and *L. bulgaricus)*

	Bacterial viability (%) after 72 h incubation		
T-2 concentrations	***L. casei shirota* (%)**	***L. acidophilus* AS1.3342 (%)**	***L. bulgaricus* (%)**
20 µg/L	13.6 a (±0.4)	19.0 a (±1.4)	6.1 a (±0.3)
50 µg/L	1.7 b (±0.1)	15.6 b (±1.0)	5.6 ab (±0.3)
100 µg/L	1.0 bc (±0.1)	10.1 c (±0.4)	5.1 ab (±0.3)
200 µg/L	0.3 c (±0.1)	3.3 d (±0.5)	4.8 b (±0.1)
250 µg/L	0.2 c (±0.0)	1.0 d (±0.0)	4.7 b (±0.1)

The percentage of cell viability for each strain was calculated by dividing the number of live cells in the toxin by the number of live cells in the respective controls. One-way ANOVA was used to test for significant differences between means within an individual concentration level. Values in individual columns with different letters are statistically different ($p < 0.05$).

4.7 Comparison between death rate of live bacterial cells and AFB_1 detoxified

As bacterial death rate increased, so did detoxification of AFB_1 increase (Fig. 35)

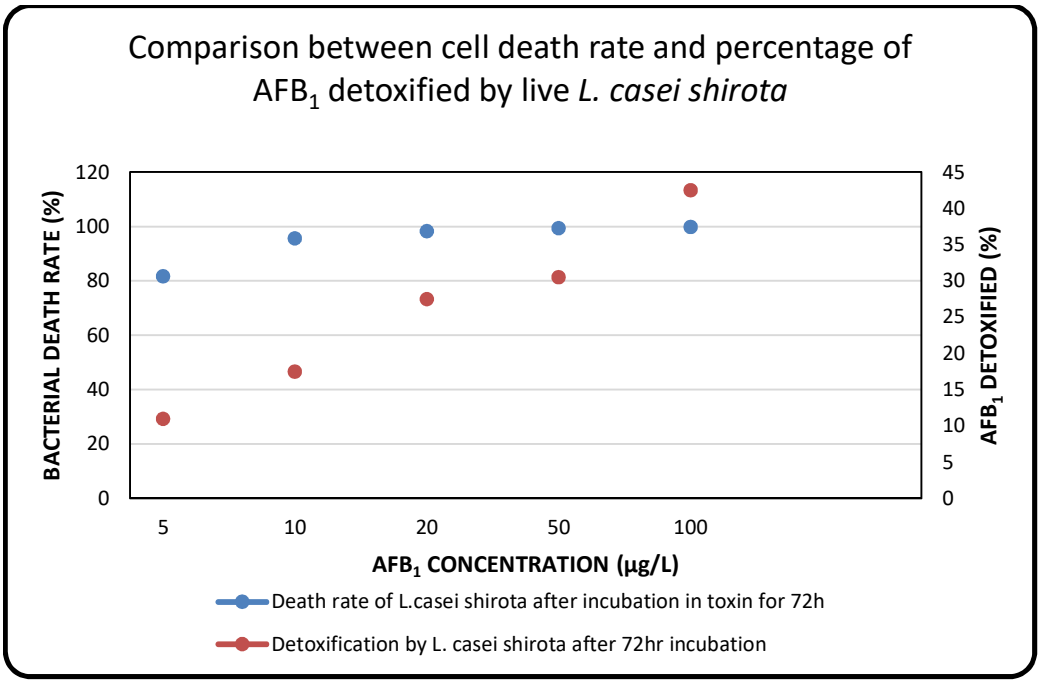

Fig. 35: A comparative profiles of bacterial death rate vs percentage of AFB_1 detoxified by *L. casei shirota* after incubation in AFB_1 for 72 h. Cells were initially cultured to 10^9 cfu/mL.

4.8 Comparison between death rate of live bacterial cells and T-2 detoxified

An increase in cell death rate resulted in an increase in percent of T-2 detoxified (Fig. 36)

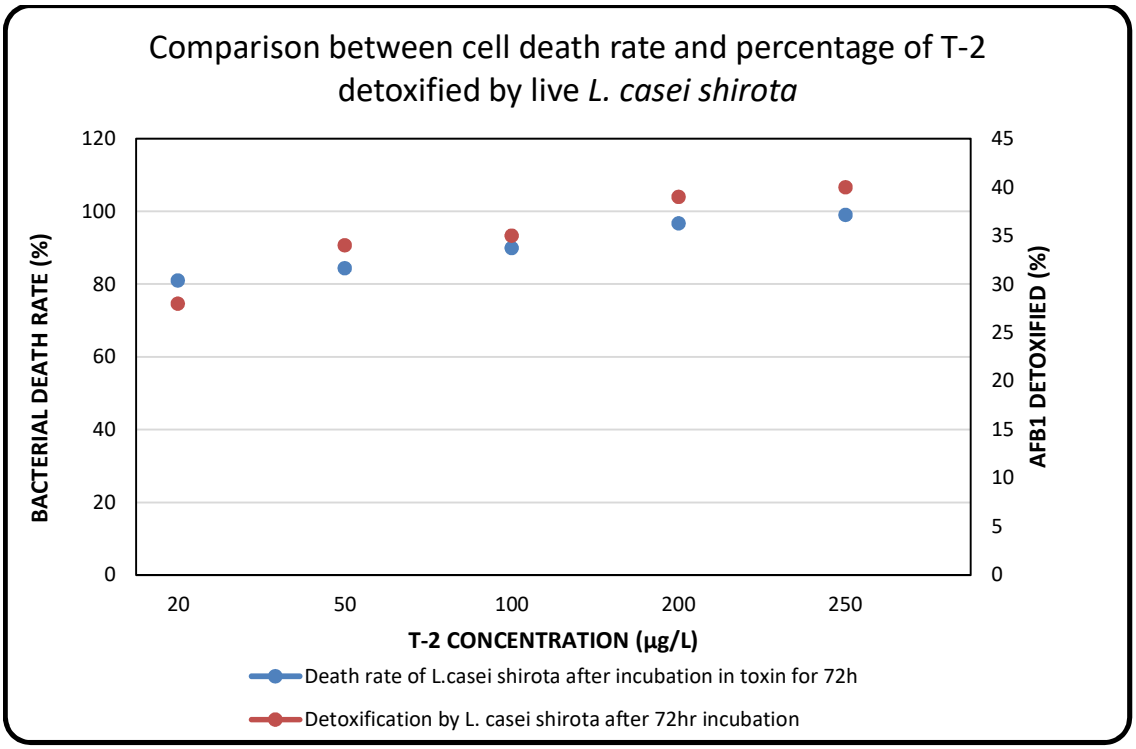

Fig. 36: A comparative profile of bacterial death rate vs percentage of T-2 detoxified by *L. casei shirota* after incubation in T-2 for 72 h. Cells were initially cultured to 10^9 cfu/mL.

Chapter 5

Discussion

The choices of incubation temperature (37°C), bacterial concentration (10^9 CFU/mL) and incubation time periods (24 & 72 h) were based on information from some previous publications. A study by El-Nezami, Kankaanpaa, Salminen, and Ahokas (1998) reported that the rate of mycotoxin removal was both temperature and bacterial concentration dependent and that maximal removal of mycotoxin occurred at 37°C. El-Nezami, Chrevatidis, Auriola, Salminen, and Mykkanen (2002) and Peltonen, El-Nezami, Haskard, Ahokas, and Salminen (2001) recommended 10^9 cfu/mL as the minimum concentration of LAB in the incubation medium that could cause a significant removal of mycotoxins. The bacterial toxin mixtures were incubated for either 24 or 72 h, a range that falls within the estimated time frame of 1–3 days for food residue to pass through the human gut (Cummings, Jenkins, & Wiggins, 1976).

The mechanism of toxin removal by LAB has been hypothesised to be by binding rather than biodegradation. For example, in an experiment by Niderkorn, Boudra, and Morgavi (2006), biodegradation did not appear to be the mode of action for removal of DON and fumonisins (B_1 and B_2) because no toxin metabolites were observed and removal was not impaired in non-viable bacteria. Similarly, in my research, binding rather than biodegradation was hypothesized as the mode of toxin removal by the LAB species. This is supported by the fact that both live and denatured cells (Fig. 31 & 34) were capable of removing AFB_1 and T-2 to a similar extent. It is also supported by the lack of biodegradation products in the AFB_1 and T-2 chromatograms (Fig. 5 & 6). This is also in agreement with observations made by El-Nezami, Polychronaki, et al. (2002) that indicated metabolites or degradation products were not detected when ZEN was incubated with *L. rhamnosus* GG. Along the same lines, Haskard, El-Nezami, Kankaanpaa, Salminen, and Ahokas (2001) showed that there was a strong evidence of effective AFB_1 removal of by all nonviable bacteria through binding rather than via metabolism. As regards a detailed investigation to reveal the binding mechanism, Lahtinen et al. (2004) isolated exopolysaccharides and a cell wall isolate containing peptidoglycan from *L. rhamnosus* strain GG and tested their ability to remove AFB_1. The *L. rhamnosus* strain GG was also subjected to various enzymatic and chemical treatments, and their effects on the binding of AFB_1 by the bacteria were confirmed. In such aforementioned research, the authors reported that there was no evidence for exopolysaccharides, cell wall proteins, Ca^{2+} or Mg^{2+} being involved in AFB_1 binding. AFB_1 binding appeared to be to the cell wall isolate, indicating that AFB_1 is bound to the cell wall peptidoglycan of *L. rhamnosus* or compounds tightly associated with the peptidoglycan. The authors reported that binding was the mechanism by which LABs detoxified the toxin from the media.

My study has revealed that LAB isolated from food can remove AFB_1 and T-2 from the media. *L. casei shirota* detoxified between 0.6 and 42.9 µg/L AFB_1 (Fig. 7) and 3.7–95.6 µg/L T-2 (Fig. 11). *L. acidophilus* AS1.3342 detoxified between 0.5 and 45.7 µg/L AFB_1 (Fig. 8) and 2.9–110.8 µg/L T-2 (Fig. 12). The mixture of CY strains detoxified 0.7–42.6 µg/L AFB_1 (Fig. 9) and 3.0–112.0 µg/L T-2 (Fig. 13). Expressed as a percentage, *L. casei shirota* detoxified 11–43% AFB_1 (Fig. 15) and 19–38% T-2 (Fig. 19). *L. acidophilus* AS1.3342 detoxified 10–46% AFB_1 (Fig. 16) and 15–45% T-2 (Fig. 20). The mixture of CY strains detoxified 14–43% AFB_1 (Fig. 17) and 15–45% T-2 (Fig. 21). There was no significant difference among the means of the three strains in toxin removal. These findings were similar to the observations made by Haskard et al. (2001) where the viable cells of *L. casei shirota*, *L. acidophilus* LC1 isolated from food and a pure culture of *L. acidophilus* ATCC 4356 were able to remove up to 21.8%, 59.7% and 48.3% of AFB_1 respectively. The results in this study also mirrored data observed by Zou et al. (2012) on the ability of four strains of LAB species and one strain of *Lactococcus* species to remove DON and T-2 from MRS broth. This study by Zou et al. (2012), demonstrated that the tested strains had the ability to remove DON and T-2. However, the rate of toxin removal depended on the bacterial strain with the strains of *L. plantarum* showing the highest T-2 removal at 19.90 ± 1.70%. In another closely related study involving assessment of the abilities of yoghurt starter cultures (*L. bulgaricus* and *Streptococcus thermophilus*) to remove aflatoxin M1 (AFM_1) from yoghurt and PBS medium, El Khoury, Atoui, and Yaghi (2011) found that yoghurt starter cultures were able to remove AFM_1 in yoghurt and PBS but the rate of removal was higher by the bacteria in yoghurt than by the PBS. Similarly, Elsanhoty, Salam, Ramadan, and Badr (2014) revealed that the addition of probiotic LAB to starter cultures increased the rate of AFM_1 removal during yoghurt production and storage. Halttunen, Collado, El-Nezami, Meriluoto, and Salminen (2008) also showed that the toxin-removal capacity of a combination of strains of LAB bacteria did not conform to the sum of toxin removal by the individual bacteria. My results are in agreement with these in that the toxin removal rate by a mixture of CY strains (*L. bulgaricus, L. acidophilus* and *Streptococcus thermophilus*) was not significantly different to the removal by the reference culture used in my experiment (Figs 18 & 22).

As shown in Figs 23 and 26, the quantity of AFB_1 and T-2 detoxified by the live cells of the FMD strain increased as toxin concentration increased. This observation supports the report by El-Nezami et al. (1998) which indicated that the quantity of AFB_1 detoxified increased with increasing concentration of the toxin. However, it contrasts with Line and Brackett (1995) who showed that the percentage of AFB_1 detoxified was lower as the toxin levels increased. The most probable explanation for the linear increase in percentage detoxified by live LAB was deemed to be the cell viability. This proposition was tested by comparing the detoxification of the two toxins by both live and denatured cells of the FMD strain and by analysing the effect of toxin concentrations on LAB. When either live or denatured cells of *L. casei shirota* were exposed to various concentrations of AFB_1, the quantity of toxin detoxified

increased as the toxin concentration increased (Figs 23 & 24). Denatured *L. casei shirota* cells detoxified a greater quantity of AFB_1 toxin than the live cells (Fig. 25). Similarly, when both the live and denatured cells of *L. casei shirota* were exposed to various concentrations of T-2, the quantity detoxified by both the denatured and live cells increased as the toxin concentration increased (Figs 26 & 27). There was a significant difference in the amount of T-2 detoxified between the live and denatured cells (Fig. 28). However, the difference was only significant at 20, 50 and 100 µg/L concentrations and not at 200 and 250 µg/L (Fig. 20). Thus 100 µg/L could be treated as the effect threshold for both the live and denatured cells. The second action to test the proposition involved mixing the viable cells with various toxin concentrations and determining LAB viability after incubation for different periods. From the experiment, it was apparent that when the viable LAB cells were mixed with various concentrations of AFB_1 or T-2 and incubated at 37°C for a total of 72 h, there was a significant decrease in cell viability with increase in toxin concentration (Tables 2 & 3). Cell viability also declined with increase in incubation time (Tables B5–B10 in appendix B). As shown in Fig. 35 and 36, detoxification rate increased with the increase in dead (non-viable) cells. Lower toxin concentrations had lower cell death resulting in lower toxin removal (absolute quantity and as a percentage). Based on these observations, it was apparent that as the cell viability decreased, the toxin removal increased. A closely related observation was made by Line and Brackett (1995), who reported that more AFB_1 were detoxified by 72-h cultures of *Flavobacterium aurantiacum* than by 24-h cultures; probably due to the presence of more dead cells in the 72-h culture than the 24-h culture.

AFB_1 and T-2 removal rates by denatured *L. casei shirota* cells were significantly higher than by the live cells (Figs 31 & 34). An experiment by Oluwafemi and Da-Silva (2009) on removal of aflatoxins by viable and heat-killed LAB species isolated from fermented maize reported a significant difference between the heated and live LAB species. It was noted that the detoxification was significantly higher in the experiment that used heat treated cells. The authors reported that autoclaving led to denaturation of bacterial proteins and enzymes leaving behind the peptidoglycan that provided a favourable binding medium. The original binding sites of the viable microorganisms may have changed by heat treatment thereby exposing new binding sites.

In my experiments, when a comparison was made between the percentage (%) and quantity (µg/L) detoxified by denatured cells of *L. casei shirota*, the absolute quantity of toxin detoxified by denatured cells increased with increase in toxin concentration (Fig. 24 & 27) while in percentage terms, there was a decrease with increase in toxin concentration but the decrease was not significant (Fig. 29 & 33). It is possible that after heat treatment, the peptidoglycan of LAB or compounds tightly associated with the peptidoglycan were already exposed, meaning the denatured LAB cells could easily reach their

absorption saturation points hence any additional toxin concentration only contributed to a reduction in percentage detoxified.

Another significant observation from this project is that LAB either in a viable or non-viable state were efficient in toxin removal and hence would be useful for treatment of digestive syndromes at least in the early stages of exposure to toxins when most of the toxin(s) are still in the gastrointestinal tract. For example, El-Nezami et al. (2000) reported that a mixture of *Lactobacillus* and *Propionibacterium* were capable of Influencing the faecal aflatoxin content in healthy Egyptian volunteers. Volunteers who were administered the probiotic preparation recorded a significant reduction in the level of AFB_1 after the second week of the trial and further reduction during the follow up period. Faecal levels of aflatoxin were thought to reflect the exposure to aflatoxin.

Chapter 6

Conclusions

In this study, it was demonstrated that LAB isolated from some foods such as fermented milk and yoghurt type foods possess the ability to detoxify AFB_1 and T-2. Toxin removal by the bacteria isolated from the foods and the reference cultures were similar. The relationship between detoxification rate and toxin concentrations was demonstrated. At a bacterial concentration of 10^9 cfu/mL, toxin removal increased with toxin concentration. However, there appeared to be a threshold for T-2 beyond which there was no significant difference in toxin removal between toxin concentrations. This was at 100 µg/L for T-2. Equally, the impact of cell viability on detoxification was highlighted. It was shown that both live and denatured cells possessed the ability to remove AFB_1 and T-2. This is of significance because it demonstrates that probiotics, despite their universally accepted definition, still have the ability to remove toxins even in a non-viable state and possibly pass health benefits to the consumers by reducing exposure to mycotoxins. Based on the findings of this project, I believe that these LAB can act as biological agents for AFB_1 and T-2 reduction by binding the mycotoxins and thereby reducing their absorption via the gastrointestinal tract into the blood. However, to ascertain this benefit, further research such as clinical trials (in vivo tests), particularly those aimed at assessing the ability of probiotic-enriched foods to reduce mycotoxin absorption into the blood and also tissue kinetic studies need to be carried out.

Chapter 7

References

Abbas, A., Valez, H., & Dobson, A. D. W. (2009). Analysis of the effect of nutritional factors on OTA and OTB biosynthesis and polyketide synthase gene expression in Aspergillus ochraceus. *International Journal of Food Microbiology, 135*(1), 22-27. doi:http://dx.doi.org/10.1016/j.ijfoodmicro.2009.07.014

Bata, A., & Lasztity, R. (1999). Detoxification of mycotoxin-contaminated food and feed by microorganisms. *Trends in Food Science & Technology, 10*(6–7), 223-228. doi:http://dx.doi.org/10.1016/S0924-2244(99)00050-3

Bennett, J. W., & Klich, M. (2003). Mycotoxins. *Clinical Microbiology Reviews, 16*(3), 497-516. doi:10.1128/cmr.16.3.497-516.2003

Beveridge, T. J., Pouwels, P. H., Sára, M., Kotiranta, A., Lounatmaa, K., Kari, K., Koval, S. F. (1997). V. Functions of S-layers. *FEMS Microbiology Reviews, 20*(1-2), 99-149. doi:10.1111/j.1574-6976.1997.tb00305.x

Bhat, R., Rai, R. V., & Karim, A. A. (2010). Mycotoxins in food and feed: Present status and future concerns. *Comprehensive Reviews in Food Science and Food Safety, 9*(1), 57-81. doi:10.1111/j.1541-4337.2009.00094.x

Binder, E. M., Tan, L. M., Chin, L. J., Handl, J., & Richard, J. (2007). Worldwide occurrence of mycotoxins in commodities, feeds and feed ingredients. *Animal Feed Science and Technology, 137*(3–4), 265-282. doi:http://dx.doi.org/10.1016/j.anifeedsci.2007.06.005

Bueno, D. J., Casale, C. H., Pizzolitto, R. P., Salvano, M. A., & Oliver, G. (2007). Physical Adsorption of Aflatoxin B1 by Lactic Acid Bacteria and S accharomyces cerevisiae: A Theoretical Model. *Journal of Food Protection®, 70*(9), 2148-2154.

Bullerman, L. B., & Bianchini, A. (2007). Stability of mycotoxins during food processing. *International Journal of Food Microbiology, 119*(1–2), 140-146. doi:http://dx.doi.org/10.1016/j.ijfoodmicro.2007.07.035

Chapot-Chartier, M.-P., & Kulakauskas, S. (2014). Cell wall structure and function in lactic acid bacteria. *Microbial cell factories, 13*(1), 1.

Chelkowski, J. (2014). *Fusarium: Mycotoxins, Taxonomy, Pathogenicity*: Elsevier.

Chen, J.-h., Cao, J.-l., Chu, Y.-l., Wang, Z.-l., Yang, Z.-t., & Wang, H.-l. (2008). T-2-induced apoptosis involving Fas, p53, Bcl-xL, Bcl-2, Bax and caspase-3 signaling pathways in human chondrocytes. *Journal of Zhejiang University Science B, 9*(6), 455-463. doi:10.1631/jzus.B0820013

Codex Alimentarius. (2014). *Code of practice for the prevention and reduction of mycotoxin contamination in cereals*. Retrieved from www.codexalimentarius.org/input/download/CXP_051e_2014.pdf.

Creppy, E. E. (2002). Update of survey, regulation and toxic effects of mycotoxins in Europe. *Toxicology Letters, 127*(1–3), 19-28. doi:http://dx.doi.org/10.1016/S0378-4274(01)00479-9

Cummings, J., Jenkins, D., & Wiggins, H. (1976). Measurement of the mean transit time of dietary residue through the human gut. *Gut, 17*(3), 210-218.

Daniels, J. M., Liu, L., Stewart, R. K., & Massey, T. E. (1990). Biotransformation of aflatoxin B_1 in rabbit lung and liver microsomes. *Carcinogenesis, 11*(5), 823-827. doi:10.1093/carcin/11.5.823

Degen, G. H., & Neumann, H.-G. (1978). The major metabolite of aflatoxin B 1 in the rat is a glutathione conjugate. *Chemico-biological interactions, 22*(2), 239-255.

Delcour, J., Ferain, T., Deghorain, M., Palumbo, E., & Hols, P. (1999). The biosynthesis and functionality of the cell-wall of lactic acid bacteria. *Antonie Van Leeuwenhoek*, *76*(1-4), 159-184.

Diaz, D.E., Whitlow, L.W., & Hagler, W.M., Jr. (2013). Mycotoxins in feed. *Feedstuffs, 85*(37), 65.

El-Nezami, H., Mykkanen, H., Kankaanpaa, P., Suomalainen, T., Salminen, S., & Ahokas, J. (2000). Ability of a mixture of Lactobacillus and Propionibacterium to influence the faecal aflatoxin content in healthy Egyptian volunteers: a pilot clinical study. *Bioscience and Microflora*, *19*(1), 41-45.El-Nezami, H., Kankaanpaa, P., Salminen, S., & Ahokas, J. (1998). Ability of dairy strains of lactic acid bacteria to bind a common food carcinogen, aflatoxin B1. *Food and Chemical Toxicology, 36*(4), 321-326. doi:http://dx.doi.org/10.1016/S0278-6915(97)00160-9

El-Nezami, H., Polychronaki, N., Salminen, S., & Mykkanen, H. (2002). Binding rather than metabolism may explain the interaction of two food-grade Lactobacillus strains with Zearalenone and Its Derivative ά-Zearalenol. *Applied and Environmental Microbiology, 68*(7), 3545-3549. doi:10.1128/aem.68.7.3545-3549.2002

El-Nezami, H. S., Chrevatidis, A., Auriola, S., Salminen, S., & Mykkanen, H. (2002). Removal of common Fusarium toxins in vitro by strains of Lactobacillus and Propionibacterium. *Food Additives & Contaminants, 19*(7), 680-686. doi:10.1080/02652030210134236

El-Nezami, H. S., Nicoletti, G., Neal, G. E., Donohue, D. C., & Ahokas, J. T. (1995). Aflatoxin M1 in human breast milk samples from Victoria, Australia and Thailand. *Food and Chemical Toxicology, 33*(3), 173-179. doi:http://dx.doi.org/10.1016/0278-6915(94)00130-G

El Khoury, A., Atoui, A., & Yaghi, J. (2011). Analysis of aflatoxin M1 in milk and yogurt and AFM1 reduction by lactic acid bacteria used in Lebanese industry. *Food Control, 22*(10), 1695-1699. doi:http://dx.doi.org/10.1016/j.foodcont.2011.04.001

Elsanhoty, R. M., Salam, S. A., Ramadan, M. F., & Badr, F. H. (2014). Detoxification of aflatoxin M1 in yoghurt using probiotics and lactic acid bacteria. *Food Control, 43*, 129-134. doi:http://dx.doi.org/10.1016/j.foodcont.2014.03.002

EU. (2001). *Fusarium toxins: T-2 and HT-2*. Retrieved from http://ec.europa.eu/food/fs/sc/scf/out88_en.pdf.

EU. (2003). *Collection of occurrence data of Fusarium toxins in food and assessment of dietary intake by the population of EU member states*. Retrieved from http://ec.europa.eu/food/fs/scoop/task3210.pdf.

EU. (2013). *Commission recommendation on the presence of T-2 and HT-2 in cereals and cereal products*. Retrieved from http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32013H0165.

FAO. (2004). *Worldwide regulations for mycotoxins in food and feed in 2003*. Retrieved from http://www.fao.org/docrep/007/y5499e/y5499e0n.htm#bm23.2.

FAO. (2008). High-level conference on world food security: The challenges of climate change and bioenergy Symposium conducted at the meeting of the Food safety and climate change: , Rome, Italy. Retrieved from http://www.fao.org/foodclimate/hlc-home/en/

FAO. (2016). *Food Safety and Quality: Mycotoxins*. Retrieved from www.fao.org/food/food-safety-quality/a-z-index/mycotoxins/en/.

FAO/WHO. (2001). Probiotics in food: Health and nutritional properties and guidelines for evaluation. ftp://ftp.fao.org/docrep/fao/009/a0512e/a0512e00.pdf

Gnonlonfin, G. J. B., Hell, K., Adjovi, Y., Fandohan, P., Koudande, D. O., Mensah, G. A., Brimer, L. (2013). A review on aflatoxin contamination and its implications in the developing world: A Sub-Saharan African Perspective. *Critical Reviews in Food Science and Nutrition, 53*(4), 349-365. doi:10.1080/10408398.2010.535718

Granato, D., Branco, G. F., Cruz, A. G., Faria, J. d. A. F., & Shah, N. P. (2010). Probiotic dairy products as functional foods. *Comprehensive Reviews in Food Science and Food Safety, 9*(5), 455-470. doi:10.1111/j.1541-4337.2010.00120.x

Groopman, J. D., Donahue, P. R., Zhu, J. Q., Chen, J. S., & Wogan, G. N. (1985). Aflatoxin metabolism in humans: Detection of metabolites and nucleic acid adducts in urine by affinity chromatography. *Proceedings of the National Academy of Sciences*, *82*(19), 6492-6496.Halttunen, T., Collado, M. C., El-Nezami, H., Meriluoto, J., & Salminen, S. (2008). Combining strains of lactic acid bacteria may reduce their toxin and heavy metal removal efficiency from aqueous solution. *Letters in Applied Microbiology, 46*(2), 160-165. doi:10.1111/j.1472-765X.2007.02276.x

Haskard, C. A., El-Nezami, H. S., Kankaanpaa, P. E., Salminen, S., & Ahokas, J. T. (2001). Surface Binding of Aflatoxin B1 by Lactic Acid Bacteria. *Applied and Environmental Microbiology, 67*(7), 3086-3091. doi:10.1128/aem.67.7.3086-3091.2001

He, J., Zhou, T., Young, J. C., Boland, G. J., & Scott, P. M. (2010). Chemical and biological transformations for detoxification of trichothecene mycotoxins in human and animal food chains: a review. *Trends in Food Science & Technology, 21*(2), 67-76. doi:http://dx.doi.org/10.1016/j.tifs.2009.08.002

Hernandez-Mendoza, A., Garcia, H. S., & Steele, J. L. (2009). Screening of *Lactobacillus casei* strains for their ability to bind aflatoxin B1. *Food and Chemical Toxicology, 47*(6), 1064-1068. doi:http://dx.doi.org/10.1016/j.fct.2009.01.042

Hernandez-Mendoza, A., Guzman-De-Peña, D., González-Córdova, A. F., Vallejo-Córdoba, B., & Garcia, H. S. (2010). In vivo assessment of the potential protective effect of *Lactobacillus casei Shirota* against aflatoxin B1. *Dairy Science & Technology, 90*(6), 729-740.

Hussein, H. M., Franich, R., Baxter, M., & Andrew, I. (1989). Naturally occurring Fusarium toxins in New Zealand maize. *Food Additives & Contaminants, 6*(1), 49-57.

ICRISAT, Ananth, S. B., & Farid, W. (2015). *Importance of aflatoxins in human and livestock health*. Retrieved from http://www.icrisat.org/aflatoxin/health.asp.

International Agency for Research on Cancer. (2002). *IARC monographs on the evaluation of carcinogenic risks to humans*. Retrieved from http://monographs.iarc.fr/ENG/Monographs/vol82/index.php.

Jouany, J. P. (2007). Methods for preventing, decontaminating and minimizing the toxicity of mycotoxins in feeds. *Animal Feed Science and Technology, 137*(3–4), 342-362. doi:http://dx.doi.org/10.1016/j.anifeedsci.2007.06.009

Kabak, B., Dobson, A. D. W., & Var, I. (2006). Strategies to prevent mycotoxin contamination of food and animal feed: A review. *Critical Reviews in Food Science and Nutrition, 46*(8), 593-619. doi:10.1080/10408390500436185

Kassim, N., Kim, K., Mtenga, A. B., Song, J. E., Liu, Q., Shim, W. B., & Chung, D. H. (2011). A preliminary study of T-2 and HT-2 in cereals sold in traditional market in South Korea. *Food Control, 22*(8), 1408-1412. doi:http://dx.doi.org/10.1016/j.foodcont.2011.02.023

Kirby, G., Wolf, C., Neal, G., Judah, D., Henderson, C., Srivatanakul, P., & Wild, C. (1993). In vitro metabolism of aflatoxin B1 by normal and tumorous liver tissue from Thailand. *Carcinogenesis, 14*(12), 2613-2620.

König, H., & Fröhlich, J. (2009). Lactic acid bacteria. In *Biology of Microorganisms on Grapes, in Must and in Wine* (pp. 3-29).

Lahtinen, S. J., Haskard, C. A., Ouwehand, A. C., Salminen, S. J., & Ahokas, J. T. (2004). Binding of aflatoxin B_1 to cell wall components of Lactobacillus rhamnosus strain GG. *Food Additives & Contaminants, 21*(2), 158-164. doi:10.1080/02652030310001639521

Lewis, L., Onsongo, M., Njapau, H., Schurz-Rogers, H., Luber, G., Kieszak, S., Misore, A. (2005). Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in eastern and central Kenya. *Environmental Health Perspectives*, 1763-1767.

Li, F.-Q., Yoshizawa, T., Kawamura, O., Luo, X.-Y., & Li, Y.-W. (2001). Aflatoxins and Fumonisins in corn from the high-incidence area for human hepatocellular carcinoma in Guangxi, China. *Journal of Agricultural and Food Chemistry, 49*(8), 4122-4126. doi:10.1021/jf010143k

Li, Y., Wang, Z., Beier, R. C., Shen, J., Smet, D. D., De Saeger, S., & Zhang, S. (2011). T-2, a Trichothecene Mycotoxin: Review of Toxicity, Metabolism, and Analytical Methods. *Journal of Agricultural and Food Chemistry, 59*(8), 3441-3453. doi:10.1021/jf200767q

Lin, W.-H., Hwang, C.-F., Chen, L.-W., & Tsen, H.-Y. (2006). Viable counts, characteristic evaluation for commercial lactic acid bacteria products. *Food Microbiology, 23*(1), 74-81. doi:http://dx.doi.org/10.1016/j.fm.2005.01.013

Lincoln University. (2016). *House Rules for the Study of Masters at Lincoln University*. Retrieved from http://www.lincoln.ac.nz/PageFiles/23364/Current%20April%20Masters-House-Rules.pdf.

Line, J., & Brackett, R. (1995). Factors affecting aflatoxin B_1 removal by Flavobacterium aurantiacum. *Journal of Food Protection®, 58*(1), 91-94.

Logrieco, A., Bottalico, A., Mulé, G., Moretti, A., & Perrone, G. (2003). Epidemiology of Toxigenic Fungi and their Associated Mycotoxins for Some Mediterranean Crops [journal article]. *European Journal of Plant Pathology, 109*(7), 645-667. doi:10.1023/a:1026033021542

Lu, P., Wu, C., Shi, Q., Wang, Y., Sun, L., Liao, J., Liu, Y. (2015). A Sensitive and Validated Method for Determination of T-2 and HT-2 Residues in Shrimp Tissues by LC-MS/MS. *Food Analytical Methods*, 1-15.

Magan, N., & Aldred, D. (2007). Post-harvest control strategies: Minimizing mycotoxins in the food chain. *International Journal of Food Microbiology, 119*(1–2), 131-139. doi:http://dx.doi.org/10.1016/j.ijfoodmicro.2007.07.034

Matumba, L., Van Poucke, C., Ediage, E. N., & De Saeger, S. (2015). Keeping mycotoxins away from the food: Does the existence of regulations have any impact in Africa? *Critical Reviews in Food Science and Nutrition*, 00-00. doi:10.1080/10408398.2014.993021

Murphy, P. A., Hendrich, S., Landgren, C., & Bryant, C. M. (2006). Food mycotoxins: An update. *Journal of food science, 71*(5), R51-R65.

Neuhaus, F. C., & Baddiley, J. (2003). A Continuum of Anionic Charge: Structures and Functions of d-Alanyl-Teichoic Acids in Gram-Positive Bacteria. *Microbiology and Molecular Biology Reviews, 67*(4), 686-723. doi:10.1128/mmbr.67.4.686-723.2003Niderkorn, V., Boudra, H., & Morgavi, D. P. (2006). Binding of Fusarium mycotoxins by fermentative bacteria in vitro. *Journal of Applied Microbiology, 101*(4), 849-856. doi:10.1111/j.1365-2672.2006.02958.x

Niderkorn, V., Morgavi, D. P., Aboab, B., Lemaire, M., & Boudra, H. (2009). Cell wall component and mycotoxin moieties involved in the binding of fumonisin B1 and B2 by lactic acid bacteria. *Journal of Applied Microbiology, 106*(3), 977-985. doi:10.1111/j.1365-2672.2008.04065.x

Nishiyama, K., Sugiyama, M., & Mukai, T. (2016). Adhesion Properties of Lactic Acid Bacteria on Intestinal Mucin. *Microorganisms, 4*(3), 34.

Nyikal, J., Misore, A., Nzioka, C., Njuguna, C., Muchiri, E., Njau, J. & Langat, A. (2004). Outbreak of aflatoxin poisoning—eastern and central provinces, Kenya, January–July 2004. *Morb Mortal Wkly Rep*, *53*(34), 790-3.

Ohta, M., Ishii, K., & Ueno, Y. (1977). Metabolism of Trichothecene Mycotoxins: I. Microsomal Deacetylation of T-2 in Animal Tissues. *Journal of Biochemistry, 82*(6), 1591-1598.

Oluwafemi, F., & Da-Silva, F. (2009). Removal of aflatoxins by viable and heat-killed Lactobacillus species isolated from fermented maize. *Journal of Applied Biosciences, 16*, 871-876.

Peltonen, K., El-Nezami, H., Haskard, C., Ahokas, J., & Salminen, S. (2001). Aflatoxin B_1 binding by dairy strains of lactic acid bacteria and Bifidobacteria. *Journal of Dairy Science, 84*(10), 2152-2156. doi:http://dx.doi.org/10.3168/jds.S0022-0302(01)74660-7

Peltonen, K. D., El-Nezami, H. S., Salminen, S. J., & Ahokas, J. T. (2000). Binding of aflatoxin B_1 by probiotic bacteria. *Journal of the Science of Food and Agriculture, 80*(13), 1942-1945. doi:10.1002/1097-0010(200010)80:13<1942::AID-JSFA741>3.0.CO;2-7

Pettersson, H., Brown, C., Hauk, J., Hoth, S., Meyer, J., & Wessels, D. (2011). Survey of T-2 and HT-2 by LC–MS/MS in oats and oat products from European oat mills in 2005–2009. *Food Additives & Contaminants. Part B, Surveillance, 4*(2), 110-115. doi:10.1080/19393210.2011.561933

Pitt, J. I. (2000). Toxigenic fungi: Which are important? *Medical Mycology, 38*(Supplement 1), 17-22. doi:10.1080/mmy.38.s1.17.22

Reddy, K. R. N., Reddy, C. S., & Muralidharan, K. (2009). Detection of Aspergillus spp. and aflatoxin B_1 in rice in India. *Food Microbiology, 26*(1), 27-31. doi:http://dx.doi.org/10.1016/j.fm.2008.07.013

Richard, J. L. (2007). Some major mycotoxins and their mycotoxicoses—An overview. *International Journal of Food Microbiology, 119*(1–2), 3-10. doi:http://dx.doi.org/10.1016/j.ijfoodmicro.2007.07.019

Shah, N. P. (2007). Functional cultures and health benefits. *International Dairy Journal, 17*(11), 1262-1277. doi:http://dx.doi.org/10.1016/j.idairyj.2007.01.014

Stiles, M. E., & Holzapfel, W. H. (1997). Lactic acid bacteria of foods and their current taxonomy. *International journal of food microbiology, 36*(1), 1-29.

Sweeney, M. J., & Dobson, A. D. (1998). Mycotoxin production by Aspergillus, Fusarium and Penicillium species. *International journal of food microbiology, 43*(3), 141-158.

Taevernier, L., Wynendaele, E., De Vreese, L., Burvenich, C., & De Spiegeleer, B. (2016). The mycotoxin definition reconsidered towards fungal cyclic depsipeptides. *Journal of Environmental Science and Health, Part C, 34*(2), 114-135. doi:10.1080/10590501.2016.1164561

Teuber, M. (1993). Lactic acid bacteria. *Biotechnology Set, Second Edition*, 325-366.

US Food and Drug Administration Office of Regulatory Affairs. (2013). *ORA Laboratory Manual: Mycotoxin analysis.* Retrieved from http://www.fda.gov/ScienceResearch/FieldScience/LaboratoryManual/default.htm.

van Egmond, H., Schothorst, R., & Jonker, M. (2007). Regulations relating to mycotoxins in food. *Analytical and Bioanalytical Chemistry, 389*(1), 147-157. doi:10.1007/s00216-007-1317-9

Visconti, A., & Mirocha, C. J. (1985). Identification of various T-2 metabolites in chicken excreta and tissues. *Applied and Environmental Microbiology, 49*(5), 1246-1250.

Wang, J.-S., Huang, T., Su, J., Liang, F., Wei, Z., Liang, Y., Sun, G. (2001). Hepatocellular carcinoma and aflatoxin exposure in Zhuqing village, Fusui county, People's Republic of China. *Cancer Epidemiology Biomarkers & Prevention, 10*(2), 143-146.

Wogan, G. N., Edwards, G. S., & Newberne, P. M. (1971). Structure-Activity Relationships in Toxicity and Carcinogenicity of Aflatoxins and Analogs. *Cancer Research, 31*(12), 1936-1942.

Woloshuk, C. P., & Shim, W.-B. (2013). Aflatoxins, fumonisins, and trichothecenes: a convergence of knowledge. *FEMS Microbiology Reviews, 37*(1), 94-109. doi:10.1111/1574-6976.12009

Yang, J., Li, J., Jiang, Y., Duan, X., Qu, H., Yang, B., Sivakumar, D. (2014). Natural occurrence, analysis, and prevention of mycotoxins in fruits and their processed products. *Critical Reviews in Food Science and Nutrition, 54*(1), 64-83. doi:10.1080/10408398.2011.569860

Yard, E. E., Daniel, J. H., Lewis, L. S., Rybak, M. E., Paliakov, E. M., Kim, A. A., Sharif, S. K. (2013). Human aflatoxin exposure in Kenya, 2007: A cross-sectional study. *Food Additives & Contaminants: Part A, 30*(7), 1322-1331. doi:10.1080/19440049.2013.789558

Zain, M. E. (2011). Impact of mycotoxins on humans and animals. *Journal of Saudi Chemical Society, 15*(2), 129-144. doi:http://dx.doi.org/10.1016/j.jscs.2010.06.006

Zou, Z.-Y., He, Z.-F., Li, H.-J., Han, P.-F., Meng, X., Zhang, Y., Tang, J. (2012). In vitro removal of deoxynivalenol and T-2 by lactic acid bacteria. *Food Science and Biotechnology, 21*(6), 1677-1683. doi:10.1007/s10068-012-0223-x

Appendix A

Health and safety hazards and control measures

Potential health and safety hazards are summarised in Table A1.

Table A 1: Potential health and safety hazards and proposed control measures

Location	Hazards	Management
Laboratory	Chemicals, mycotoxins and microorganisms	Use protective clothing: lab coats, closed footwear, nose mask, hand gloves, goggles or visors as required. Follow instructions on the label of containers. Waste to be disposed of in the correct bags. Microbe waste only in the bio-hazard bin. Chemicals to be stored in the appropriate storage facilities with appropriate labelling. Hands to be washed after all lab work is completed
Laboratory	UV light on in biosafety cabinet	UV light to be switched off before working in the biosafety cabinet
Laboratory	Back, wrist and eye pain from microscope work	Ensure seat height is correctly adjusted and supportive. If using glasses, ensure they are detoxified before observing through the microscope lens. Adjourn for short breaks
Work station	Hand, back and eye strain using computer for prolonged periods	Adjust chair to correct height and in position with respect to monitor, keyboard and working table. Obtain appropriate supports for feet and back. Adjourn for short breaks
Occupational Overuse Syndrome	Performing a repetitive task over a long time	Regular breaks and undertaking alternate tasks
Travels	Road motor accidents	When travelling in a personal car, ensure the vehicle is safe and fully certified. When using hired car, ensure the vehicle is safe, fully certified and driven by approved licence-holding driver

Appendix B

Summary of statistical analysis

Table B1: Statistical results for AFB_1 detoxification rate at 5 µg/L (FMD strain, CY strains and Reference strain)

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Bacteria	2	17.33	8.667	2.17	0.262
Error	3	12.00	4.000		
Total	5	29.33			
S	R-sq	R-sq(adj)	R-sq(pred)		
2	59.09%	31.82%	0.00%		

The detoxification rate among the means of the three treatments (FMD strain, CY strains and Reference strain) were not significantly different ($p < 0.05$).

Table B2: Statistical results for T-2 detoxification rate at 20 µg/L (FMD strain, CY strains and Reference strain)

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Bacteria	2	24.33	12.167	2.92	0.198
Error	3	12.50	4.167		
Total	5	36.83			
S	R-sq	R-sq(adj)	R-sq(pred)		
2.04124	66.06%	43.44%	0.00%		

The detoxification rate among the means of the three treatments (FMD strain, CY strains and Reference strain) were not significantly different ($p < 0.05$).

Table B3: Statistical results for detoxification of 5 µg/L of AFB_1 by the live and denatured cells of *L. casei shirota*

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Bacteria	1	2601.00	2601.00	5202.00	0.000
Error	2	1.00	0.50		
Total	3	2602.00			
S	R-sq	R-sq(adj)	R-sq(pred)		
0.707107	99.96%	99.94%	99.85%		

The detoxification rates between the live and denatured cells were significantly different ($p < 0.05$).

Table B4: Statistical results for detoxification of 20 µg/L of T-2 by the live and denatured cells of *L. casei shirota*

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Bacteria	1	625.000	625.000	625.00	0.002
Error	2	2.000	1.000		
Total	3	627.000			
S	R-sq	R-sq(adj)	R-sq(pred)		
1	99.68%	99.52%	98.72%		

There was a significant difference between the live and denatured cells in detoxification of T-2.

Table B5: Viability of *L. casei shirota* exposed to various concentrations of AFB_1 and in control

L. casei shirota	**AFB_1 concentrations**	**Bacterial load (cfu/mL) after incubation with AFB_1**			**Bacterial load in control sample (cfu/mL) after incubation**		
Incubation time		**24 h**	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
Toxin concentrations	5 µg/L	$2.8x10^{8a}$ (5.1)	$2.9x10^{7a}$ (2.5)	$8.3x10^{6a}$ (4.3)	$3.8x10^{9}$ (9.4)	$2.9x10^{8}$ (12.4)	$4.5x10^{7}$ (15.7)
	10 µg/L	$1.50x10^{8b}$ (4.9)	$2.0x10^{7b}$ (7.1)	$2.0x10^{6b}$ (1.8)			
	20 µg/L	$1.1x10^{8c}$ (9.9)	$3.9x10^{6c}$ (3.6)	$7.5x10^{5c}$ (9.4)			
	50 µg/L	$8.7x10^{7cd}$ (4.9)	$3.0x10^{6cd}$ (7.2)	$2.5x10^{5c}$ (28.3)			
	100 µg/L	$5.6x10^{7d}$ (11.5)	$5.3x10^{5d}$ (6.7)	$1.0x10^{5\,d}$ (14.1)			

Each value of bacterial load is a mean of duplicate assays using the standard plate count method. The samples were incubated at 37°C. Pre-incubation bacterial load was 8.3×10^9 cfu/mL. Values in brackets are coefficient of variation expressed as percentage. Values in individual columns with different letters are statistically different ($p < 0.05$). All the controls were significantly different from their respective treatments.

Table B6: Viability of *L. casei shirota* exposed to various concentrations of T-2 and in control

L. casei shirota	T-2 Concentrations	Bacterial load (cfu/mL) after incubation with T-2			Bacterial load in control sample (cfu/mL) after incubation		
Incubation time		**24 h**	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
Toxin concentrations	20 µg/L	$1.4x10^{9a}$ (2.3)	$2.4x10^{7a}$ (6.7)	$4.9x10^{6a}$ (8.3)	$3.7x10^{9}$ (5.8)	$3.6x10^{8}$ (17.9)	$3.6x10^{7}$ (15.7)
	50 µg/L	$8.1x10^{8b}$ (8.3)	$1.1x10^{7b}$ (8.3)	$6.2x10^{5b}$ (18.4)			
	100 µg/L	$1.8x10^{8c}$ (4.0)	$9.2x10^{6bc}$ (2.3)	$3.8x10^{5bc}$ (9.4)			
	200 µg/L	$8.5x10^{7d}$ (0.9)	$2.6x10^{6cd}$ (9.9)	$1.2x10^{5cd}$ (3.4)			
	250 µg/L	$6.1x10^{7d}$ (3.0)	$1.1x10^{6d}$ (15.0)	$8.5x10^{4d}$ (2.9)			

Each value of bacterial load is a mean of duplicate assays using the standard plate count method. The samples were incubated at 37°C. Pre-incubation bacterial load was 8.3×10^{9} CFU/mL. Values in brackets are coefficient of variation expressed as percentage. Values in individual columns with different letters are statistically different ($p < 0.05$). All the controls were significantly different from their respective treatments.

Table B7: Viability of *L. acidophilus* AS1.3342 in various concentrations of AFB_1 and control

L. acidophilus AS1.3342	AFB_1 Concentrations	Bacterial Load (cfu/mL) after incubation with AFB_1			Bacterial load in control sample (cfu/mL) after incubation		
Incubation time		**24 h**	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
Toxin concentrations	5 µg/L	$3.2x10^{7a}$ (6.7)	$5.9x10^{6a}$ (2.4)	$1.1x10^{6a}$ (12.9)	$5.9x10^{8}$ (3.6)	$5.8x10^{7}$ (6.1)	$7.6x10^{6}$ (11.2)
	10 µg/L	$2.5x10^{7b}$ (2.9)	$4.3x10^{6b}$ (8.3)	$9.5x10^{5a}$ (7.4)			
	20 µg/L	$7.8x10^{6c}$ (4.6)	$8.8x10^{5c}$ (4.0)	$1.1x10^{5b}$ (12.9)			
	50 µg/L	$2.5x10^{6c}$ (5.7)	$2.5x10^{5cd}$ (28.3)	$8.8x10^{4b}$ (4.0)			
	100 µg/L	$7.6x10^{6d}$ (7.4)	$1.1x10^{5d}$ (12.9)	$2.5x10^{4b}$ (28.3)			

Each value of bacterial load is a mean of duplicate assays using the standard plate count method. Pre-incubation bacterial load was 3.8×10^{9} CFU/mL. The samples were incubated at 37°C. Values in brackets are coefficient of variation expressed as percentage. Values in individual columns with different letters are statistically different ($p < 0.05$). All the controls were significantly different from their respective treatments.

Table B8: Viability of *L. acidophilus* AS1.3342 in various concentration of T-2 and control

***L. acidophilus* AS1.3342**	**T-2 concentrations**	**Bacterial load (cfu/mL) after incubation with T-2**			**Bacterial load in control sample (cfu/mL) after incubation**		
Incubation time		**24 h**	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
Toxin concentrations	20 µg/L	$3.3x10^{8a}$ (3.9)	$3.0x10^{7a}$ (0.0)	$4.1x10^{6a}$ (1.0)	$5.8 x10^{8}$ (1.2)	$6.3x10^{7}$ (6.7)	$6.8x10^{6}$ (5.2)
	50 µg/L	$2.6x10^{8\,b}$ (7.4)	$1.1x10^{7b}$ (2.3)	$1.1x10^{6b}$ (15.7)			
	100 µg/L	$8.6x10^{7\,c}$ (7.4)	$7.1x10^{6c}$ (2.0)	$6.8x10^{5c}$ (4.2)			
	200 µg/L	$5.7x10^{7\,d}$ (2.8)	$3.1x10^{6d}$ (12.9)	$2.3x10^{5d}$ (6.7)			
	250 µg/L	$7.2x10^{6\,e}$ (2.2)	$8.2x10^{5d}$ (2.4)	$7.1x10^{4d}$ (1.7)			

Each value of bacterial load is a mean of duplicate assays in the standard plate count method. The samples were incubated at 37°C. Pre-incubation bacterial load was 3.8×10^{9} CFU/mL. Values in brackets are coefficient of variation expressed as percentage. Values in individual columns with different letters are statistically different ($p < 0.05$). All the controls were significantly different from their respective treatments.

Table B9: Viability of *L. bulgaricus* in various concentrations of AFB_1 and in control

L. bulgaricus	**AFB_1 Concentrations**	**Bacterial Load (cfu/mL) after incubation with AFB_1**			**Bacterial load in control sample (cfu/mL) after incubation**		
Incubation time		**24 h**	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
Toxin concentrations	5 µg/L	$4.3 x10^{7\,a}$ (1.7)	$3.9x10^{6\,c}$ (1.8)	$2.0x10^{6\,a}$ (7.1)	$4.4x10^{8}$ (1.6)	$3.2x10^{7}$ (6.7)	$2.5x10^{6}$ (8.7)
	10 µg/L	$4.0x10^{7\,ab}$ (1.8)	$3.6x10^{6\,ab}$ (3.9)	$1.9x10^{6ab}$ (7.4)			
	20 µg/L	$3.7x10^{7\,bc}$ (3.8)	$3.5x10^{6\,ab}$ (4.0)	$1.5x10^{6bc}$ (4.9)			
	50 µg/L	$3.7x10^{7\,bc}$ (1.9)	$3.5x10^{6\,ab}$ (2.0)	$1.1x10^{6\,c}$ (12.9)			
	100 µg/L	$3.4x10^{7\,c}$ (2.1)	$3.3x10^{6\,b}$ (2.2)	$1.0x10^{6\,c}$ (9.6)			

Each value of bacterial load is a mean of duplicate assays using the standard plate count method. The samples were incubated at 37°C. Pre-incubation bacterial load was 4.6×10^{9} CFU/mL. Values in brackets are coefficient of variation in percentage. Values in individual columns with different letters are statistically different ($p < 0.05$). All the controls were significantly different from their respective treatments.

Table B10: Viability of *L. bulgaricus* in various concentrations of T-2 and in control

L. bulgaricus	T-2 concentrations	Bacterial load (cfu/mL) after incubation with T-2			Control (bacterial load in cfu/mL after incubation		
Incubation time		**24 h**	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
Toxin concentrations	20 µg/L	$4.1x10^{8\ a}$ (2.2)	$3.0x10^{7\ a}$ (6.4)	$1.3x10^{6a}$ (1.5)	$4.5x10^{8}$ (3.1)	$3.0x10^{7A}$ (2.4)	$2.1x10^{6}$ (3.4)
	50 µg/L	$3.8x10^{8\ ab}$ (2.1)	$2.9x10^{7\ ab}$ (6.1)	$1.2x10^{6\ ab}$ (7.4)			
	100 µg/L	$3.6x10^{8\ bc}$ (0.0)	$2.4x10^{7\ abc}$ (11.8)	$1.1x10^{6ab}$ (6.7)			
	200 µg/L	$3.4x10^{8\ cd}$ (3.7)	$2.3x10^{7bc}$ (2.5)	$9.5x10^{5b}$ (6.1)			
	250 µg/L	$3.2x10^{8\ d}$ (1.7)	$2.2x10^{7bc}$ (4.7)	$9.7x10^{5b}$ (5.7)			

Each value of bacterial load is a mean of duplicate assay using the standard plate count method. The samples were incubated at 37°C. Pre-incubation bacterial load was 4.6×10^{9} CFU/mL. Values in brackets are coefficient of variation in percentage. Values in individual columns with different letters are statistically different ($p < 0.05$). All the controls except the ones at 48 h incubation were significantly different from the treated samples.

Appendix C

Pictorial display of the research

In this section, some of the key analyses that were undertaken in this research are demonstrated in pictorial form. The key aspects covered include: bacterial isolation process, bacterial growth curves using OD_{600} and the standard plate count method, cellcharacterization, cell cleaning and suspension in PBS, mixing of toxin with bacteria, incubation and toxin extraction. Finally, the toxin assay is demonstrated in pictorial form.

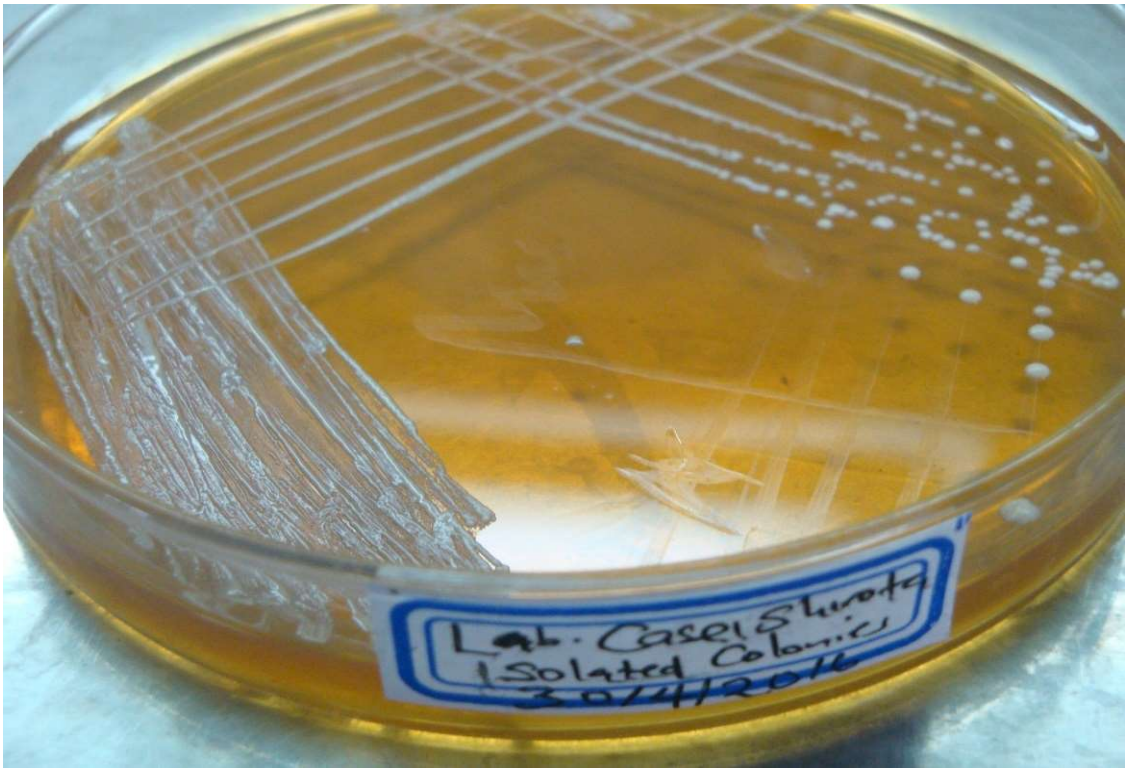

Fig. C1: Isolation of LAB in the Food Microbiology Laboratory, College of Food Science and Technology, Guangdong Ocean University, China.

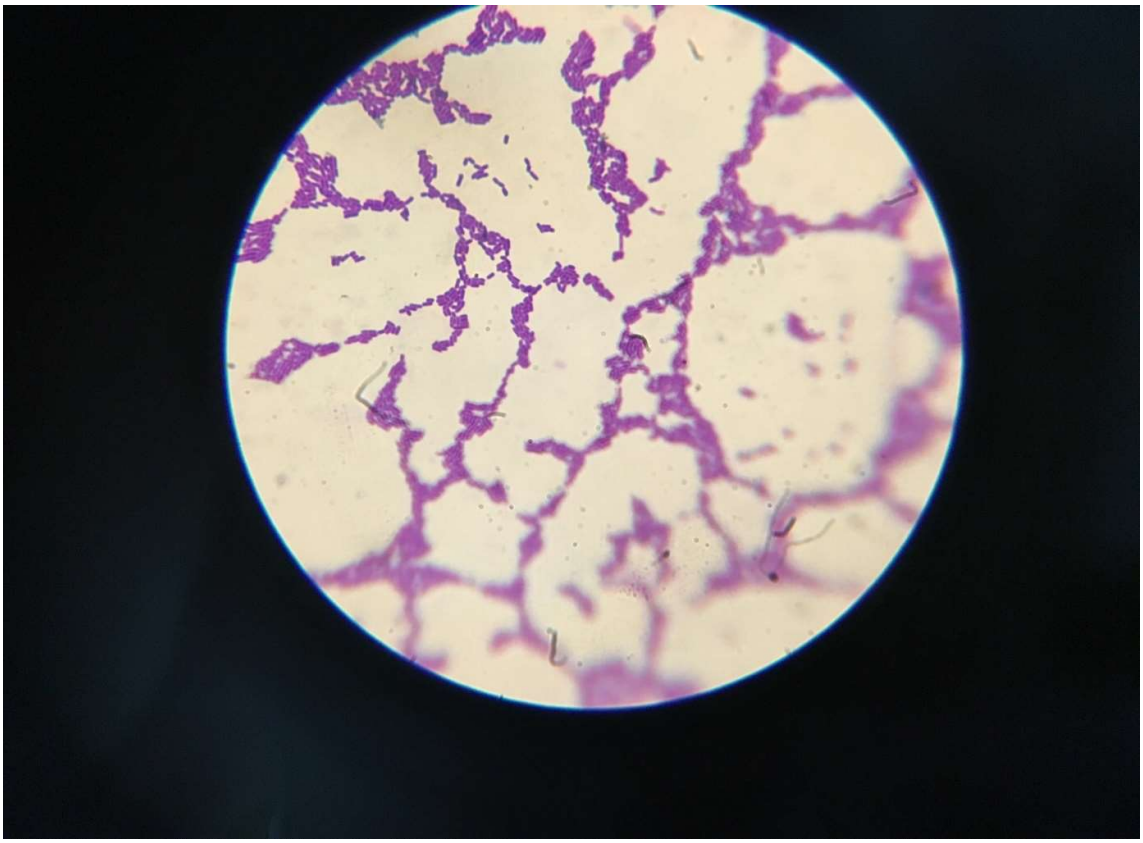

Fig. C2: Gram stain for *Streptococcus thermophilus* isolated from CY: Gram-positive and spherical-shaped cells that occur in chains resembling a string of beads.

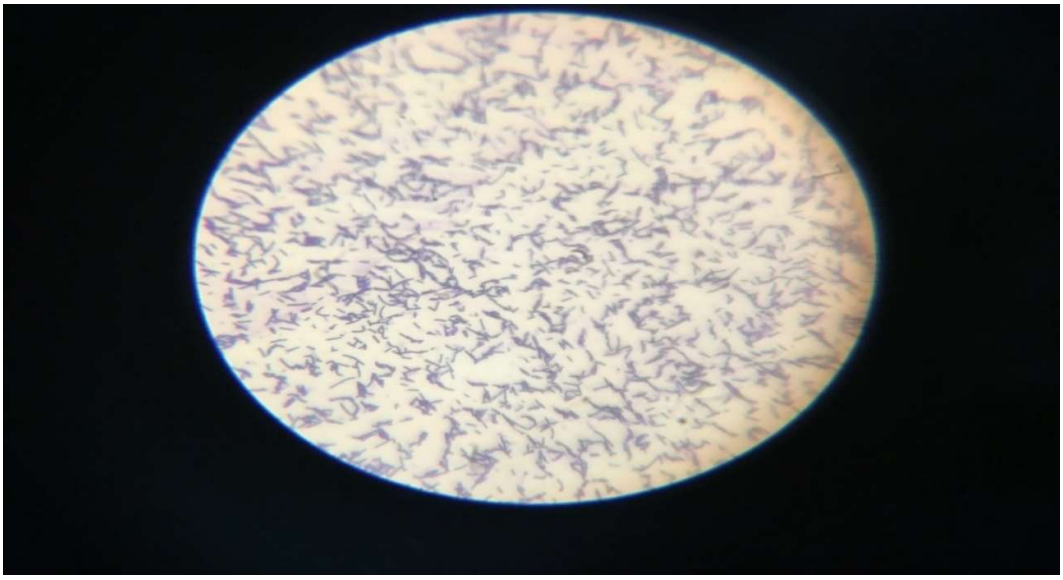

Fig. C3: Gram stain for *L. acidophilus;* Gram-positive and rod-shaped cells. The cells appear singly, in pairs or in short chains.

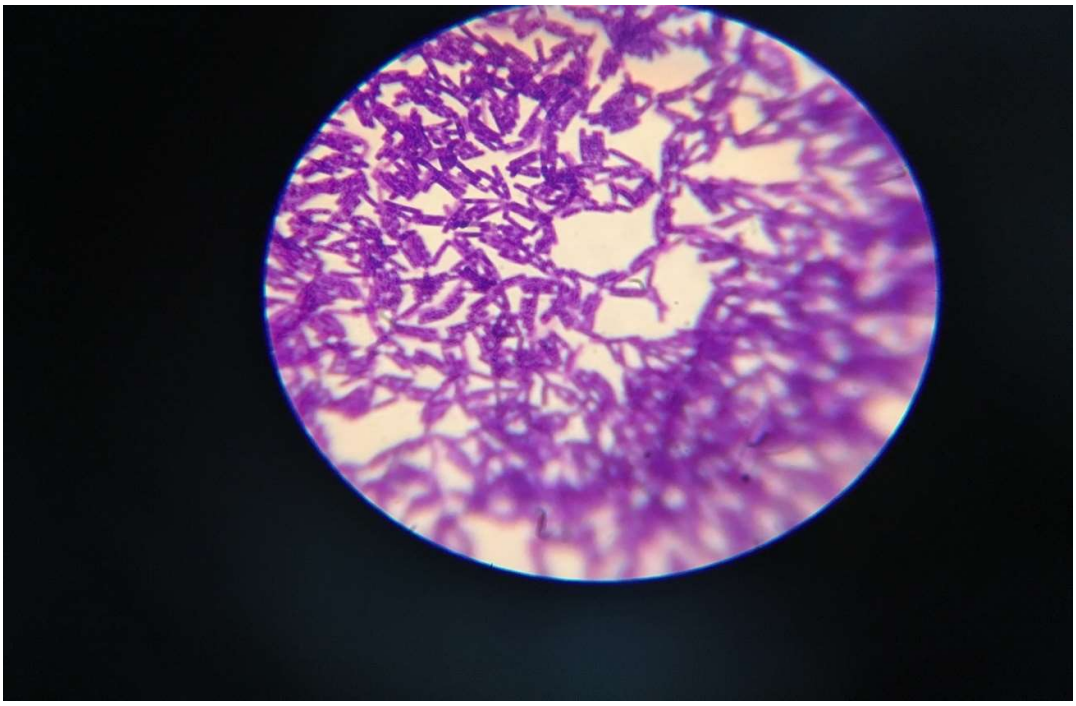

Fig. C4: Gram stain for *L. bulgaricus* isolated from CY; Gram-positive and rod-shaped cells.

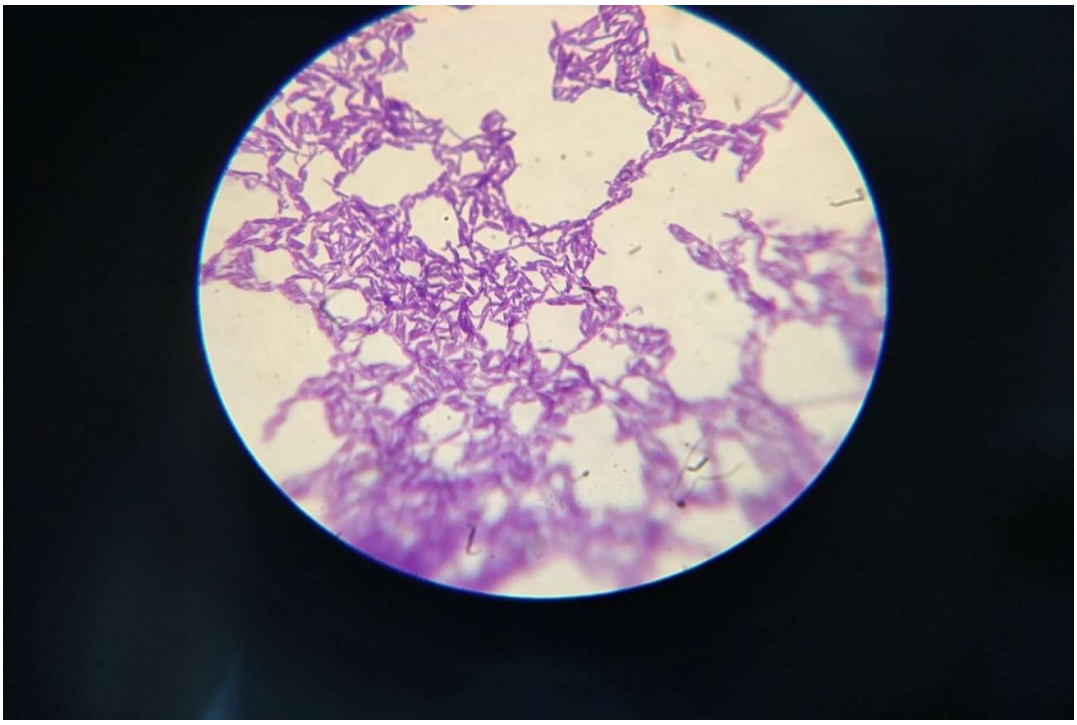

Fig. C5: Gram stain for *L. casei shirota* isolated from FMD; Gram-positive, rod-shaped cells.

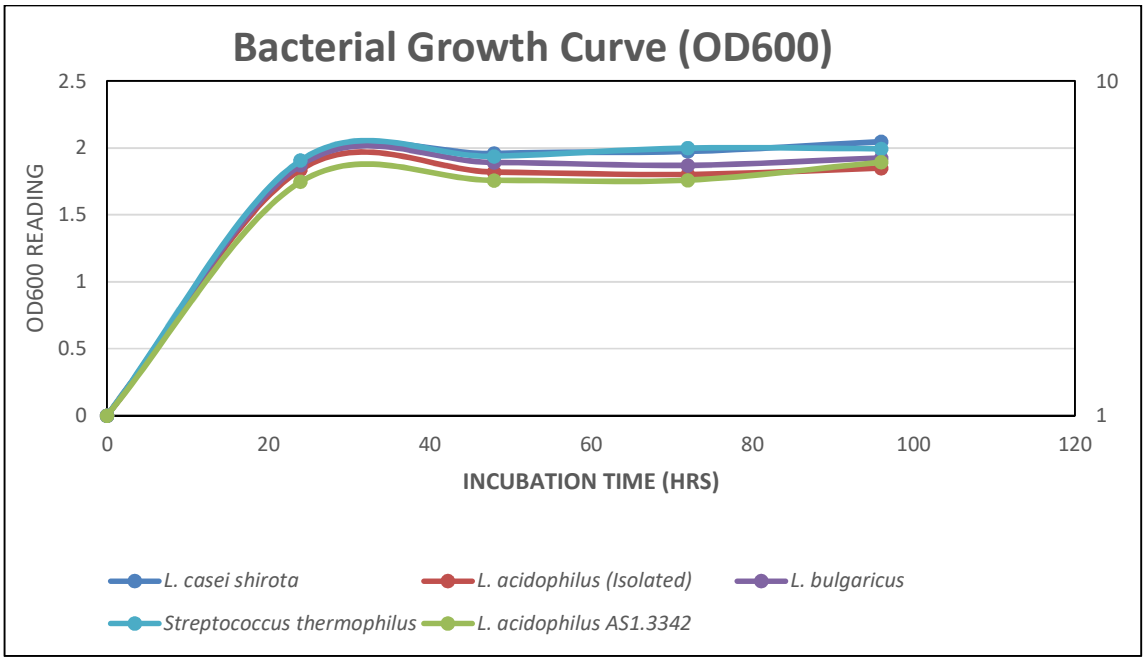

Fig. C6: Bacterial growth curve using optical density (OD) at 600 nm; five bacterial cells grown in MRS broth, incubated at 37°C, under anaerobic conditions.

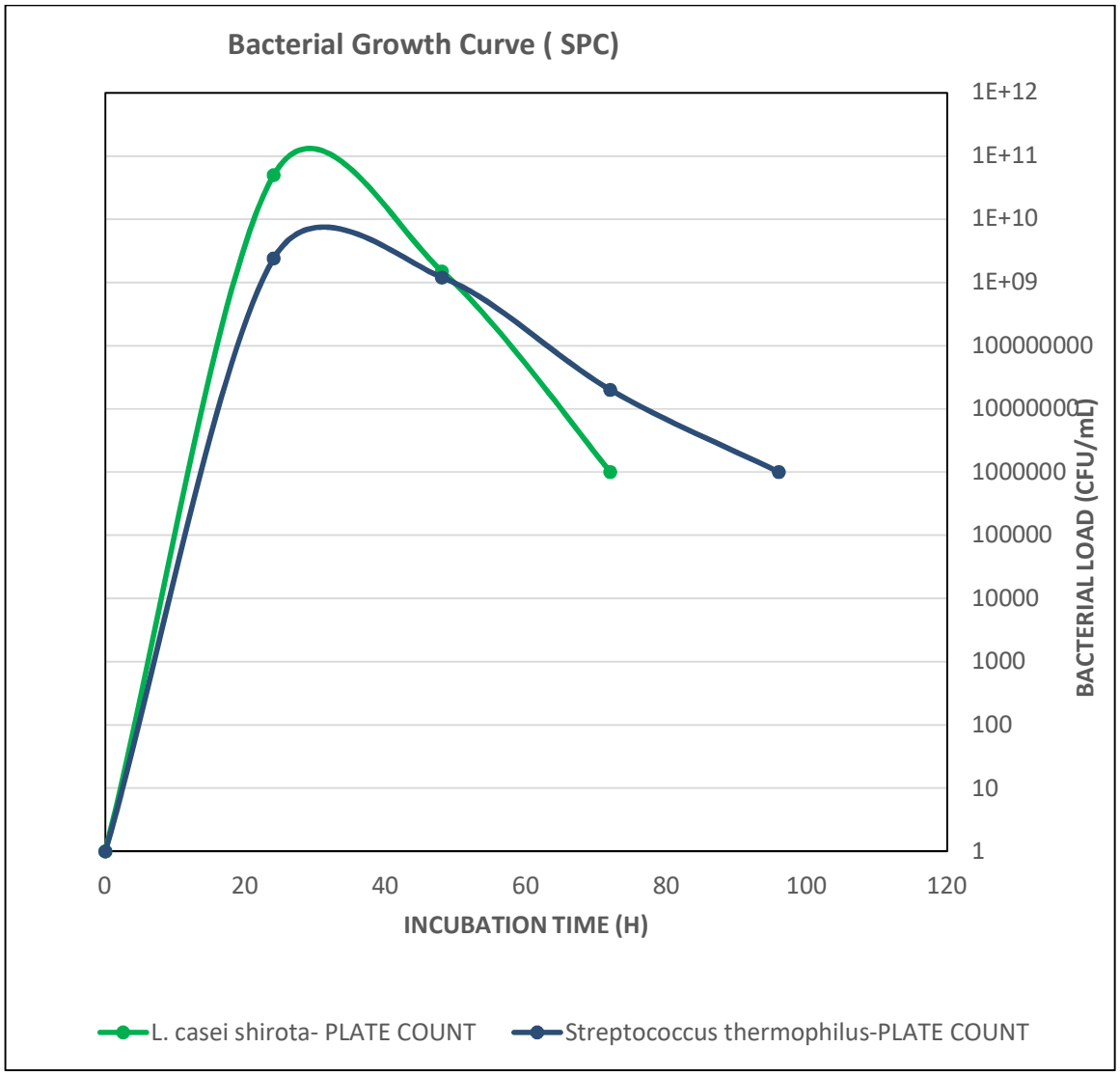

Fig. C7: Bacterial growth curve using the standard plate count method (SPC)*; Lactobacillus casei shirota* and *Streptococcus thermophilus* spread on MRS agar, incubated at 37°C, under anaerobic conditions; cells reported as CFU/mL.

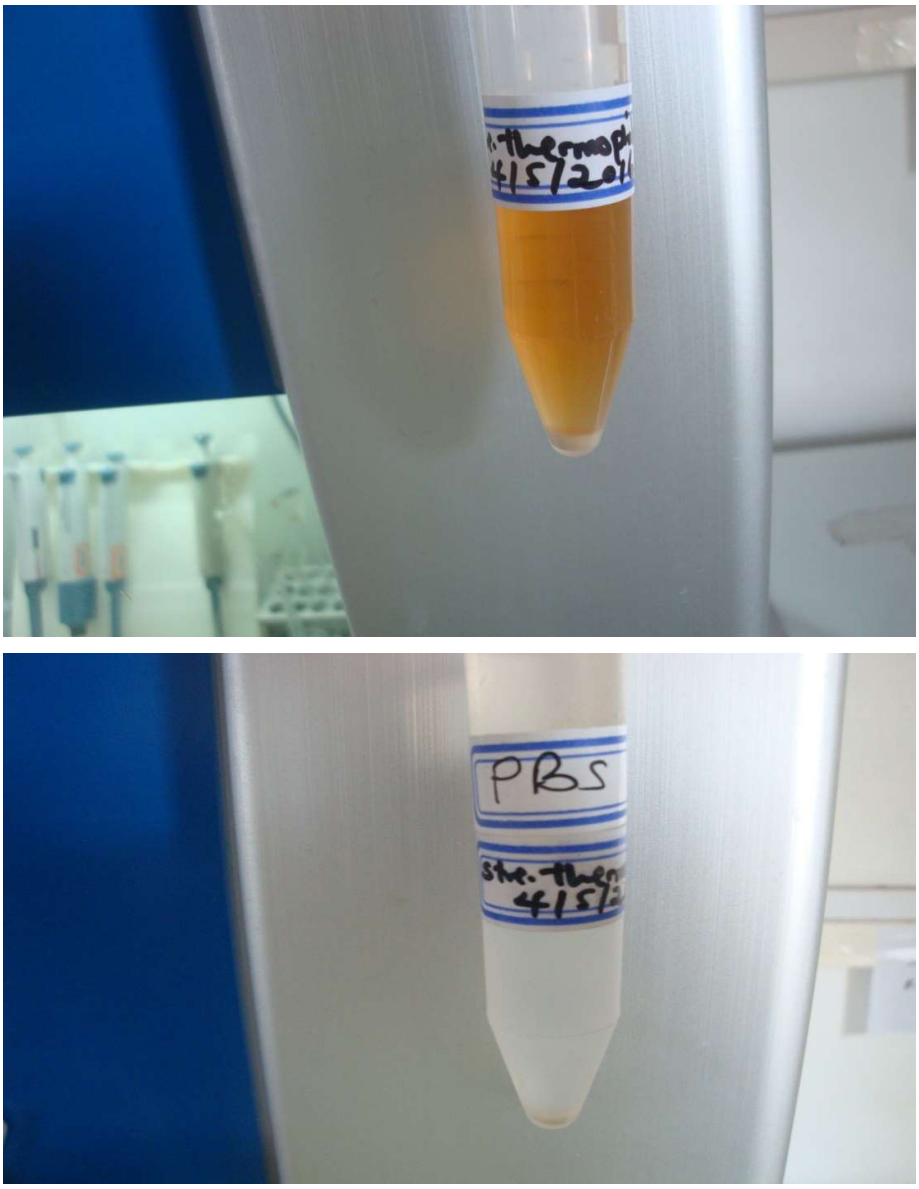

Fig. C8: Centrifugal cleaning of LAB cells; cells cultured in MRS broth then cleaned with PBS; 7000 x g, 4°C, 5 min; Food Microbiology Laboratory; College of Food Science and Technology, Guangdong Ocean University, China.

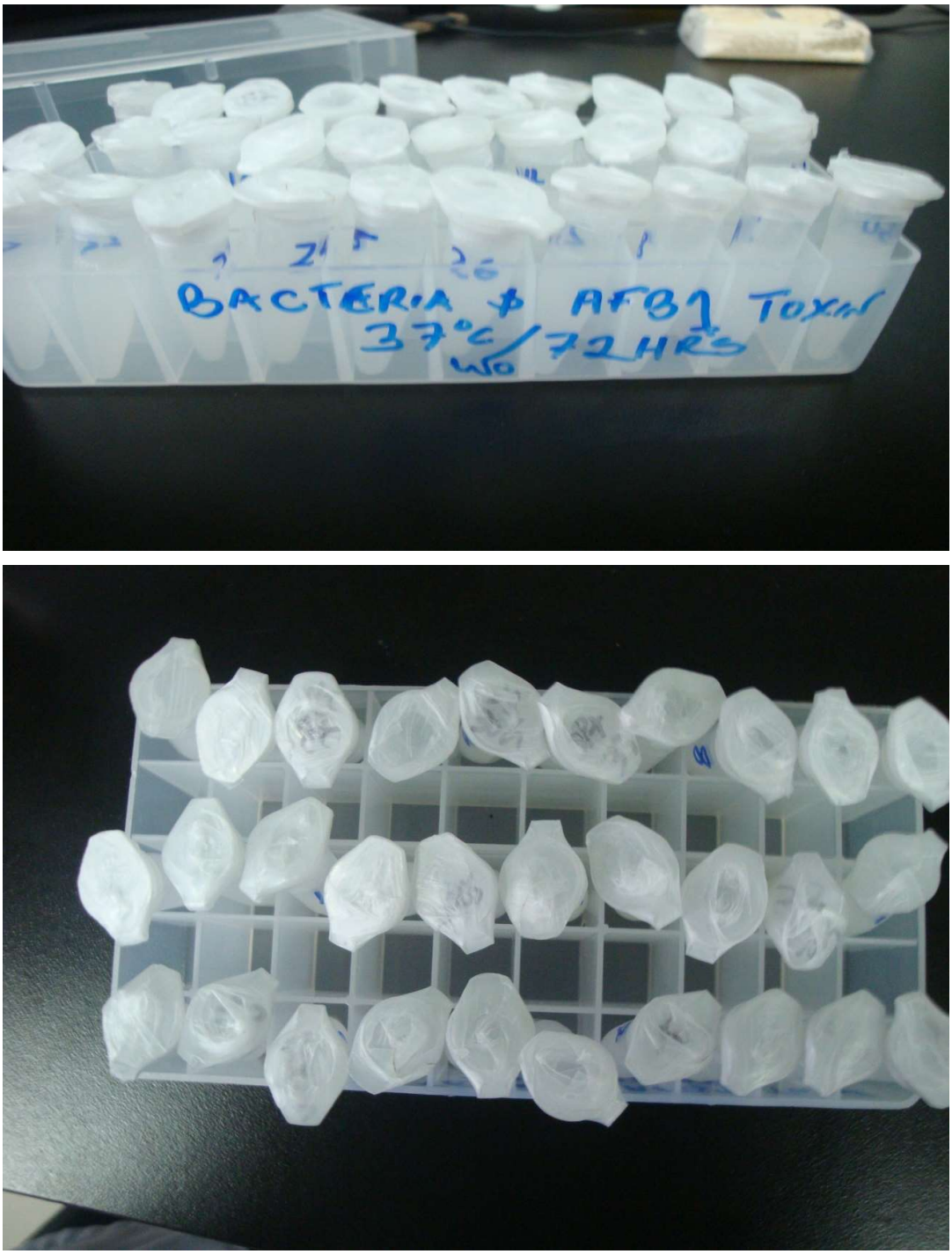

Fig. C9: Toxin-bacteria mixture; incubated at 37°C, for 72 h; toxin extracted after incubation; Food Toxicology Laboratory; College of Food Science and Technology, Guangdong Ocean University, China.

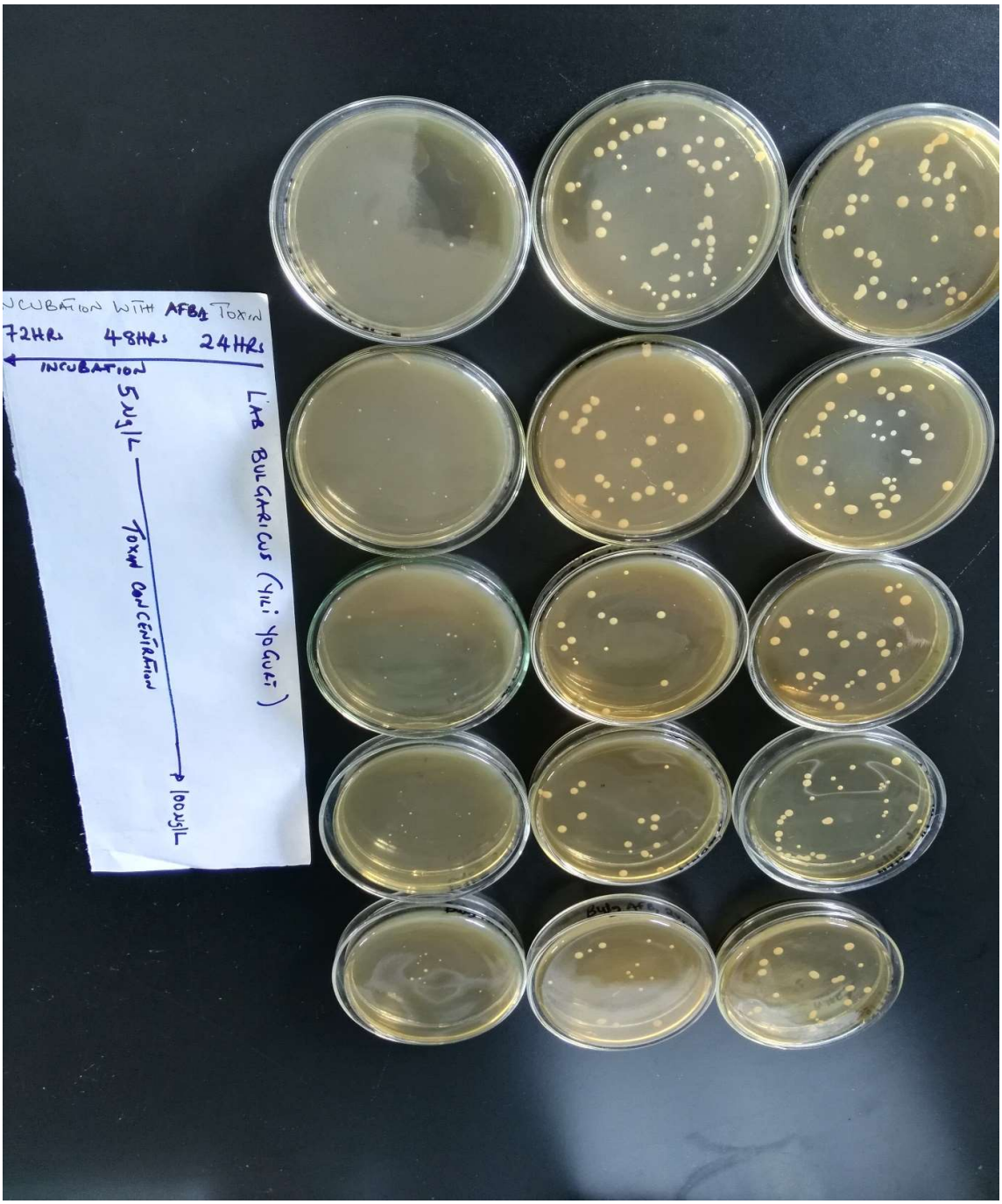

Fig. C10: Cell viability count; *L. bulgaricus* mixed with various concentrations of AFB_1; toxin-bacteria mixtures incubated at 37°C and spread on MRS agar after 24, 48 and 72 h; cells reported as CFU/mL.

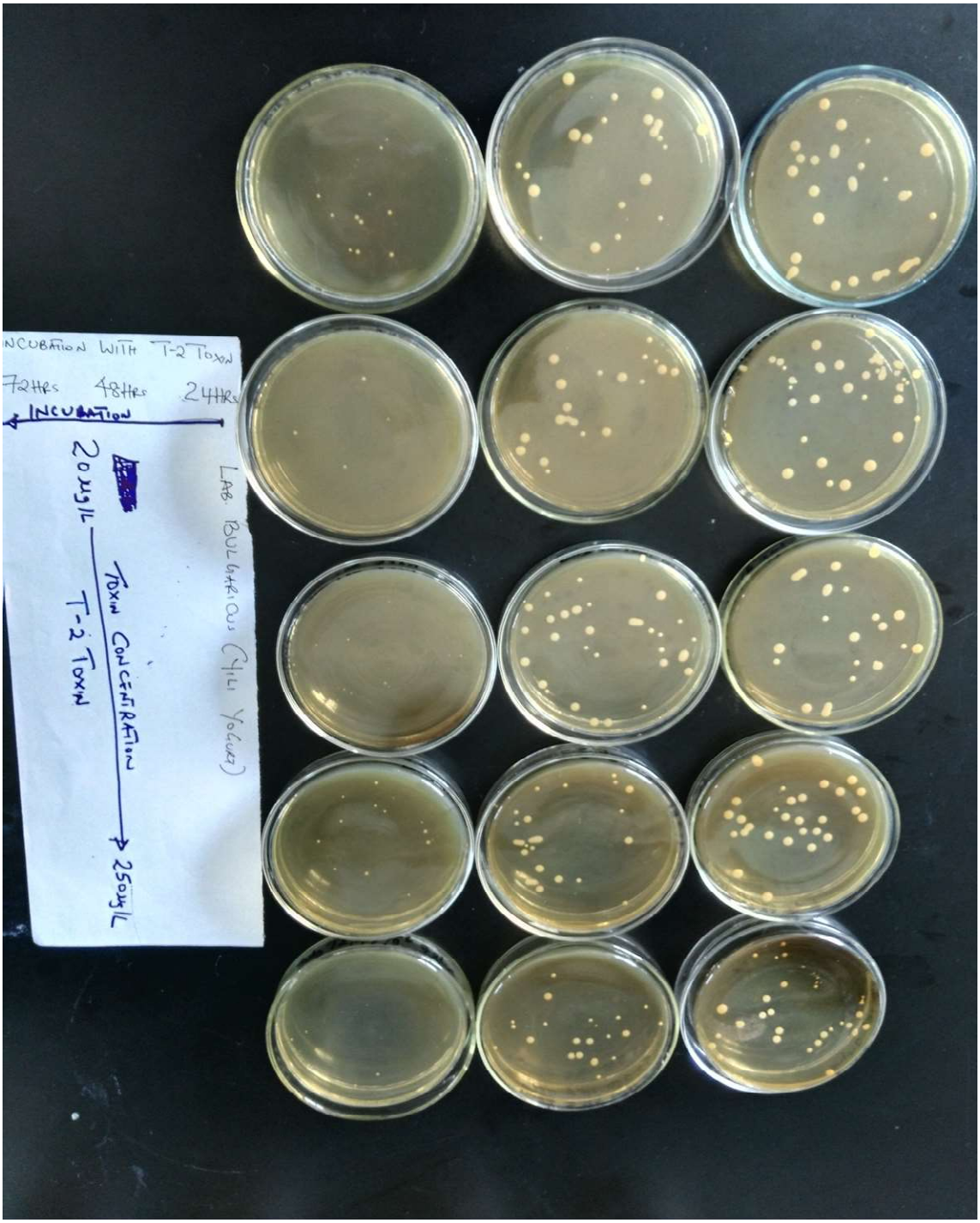

Fig. C11: Sample of cell viability count; *L. bulgaricus* mixed with various concentrations of T-2; toxin-bacteria mixtures incubated at 37°C and spread on MRS agar after 24, 48 and 72 h; cells reported as CFU/mL.