Reducing fumonisin exposure in African fermented maize-based foods with lactic acid bacteria

By

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Submitted in partial fulfilment of the requirements for the degree of

PHILOSOPHIAE DOCTOR **(Microbiology)**

In the Faculty of Natural and Agricultural Sciences.

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2018

Declaration

I declare that the thesis which I hereby submit at the University of Pretoria for the award of the degree PhD (Microbiology) is my work and has not been submitted by me for a degree to any other university or institution of higher education.

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July 2018

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List of Abbreviations

ANOVA	One-way analysis of variance
ATCC	American Type Culture Collection
ARVs	Anti-retroviral drugs
CLSM	Confocal laser scanning microscopy
CO_2	Carbon dioxide
CPB	Citrate phosphate buffer
DNA	Deoxyribonucleic acid
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FB_3	Fumonisin B_3
FB_4	Fumonisin B_4
GRAS	Generally recognised as safe
HPLC	High pressure liquid chromatography
IPCS	International Programme on Chemical Safety
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LAB	Lactic acid bacteria
MRS	Man, Rogosa and Sharpe
NDA	Naphthalene-2, 3-dicarboxaldehyde
ODMBA	Octadecyl dimethylbenzyl ammonium
OPA	o-phthaldialdehyde

PBS	Phosphate buffered saline
PMTDI	Provisional Maximum Tolerable Daily Intake
SRB	Sulforhodamine B
WHO	World Health Organization
ZEN	Zearalenone

Acknowledgements

Acknowledgements

My deepest gratitude goes to my three supervisors: Prof. Elna Buys, Prof. Mapitsi Thantsha and Dr. Catherine Brabet for their understanding, invaluable support, immense encouragement and unwavering determination and perseverance with this degree and myself.

I would like to acknowledge and thank the Council for Scientific and Industrial Research (CSIR) and Centre de Cooperation Internationale en Recherché Agronomique pour le Développement (CIRAD) for use of their facilities for the laboratory work conducted throughout all the chapters.

I would like to acknowledge and thank Mr. Alan Hall from the Laboratory for Microscopy and Microanalysis at the University of Pretoria (UP) for his invaluable assistance, time, insightful discussions and experience with the Zeiss LSM 510 META confocal laser scanning microscope for the work conducted in Chapter Two.

I would like to acknowledge funding received from the European Union (FP7 245–025) called African Food Revisited by Research (AFTER - http://www.afterfp7.eu/) and from a French-South African PHC Protea project (Project N° 29769VL) for laboratory work conducted in Chapters Three and Four.

I would like to acknowledge and thank Mrs. Renee Koen from Built Environment, the Council of Scientific and Industrial Research (CSIR), for her assistance in performing the statistical analyses presented in Chapters Three, Four and Five. I would also like to thank her for her patience and interpretation of the statistical results, which improved the quality of this thesis.

I would like to acknowledge and thank Mrs. Natasha Koleshnikova from Biosciences, the Council of Scientific and Industrial Research (CSIR), for her unmatched skills in cytotoxicity testing presented in Chapter Five.

My family and friends (Mrs. Marija Kvas de Echeverri, Dr. Kersch Naidoo, Mr. Khai Cheong, Ms. Dineshree Naidoo, Mr. David Pretorius, Mr. Sadique Mahommed), for their infinite support and encouragement throughout this degree programme.

Last but not least, God, for giving me the strength and courage to complete this degree programme.

Doctoral Thesis

Abstract

Abstract

Maize, rice, wheat and barley are the world's four major cereal crops. In Africa, maize is the major staple cereal crop for the majority of the population and most of the traditional fermented products are maize-based (e.g. ogi, kenkey, mawe and mahewu). Similar to other cereals, maize is also at risk of infestation by mycotoxigenic fungi. International studies have indicated that fumonisins (64%) take the lead in mycotoxins contamination worldwide followed by deoxynivalenol (59%), zearalenone (45%), aflatoxin (33%) and ochratoxin A (28%). Various African studies have indicated fumonisins occur in maize at levels that can exceed 10 µg/g. Fumonisins are most widely known to cause leucoencephalomalacia in horses and high levels of fumonisin B_1 (FB_1) has been linked to increased incidence of oesophageal cancer in Eastern Cape formerly known as Transkei. Further research has shown the consumption of FB_1 by pregnant women can cause birth defects. Due to their structural similarity to sphingonine and sphingosine, fumonisins interfere with sphingolipid metabolism and this result in liver disease and tumours in the liver and kidneys. Many methods have been investigated for the detoxification of mycotoxins. A new method of detoxification has recently been developed wherein some mycotoxins have a natural affinity to bind to microorganisms. Recent studies have indicated that probiotics, specifically lactic acid bacteria (LAB), have a natural ability to bind to fumonisins and introducing a detoxifying effect on the fermented product. In this project, we investigated the ability of the following dominant probiotic LAB strains isolated from traditional fermented maize-based products (ogi and mahewu) namely, *Lactobacillus plantarum* FS2, *L. delbrueckii* subsp. *delbrueckii* CIP 57.8T and *Pediococcus pentosaceus* D39, against a positive control strain, *L. plantarum* R1096, to reduce the toxicity of fumonisins (B_1 and B_2).

In completion of the main objective, each LAB strain was evaluated for its binding ability. Visualization of the binding interaction was achieved by derivatization of the fumonisins and interaction with the non-fluorescent LAB cells (viable and non-viable) under confocal laser scanning microscopy (CLSM) with the appropriate controls in place. Results provided physical evidence that the fluorescent fumonisins (B_1 and B_2) bound to the LAB cells externally, as both the viable and non-viable cells fluoresced green under CLSM. To the best of our knowledge, this is the first study to visualize the interaction between LAB and fumonisins. Each LAB strain was evaluated to quantify the binding of fumonisin molecules to a certain number of bacterial cells, proof of adsorption as well as reproducibility and

repeatability. The high binding percentages obtained indicated successful binding strength being achieved across all LAB strains for both FB_1 and FB_2. For viable cells, FB_1 bound the most to *L. plantarum* FS2 but the least to *P. pentosaceus* D39, whereas, the highest binding for FB_2 was with *L. plantarum* R1096 but the least with *L. delbrueckii* CIP 57.8 T. Similarly, non-viable cells of *L. plantarum* FS2 indicated the highest binding to FB_1 and FB_2, while *P. pentosaceus* D39 and *L. delbrueckii* CIP 57.8 T bound the least to FB_1 and FB_2, respectively. Overall, non-viable cells bound more to FB_1 and FB_2 in comparison to viable cells due to the heat shock causing the cell wall to disrupt exposing more favourable binding sites to FB_1 and FB_2. Binding occurred with viable and non-viable cells which confirmed the results seen with the visualization of binding. The binding strength was repeated in two independent trials to establish repeatability and reproducibility where the statistical analyses indicated no significant differences.

To test the stability of the LAB – fumonisin complex, the main parameters were based on the fermentation process of ogi: incubation temperature of 30 ^{o}C and decreasing pH from 6 to 4 that occurs in a natural ogi fermentation process; same decreasing pH values and optimal growth temperature of the LAB strains which is 37 ^{o}C; simulated gastric condition of a lower pH of 2 and temperature of 37 ^{o}C and storage period of 7 days at pH 4 at 30 ^{o}C were selected. Results indicated that whether ogi fermentation process occurs at 30^{o}C or 37 ^{o}C, the binding strength for both fumonisins was still very high, in addition, as the pH decreases it can be seen that the binding for both the fumonisins increased. Even at the lower pH of 2 and incubation temperature of 37 ^{o}C, binding was quite high and the complex was still stable. For the storage conditions (7 days at pH 4 and 30 ^{o}C), contrary to previous binding strength results in this project, *L. plantarum* R1096 was the only strain that increased in binding strength whereas the other strains decreased in binding. Generally, as pH decreased the binding increased, but across all strains, FB_2 was bound more than FB_1.

Due to the liver being the main site of toxicity, the bound complex was evaluated on HepG2 cell line with controls and the LAB strains in order to give a comparative analysis and have a clear understanding of its toxicity *in vitro* for the objective of reducing the toxicity of fumonisins by use of LAB strains. HepG2 cells were observed in the presence of fumonisin only, LAB cells only and then with a combination of fumonisin and LAB cells to assess toxicity using the sulforhodamine B (SRB) assay. The results indicated that the LAB cells did not attach, aggregate or disrupt the HepG2 cells as the absorbance readings remained constant

throughout the 24-48 h incubation period. Observation of the HepG2 cells exposed to the fumonisins alone indicated the IC_{50} value of 308.6 µg/ml. The comparative observation of the HepG2 cells exposed to a combination of the fumonisins and LAB cells indicated that the LAB cells "protected" the HepG2 cells by binding to the fumonisins and increasing the IC_{50} values. *L. plantarum* R1096 "protected' the HepG2 cells the most by increasing the IC_{50} value to 903.1 µg/ml followed by *L. delbrueckii* CIP57.8T at 857.3 µg/ml, similarly with *L. plantarum* FS2 at 856.5 µg/ml and the least by *P. pentosaceus* D39 at 701.4 µg/ml. All LAB strains had more than doubled the IC_{50} value by being present and binding to the fumonisins.

In conclusion, *L. plantarum* FS2, *L. delbrueckii* CIP 57.8T and *P. pentosaceus* D39, test strains isolated from African traditional fermented foods, have been successfully tested against the positive control strain *L. plantarum* R1096 and can be used as detoxifying agents in reducing the toxicity of fumonisin B_1 and B_2 in fermented cereal based products.

Chapter One

Literature Review: Lactic acid bacterial interactions with fumonisins

1.1. Introduction

A wide variety of cereal crops are produced nationally and internationally, the most common being maize, wheat, rice and sorghum (Oluwakemi and Omodele, 2015). These cereal crops are staple foods and due to the import and export value of the commodity forms the economical basis in a country. Cereals, however, are generally at risk of infection by mycotoxigenic fungi that produce a wide range of secondary metabolites some of which are mycotoxins. These mycotoxins are insidious poisons and have been proven to affect human health (Reddy *et al.*, 2010). Mycotoxigenic fungi are more common in humid and semi-humid areas due to their defining requirement for moisture (Chulze, 2010).

In developing countries, the staple food of the population is usually a cereal e.g. maize in South Africa. As a consequence of poverty, there is increased poor food security and malnutrition and exposure to mycotoxins is quite high (Marasas *et al.*, 2008). Traditional fermented cereal foods are cheap, relatively easy to prepare and readily available food source.

Many methods have been undertaken to reduce or deactivate mycotoxins in foods, mostly with little or no effect. An atoxigenic strain has been used as a competitive bio preservative on maize, but this does not eliminate the possibility that the strain might regain its toxicity (Reddy *et al.*, 2010). Newer technologies are being investigated, one of which is the utilisation of lactic acid bacteria (LAB) to remove and/or deactivate mycotoxins.

Some LAB often acts as probiotics with a proven beneficial effect on human health (Salminen *et al.*, 2010). Probiotics have been associated with the alleviation of gastro-enteritis, irritable bowel syndrome and many others (Goldin, 2010) as well as reducing the side-effects and improving the efficacy of anti-retroviral drugs (ARVs) (Yan and Polk, 2010). The mechanism of action of LAB is the secretion of compounds which improve nutrient absorption by the mucosal membranes in the digestive system. In addition to their probiotic effect, thus far experimental evidence has suggested that LAB can bind to and remove mycotoxins from the body (Gratz *et al.*, 2010). For example Gratz *et al.* (2007) was able to demonstrate *in vitro* that *L. rhamnosus* GG can bind aflatoxin B_1 that has been known to interfere with the intestinal mucosal barrier.

1.2. Cereals

Cereals have been part of the world since crop cultivation became known. References to grain crops date as far back as before the time of Christ (Goldin, 2010). Due to its longevity in the world, humans have introduced different ways in which to utilise cereals and produce many cereal products using innovative methods and processes. Cereals are commonly consumed as fermented products rather than unfermented/raw cereal products in Africa (Soro-Yao, 2014). A brief discussion of unfermented cereals will be followed by an in-depth discussion of selected African fermented cereal products.

1.2.1. Unfermented cereals

Unfermented or raw cereal products generally refer to a cereal crop in its natural state without processing by fermentation that result in a change of the food matrix. These cereals are usually ground to make different flours or crushed/rolled to make flakes. Milling results in kernels being separated into different seed components which are used in and for different products. Generally, a maize kernel is separated during processing which results in the germ being used for oil, the gluten to enrich animal feeds and starch in the production of glucose, dextrin and alcohol (Reis *et al.*, 2017).

Some of the most common unfermented cereal products are popped and puffed rice grains, unfermented bread, couscous, dumplings, gruels and a variety of porridges (Taylor and Emmambux, 2008). Popped and puffed rice are rice kernels that are swollen and roasted till it "pops" or "puffs". Couscous is a variety of seeds that are cooked or roasted together. Most flours are cooked in different ways to produce different consistencies forming "gruel" to a more semi-solid consistency of porridge, ranging from alkaline to sour. Pap is produced from the flour of ground cereal grains; it is a bland, soft or semi-liquid food that is suitable for babies or convalescents. A more solid variety of pap is produced for adults to consume at home.

1.2.2. Fermented cereals

Most cereals are processed in some form or another to enhance the palatability of the grains; it is very rare that certain grains are consumed "raw" e.g. flax seed. Fermented cereal products have been enhanced by microorganisms grown directly on the cereal substrate. This production process is widely known as a "household art" passed down through many

generations of families and produce an intricate part of diet in our foods (Egwim *et al.*, 2013). The distinctiveness of a fermented food depends on the diversity of raw cereal substrates used and the different methods of preparation which produces varying and excellent/delightful sensory qualities in the finished fermented product. The acidic pH nature of fermentation enhances activity of microbial enzymes which reduces anti-nutrients and increases the bioavailability of minerals and other nutrients (Chelule *et al.*, 2010). Fermentation also has the added advantage of lowering the pH which inhibits pathogen contamination, thereby extending the shelf life of the product. Some microorganisms, namely LAB, used in fermentation have antimicrobial activities against food poisoning bacteria e.g. *Lactobacillus lactis* subsp. *lactis* produces nisin which prevents the growth of C*lostridium* and *Bacillus* spores (Chelule *et al.*, 2010).

The most well-known fermented products of the world are alcohol, wine, vinegar, olives, yoghurt, bread and cheese. Certain fermented products are produced as delicacies, native to specific areas of the world such as atole (produced from maize), chica (alcoholic beverage produced from pineapple, barley steep liquor, and black maize dough), pozol (produced from fermented corn dough) and jamin-bang (bread produced from maize fermented) in the Americas. In Europe, there is elderberry wine (produced from elderberries) and boza (produced from fermented maize and wheat). Asia is known for adai (mixture of fermented lentils), miso (a traditional Japanese seasoning produced by fermenting soybeans with salt and koji) and rabdi (sweet, condensed-milk-based dish) (Tamang *et al.*, 2016). In Africa the most common fermented cereal products are: akpan and gowé in Benin, ogi in Nigeria and mahewu in South Africa, which is discussed, below, in detail.

The fermented cereal products discussed here are produced from maize, sorghum and wheat with a natural spontaneous fermentation process or inoculated with old stock to initiate fermentation. During a previous study (Dawlal *et al.*, 2010), it was established that South African maize cultivars are mainly contaminated by *Fusarium verticillioides*. This fungus produces the mycotoxin, fumonisin, which is also found to be the most prevalent in maize (Dawlal *et al*., 2010). The high occurrence and levels of fumonisins in maize have been reported by several studies in Africa (Vismer *et al.* 2015; Mngqawa *et al.,* 2016; Udomkun *et al.*, 2017) with levels that can exceed 10 µg/g (Okeke *et al.,* 2015; Chilaka *et al.,* 2016). A study of mycotoxins found in cereal grains over a period of 3 years indicated the prevalence

of the following mycotoxins: fumonisin (64%), deoxynivalenol (59%), zearelenone (45%), aflatoxin (33%) and ochratoxin A (28%) (Rodriguez and Naehrer, 2012). Usually contaminated cereals are declared unfit for human consumption and thus considered suitable for animal feeding. In third world countries where food is scarce and subsistence farming is the only source of food, contaminated cereals are consumed, irrespective of quality considerations (Marasas *et al*., 2008). The International Programme on Chemical Safety (IPCS, 2011) states that European fumonisin daily intake is 0.2 µg/kg body weight whereas African fumonisin daily intake is 200 µg/kg body weight which exceeds the Provisional Maximum Tolerable Daily Intake (PMTDI) of 2 µg/kg bw/day (JECFA, 2001).

1.2.2.1. Akpan

Akpan is produced as a fermented cereal delicacy in Benin. Ogi or mawé are the fermented products that akpan is made from. These base products are made from maize although sorghum is also used (Nago, 1989). Processes for the production of akpan include steeping, wet milling, wet sieving, decanting and fermentation (Nago *et al.*, 1998). Akpan is consumed as a beverage where the ogi is mashed and pre-cooked and additives such as milk, sugar and ice are added before consumption. This beverage is consumed at any time, usually as a snack. Research on the literature has shown that microorganisms used in the fermentation of akpan have yet to be isolated and identified.

1.2.2.2. Gowé

Gowé is a fermented cereal product produced in Benin made from maize or sorghum ogi. Gowé is consumed as a beverage with sugar, ice and milk during hot periods and during the fasting periods of Ramadaan. Different processes are used in the production of gowé such as malting, fermentation and cooking (Vieira-Dalodé *et al.*, 2008). Microbiological analysis of the food product has shown LAB to be the most dominant microorganisms in gowé (Vieira-Dalodé *et al.*, 2007).

1.2.2.3. Ogi

Ogi is a fermented cereal product produced from maize or sorghum. It forms the basis of most traditional African fermented cereal products e.g. akpan, kenkey, agidi, kafa, koko etc. (Nago *et al.*, 1998; Blandino *et al.*, 2003). Ogi has the reputation of being the most popular traditional weaning food for infants as well as being a nutritious, easily consumable product

for convalescents (Teniola and Odunfa, 2002). Production of ogi involves a 3 day steeping process, followed by wet milling and wet sieving where the slurry is allowed to ferment for a further 2 days (Fayemi and Buys, 2017; Fayemi *et al.*, 2017). Lactic acid bacteria have been shown to be the most dominant microorganisms in the microbiological analysis of the product, particularly the *Lactobacilli* (Adebayo-tayo and Onilude, 2008; Omemu, 2011; Akinleye *et al.,* 2014; Fayemi and Buys, 2017; Fayemi *et al.*, 2017).

1.2.2.4. Mahewu

In South Africa, the most commonly known fermented cereal product is mageu or amahewu (Zulu), which is a fermented beverage made from maize. It is prepared from maize porridge which is mixed with sorghum, millet or wheat. This is then boiled with occasional stirring for 10-15 mins and then allowed to cool to ~40 ^{o}C (Chelule *et al.*, 2010). Spontaneous fermentation occurs from the natural flora (Blandino *et al.*, 2003) or the batch is supplemented with old stock to initiate fermentation (Chelule *et al.*, 2010). The mixture is then allowed to ferment for 1-3 days in a warm place. Mahewu is usually served as a refreshing drink for both adults and children.

1.3. Mycotoxins

Mycotoxins are mainly fungal secondary metabolites that have the ability to impair human and animal health. These are low molecular weight chemical compounds, which are not necessarily detected by antigens and hence often produce no obvious initial symptoms. Some of the major mycotoxins are aflatoxins, deoxynivalenol, fumonisins, ochratoxin A, patulin and zearalenone (Ahangarkani *et al.,* 2014) as they are mainly found in food.

In this study, the interaction and binding between lactic acid bacteria (LAB) and fumonisin B_1 and B_2 will be investigated.

1.3.1. Fumonisins

The discovery and structural elucidation of fumonisins were first reported by two independent groups of South African researchers, Bezuidenhout *et al.* (1988) and Gelderblom *et al.* (1988). Based on their chemical structures, fumonisins are categorized to many analogues and the most important include: fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), fumonisin B_3 (FB_3) and fumonisin B_4 (FB_4). They are produced primarily by *Fusarium*

verticillioides and other *Fusarium* species such as *F. gramineraum, F. napiforme, F. dlamini F. nygami* and *Aspergillus niger* (Ahangarkani *et al.,* 2014). These fungi are most commonly associated with maize contamination worldwide (EFSA, 2005).

Fumonisin B_1, also known as macrofusine is the most dominant and toxic of all the fumonisin analogues. Fumonisin B1 has an empirical formula of $C_{34}H_{59}NO_{15}$ (Fig. 1) with a relative molecular mass of 721 g/mol. As a pure substance it is seen as a white to off white powder.

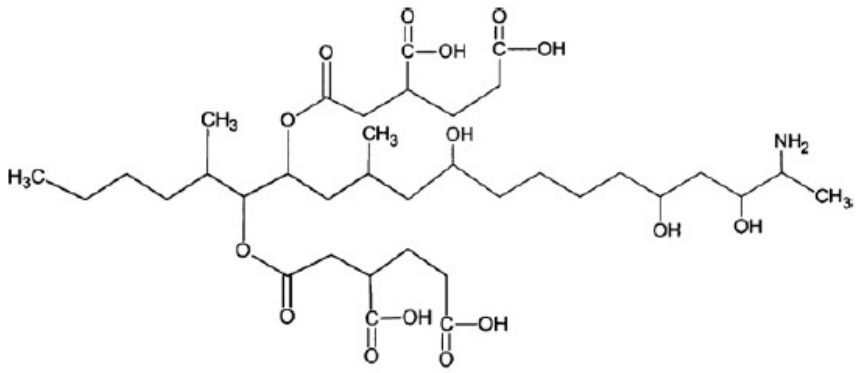

Figure 1.1: Chemical structure of fumonisin B_1 (FB_1) (Zain, 2011)

Fumonisin B_2 is a homolog of fumonisin B_1 that lacks one of the free hydroxyl groups on the backbone of C_{10}. It has an empirical formula of $C_{34}H_{59}NO_{14}$ (Fig. 2) with a relative molecular mass of 705.8 g/mol. Fumonisin B_2 can be seen as an off white solid powder, as a pure substance.

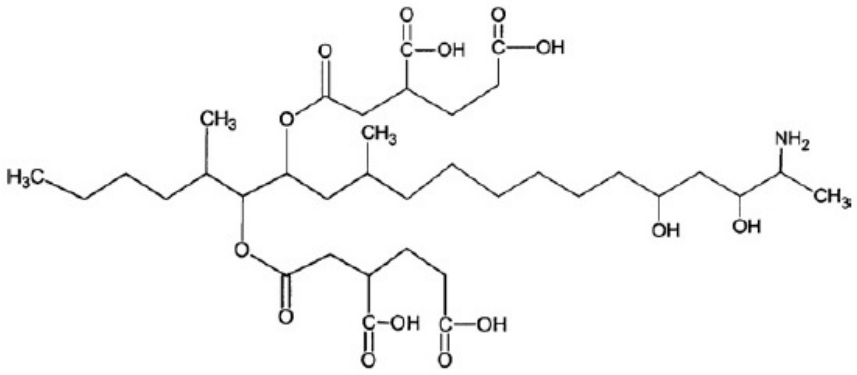

Figure 1.2: Chemical structure of fumonisin B_2 (FB_2) (Zain, 2011)

1.3.2. Health effects

Consumption of contaminated crops or foods prepared from contaminated raw materials can cause critical or protracted toxicity in humans and animals. This can include immunological effects, organ-specific toxicity, cancer and in severe cases, causes death (Ahangarkani *et al.,* 2014).

Mycotoxins are able to work with one another to produce different toxic effects in humans and animals as they are able to produce additive, synergistic or antagonistic reactions due to their various arrangements, including their different mechanisms of action, toxicity, origins and synthetic pathways as well as route of exposure (Smith *et al.,* 2016). Various mycosis can occur, from superficial skin disease (e.g. tinea) to invasive organ pathology (e.g. pulmonary aspergillosis) especially to those in patients that are immune compromised (Fung and Clark, 2004).

Fumonisin B_1 (FB_1) is the most copiously produced and most toxic fumonisin (Kumar *et al.*, 2008) and is linked to areas with high incidence of this cancer in Eastern Cape formerly known as Transkei (Sydenham *et al.*, 1990). According to Gelderblom *et al.* (1996), FB_1 are hepatotoxic and has a carcinogenic affect in rats. The widely known effect of FB_1 is leucoencephalomalacia in horses (Marasas *et al.*, 1976) but they have also been known to cause severe mortality in broiler chickens (Javed *et al.*, 1993), pulmonary oedema in pigs and duodenitis/proximal jejunitis in horses older than 2 years (D'Mello *et al.*, 1999). The consumption of FB_1 by pregnant women can cause birth defects in humans (Hendricks, 1999). Fumonisins interfere with sphingolipid metabolism (Merrill *et al.*, 2001) that can result in liver disease and tumors in the liver and kidneys (Richard, 2007).

In third world countries, where food is scarce, children are distinctly vulnerable to be exposed to mycotoxins due to a greater degree of food shortage. The mycotoxins effects are severe in children compared to adults. Children grow and develop, and therefore have a larger scale of cell production which results in a greater change in their bodies (Sherif *et al*., 2009). According to Anderson *et al.* (2000) and Barton *et al.* (2005), young children exposed to chemicals that act through a mutagenic mode of action have an increased susceptibility

towards the toxin and more time to develop a chronic disease. The extent of damage in children is also affected by extent of exposure and the toxicity of each mycotoxin consumed (Sherif *et al.,* 2009).

1.3.3. Decontamination

Most mycotoxins are heat stable compounds and are thus not easily destroyed during the cooking processes (Temba *et al.,* 2016). Mycotoxins are also invisible to the naked eye, odourless and tasteless making their detection impossible. They are generally also chemically very stable, resistant to storage and processing conditions (Valiuškaitė *et al.*, 2006).

The application of physical methods to reduce mycotoxin levels is time-consuming and also requires additional facilities. Physical methods of decontamination include mechanical separation (Grenier *et al.,* 2014), density segregation (Matumba *et al.,* 2015; Karlovsky *et al.*, 2016), thermal inactivation (Karlovsky *et al.*, 2016), irradiation (Karlovsky *et al.*, 2016) and solvent extraction (Grenier *et al.,* 2014; Karlovsky *et al.*, 2016). The efficiency of the methods highly depends on the level of contamination and the distribution of the mycotoxins throughout the grain (Grenier *et al.,* 2014; Karlovsky *et al.*, 2016). The practical application is very limited due to relatively high costs and removal or destruction of essential nutrients in the feed.

Regardless of inherent stability of mycotoxins to high temperatures, one of the techniques that are applied to reduce their levels is the application of high temperatures. It is generally noted that the higher the temperature, the greater the reduction in mycotoxin levels although heat does not completely eliminate mycotoxins (Bullerman and Bianchini, 2007; Temba *et al.,* 2016). Some mycotoxins are quite thermostable like FB_1 (Temba *et al.,* 2016), at a temperature of 100-120 ^{o}C. An increase of heat (above 120^{o}C) during the cooking process causes a reduction of the mycotoxin due to the interaction of pH, duration, temperature, sugar and water content. Even though the cooking process causes the breakdown of fumonisin, it is still able to retain its biological activity (Munkvold and Desjardins, 1997; Temba *et al.,* 2016).

Different combinations of temperature and ammoniation were applied to fumonisin decontamination. The combination of low temperature and ammoniation was unsuccessful

(Sydenham *et al.*, 1995) whereas this was possible when a combination of combination of high temperature and ammoniation was applied (Park *et al.*, 1992). The process of fermentation was also investigated which showed negligible degradation of fumonisin B_1 in contaminated maize for ethanol fermentation (Bothast *et al.*, 1992). According to Aziz *et al.* (2007) the method of irradiation yielded better results in fumonisin decontamination. Results indicated the higher the level of irradiation, the lower the growth of *Fusarium* species in the maize and at 5 kGy, fumonisin B_1 was deactivated.

The application of biological methods to fumonisin decontamination also yielded unsuccessful to semi-successful results. Adsorption methods for fumonisin B_1 showed a greater affinity for its removal. Dakovic *et al.* (2007) utilized natural clinoptilolite-rich zeolitic tuff, modified with octadecyl dimethylbenzyl ammonium (ODMBA). Results indicated that the highest adsorption of fumonisin B1 was obtained when the zeolite surface was fully covered with ODMBA. Analysis of results also indicated the charge of fumonisin B_1 (cationic or anionic) also plays a role in adsorption. There is also the possibility of more than one adsorption mechanism relevant for fumonisin B_1 adsorption (Dakovic *et al.*, 2007).

Microorganisms were also investigated as an alternative method of fumonisin decontamination with successful results. Niderkorn *et al.* (2006) screened fermentative bacteria such as *Lactobacilli, Lactococci, Streptococci* and *Enterococci* for their ability to bind deoxynivalenol, zearalenone and fumonisin. As found, *Streptococcus* and *Enterococcus* were able to bind 24% of fumonisin B1 and 62% of fumonisin B2, respectively. *Lactobacilli* constituted two-third of the group but the binding activity was less than that of *Streptococcus* and *Enterococcus.* In a further investigation, Niderkorn *et al.* (2009) examined the binding activity of fumonisins to cell wall components of LAB. Results indicated that peptidoglycan played an important role in binding of fumonisins. Niderkorn *et al.* (2009) have indicated that further investigations are required into the selection of specific LAB strains that bind fumonisins, the stability of the interaction between fumonisins and LAB, whereby investigations should be supported by *in vitro* work.

1.4. Prebiotics, synbiotics and probiotics

The study of functional foods has shown a clear relation between the food we eat and our health. Functional foods contribute towards the maintenance of health and prevention of a

broad range of illnesses but do not cure diseases (Mann and Truswell, 2016). Consumers are becoming more aware of this link between health and nutrition, leading to the demand for more functional foods. Due to the continual rise of medical costs, consumers prefer to prevent rather than cure illnesses. Prebiotics, synbiotics and probiotics found naturally in functional foods, tend to fill this gap for consumers although more research is required on the role of prebiotics and synbiotics in the human gut system.

1.4.1. Prebiotics

"Prebiotics are non-digestible, fermentable dietary carbohydrates that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon" as defined by Gibson and Roberfroid (1995). Some examples of common prebiotics are inulin type fructans, fructo-oligosaccharides, oligofructose, high molecular weight inulin and galacto-oligosaccharides (Roberfroid *et al.*, 2010). These prebiotics generally modify viscosity, emulsification capacity, gel formation, freezing point and colour of foods. Confirmed advantageous effects of prebiotics are the following: non-digestible with a low energy value, increase stool volume, modulate the colonic flora by stimulation of beneficial (LAB) bacteria and inhibition of "undesirable" bacterial pathogens such as *Clostridium* and *Bacteriodes* (Holzapfel and Schillinger, 2002). Thus far, prebiotics need to be investigated in-depth on associated disadvantages.

1.4.2. Synbiotics

A synbiotic is a combination of a prebiotic and a probiotic to effectively enhance and promote healthy gut interaction with the probiotic microorganism (Holzapfel and Schillinger, 2002). The prebiotic and probiotic used to produce the synbiotic are usually strain specific and oligosaccharide specific (Saad *et al.*, 2013). Synbiotics target the small and large intestines where the prebiotic carbohydrate is utilised by the probiotic strain and selectively promotes its proliferation and growth in the gut system.

The grouping of a prebiotic and probiotic into one product provides advantages of both, beyond those of either of them (Holzapfel and Schillinger, 2002). These advantages include stimulating immunity, treatment of diarrhoea in children and elderly people, enhancing intestinal barrier and remission of intestinal inflammation (Saad *et al.*, 2013). Due to osmotic potential and/or excessive fermentation of the synbiotic, some undesirable effects occur such

as excessive flatus, bloating and abdominal cramps (Holzapfel and Schillinger, 2002).

1.4.3. Probiotics

Probiotics are defined by World Health Organization (WHO) as "live microorganisms which when administered in adequate amounts confer a health benefit on the host". Many positive health benefits are associated with probiotics such as their resistance to gastric acidity and bile toxicity which reduce lactose intolerance (Kechagia, 2013). Their ability to transiently colonise the gastrointestinal tract and inhibit intestinal pathogens alleviate gastroenteritis and other gastro-associated illnesses (Sanchez *et al.*, 2009). Non-pathogenic, non-invasive lactic acid bacterial strains are used as bacterial vectors that deliver antigen to mucousal and systemic immune systems, generating specific antibody responses in serum and secretions (da Silva *et al*., 2014). By stimulation of the immune system, probiotics are also able to alleviate allergic reactions. Added benefits are lowering of cholesterols, prevention of dental caries and treatment and prevention of cancer by deactivation of mycotoxins (Goldin, 2010).

To the best of our knowledge no risks on probiotics overdose in humans have been reported (Figueroa-González *et al.,* 2011). Very few disadvantages are associated with probiotics, but probiotics need to be correctly identified and classified by use of molecular identification and typing techniques. The misidentification of a strain can result in its incorrect usage with detrimental health effects (Sanchez *et al.,* 2009).

1.4.3.1. Lactic acid bacteria (LAB)

Lactic acid bacteria are considered to have a generally recognised as safe (GRAS) status and continue to be the most widely used probiotics. Most probiotics strains have been isolated from the gut system, which makes the strains non-culturable or difficult to culture (Gratz *et al.*, 2010). Strains to be recognised as probiotics need to meet growth requirements and technological parameters of having good growth in industrial media, having moderate resistance to aerobic conditions and resistance to freezing/drying processes (Markowiak and Śliżewska, 2017). The major groups of probiotic strains come from the genera *Lactobacillus* with 23 strains, 5 strains from *Bifidobacterium*, 2 from *Escherichia coli*, 1 each from *Bacillus, Streptococcus, Enterococcus, Lactococcus* and 1 yeast strain, *Saccharomyces boulardii* (Goldin, 2010).

The bacteria that populate the lactic acid bacterial group are defined by their cell surface properties (Kleerebezem, 2010), growth at different temperatures, and conformation of the lactic acid produced, ability to grow at high salt concentrations, and acid or alkaline tolerance (Papadimitriou, 2016). They are non-spore forming, gram positive, non-motile and vary from long to short rods. Lactic acid bacteria are facultative anaerobes able to grow in an aerobic or anaerobic environment (Gratz *et al.*, 2010). One of the defining characteristics of LAB is the ability to convert sugars to lactic acid by fermentation hence the name LAB such as *Lactobacillus plantarum, L. brevis, L. delbrueckii, Pediococcus pentosaceus, Lactococcus lactis* etc.

Literature search has indicated that LAB has a natural affinity to bind to fumonisins B_1 and B_2 (Niderkorn *et al.,* 2006). These authors showed the natural ability of 29 (26 strains of LAB and 3 strains of propionic acid bacteria) different bacterial species to bind fumonisins B_1 and/or B_2. The LAB indicated a higher interaction with fumonisins B_1 and B_2 than any other bacterial strains tested.

Adebayo-tayo and Onilude (2008) shown that the dominant LAB found in Nigerian ogi were *L. plantarum* (30.8%) and *L. delbrueckii* (15.4%) with other LAB strains, yeast and fungi comprising the minority. This research was confirmed further by Afolabi and Akintokin (2008) with *L. plantarum* (32.4%) and *L. delbrueckii* (14.5%) comprising the major species. Microbiological research of Nigerian ogi by Omemu (2011) and Lahtinen *et al.* (2011) have shown *L. plantarum* as the dominant species followed by *L. fermentum*, *P. pentosaceus* and *L. brevis.* Further research by Fayemi and Buys, (2017) and Fayemi *et al.* (2017) has indicated *L. plantarum* FS2 and *P. pentosaceus* D39 as the most prevalent in Nigerian ogi. According to Akinleye *et al.* (2014), *L. plantarum* (10.31%), *L. fermentum* (9.28%) and *Staphylococcus aureus* (9.28%) are the dominant species in Nigerian ogi with a variety of fungal species comprising the minority.

Lactobacillus plantarum R1096 was selected as positive control strain from the Niderkorn PhD Thesis (2007) as it had amongst the highest binding of FB_1 and FB_2. *L. plantarum* FS2*, L. delbreuckii ssp. delbreuckii* CIP 57.8T and *P. pentosaceus* D39 were selected as test strains.

In this literature review chapter, the 2 genera namely, *Lactobacillus* and *Pediococcus,* are reviewed.

1.4.3.1.1. Genus: *Lactobacillus* Beijerinck 1901

The scientific classification of the genus: *Lactobacillus* is as follows: Phylum: Firmicutes, Class: Bacilli, Order: Lactobacillales, Family: Lactobacillaceae.

These LAB are most active in the production of yoghurt, cheese, pickles, beer and other fermented foods. Their ability to produce lactic acid modifies the environment to acidic, which inhibits growth of harmful bacteria. Bacterial strains from the *Lactobacillus* genus have potential therapeutic properties which are anti-inflammatory and anti-cancer.

In this study 2 strains from the genus will be investigated i.e. *L. plantarum* FS2 and *L. delbrueckii* CIP 57.8T.

Lactobacillus plantarum strains grow in de Man, Rogosa and Sharpe (MRS) (de Man *et al.,* 1960) broth and agar, are facultative anaerobic (Smetanková, 2012), have a temperature growth range of 10 - 40 ^{o}C (Pederson, 1935) with an optimum growth temperature at 37 ^{o}C (Smetanková, 2012). Defining macroscopic characteristics of the strain are that colonies grown on the agar appear circular, white, small (2-5 mm), entire margin, convex, smoothand glistening; are commonly opaque with no pigment observed. Microscopically, the bacterial cells are straight rods with rounded ends; occur singly, pairs or short chains; are non-motile with 0.19 - 1.2 µm x 3 – 8 µm cell size.

It is one of the most commonly known probiotic strains. *L. plantarum* occurs spontaneously in large numbers in most lactic acid fermented foods especially when it is based on plant material (Molin, 2010). These strains have the ability to survive gastric transit and can colonize the intestinal tract of humans and animals (de Vries *et al.,* 2006; Santarmaki *et al.,* 2017). Research conducted by Cunningham-Rundles *et al.* (2000) showed that the inclusion of *L. plantarum* into the diet of children exposed to HIV has improved natural immune response. Further research with placebo studies of *L. plantarum* by Nobaek *et al.* (2000) and Niedzielin *et al.* (2001) demonstrated a reduction in symptoms associated with irritable bowel syndrome.

Like the other *Lactobacilli* strains, *L. delbrueckii* also grows in de Man, Rogosa and Sharpe (MRS) broth and agar (de Man *et al.,* 1960), is a facultative anaerobic, has a temperature growth range of 2 – 53 ^{o}C (Hammes, 2011) with an optimum growth temperature at 37 ^{o}C (ATCC, 2013). The macroscopic characteristics of the strain grown on agar plate are that it has circular white colonies of average size 2 – 3 mm, filamentous margin, convex, smooth and glistening, colonies are opaque with no pigment observed. When viewed under white light, microscopically, the cells appear to be long rod shaped, that occur singly or in short chains, non-motile with a cell size of 0.5 - 1.2 µm x 1.0 - 10.0 µm.

This strain has been isolated from yoghurt, Swiss and Italian type cheeses (Leroy and de Vuyst, 2004). The use of *L. delbrueckii* in yoghurts provides a technical advantage for the prevention of over acidification in the product. This probiotic also aides the repair and equilibrium of the gut intestinal microflora (Lyoferm, 2014). According to Ruiz *et al.* (2013), *L. delbrueckii* is highly resistant to bile salts, gastrointestinal enzymes and has adhesion properties. It has high aggressive actions against pathogenic and food spoilage microorganisms (Lyoferm, 2014). *L. delbrueckii* provides a nutritional advantage by producing B-group vitamins e.g. folic acid (Rossi *et al.,* 2016).

1.4.3.1.2. Genus: *Pediococcus*

The scientific classification of the genus: *Pediococcus* is as follows: Phylum: Firmicutes, Class: Bacilli, Order: Lactobacillales, Family: Lactobacillaceae.

The strains from the genus *Pediococcus* are used in the making of cheeses and yoghurts, in the fermentation of cabbage to sauerkraut, although they are considered to be spoilage contaminants of beer and wine, most often, the strains are used as silage inoculants.

Pediococcus pentosaceus grows in de Man, Rogosa and Sharpe (MRS) broth and agar (de Man *et al.,* 1960), are facultative anaerobic, have a temperature growth range of 15 – 42 ^{o}C with an optimum growth temperature at 37 ^{o}C (Semjonovs and Zikmanis, 2010). The macroscopic characteristics of the strain grown on agar plate are as follows: circular, white colonies of average size 1 – 2 mm, entire margin, convex, smooth and glistening, colonies are opaque with no pigment observed. *Pediococcus pentosaceus* has a distinctive microscopic

characteristic in that the strain divides alternatively in two perpendicular directions to form tetrads (Semjonovs and Zikmanis, 2010). The cells tend to be spherical to occasionally ovoid, non-motile with a cell size of 0.5 – 1 μm.

This strain has high bile tolerance, β-galactosidase activity, acid tolerance, adherence ability and antibacterial property which make it a good candidate as a probiotic (Sukumar and Ghosh, 2010). Pediocin antibacterial activity isolated from *P. pentosaceus* was characterized by Osmanagaoglu *et al.* (2001 and 2011) showing the inhibition of *Listeria monocytogenes*. Further research conducted by Vidyasagar and Jeevaratnam (2013), showed that *P. pentosaceus* has strong auto-aggregation and co-aggregation against *L. monocytogenes*, has effective inhibition against intestinal gram positive and negative pathogens and that the use of live cultures can effectively lower cholesterol.

The fumonisin (B_1 and B_2) binding interaction of these 4 strains i.e. *L. plantarum* (Positive control) R1096*, L. plantarum* FS2*, P. pentosaceus* D39 (Test strains isolated from ogi) and *L. delbrueckii* CIP 57.8T will be investigated further in this project.

For this literature review, a brief explanation is given for prebiotics, synbiotics and its involvement with probiotics. Due to the focus and scope of this study, probiotics are reviewed in more detail.

1.5. Lactic acid bacterial interactions

Even though this project focuses on the interaction between LAB and fumonisins (B_1 and B_2), this particular interaction cannot be examined in isolation (Shetty and Jespersen, 2006). Each probiotic can produce different effects which depend on the probiotics' metabolic properties, diverse molecules present on its surface and on the components that it is able to secrete (Oelschlaeger, 2010). Further investigation has shown that constituents of the bacterial cell wall namely, peptidoglycan (Niderkorn *et al.,*2006) or deoxyribonucleic acid (DNA) might be important for the probiotic to be effective (Oelschlaeger, 2010). All these properties, individually or mutually, establish the probiotic action and therefore its effectiveness in application for prevention and/or treatment of certain diseases (Oelschlaeger, 2010). There are 3 modes of probiotic action, which involve illness protection, prevention of cancer and stabilisation of the physiological balance between intestinal microbiota and the

host. In the gastrointestinal environment, LAB interacts with intestinal microbiota, different compounds/chemicals/toxins and the host's intestinal wall receptors, initiating different reactions (See Fig. 1.3).

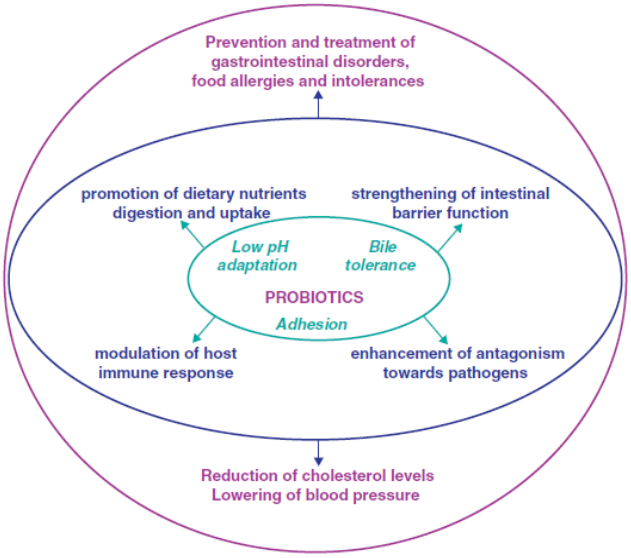

Figure 1.3: Different lactic acid bacterial interactions (Siciliano and Mazzeo, 2012)

1.5.1. Lactic acid bacterial interactions with other bacteria

Lactic acid bacteria interact directly with other bacteria (Oelschlaeger¸ 2010). This interaction occurs with the LAB producing bacteriocins such as short chain fatty acids (e.g. lactic acid) and hydrogen peroxide (Wohlgemuth *et al.*, 2010) which inhibit the growth and replication of pathogens (Maqueda *et al.,* 2008). According to Siciliano and Mazzeo (2012), LAB also produces derivatives of bile salts, namely deconjugated bile acids, which have a higher antimicrobial activity than bile salts. Intestinal microbiota and pathogens are always in competition for resources as it is quite limited in the gut environment. According to Bruyneel *et al.* (1989) and Imbert and Blondeau (1998), almost all bacteria require iron as a nutrient except for *Lactobacilli*, that are able to grow without iron in the gut environment. Another advantage of LAB is the ability to modulate the gut microbiota composition in the gut environment. For example, according to Yamano *et al.* (2006), *Lactobacilli* are able to increase in the gut environment after ingestion. Lactic acid bacteria are also able to establish a biofilm that prevents the adhesion of pathogens thereby protecting the intestinal barrier (Oelschlaeger¸ 2010).

1.5.2. Lactic acid bacterial interactions with mycotoxins

A further advantageous interaction of LAB is its ability to protect against cyanobacteria and fungal toxins which are considered to be tumour promoters (Oelschlaeger, 2010). Some studies have shown that the deactivation of mycotoxins is due to binding rather than metabolism (El-Nezami *et al.*, 2002; Dalie *et al.*, 2010). Binding between LAB and mycotoxin depends on the concentration of the toxin, cell density, viability of bacteria and pH value. Some studies have shown that non-viable LAB have the same binding capacity as viable LAB, but the stability/permanency of the binding are yet to be evaluated (Fuchs *et al.*, 2008).

Literature surveys conducted have illustrated the fact that most lactic acid bacterial strains are used for binding mycotoxins (Dalié *et al.,* 2010; Ahlberg *et al.,* 2015), such as aflatoxin (Haskard *et al.*, 2001; Salim *et al.*, 2011); aflatoxin B_1 (El-Nezami *et al.,*1998); ochratoxin A (OTA) (Fuchs *et al.*, 2008), zearalenone (ZEN) and α-zearalenol (El-Nezami *et al.,* 2002); deoxynivalenol (DON), nivalenol (NIV), ZEN, FB_1 and FB_2 (Niderkorn *et al.,* 2006), AFB_1 and patulin (Topcu *et al.,* 2010), and FB_1 and FB_2 (Zhao *et al.,* 2016). Studies conducted by Niderkorn *et al.* (2006) and Dalie *et al.* (2010) have illustrated that LAB bind fumonisins and that deactivation of fumonisins occurs to a certain degree with adhesion to LAB cell wall components occurring rather than covalent binding or metabolism (Niderkorn *et al.*, 2009; Dalié *et al.*, 2010; Zhao *et al.*, 2016). Peptidoglycans, teichoic acids, proteins and polysaccharides are the components that make-up a Gram positive cell wall structure (Delcour *et al.*, 1999; Chapot-Chartier and Kulakauskas, 2014). The components of the cell wall, the electrostatic charge of the cell wall and the electrostatic charge of the molecules can all play a role in the binding interaction that occur between the two.

The chemistry, binding and stability of the bound complex under harsh conditions of the gut have yet to be investigated (Shetty and Jespersen, 2006). Gratz *et al.* (2010) have shown that the bound complex is rapidly excreted from the gut system but *in vitro* studies have yet to be supported by *in vivo* studies to establish the level of toxicity of the bound complex.

1.5.3. Lactic acid bacterial interactions with intestinal cells

Lactic acid bacteria are able to interact/bind to the host cells/epithelium, and elicit responses

that affect the host's immune system with products like metabolites, cell wall components and DNA (Oelschlaeger, 2010). This adhesion interaction of the LAB with gut epithelial cells and gut associated immune cells further trigger a signal cascade that modulates the immune system (Siciliano and Mazzeo, 2012). According to Mack *et al.* (2003), this adhesion also blocks the binding of pathogens to the epithelial cells. Research conducted by Roos and Jonnson (2002) indicated that the LAB are able to outcompete pathogens for the same binding receptor on the epithelial cells thereby prohibiting pathogens from binding. Whereas, Sanchez *et al.* (2011) shown that *L. plantarum* is able to produce proteins that give the bacterium the ability to bind to more surfaces than just the epithelial cell. The metabolites/soluble factors released by the LAB can also trigger a signal cascade. This strengthens the gut epithelial barrier to pathogens (Oelschlaeger, 2010) and amplifies the immune response to tumor tissue (Hirayama and Rafter, 2000).

From the above information, LAB are well designed to protect and aid against pathogens in the gut environment. It is suggested that with further research, designer LAB will soon be able to carry all these traits as well as be able to implement them in the gut environment. In view of this project, review of literature has indicated these areas for further investigation. The binding interaction that occurs between LAB and fumonisin can be evaluated in terms of stability and toxicity. Stability of the bound complex can be determined *in vitro* with neat combinations of optimized temperature, incubation temperature, pH. This will give an indication of the stability of the bound complexes in different environments. Such studies can then be finalized by testing the toxicity of the bound complex on human cell line.

1.6. Toxicological studies with regard to cell lines

Cell lines are cell cultures which are grown outside their natural environment under controlled conditions. These cells are usually taken from multicellular eukaryotes and used to test their reaction to certain substances/compounds. There are 2 major types of cell lines applied in experiments. Firstly, primary cell lines which are those cells that are directly cultured from a subject. They generally have a limited lifespan as after a certain number of population doublings, they stop dividing while retaining viability (Hayflick, 1979). Secondly, there are immortalized cell lines that have the ability to double population size indefinitely through random mutation or deliberate modification (Hayflick and Moorhead, 1961). There are many cell lines that have been established as a representative of a particular cell type.

Cell cultures require specific temperatures and gas mixtures in order to grow and be maintained. A typical culture condition for mammalian cells is 37 ^{o}C with 5% CO_2 in a cell incubator (BioRad, 2012). Every cell type's culture conditions varies widely. Different growth media with a variation of culture conditions result in different phenotypes of cultures.

Use of cell lines in experiments to determine stability and toxicity of a compound are frequently applied in science (Freshney, 2006). They are low cost, not labour intensive, with moderate throughput capabilities. It is the only system that mathematically determines synergy, additivity and antagonism of a test compound. What makes the use of cell lines so advantageous is the ability to test different parameters e.g. temperature, pH, etc. for the cells reaction against the test compound without endangering any test subject. It also provides the ability to easily manipulate the system to extremes (e.g. very high temperatures or high pH and vice versa) to which a live test subject might not be able to survive or adapt to. The added advantage is that results obtained from the cell culture is wholly dependent on the test compound, one does not run the risk of contamination by other compounds (ATCC, 2012a). Another unique advantage of cell lines is that the cells react/behave with the test compound in the same manner as it would in its natural environment i.e the body, which provides scientists with a clear and in-depth look into the cell behaviour without the complications of extracting samples (Ekwell, 1990).

There are very few disadvantages regarding cell lines, one is that cells may undergo transformation to allow for *in vitro* growth which might change the outcome of the trial. Some compounds may require metabolic activation. Nutrient depletion in growth medium can result in cells dying (ATCC, 2012b). Due to the cells being in a stable environment, the accumulation of dead cells can also contribute to cell cultures dying.

Cell lines have been used to test the toxicity of fumonisins. Creppy *et al.* (2004) tested fumonisins and ochratoxin A in rat brain cells, human intestinal cells and green monkey kidney cells. Results indicated that human intestinal cells are less sensitive to fumonisin B1. Cytotoxicity results obtained from *in vitro* testing can predict acute toxicity of substances *in vivo.* Mokoena *et al.* (2005) tested the reduction of aflatoxin and zearalenone by lactic acid bacteria in fermented maize meal followed by cytotoxicity testing on SNO human

oesophageal carcinoma cells. Results indicated that after fermentation, the compounds had a reduced toxicity to the SNO cells. A comparative study of toxicity and oxidative stress induced by deoxynivalenol, zearalenone and fumonisin B_1 in human intestinal cells was carried out by Kouadia *et al.* (2005). The neutral red test was used to determine cytotoxicity of which live cells accumulate the red dye across cell membranes and the absorbance levels can be measured for viability of cells. Results indicated fumonisin B_1 had the highest cytotoxicity. Research conducted by Mckean *et al.* (2006) tested the combination of aflatoxin B_1 and fumonisin B_1 in animal (rat and mosquitofish cells) and human cells (BEAS-2B and HepG2). These 2 toxins had a strong additive reaction in the animal cells but no human cell line was found to be sensitive to fumonisin B_1. IC_{50} values were determined for all the toxins and cell lines.

A review of the literature conducted to date illustrates no research has been reported on the toxicity of fumonisin in conjunction with LAB.

1.7. Conclusion

Most fermented cereal products consumed in Africa are home-made using cereals that are likely contaminated with mycotoxins. These products are then consumed at home and also sold in cities to generate income. Mycotoxins are known secondary fungal metabolites that are hepatotoxic, carcinogenic and teratogenic. Studies have indicated that in Africa the most prevalent mycotoxin found in cereal grains are fumonisins (Dawlal *et al.*, 2010), specifically fumonisin B_1 and fumonisin B_2 (Vismer *et al.* 2015; Mngqawa *et al.,* 2016; Udomkun *et al.*, 2017) with levels that can exceed 10 µg/g (Okeke *et al.,* 2015; Chilaka *et al.,* 2016).

This project proposes to investigate the binding interaction that occurs between LAB and fumonisins to form a stable bound complex. Further testing of the bound complex will examine the stability and toxicity. This will allow recommendations to be made into the re-engineering of fermented cereal-based products in order to facilitate selection of the best LAB that can be able to produce a fermented product with the correct sensory properties while at the same time reduce toxicity and limit fumonisin ingestion among the consumers.

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Doctoral Thesis

Problem statement, Hypothesis and Objectives

Problem Statement

Cereal grains form the majority of humanity's food supply and they include, maize, wheat, rice and barley (Oluwakemi and Omodele, 2015). Maize is the staple crop of South Africa consumed in one form or another by the majority of the African population. Cereal grains are prone to infestation by mycotoxigenic fungi and their corresponding mycotoxins. According to Rodriguez and Naehrer (2012) these mycotoxins contamination in cereal grain have the following degree of prevalence worldwide: fumonisins (64%), deoxynivalenol (59%), zearalenone (45%), aflatoxin (33%) and ochratoxin A (28%). Fumonisins in maize have levels that can exceed 10 µg/g in various African studies (Chilaka *et al.,* 2016). These mycotoxins have been linked to oesophageal cancer in the Eastern Cape area, SA (Sydenham *et al.*, 1990). They are well known for causing leucoencephalomalacia in horses (Marasas *et al.*, 1976). The most disturbing factor is their structural similarity to sphingonine and sphingosine (Stockmann-Juvala and Savolainen, 2008) and their ability to interfere in sphingolipid metabolism. This can result in liver disease and tumours in liver and kidneys. Many methods have been investigated to detoxify mycotoxins. A new method of detoxification has recently been developed where some mycotoxins have a natural affinity to bind to microorganisms (Niderkorn *et al.,* 2006; Topcu *et al.,* 2010; Salim *et al.*, 2011; Zhao *et al.,* 2016). Recent studies have indicated that probiotics, specifically LAB, have a natural ability to bind to fumonisins and produce a natural detoxifying effect on the fermented product (Niderkorn *et al.,* 2006; Zhao *et al.,* 2016). Maize forms the base cereal product of most traditional fermented products, such as ogi and mahewu, of which the dominant microorganisms driving the fermentation are probiotic, LAB strains (Tamang *et al.,* 2016). The project proposed here focuses on the ability of the following dominant probiotic LAB strains isolated from traditional fermented maize based products (ogi and mahewu) namely, *Lactobacillus plantarum* FS2, *L. delbrueckii* subsp. *delbrueckii* CIP 57.8T and *Pediococcus pentosaceus* D39, against a positive control strain, *L. plantarum* R1096 to investigate its potential in mycotoxin control, specifically the control and/or reduction of fumonisin toxicity. The binding interaction between the LAB and fumonisins can be investigated along with the stability of the resulting bound complex. The protective effect of the LAB strains to reduce the toxicity of fumonisins in HepG2 cells can also be investigated *in vitro.*

Hypothesis 1:

It is hypothesised that due to the fumonisin being able to bind to LAB as previously indicated (Niderkorn *et al.,* 2009) and due to its natural affinity for microorganisms, that the fumonisins will bind to the selected LAB isolated from traditional fermented cereal products in this project. It has also been shown that LAB are able to bind multiple fumonisins (B_1 and B_2) molecules (Niderkorn *et al.,* 2009). Due to the minute size of the molecules, it has been hypothesized that fluorescent tagging of the molecules could prove to be a solution to visualize binding interaction. Based on this hypothesis we propose to use two different derivatizing agents: naphthalene-2, 3-dicarboxaldehyde (NDA) (Maragos and Richard, 1994) and o-phthaldialdehyde (OPA) (Dasko *et al*., 2005). Owing to the stability of the derivatizing agent at room temperature for the confocal laser scanning microscopy (CLSM) work, naphthalene-2, 3-dicarboxaldehyde (NDA) will be used and OPA will be used for the quantification via high pressure liquid chromatography (HPLC) to determine the number of bound fumonisins per predetermined number of LAB cells. Conventional microbiology methods will be used to approve/disapprove the discrepancy between viable and non-viable LAB cells due to the difficulty in using dual fluorescent probes simultaneously (Zotta *et al.,* 2012). The quantification of bound fumonisins to obtain the baseline binding interaction for the viable and non viable cells will be based on Niderkorn's protocols (Niderkorn *et al.,* 2006 a, b).

Objective 1:

For objective one, we plan to determine the physical visual and quantify the binding interaction that occurs between fumonisins (B_1 and B_2) and LAB, namely *L. plantarum* FS2, *L. delbrueckii* subsp. *delbrueckii* CIP 57.8T and *P. pentosaceus* D39, isolated from traditional fermented maize-based products (ogi and mahewu) against the positive control strain, *L. plantarum* R1096 by using CLSM and HPLC with the aim to demonstrate that LAB strains isolated from traditional fermented African cereal products have the ability to reduce the toxicity of fumonisins in food.

Hypothesis 2:

Niderkorn *et al.* (2006 and 2009) illustrated that LAB strains are able to bind multiple fumonisins (B_1 and B_2) molecules. Based on this premise, the researchers demonstrated the stability of the bound complex on different parameters using an experimental model that

simulated the acidic conditions of silages with various LAB strains against a positive control *Lactobacillus rhamnosus* GG strain (Niderkorn *et al.*, 2006). It is hypothesised to use a similar model, to the demonstrate the binding stability of LAB – fumonisin complex utilising the selected LAB isolated from traditional fermented cereal products in this project in conditions simulating ogi fermentation parameters against a positive control strain selected from the Niderkorn article, *Lactobacillus plantarum* R1096.

Objective 2:

For objective two, we plan to determine the *in vitro* interaction between the dominate LAB strains (*L. plantarum* R1096, *L. plantarum* FS2, *L. delbrueckii* subsp. *delbrueckii* CIP 57.8T and *P. pentosaceus* D39) in African traditional fermented maize based foods (ogi and mahewu) and fumonisins (B_1 and B_2) including the stability of the bound complex under varying pH, temperature, and post fermentation conditions of storage and lower pH of 2 and incubation temperature of 37^{o}C.

Hypothesis 3:

Fumonisins interfere with sphingolipid metabolism resulting in liver disease and tumours in the liver and kidneys, due to their structural similarity to sphingonine and sphingosine (Stockmann-Juvala and Savolainen, 2008). It is hypothesised that in the presence of LAB cells the fumonisins would have a reduced toxicity towards the liver cells due to their natural affinity to bind to the LAB cells based on the Sulforhodamine B (SRB) assay (Skehan *et al.,* 1990). It is hypothesised that the binding interaction between the LAB cells and the fumonisins alters the configuration of the fumonisins thereby preventing the interaction and / or absorption into the liver cell, limiting exposure to fumonisins among consumers.

Objective 3:

For objective three, we plan to determine the effect of LAB isolates (*L. plantarum* R1096, *L. plantarum* FS2, *L. delbrueckii* subsp. *delbrueckii* CIP 57.8T and *P. pentosaceus* D39) from traditionally fermented cereals, on toxicity of fumonisins (B_1 and B_2) to HepG2 cells *in vitro* using the SRB assay, with the aim of reducing the toxicity among consumers using LAB strains.

Chapter Two

Visualization and quantification of fumonisins bound by viable and non-viable lactic acid bacteria isolated from traditional fermented maize-based products, ogi and mahewu

2.1. Abstract

In Africa, consumption of food contaminated with fumonisins has a negative influence on the health of both humans (causing tumours in the liver and kidneys, well known for oesophageal cancer in Eastern Cape in South Africa) and animals (leucoencephalomalacia in horses). Lactic acid bacteria (LAB) have emerged as a promising natural detoxification agent against mycotoxins. The aim of this study was to visualize and quantify the interaction between fumonisins (fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2)) and LAB, namely *Lactobacillus plantarum* FS2, *L. delbrueckii* subsp. *delbrueckii* CIP 57.8T and *Pediococcus pentosaceus* D39, isolated from traditional fermented maize-based products (ogi and mahewu) against a positive control strain, *L. plantarum* R1096 by using confocal laser scanning microscopy (CLSM) and high performance liquid chromatography (HPLC), respectively. Fumonisins were derivatized using naphthalene-2, 3-dicarboxaldehyde (NDA) and then combined with non-fluorescent LAB cells (viable and non-viable). Confocal scanning microscopy showed the derivatized green fluorescent fumonisins binding to the surface of each of the LAB cells thereby making the entire LAB cell to fluoresce green due to binding of multiple fumonisin molecules to the external surface of the LAB cell. For the quantification of bound fumonisins, viable and non-viable cells were incubated in presence of predetermined concentrations of fumonisins and then the level of fumonisin in the suspension was determined. For viable cells, *L. plantarum* FS2 bound the highest levels of FB_1 while *P. pentosaceus* D39 the least amount of fumonisin. The highest levels of FB_2 were bound by *L. plantarum* R1096 and the least by *L. delbrueckii* CIP 57.8T. For non-viable cells, highest levels of both fumonisins were bound by *L. plantarum* FS2 while the lowest amounts were bound by *P. pentosaceus* D39 and *L. delbrueckii* CIP 57.8T for FB_1 and FB_2, respectively. The results indicate that LAB isolates from fermented maize based beverages bind fumonisins and thus present a potential strategy for thereby limiting exposure among humans. To the best of our knowledge, this is the first study to visualize the interaction between LAB and fumonisins.

2.2. Introduction

Mycotoxins are known to be fungal secondary metabolites that have the ability to impair human and animal health. Most mycotoxins are heat stable compounds, not easily destroyed by the cooking processes (Temba *et al.,* 2016) and also chemically very stable, resistant to storage and processing conditions (Grenier *et al.,* 2014; Karlovsky *et al.*, 2016) which make their decontamination/detoxification complicated and difficult. LABs are the most dominant microorganisms found in traditional fermented cereal products with the natural affinity to bind mycotoxins. Based on the ability of non-viable cells to also bind toxins, some studies have demonstrated the deactivation of mycotoxins as a result of binding rather than metabolism (El-Nezami *et al.*, 2002; Fuchs *et al.*, 2008; Dalié *et al.,* 2010). Most LAB strains are used to bind aflatoxin (Haskard *et al.*, 2001; Salim *et al.*, 2011); ochratoxin A (Fuchs *et al.*, 2008) and zearalenone (El-Nezami *et al.,* 2002), whereas Niderkorn *et al.* (2006a) and Dalié *et al.* (2010) have illustrated that LAB bind to and deactivate fumonisins.

Binding by definition means the uniting or securing of two components together and in the context of this project, binding would mean the uniting of the fumonisin and the LAB cell. To date, visualization of the binding interaction between the two components has not been achieved and/or published due to the difficulty in finding and selecting appropriate fluorescent probes that could allow for the simultaneous detection of mycotoxin and LAB (Zotta *et al.,* 2012). The added difficulty is being able to demonstrate the concept of viable and non-viable cells with fluorescent probes, as cells stained and deemed to be "viable" or "non-viable" are actually due to the probes being able to permeate a damaged or undamaged membrane and does not necessarily dictate that the cell is "viable" or "non-viable" (Zotta *et al.,* 2012). In this project, the objective was to fluorescently tag one component (i.e. the fumonisins) and demonstrate the non-viability of the LAB cells by conventional microbiology methods. This would resolve the issue of dual fluorescent staining and visualization.

In the removal of fumonisin, binding as the main mechanism could possibly involve adhesion to cell wall components rather than covalent binding or metabolism (Niderkorn *et al.*, 2009; Dalié *et al.,* 2010; Zhao *et al.*, 2016). It has been reported that peptidoglycans of LAB cell wall are the main binding sites for fumonisins, their structural integrity is essential and their amino acid sequence plays an important role in enhancing the efficiency of the binding

mechanism.

Once we establish that the strains bind the fumonisins by the visualization of binding interaction then the challenge is to determine quantitatively the difference in binding between FB_1 and FB_2 as it is hypothesized that both molecules would compete for the same binding site, being dependent on the similarity of their chemical structures (Beier and Stanker, 1997). Furthermore investigating the parameters of viability vs non-viability of the LAB cells in quantification of fumonisin binding will also give a better understanding as to the mechanism of binding.

Therefore, the objective of this study was to visualize and quantify the interaction between fumonisins and LAB, namely *L. plantarum* FS2, *L. delbrueckii* subsp. *delbrueckii* CIP 57.8T and *P. pentosaceus* D39, isolated from traditional fermented maize-based products (ogi and mahewu) with *L. plantarum* R1096 used as the positive control strain on confocal laser scanning microscopy (CLSM) and high performance liquid chromatography (HPLC), respectively. This is with the view to demonstrate that LAB strains isolated from traditional fermented African cereal products have the ability to reduce the toxicity of fumonisins in food.

2.3. Materials and methods

2.3.1. LAB cultures

Three LAB cultures (*L. plantarum* FS2, *L. delbrueckii* subsp. *delbrueckii* CIP 57.8T and *P. pentosaceus* D39) (Fayemi and Buys, 2017; Fayemi *et al.*, 2017) originally isolated from the traditional African fermented cereal products: ogi and mahewu, were used in the current study. *L. plantarum* R1096, which was shown by Niderkorn *et al*. (2006a) to have ability to bind fumonisins, was used as a positive control. Table 2.1 shows the maintenance and optimal growth conditions for the LAB strains used.

All LAB strains were cultivated and stored on MRS agar (de Man *et al.,* 1960) slants at 4 ^{o}C for 3 months and for long term conservation, cryopreserved at -80 ^{o}C in 12.5% glycerol (Jacobs, 1991) and on microbeads (MAST Diagnostics, France).

Table 2.1: Lactic acid bacterial strains selected for binding with fumonisin (B_1 and B_2)

Strain	Culture Collection	Strain Type	Format Received	Format Maintained	Medium (Agar and Broth)	Incubation period (Hours)	Growth Temperature (^oC)	Optimal Growth Temperature (^oC)
***Lactobacillus plantarum* R1096**	Lallemand SAS, France	Positive control	Petri-dish for single colonies	Cryo-preserved vial in MAST (x2) and glycerol (x4)	MRS (Smetanková, 2012)	24-48	10-40 (Pederson 1935) (Facultative anaerobic) (Smetanková, 2012)	37 (Smetanková, 2012)
***L. plantarum* FS2**	Isolated from Nigerian ogi	Test strain	Cryo-preserved vial (x1) MAST	Cryo-preserved vial in MAST (x2) and glycerol (x4)	MRS (Smetanková, 2012)	24-48	10-40 (Pederson 1935) (Facultative anaerobic) (Smetanková, 2012)	37 (Smetanková, 2012)
***Pediococcus pentosaceus* D39**	Isolated from Nigerian ogi	Test strain	Cryo-preserved vial (x1) MAST	Cryo-preserved vial in MAST (x2) and glycerol (x4)	MRS (Semjonovs, 2010)	24-48	15-42 (Facultative anaerobic) (Semjonovs, 2010)	37 (Semjonovs, 2010)
***L. delbrueckii* ssp. *delbrueckii* CIP 57.8T**	Pasteur Institute	Test strain	Cryo-preserved vial	Cryo-preserved vial in MAST (x2) and glycerol (x4)	MRS (Hammes and Hertel, 2011)	24-48	2-53 (Facultative anaerobic) (Hammes and Hertel, 2011)	37 (ATCC, 2013)

2.3.2. Visualization of binding interaction between fumonisins and LAB cells (viable and non-viable)

2.3.2.1. Culturing and harvest of viable LAB cells

LAB cultures were obtained by streaking out for single colonies onto MRS agar plates. A single colony from the 24 h plate culture was aseptically inoculated into 2 ml Eppendorf tubes containing 1.8 ml of MRS broth and incubated for 24 h at 37 ^{o}C. The bacterial cells were then harvested by centrifugation at 3000 rpm for 10 min at <10 ^{o}C. The harvested cells were subsequently washed in phosphate buffered saline (PBS) and then re-suspended in 0.05 M sodium phosphate buffer (pH 6.6) for use in visualization experiments.

2.3.2.2. Preparation of non-viable LAB cells

The LAB cultures were inoculated into 2ml Eppendorf tubes containing 1.8 ml of MRS broth and incubated for 24 h at 37 ^{o}C. The Eppendorf tubes were then heat-treated in a water bath at 80 ^{o}C for 10 min and immediately cooled on ice. The cells were then harvested and treated in a similar manner as for viable cells (Section 2.3.2.1). To verify non-viability of the cells, 100 µl of cell suspension was spread onto MRS agar plates and incubated at 37 ^{o}C for 24 h.

2.3.2.3. Preparation of fumonisin solutions

Fumonisin B_1 (Product no: F1147) and fumonisin B_2 (Product no: F3771) were obtained from Sigma Aldrich (South Africa). The standards of fumonisins were each diluted with 8 ml of acetonitrile: water (1:1 v/v), to obtain a concentration of 125 µg/ml stock solution. All solutions were stored at -20 ^{o}C.

2.3.2.4. Derivatization of fumonisins

Derivatization of FB_1 and FB_2 was done according to the method of Maragos and Richard (1994). Briefly, the fumonisin (1 ml of 125 µg/ml) was derivatized at 60 ^{o}C for 15 min using a mixture of 1 ml of 0.05 M sodium borate (pH 9.5), 200 µl of sodium cyanide (0.1 mg/ml) (Sigma Aldrich) and 200 µl of naphthalene-2,3-dicarboxaldehyde (NDA) (0.25 mg/ml) (Sigma Aldrich).

2.3.2.5. Visualization of interaction between LAB cells and fumonisins

Exactly, 0.5 µl of LAB cells, both viable and non-viable, were mixed with 0.5 µl of derivatized fumonisin (FB_1 and /or FB_2) onto a microscope slide and visualized with white

light and CLSM. The mixture was immediately visualized using Zeiss LSM 510 META confocal laser scanning microscope at an excitation and emission wavelengths of 488 and 505 nm, respectively. For control samples, LAB cells (0.5 µl) or derivatized fumonisin (0.5 µl) alone were spotted onto a clean microscope slide and visualized using CLSM.

2.3.3. Quantification of fumonisins bound by viable and non-viable LAB cells

2.3.3.1. Culture and harvest of LAB cells

Culturing and harvesting of LAB cells were performed according to the method described by Niderkorn *et al.* (2006a), with minor modifications. Briefly, a single colony of each LAB strain from a 24 h MRS agar plate culture was inoculated into four Erlenmeyer flasks containing 200 ml of MRS broth and incubated for 24 h at 37 ^{o}C. All cultures were homogenized and aliquots of 2 ml each were removed for quantification of LAB cell concentration. A standardized concentration of LAB cells (10^{13} cfu/ml) was systematically obtained and used for all strains across all tests.

Post quantification, for each LAB strain, 2 of the broth cultures were then heat-treated in a water bath at 80 ^{o}C for 10 min and immediately cooled on ice. Non-viability of cells were verified as stated (Section 2.3.2.2).

2.3.3.2. Determination of LAB cell concentration

For each LAB strain, the LAB cell concentrations were estimated by determining the absorbance (OD_{600}). The absorbance was then compared to the standard curves previously obtained per LAB strain by linking colony forming units (cfu) per ml from plate counts and absorbance measurements over a range of LAB concentrations.

2.3.3.3. Preparation of fumonisin solutions

Fumonisins (B_1 (F1147) and B_2 (F3771)) (Sigma Aldrich, France) were respectively diluted with 10 ml of acetonitrile: water (1:1; v/v) to give a concentration of 250 µg/ml, aliquoted into 40 amber vials (holding capacity of 2 ml each) and stored at -20 ^{o}C. A standardized concentration of 10 µg/ml for each fumonisin was used throughout the experiment according to Niderkorn *et al.* (2006a).

2.3.3.4. Binding quantification of LAB cells and fumonisins (B1 and B2)

Subsequent to quantification of LAB cell concentration, 250 ml of each culture were centrifuged at 3000 x g for 10 min at <10 ^{o}C. The supernatant was discarded and the bacterial cells re-suspended and washed (x3) in 100 ml of 0.01M phosphate buffer solution (PBS) of pH 7.4. The bacterial pellet from one of the two broth cultures was re-suspended in 2 ml of 0.1 M citrate phosphate buffer (CPB) (pH of 4) containing 10 µg/ml of FB_1 and 10 µg/ml of FB_2 (fumonisin buffer solution). The sample was then split into 1 ml each and transferred to a 2 ml Eppendorf tube (test sample in duplicate). The negative control, excluding FB_1 and FB_2, was treated similarly. All samples were incubated at 30 ^{o}C for 1h with agitation (480 rpm) and for 23 h without agitation. The samples were then centrifuged at 3000 x g for 10min at < 5 °C, supernatants transferred to 2 ml amber vials and stored at 4 ^{o}C until the analysis of fumonisins (B_1 and B_2) using high performance liquid chromatography (HPLC).

2.3.3.5. Quantification of bound fumonisin using HPLC

The supernatants of all the samples (test samples, positive and negative controls) were quantified for fumonisin (B_1 and B_2) by HPLC coupled with fluorescence detector (Shimadzu RF 20A, Japan) after derivatization with *o*-phthaldialdehyde (OPA) where 100 µl of derivatization solution was added to 100 µl of sample via the auto-sampler. Prior to injection, the contents were mixed and allowed to react for 2 min. The derivatization solution was prepared by mixing 120 mg of OPA (Sigma-Aldrich), 3 ml of 100% methanol, 15 ml of 0.1 M Borate buffer pH 10 and 179 µl of 1-thioglycerol (Sigma-Aldrich), and left overnight in the dark at ambient temperature. The operating conditions were as follows: injection volume of 100 µl of each sample after fumonisin derivatization; C18 reverse-phase column, Uptisphere type, ODB, 5 µm particle size, 250 x 4.6 mm, with identical pre-column, thermostatically controlled at 35 °C; isocratic flow rate of 1 mL/min; mobile phase A was acetonitrile-glacial acetic acid (99:1; v/v) and mobile phase B was ultrapure water-glacial acetic acid (99:1; v/v), with gradient conditions: mobile phase A: 41% (0-9 min), 61% (9-16 min), 100% (16-20 min), 41% (20-25 min); fluorescence detection for fumonisins was set at 335 nm excitation and 440 nm emission (Ware *et al.*, 2017). The retention times were 11 min for FB_1 and 15 min for FB_2. The fumonisin contents were calculated from calibration curves established for FB_1 and FB_2 with standard solutions (TSL-204 and TSL-205 respectively, Biopharm Rhône Ltd, Glasgow, UK). If the measured fumonisin content in a sample was higher than the highest calibration point, the sample was diluted in 0.1 M Borate buffer (pH

10) and analysed again by HPLC. The percentage of bound fumonisin (FB_1 or FB_2) by the different LAB strains was calculated using the following equation:

$$\text{Bound fumonisin } (FB_1 \text{ or } FB_2)\ (\%) = 100 \times \left(1 - \frac{\text{Peak area of fumonisin in the supernatant}}{\text{Peak area of fumonisin in positive control}}\right)$$

2.3.3.6. Statistical analysis

Statistical analyses were carried out on the data obtained for the baseline binding strength, the binding ratio between viable and non-viable cells and for the reproducibility between strains. The Mann Whitney U test (also called the Mann Whitney Wilcoxon Test or the Wilcoxon Rank Sum Test) was used to test whether there was a difference between the amount of FB_1 and FB_2 bound for the 1) baseline quantification and 2) reproducibility for the different trials. A one-way analysis of variance (ANOVA) was carried out to determine the overall difference between strains and within viable and non-viable cells for FB_1 and FB_2. The Mann Whitney U test was used for testing differences between viable and non-viable cell bindings. The statistical software SAS (version 9.4) was used to carry out the statistical tests and a significance level of 95% was applied.

2.4. Results

2.4.1. Visualization of binding interaction between fumonisins and LAB cells

2.4.1.1. Visualization of derivatized fumonisins

Visualization of both fumonisins under conventional white light did not show any fluorescence (Fig 2.1 A and B). However, upon visualization under CLSM both FB_1 and FB_2 fluoresced green (Fig 2.1 C and D).

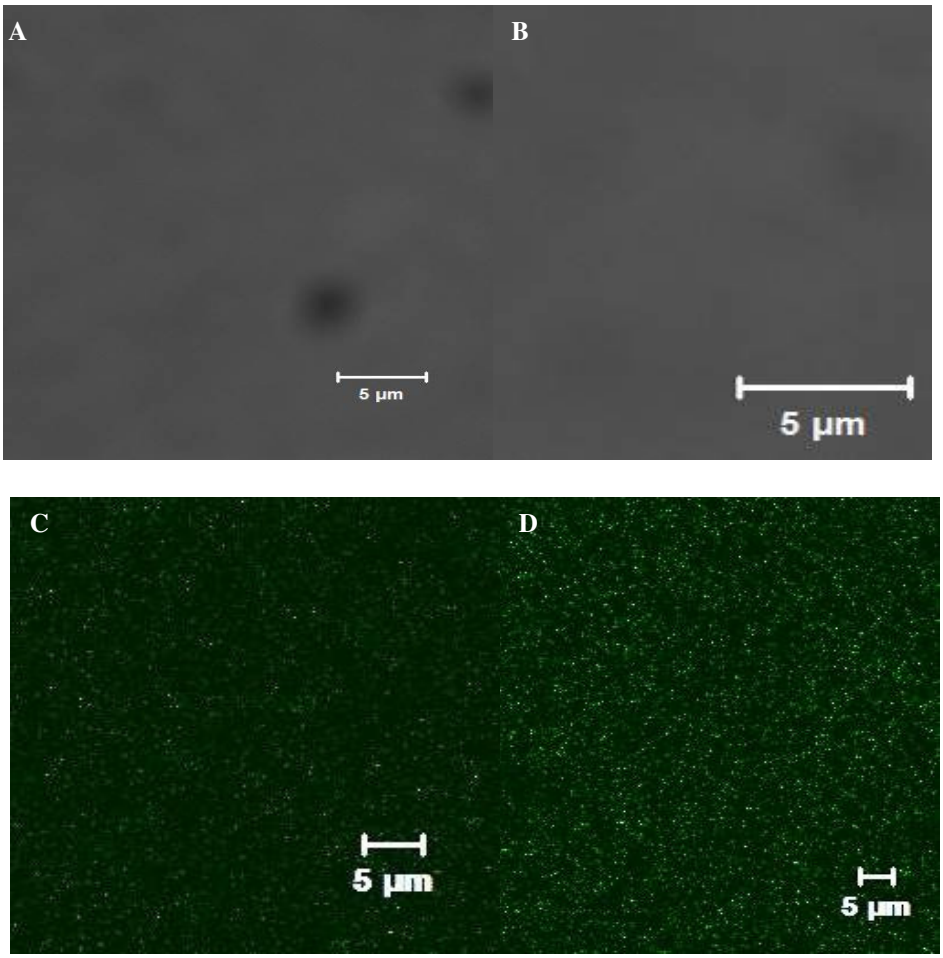
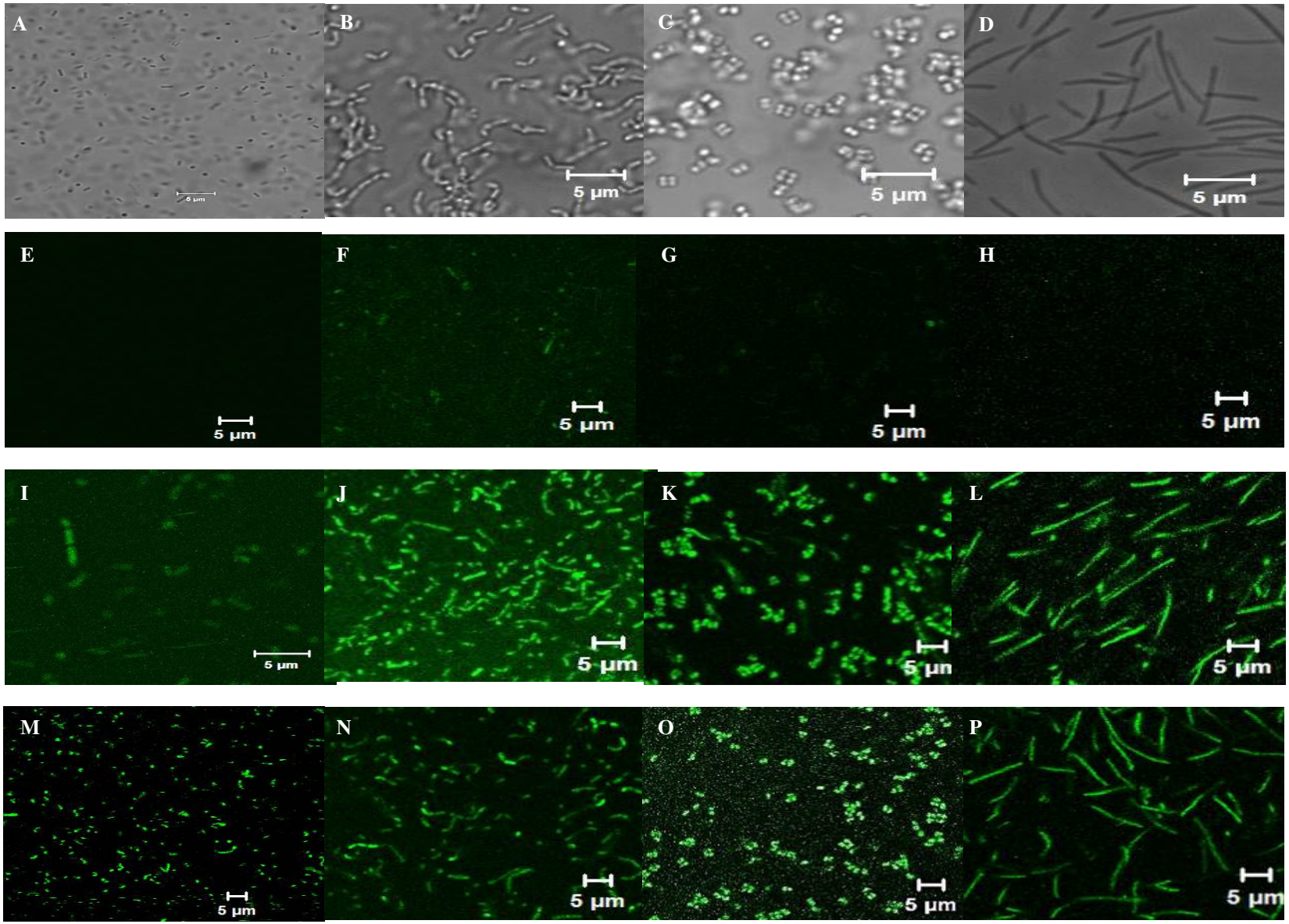


Figure 2.1 Derivatized fumonisins under conventional white light:(A): FB_1, (B) FB_2 and CLSM: (C): FB_1, (D) FB_2

2.4.1.2. Visualization of interaction between viable LAB cells and fumonisins

L. plantarum R1096 cells viewed under white light indicated that cells were present and were non-aggregated (Fig. 2.2 A). When the same cells were viewed under CLSM it was observed they were non-fluorescent (Fig. 2.2 E). However, visualization of the cells mixed with derivatized fumonisins caused them to appear fluorescent green under CLSM (Fig. 2.2 I and M). Fluorescence was more intense for cells mixed with FB_2 than those mixed with FB_1. Similar observations were made for *L. plantarum* FS2 cells whereby under white light the cells were non-aggregated (Fig. 2.2 B) and non-fluorescent (Fig. 2.2 F). These cells were observed to be "coated" with the fluorescing FB_1 and FB_2 (Fig. 2.2. J and N) under CLSM indicating binding interaction on the surface of the cell wall. *P. pentosaceus* D39 were observed to be non-fluorescent and arranged in tetrads under white light (Fig. 2.2 C and G). As was the case for *L. plantarum* FS2 cells, cells of *P. pentosaceus* D39 combined with the derivatized fumonisins were bound externally to the LAB cell wall, "coating" and causing the tetrads to fluoresce green (Fig. 2.2 K and O). Contrary to the *L. plantarum* FS2 and *P. pentosaceus* D39 strains, *L. delbrueckii* CIP 57.8T had the largest, most elongated cell size and surface area, viewed under white light, the cells were non-aggregated (Fig. 2.2 D) and non-fluorescent (Fig. 2.2 H), once mixed with the derivatized fumonisins they also appeared fluorescent green under CLSM (Fig. 2.2 L and P).


A
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C
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5 µm
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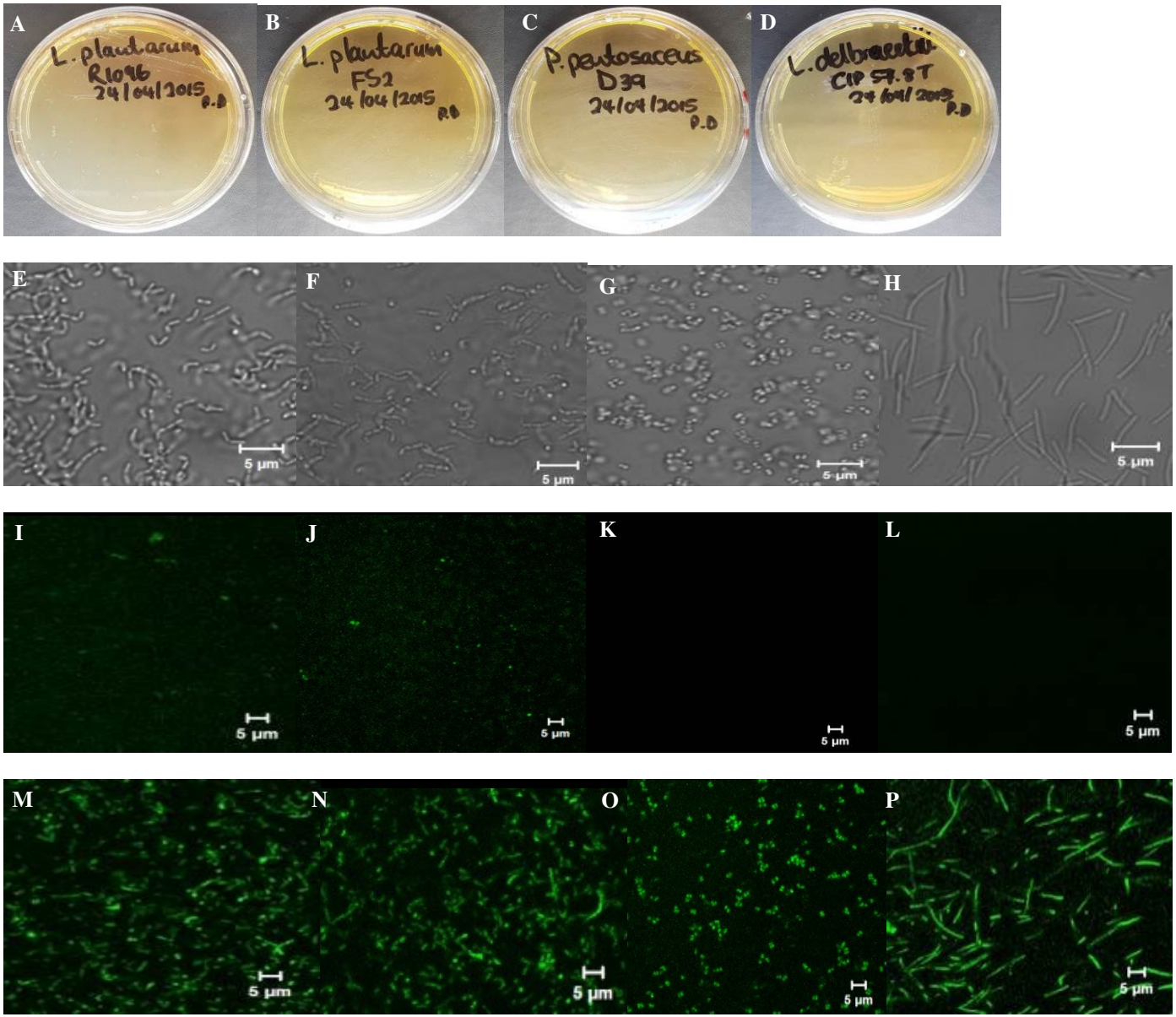
5 µm

Figure 2.2: Visualization of interaction between viable LAB cells and fumonisins. Viable cells visualized under white light: *L. plantarum* R1096 (A), *L. plantarum* FS2 (B)**,** *P. pentosaceus* D39 (C) and *L. delbrueckii* CIP 57.8T (D). Viable cells only, viewed under CLSM: *L. plantarum* R1096 (E), *L. plantarum* FS2 (F)**,** *P. pentosaceus* D39 (G) and *L. delbrueckii* CIP 57.8T (H). Viable cells mixed with derivatized FB_1 viewed under CLSM: *L. plantarum* R1096 (I), *L. plantarum* FS2 (J)**,** *P. pentosaceus* D39 (K) and *L. delbrueckii* CIP 57.8T (L). Viable cells mixed with derivatized FB_2 viewed under CLSM: *L. plantarum* R1096 (M), *L. plantarum* FS2 (N)**,** *P. pentosaceus* D39 (O) and *L. delbrueckii* CIP 57.8T (P).

2.4.1.3. Visualization of interaction between non-viable LAB cells and fumonisins

For non-viable cells, due to the difficulty in viewing dual fluorescence simultaneously under CLSM, to confirm the non-viability of the LAB cells, 100 µl of cell suspension was plated out and incubated for 24-48 hours under anaerobic conditions at 37 ^{o}C (Fig. 2.3 A) for *L. plantarum* R1096, *L. plantarum* FS2 (Fig. 2.3. B), *P. pentosaceus* D39 (Fig. 2.3. C) and *L. delbrueckii* CIP57.8T (Fig. 2.3 D). *L. plantarum* R1096 cells were viewed under white light (Fig. 2.3 E) which showed cell aggregation due to cell death and debris clumping, more so than was observed for *L. plantarum* FS2 (Fig. 2.3 F). Even in cell death *L. plantarum* R1096 (Fig. 2.3 I) and *L. plantarum* FS2 (Fig. 2.3. J) retained no natural fluorescence. Nevertheless, even though being non-viable cells, once mixed with derivatized fumonisins, the cells retain the ability to bind fumonisins. These cells were observed to be "coated" with the fluorescing FB_1 for *L. plantarum* R1096 (Fig. 2.3 M) and *L. plantarum* FS2 (Fig. 2.3 N) and for FB2, Fig 2.3 Q for *L. plantarum* R1096 and Fig. 2.3 R for *L. plantarum* FS2. Similarly, *P. pentosaceus* D39, when viewed under white light (Fig. 2.3 G) indicated a similar degree of aggregation of tetrad formation as to viable cells and also indicated no natural fluorescence under CLSM (Fig. 2.3 K). Although when combined with derivatized fumonisins, the tetrad cells fluoresced green with the FB_1 (Fig. 2.3 O) and FB2 (Fig. 2.3 S) binding to the cell walls externally. Heat shocking to kill the cells seems to have no external effect on the *L. delbrueckii* CIP 57.8T cells viewed under white light as no aggregation or cell debris/clumping could be seen (Fig. 2.3 H), including no natural fluorescence (Fig. 2.3 L). Consistent to viable cells mixed with derivatized fumonisins caused the elongated cells to be coated and fluoresced green under CLSM (Fig. 2.3 P and T).


A
L. plantarum R1096 24/04/2015 P.D
B
L. plantarum FS2 24/04/2015 P.D
C
P. pentosaceus D39 24/04/2015 P.D
D
L. delbrueckii CIP 57.8T 24/04/2015 P.D
E
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5 µm

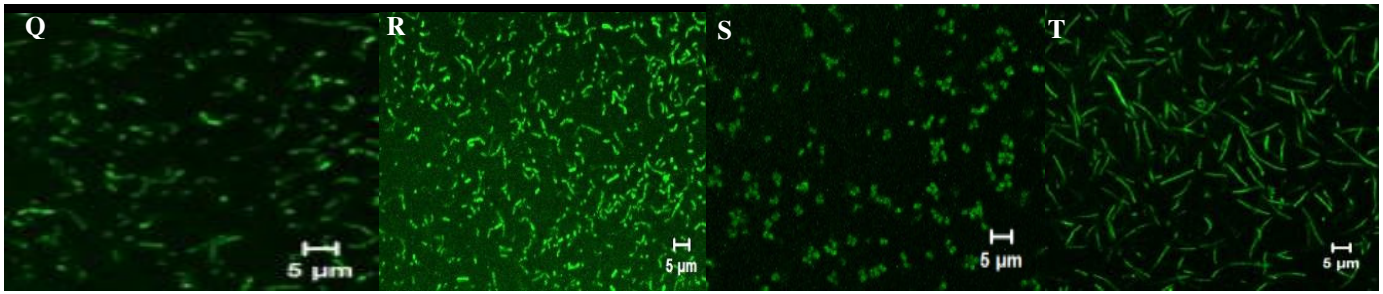

Figure 2.3: Visualization of interaction between non-viable LAB cells and fumonisins. Agar plates of non-viable cells: *L. plantarum* R1096 (A), *L. plantarum* FS2 (B), *P. pentosaceus* D39 (C) and *L. delbrueckii* CIP 57.8T (D). Non-viable cells visualized under white light: *L. plantarum* R1096 (E), *L. plantarum* FS2 (F), *P. pentosaceus* D39 (G) and *L. delbrueckii* CIP 57.8T (H). Non-viable cells only, viewed under CLSM: *L. plantarum* R1096 (I), *L. plantarum* FS2 (J)**,** *P. pentosaceus* D39 (K) and *L. delbrueckii* CIP 57.8T (L). Non-viable cells mixed with derivatized FB_1 viewed under CLSM: *L. plantarum* R1096 (M), *L. plantarum* FS2 (N)**,** *P. pentosaceus* D39 (O) and *L. delbrueckii* CIP 57.8T (P). Non-viable cells mixed with derivatized FB_2 viewed under CLSM: *L. plantarum* R1096 (Q), *L. plantarum* FS2 (R)**,** *P. pentosaceus* D39 (S) and *L. delbrueckii* CIP 57.8T (T).

2.4.2. Quantification of fumonisins

2.4.2.1. Quantification of fumonisins bound by viable LAB cells

In the fermentation of ogi, the baseline parameter of pH is 4 and temperature is 30 ^{o}C, these parameters were used to obtain baseline quantification of binding between the LAB strains and fumonisins (B_1 and B_2). Figure 2.4 illustrates, graphically, the binding strength between FB_1 and FB_2 as well as the difference in binding strength between the LAB strains. Binding strength for FB_1 was the highest at 57% with the test strain *L. plantarum* FS2, 54% for *L. delbrueckii* CIP 57.8 T, 51% for *L. plantarum* R1096 and the least binding of 38% for *P. pentosaceus.* In overall comparison of FB_1 to FB_2, there was a statistically significant difference in binding strength detected (P = 0.0009), with the *L. plantarum* (R 1096) binding 93%, *L. plantarum* FS2 binding 92%, *P. pentosaceus* binding 85% and *L. delbrueckii* CIP 57.8T binding the least at 81%. The greatest difference in binding of the fumonisin B1 and B2 molecules was for *P. pentosaceus* that showed a difference of 47% in binding and the least difference was shown by *L. delbrueckii* CIP 57.8T. Overall, *L. plantarum* R1096 bound the most with *P. pentosaceus* D39 binding the least FB_1 and FB_2.

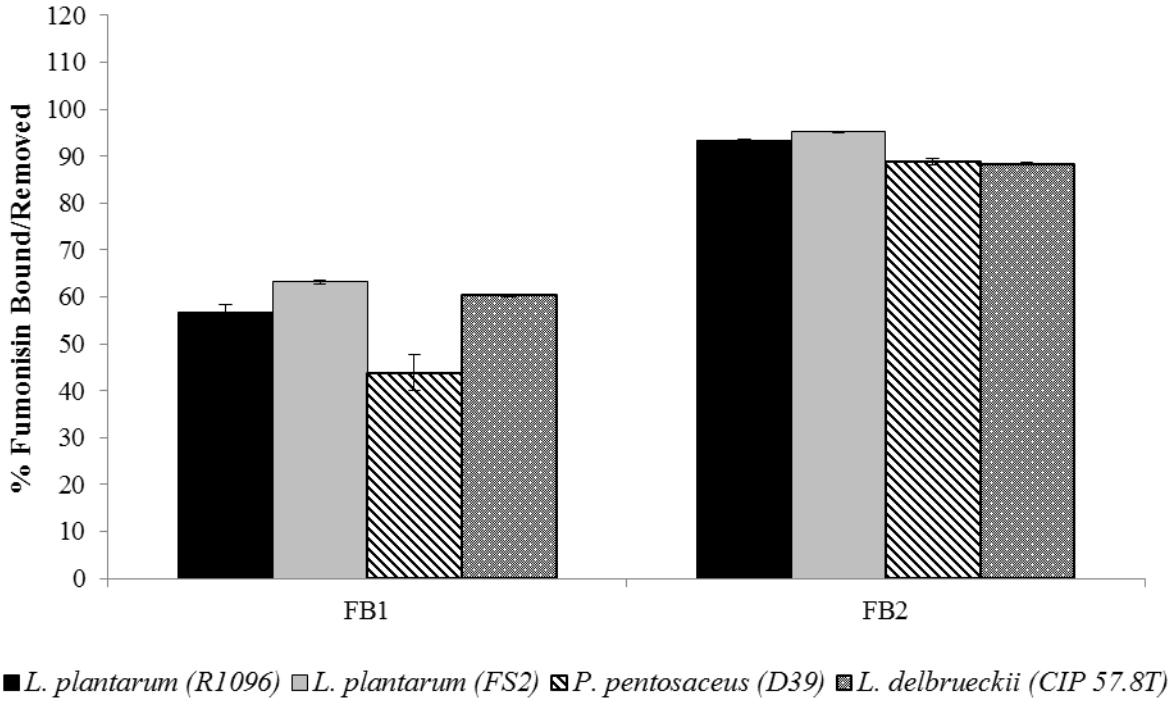

Figure 2.4: Effect of pH of 4 and incubation temperature of 30 ^{o}C on the percentage of fumonisins (B_1 and B_2) bound by viable cells of LAB strains

2.4.2.2. Comparison of fumonisin levels bound by viable and non-viable cells LAB cells

Binding strength between viable cells was compared to that of non-viable LAB cells as a means of differentiating the mechanism of binding i.e. adsorption vs biotransformation (Fig. 2.5). The ANOVA test for an overall difference, carried out to test for differences among all the strains but separately for FB_1 and FB_2, showed statistical significant difference only for FB_2 ($P = 0.0015$). Within the viable cells, binding of both FB_1 ($P = 0.0026$) and FB_2 ($P = 0.0002$) showed statistically significant differences between all strains whereby *L. plantarum* FS2 had the highest binding of FB_1 at 63% and FB_2 at 95% whereas *P. pentosaceus* D39 had the least binding at 43% for FB_1 and *L. delbrueckii* CIP 57.8T, 88% for FB_2. Statistically significant differences were seen for FB_1 ($P < 0.0001$) and for FB_2 (P value: 0.0083) when comparing all strains for the non-viable cells with *L. plantarum* R1096 binding the most FB_1 (77%) and FB_2 (96%) and *L. delbrueckii* CIP 57.8T binding the least FB_1 (62%) and FB_2 (88%). The Mann Whitney U test indicated a significant statistical difference between the binding for the viable and non-viable cells for FB_1 (P value: 0.0375) but not for FB_2 ($P = 0.3843$). The Mann-Whitney test was used to test for differences if there were only two groups, e.g. viable and non-viable cells, but that the ANOVA was used to detect differences between more than two groups, e.g. the four strains.

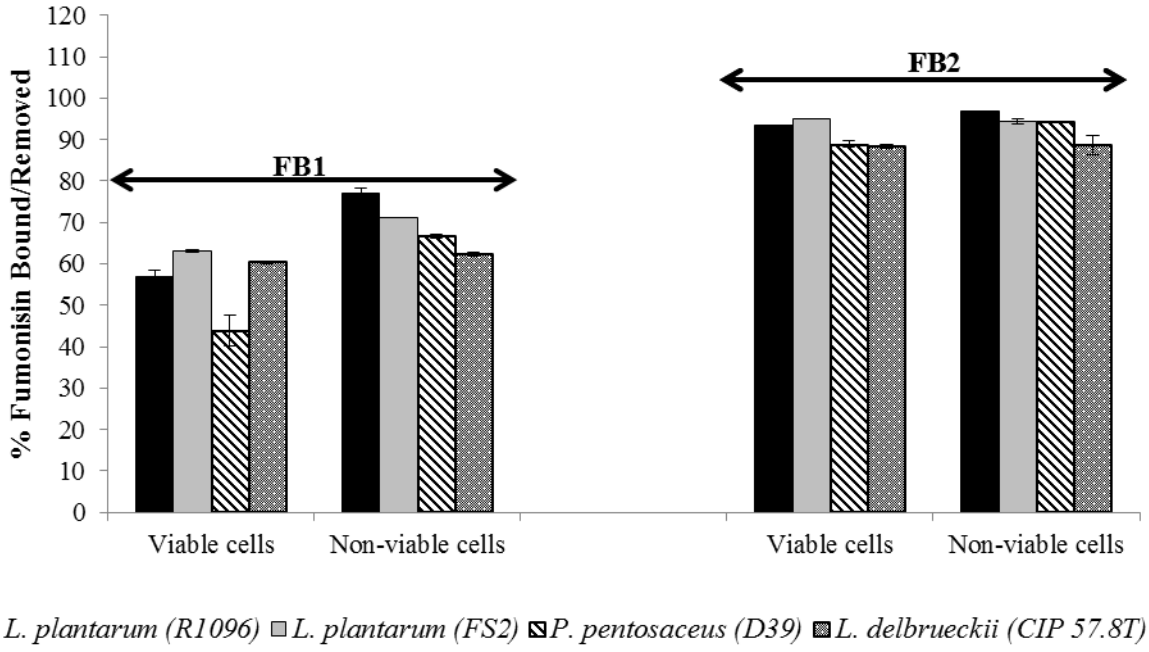

Figure 2.5: Comparison of fumonisin (B_1 and B_2) levels bound by viable and non-viable LAB cells at pH 4 and incubation temperature of 30 ^{o}C

2.4.2.3. Reproducibility of fumonisin binding capacity by viable LAB cells

Figure 2.6 compares the percentage of bound FB_1 and FB_2 bound by each LAB strain in 2 independent experiments to show reproducibility and reliability of results. The results indicate that the amount of FB_1 bound by each LAB strain was always lower than levels of FB_2 bound. The efficiency of FB_1 bound by the different strains was as follows: *L. plantarum* FS2 > *L. delbrueckii* CIP 57.8T > *L. plantarum* R1096 > *P. pentosaceus* D39. For FB_2, the levels of the fumonisin bound by the LAB strains compared to each other were as follows: *L. plantarum* R1096 > *L. plantarum* FS2 > *L. delbrueckii* CIP 57.8T > *P. pentosaceus* D39 for trial 1, while for trial 2 the strains compared to each as follows: *L. plantarum* FS2 > *L. plantarum* R1096 > *P. pentosaceus* D39 > *L. delbrueckii* CIP 57.8T. Although the different LAB cells were not consistent with how they compare based on the level of bound FB_2, the amounts based by different cells were very similar. Furthermore, Mann Whitney U test indicated that there were no significant differences between levels of FB_1 and FB_2 bound by each LAB strain in each trial ($P > 0.05$). This indicates the reproducibility and reliability of the results obtained.

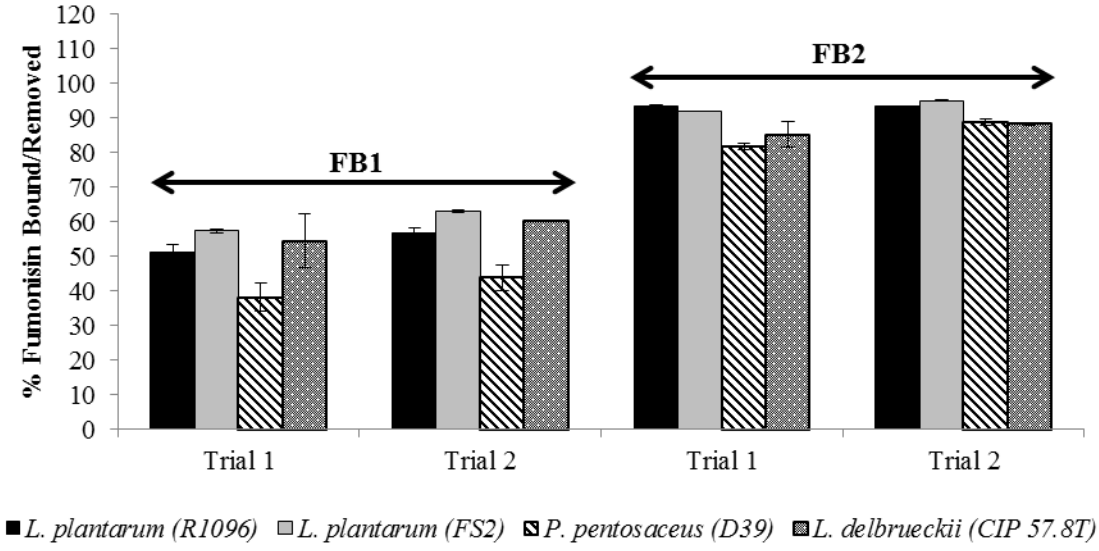

Figure 2.6: Comparison of fumonisin (B_1 and B_2) levels bound by viable LAB strains at pH 4 and incubation temperature of 30 ^{o}C in independent experiments (Trial 1 and Trial 2)

2.5. Discussion

The objective of this study was to visualize and quantify the interaction between fumonisins and LAB isolated from traditional fermented maize-based products (ogi and mahewu) by using confocal laser scanning microscopy (CLSM) and high performance liquid chromatography (HPLC) with the aim to show that LAB strains isolated from traditional fermented African cereal products have the ability to reduce the toxicity of fumonisins in food. Comparison of the various images recorded under white light and CLSM in the various combinations with each of the strains and derivatized FB_1 and FB_2, indicated successful binding interaction.

Different methods were investigated in the ability to fluorescently tag both the LAB cells and the fumonisins with opposing/dual fluorescence to be able to visualise the fluorescent binding interaction with CLSM. We were able to fluorescently tag natural, viable LAB cells. Unfortunately, the fluorescence with CLSM for the viable LAB cells was green, the same as the derivatized fumonisins and no difference in emission intensity was observed. We also showed that the LAB cells were truly non-viable after the heat shock treatment and had not just sustained damage to the membrane, as discussed by Zotta *et al.* (2012), these probes permeate damaged membranes and cannot verify true viability vs non-viability of the cell itself. The ability to fluorescently tag one component (derivatize the fumonisins) and maintain the other component as non-fluorescent (LAB cells), assisted in proving the binding visualization with controls in place.

Clarification of the mechanism of toxin removal has been the subject of many experiments i.e. adsorption vs biotransformation. Numerous studies have indicated the removal mechanism to be binding which involved adhesion of the fumonisin to LAB cell wall components (Niderkorn *et al.*, 2009; Dalié *et al.,* 2010; Zhao *et al.*, 2016). We showed that active growth of LAB was not required for binding or interaction with fumonisins. Since no active metabolism is required, we confirm that fumonisins bind to the LAB cells and that it is not due to biodegradation, which would only be possible with viable cells. This can also be seen in our results with the non-viable cells whereby visually the derivatized fumonisins can be seen binding externally to the LAB cell walls of the strains and quantitatively, decreasing FB_1 bound *L. plantarum* R1096 < *L. plantarum* FS2 < *P. pentosaceus* D39 and < *L. delbrueckii* CIP 57.8T, similarly for FB_2. Previous studies also reported that deactivation of

the mycotoxins by LAB was due to binding rather than metabolism (El-Nezami *et al.*¸2002; Dalié *et al.*, 2010).

The physicochemical properties of the cell wall are determined by the structural organization of the cell wall, chemical properties of the surface constituents and conformation of the surface macromolecules (Schar-Zammaretti and Ubbink, 2003). Due to the difference in binding ratios between the viable and non-viable cells, it is hypothesised that the heat shock treatment caused the components in the LAB cell wall to begin denaturing or disintegration, allowing more components of the cell wall to become "available" as favourable binding sites for FB_1 and FB_2 (Delcour *et al.*, 1999). Observation of the cell wall surface area would lead to the assumption that the larger the surface the area, the higher the degree of binding. Comparison of the strains indicated *L. delbrueckii* CIP 57.8T to have the largest, elongated cell size and surface area (0.5 - 0.8 x 2.0 - 9.0 µm) (Hammes and Hertel, 2011), which should then have bound the most FB_1 and FB_2, but the results indicate otherwise. In fact, results indicate for both FB_1 and FB_2, for the viable cells, *L. plantarum* FS2 bound the most fumonisins whereas *L. delbrueckii* CIP57.8T bound the second highest only for FB_1 but the least for FB_2, indicating that electrostatic potential was more favourable between the two molecules for the binding interaction for *L. plantarum* rather than for *L. delbrueckii.* Comparatively, for the non-viable cells, *L. plantarum* R1096 bound the most fumonisins and *L. delbrueckii* the least, this could be due to the fact that when under heat stress, *L. plantarum* cells are easier to disrupt/disintegrate cell wall structure and therefore provide more favourable binding sites than *L. delbrueckii.* The genetic differences between the LAB strains such as varying number of peptidoglycans, teichoic acids, proteins and polysaccharides, could also contribute to differences in amounts of fumonisin binding between the strains (Schar-Zammaretti and Ubbink, 2003) as each strain has its own unique genetic sequence which also allow for strain identification (Niderkorn *et al.*, 2009; Zhao *et al.*, 2016). More in depth research needs to be undertaken into each specific cell wall structure and the manner in which binding occurs in viable cells. Additionally, the means in which cell wall disruption/disintegration occurs in order for binding to ensue in non-viable cells, as can be seen from the varying binding results obtained per LAB strain.

Quantification of the binding ratio for FB_1 and FB_2 were repeated with viable cells for each LAB strain in order to observe the binding ratio behaviour of each of the strains and to ascertain the reproducibility of the experiments and reliability of binding results obtained.

Statistical analysis of the results indicated that no significant differences were obtained in the duplicated experiments; this is confirmed from the quantitative results obtained from both the independent trials, that repetitively, for FB_1 and FB_2, the LAB strains bound the same amount, meaning that the binding site is fixed for both fumonisin molecules. This indicates that these strains would bind the same amount of fumonisins if used in future applications for detoxification purposes.

The structural conformation and charge of the fumonisin molecules also contributes to the binding, indicating that these parameters are non-changeable/fixed on the molecule. Beier *et al.* (1995) showed that FB_1 and FB_2 have a cage-like structure, where each molecule folds back onto itself. It was shown in a different study that both fumonisin molecules appear to be oblong in shape, rather than linear as expected (Beier and Stanker, 1997). Additional information indicated that both molecules carry different surface electrostatic potentials from each other, which indicates that each molecule would have preferential, different binding sites on the LAB cell wall, demonstrating the misconception that both molecules compete for the same binding sites on the LAB cell wall. It was expected for both the molecules to have the same binding site and therefore to compete for binding as reported by El-Nezami *et al.* (2002) for zearalenone and α-zearalenol, where both toxins competed for the same binding site. The difference in chemical structure between the FB_1 and FB_2 molecules is an additional hydroxyl group in C_{10} for FB_1. However, the results obtained here and in other studies (Niderkorn *et al.,* 2006a, b; Zhao *et al.,* 2016) indicate higher binding for FB_2 than FB_1. It was suggested that the hydrogen bond between this extra hydroxyl group and a carboxyl group resulted in a spatial conformation change, which may limit the accessibility of FB_1 to the binding sites of LAB cell walls (Niderkorn *et al.* (2006a and 2009)) which decreased its binding affinity. The other reason could be that FB_2 has a more "slimmer" design/fit when compared to that of FB_1 and could be easier to fit into spaces on the viable LAB cell wall than FB_1 but more so on a non-viable cell wall that has begun to disintegrate.

The binding interaction that occurs between the LAB cells and fumonisins is mediated by both long range (steric and electrostatic interactions) and short range (Van Der Waals, Lewis acid-base, hydrogen bonding and bio specific interactions) forces (Burgain *et al.*, 2014b). These forces allow the bacterial adhesion to occur in 2 steps: 1) non-specific and reversible interaction and 2) specific and non-reversible interaction (Burgain *et al.* 2014a). Physical photographic observation of the binding visualization (for both viable and non-viable LAB

cells) with fumonisin molecules (B_1 and B_2), indicates similar binding sites, as images of each strain with CLSM has shown the entire cell to be fluorescing green. It is recommended that further in-depth study using atomic force microscopy (Dufrene, 2014) be performed as it will enable visualization of the interaction and confirm the site of binding per toxin.

The binding strength results obtained indicate the ability of viable LAB cells to have a high binding of FB_1 and FB_2. These LAB strains can be utilised as a detoxifying agent in fermented maize based cereal products to reduce toxicity of fumonisins and confer probiotic benefits in the gastrointestinal system of the host.

2.6. Conclusion

L. plantarum FS2*, L. delbrueckii* ssp. *delbrueckii* CIP 57.8 T and *P. pentosaceus* D39 isolated from traditional fermented maize based products (ogi and mahewu) were shown to bind fumonisins (B_1 and B_2), visually and quantitatively. These LAB strains have the ability to improve the safety of traditional fermented cereal products by reducing the toxicity of fumonisins in food. Due to both fumonisins fluorescing the same, visually, no discrepancies could be seen between the strains to determine any differences. Quantitatively, *L. plantarum* FS2 bound the most FB_1 and *P. pentosaceus* D39, the least, for viable LAB cells, whereas, the highest binding for FB_2 was with *L. plantarum* R1096 and the least with *L. delbrueckii* CIP 57.8 T. Quantification of binding ratio for the non-viable cells indicated the highest binding of FB_1 and FB_2 to be *L. plantarum* FS2 and the least binding for FB_1 to be *P. pentosaceus* D39 and *L. delbrueckii* CIP 57.8 T for FB_2. Non-viable cells bound the most FB_1 and FB_2 in comparison to viable cells. To our knowledge, this is the first study to use CLSM to visualize the interaction between LAB and fumonisins. For future and further in-depth study of binding visualization, it is recommended to use atomic force microscopy to determine binding interaction and specificity per site and per toxin. High binding strength was observed for all viable LAB cells, in conclusion, it is suggested for these strains to be utilised in the fermentation process of traditional fermented products, specifically, ogi and mahewu.

2.7. References

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Chapter Three

Potential of lactic acid bacteria for the reduction of fumonisin exposure in African fermented maize-based foods

This chapter is published as an article in the **World Mycotoxin Journal** with the following reference:

Dawlal, P., Brabet, C., Thantsha, M.S. and Buys, E.M. **2017**. Potential of lactic acid bacteria for the reduction of fumonisin exposure in African fermented maize based foods. *World Mycotoxin Journal.* 10(**4**): 309-318.

3.1. Abstract:

Maize, which contributes to a large portion of the African diet and serves as the base substrate for many fermented cereal products, has been reported to be contaminated with fumonisins. This study aimed to evaluate the *in vitro* ability of predominant lactic acid bacteria (LAB) in African traditional fermented maize based foods (ogi and mahewu) to bind fumonisin B_1 (FB_1) and B_2 (FB_2), as well as the stability of the complex at different pH and temperatures conditions, particularly observed during ogi fermentation and during storage at varying conditions (time, temperature). The percentage of bound fumonisins was calculated after analysing the level of fumonisins not bound to LAB after a certain incubation time, by HPLC. The results revealed the ability of all tested LAB strains to bind both fumonisins, with binding efficiencies varying between strains and higher for FB_2. Binding of fumonisins increased with a decrease in pH from 6 to 4 (observed during the ogi fermentation process) and from 4 to 2 (acidic pH in the stomach), and an increase in temperature (from 30 to 37 °C). The percentage of FB_1 and FB_2 bound to LAB at pH 4 decreased after 6 days of storage at 30 °C for all LAB strains, except for *Lactobacillus plantarum* (R1096) for which it increased. *Lactobacillus* species (*L. plantarum* and *Lactobacillus delbrueckii*) were the most efficient in binding FB_1 and FB_2, whereas *Pediococcus sp*. was less efficient. Therefore, the *Lactobacillus* strains tested in this study can be recommended as potential starter cultures for African traditional fermented maize based foods having detoxifying and probiotic properties.

3.2. Introduction

The majority of humanity's food supply consists of a wide variety of cereal grains, with maize, rice, wheat and barley being the world's four major ones (FAO, 2014; Pereira *et al.,* 2014). In Africa, maize is the most produced cereal crop and a major staple food for the majority of the population (FAO, 2014; Macauley and Ramadjita, 2015), and traditional fermented maize-based products (e.g. ogi, kenkey, mawe and mahewu) are particularly important (FAO, 1999). Maize, as with other cereals, is at risk of infection by mycotoxigenic fungi and their related mycotoxins (Pereira *et al.,* 2014). Mycotoxins are secondary fungal metabolites, with major concern for public health since they can cause acute or chronic intoxications to humans, which are sometimes fatal, due to their various toxic effects (carcinogenic, hepatotoxic, nephrotoxic, neurotoxic, immunotoxic and oestrogenic) (AFSSA, 2006; Zain, 2011). Most mycotoxins are chemically and heat stable, resilient to storage and processing conditions. In particular, they are not easily destroyed by cooking processes (Valiuškaitė *et al.,* 2006; Kabak, 2009;). In countries where food is scarce and subsistence farming provides the major source of food, cereals contaminated with mycotoxins are consumed (Marasas *et al.,* 2008). Moreover, traditional fermented cereal foods are cheap and relatively easy to prepare and this accounts for their popularity amongst most African population groups. As a consequence of poverty, poor food security and malnutrition, exposure to mycotoxins is high in Africa (Marasas *et al.,* 2008).

Several studies have reported the occurrence of mycotoxins in maize, in particular in Africa, fumonisins being among the most prevalent ones with high contamination rates and levels that can exceed 10 μg/g (Okeke *et al.,* 2015; Vismer *et al.,* 2015; Chilaka *et al.,* 2016; Mngqawa *et al.,* 2016; Udomkun *et al.,* 2017;). Fumonisins are a group of mycotoxins primarily produced by *Fusarium verticillioides* and *Fusarium proliferatum* (Dawlal *et al.,* 2010; Wild *et al.,* 2015; Ferrigo *et al.,* 2016). They consist of a long hydroxylated hydrocarbon chain (20 carbon atoms) containing methyl and amino groups in which the hydroxyl groups on C_{14} and C_{15} are esterified with tricarboxylic acid (TCA) (Beier *et al.,* 1995; Beier and Stanker, 1997; Abrunhosa *et al.,* 2016). Fumonisins B_1 (FB_1) and B_2 (FB_2) are the most commonly found fumonisins in food and feed, with FB_1 the most abundant (Wild *et al.,* 2015; Abrunhosa *et al.,* 2016; Ferrigo *et al.,* 2016). FB_1 is also the most toxic fumonisin. An exposure to this toxin has been associated with high incidence of human oesophageal cancer in various parts of Africa (Zain, 2011).

Different methods have been developed for the reduction and detoxification of mycotoxins in food and feed (Reddy *et al.,* 2010; Jard *et al.,* 2011; Karlovsky *et al.,* 2016). Among these methods, the use of lactic acid bacteria (LAB) is regarded as a promising approach due to their ability to bind mycotoxins, including fumonisins (Dalié *et al.,* 2010). Niderkorn *et al.* (2006a,b) investigated in vitro the interaction between fermentative bacteria and FB_1 and FB_2, and they reported that LAB belonging to the genera *Lactobacillus, Pediococcus, Streptococcus, Lactococcus* and *Leuconostoc* are able to bind fumonisins, but with great differences among the strains. Binding efficiency was higher for FB_2 and affected by pH, with binding nearly 0% at neutral pH. In a further investigation, Niderkorn *et al.* (2009) identified peptidoglycan and TCA as important components of the LAB cell wall and fumonisins, respectively, involved in the binding interaction. These studies revealed a field of research that can provide unique, dual benefits in the area of African fermented maize based foods, as LAB are generally recognised as probiotic strains and being a safe, possibly stable, fumonisin detoxifying agent.

Ogi is an acid fermented cereal gruel made mainly from maize and consumed widely in West African countries (e.g. Nigeria, Benin, Togo and Ghana) where it is a staple food, used as infant weaning foods, breakfast cereal and convenient meal for sick and convalescent individuals. The traditional process of ogi consists of grain steeping in water for 1 to 3 days, wet milling, wet sieving to remove bran, decanting and fermentation of the sievate for 1 to 3 days (Nago *et al.,* 1998; Nwosu and Oyeka, 1998; Ijabadeniyi, 2007; Omemu, 2011; Fayemi, 2016). During the fermentation step, it was reported that the temperature of fermenting maize remains relatively constant between 28 and 30 °C (Omemu, 2011; Fayemi, 2016) and the pH decreases from approximatively 6 to 4 (Nwosu and Oyeka, 1998; Omemu, 2011; Fayemi, 2016). Microbiological studies also showed the major involvement of LAB in ogi fermentation (Agati *et al.,* 1998; Nago *et al.,* 1998; Fayemi, 2016;), with LAB population increasing from 4.3×10^{10} to 7.9×10^{10} cfu/g (Omemu, 2011) or even reaching values of 10^9 cfu/g (Nago *et al.,* 1998). The predominant LAB included *Lactobacillus plantarum, Lactobacillus delbrueckii, Lactobacillus brevis, Lactobacillus fermentum* and its biotype *Lactobacillus cellobiosus,* and *Pediococcus pentosaceus* (Nago *et al.,* 1998; Nwosu and Oyeka, 1998; Ijabadeniyi, 2007; Adebayo-tayo and Onilude, 2008; Afolabi and Akintokun, 2008; Omemu, 2011 Fayemi, 2016). Mugcoba (2001), Holzapfel and Taljaard (2004) and Katangole (2008) have all indicated *L. delbrueckii* and *L. brevis* to be the most prevalent LAB

during the fermentation of mahewu which is a South African traditional maize based product similar to ogi.

The aim of this study was to evaluate the interaction between predominant LAB in African traditional fermented maize-based foods (ogi and mahewu) and FB_1 and FB_2 in vitro, as well as the stability of the bound complex at different pH and temperatures, in particular observed during ogi fermentation, and under its storage conditions (time and temperature).

3.3. Materials and Methods

3.3.1. LAB strains and media

Three LAB strains belonging to species that predominate in African traditional fermented maize based foods (ogi and mahewu) were tested in this study: *L. plantarum* (FS2) and *P. pentosaceus* (D39), with probiotic characteristics, isolated from Nigerian ogi (Fayemi, 2016), and *L. delbrueckii* ssp. *delbrueckii* (CIP 57.8T) isolated from sour grain mash and obtained from the Pasteur Institute (Paris, France). *L. plantarum* (R1096) was obtained from Lallemand SAS, (Blagnac, France) was selected as positive control strain as it had amongst the highest binding of FB_1 and FB_2 (Niderkorn et al., 2006a).

De Man, Rogosa and Sharpe (MRS) Broth (Biokar, Pantin, France) and MRS agar (Biokar) (De Man *et al.,* 1960) were used for the culture of LAB strains. All LAB strains were cultivated and stored on MRS agar slants at 4 °C for 3 months, and for long term conservation, cryopreserved at -80 °C in 12.5% glycerol (Jacobs, 1991) and on microbeads (MAST Diagnostics, Amiens, France).

3.3.2. Fumonisins B_1 and B_2

FB_1 and FB_2 were purchased from Sigma Aldrich (St. Quentin Fallavier, France). 10 mg of each mycotoxin was dissolved in 10 ml of 50% acetonitrile to obtain 40 amber vials with a concentration of 250 µg/ml which was stored at -20 °C. A standardised concentration of 10 µg/ml for each fumonisin was used across all tests during experimentation according to the method developed by Niderkorn et al. (2006a).

3.3.3. Determination of the binding ratio between LAB and fumonisins

The method described by Niderkorn *et al.* (2006a) was used in this study with a few modifications. For each LAB strain, two Erlenmeyer flasks containing 200 ml of MRS broth were inoculated with a fresh, single colony from 24-48 h culture on MRS agar, and incubated at 37 °C (optimal growth temperature) for 24 h. At the end of the incubation time, the two broth cultures were homogenised and an aliquot of 2 ml was used for quantifying the LAB concentration. A standardised concentration of LAB cells (10^{13} cfu/ml) was systematically obtained and used for all strains throughout the experiment.

Thereafter, the two broth cultures were each aseptically transferred to a sterile 250 ml centrifuge bottle and centrifuged at 3,000×g for 10 min at <10 °C. The supernatant was removed and the LAB cell pellet was re-suspended and washed (3×) in 100 ml of 0.01 M phosphate buffered saline (PBS) pH 7.4 by mixing then centrifuging under the same conditions. After the last wash step, the bacterial pellet from one of the two broth cultures was re-suspended in 2 ml of 0.1 M citrate phosphate buffer (CPB) pH 4 containing 10 µg/ml FB_1 and 10 µg/ml FB_2 (fumonisin buffer solution). After homogenisation with a vortex, the 2 ml were split into 1 ml each and transferred to a 2 ml Eppendorf tube (test samples in duplicate). The bacterial pellet from the second broth culture was re-suspended in 2 ml of 0.1 M CPB pH 4 and after homogenisation, 1 ml was transferred to a 2 ml Eppendorf tube (negative control sample excluding FB_1 and FB_2). For the positive control samples (excluding LAB cells), 1 ml of fumonisin buffer solution was transferred to a 2 ml Eppendorf tube in duplicate. All the samples were incubated at 30 °C for 1 h with agitation (480 rpm) and for23 h without agitation, then centrifuged at 3,000×g for 10 min at <5 °C. The supernatants were each transferred to 2 ml amber vials and stored at 4 °C until the analysis of FB_1 and FB_2 using HPLC.

3.3.4. Determination of LAB concentration

LAB concentration in the culture broth was estimated by measuring the absorbance at 600 nm (OD600) then comparing to standard curves previously obtained for each LAB strain by relating colony forming units (cfu) per ml from plate counts and absorbance measurements over an array of LAB concentrations.

3.3.5. Effect of pH and temperature on the binding ratio

As ogi fermentation occurs around 30 °C with pH decreasing from approximatively 6 to 4, the binding ratio between LAB and FB_1 and FB_2 was also determined at pH 5 and 6. For that, the method detailed above (binding ratio method) was repeated with variation in pH of the fumonisin buffer solution. Tests were performed using 0.1 M CPB which was adjusted to pH 5 and 6.

The binding ratio method was also modified to simulate the optimal growth temperature of LAB strains, i.e. 37 °C, as this may influence the dominance of the strains during the fermentation of maize based foods, such as ogi and mahewu. Samples were prepared as detailed above using 0.1 M CPB which was adjusted to pH 4, 5 and 6, individually. Each set of samples at different pH was incubated at 37 °C for 1 h with agitation (480 rpm) and for 23 h without agitation, thereafter supernatants were recovered and stored at 4 °C until analysis by HPLC.

The binding ratio was also determined at a lower pH of 2 and an incubation temperature of 37 °C, found during human gastric digestion. Samples were prepared as previously indicated using 0.1 M CPB which was adjusted to pH 2. After incubation at 37 °C for 1 h with agitation (480 rpm) and for 23 h without agitation, supernatants were recovered and stored at 4 °C until analysis by HPLC.

3.3.6. Effect of simulated ogi storage conditions *in vitro* on binding stability

Preparation of samples followed the initial binding ratio method mentioned above in 0.1 M CPB at pH 4. The samples were then incubated at 30 °C for 1 h with agitation (480 rpm) and without agitation for 23 h, followed by 6 days to simulate ogi storage conditions. Supernatants were recovered and stored at 4 °C until fumonisin analysis by HPLC.

3.3.7. Fumonisin analysis by HPLC

FB_1 and FB_2 were quantified in the supernatants of all samples (test samples, positive and negative controls) stored at 4 °C by HPLC coupled with a fluorescence detector (Shimadzu RF 20A, Tokyo, Japan) after derivatisation with o-phthaldialdehyde (OPA). For fumonisin derivatisation, 100 µl of derivatisation solution was added to 100 µl of sample. The contents were mixed and allowed to react for 2 min prior to injection. The derivatisation solution was prepared by mixing 120 mg of OPA (Sigma-Aldrich), 3 ml 179 µl of 1-thioglycerol (Sigma-

Aldrich), and left overnight in the dark at room temperature prior to use. The operating conditions were as follows: injection volume of 100 µl of each sample after fumonisin derivatisation; Uptisphere type ODB C18 reverse-phase column, 5 µm particle size, 250×4.6 mm, with identical pre-column, thermostatically controlled at 35 °C (Swiss labs, Basel, Switzerland); isocratic flow rate of 1 ml/min; mobile phase A was acetonitrile-glacial acetic acid (99:1; v/v) and mobile phase B was ultrapure water glacial acetic acid (99:1, v/v), with gradient conditions: mobile phase A: 41% (0-9 mins), 61% (9-16 min), 100% (16-20 min), 41% (20-25 min); fluorescence detection for fumonisins was set at 335 nm excitation and 440 nm emission (Ware et al., 2017). The retention times were 11 min for FB_1 and 15 min for FB_2. The fumonisin contents were calculated from calibration curves established for FB_1 and FB_2 with standard solutions (TSL-204 and TSL-205, respectively; Biopharm Rhône Ltd, Glasgow, UK). If the measured fumonisin content in a sample was higher than the highest calibration point, the sample was diluted in 0.1 M borate buffer (pH 10) and analysed again by HPLC. The percentage of bound fumonisin (FB_1 or FB_2) by the different LAB strains was calculated using the following equation:

$$\text{Bound fumonisin } (FB_1 \text{ or } FB_2)\ (\%) = 100 \times \left(1 - \frac{\text{Peak area of fumonisin in the supernatant}}{\text{Peak area of fumonisin in positive control}}\right)$$

The HPLC method was validated by measuring the linearity, recovery, matrix effect, limit of detection (LOD) and limit of quantification (LOQ), as well as the intraday (repeatability) and interday (reproducibility) precisions for FB_1 and FB_2. The linearity was evaluated for FB_1 and FB_2 using the calibration curve of each standard (TSL-204 for FB_1 and TSL-205 for FB_2) at six concentration levels, ranging from 5 to 500 ng/ml for each fumonisin. The recovery was calculated by comparison of the response obtained for each fumonisin with the HPLC method and the theoretical fumonisin level in the positive control at the different tested pH (2, 4, 5 and 6). The intraday and interday precisions were determined by calculating the relative standard deviation (RSD_r for repeatability and RSD_R for reproducibility) obtained from results generated from the analysis of positive controls at the different tested pH (6 determinations in the same day for repeatability; 2 determinations on 5 consecutive days for the reproducibility). The LOD and LOQ were determined from a signal to noise ratio of 3 and 10 respectively.

3.3.8. Statistical analysis

All experiments were performed in duplicate and results analysed using a two-way analysis of variance (ANOVA) to determine whether the tested parameters (pH, incubation temperature, storage time and LAB strain) affected the binding interaction and stability between the LAB strains and FB_1 and FB_2. A t-test was performed to examine significant differences between pH, incubation temperatures (30 and 37 °C), storage time, LAB strains and FB_1 and FB_2. Where significant differences were found on the ANOVA test, the Tukey's Honest Significance Difference (HSD) Test based on the studentized range distribution was used to determine for which LAB strains tested parameters there were significant differences.

3.4. Results

3.4.1. Validation of the HPLC method for FB_1 and FB_2 analysis

The performance parameters of the method are given in Table 3.1. For both fumonisins, the linearity was good (R2 of 0.9970 for FB_1 and 0.9976 for FB_2) in the range of between 5 and 500 ng /ml. The LOD and LOQ were 0.3 and 1 ng/ml, respectively, for both FB_1 and FB_2. The recoveries calculated from results obtained from the analysis of positive controls prepared in CPB at different pH (2, 4, 5 and 6) were higher for FB_1 (75- 117%) than for FB_2 (63-71%) and, for both toxins, within the range (60-120%) recommended by European Commission (EC) Regulation No 401/2006 (EC, 2006). The repeatability and reproducibility of the method were satisfactory at all tested pH with RSD_r and RSD_R being lower than the recommended values by EC Regulation No 401/2006 (30 and 60%, respectively).

3.4.2. Effect of pH and temperature on LAB – fumonisin (B_1 and B_2) complex

Baseline quantification of the binding ratio completed at 30 °C with pH 4, was indicative of fumonisin (FB_1 and FB_2) binding occurring for all 4 LAB strains. The ANOVA carried out on the 48 binding ratio values recorded for FB_1 and FB_2 indicated that LAB strain, pH and temperature had significant effects on the percentage of bound FB_1 and FB_2 (Figure 3.1A, B). Analysis of the quantification of binding ratio in conditions simulating ogi fermentation parameters (30 °C with pH decreasing from 6 to 4) showed that for both FB_1 and FB_2, the binding occurred at pH 6 (start of fermentation) and the percentage of bound fumonisins increased as pH decreased to 5 and 4 (end of fermentation) (Figure 3.1A) (pH significant for FB_1 and FB_2 with all P-values <0.0001). The same pattern was observed at 37 °C (Figure 3.1B), but the percentage of bound FB_1 and FB_2 was higher at 37 °C than at 30 °C (P <0.0001

for FB_1 and P <0.0001 for FB_2). Therefore, all 4 LAB strains were able to bind both fumonisins under pH and temperature conditions tested with strain significant for both FB_1 and FB_2 (all P-values <0.0001), but with binding efficiency higher for FB2 (P <0.0001).

The Tukey's HSD test indicated significant differences among the 4 LAB strains and the pH levels, depending on the type of fumonisin (FB_1 or FB_2) and the incubation temperature. At 30 °C, the percentage of bound FB_1 by *P. pentosaceus* (D39) was significantly lower (23% at pH 6 and 38% at pH 4) than by *Lactobacillus* species: *L. plantarum* (R1096) (42% at pH 6 and 51% at pH 4), *L. plantarum* (FS2) (46% at pH 6 and 57% at pH 4) and *L. delbrueckii* (CIP 57.8 T) (52% at pH 6 and 55% at pH 4). At 37 °C, the percentage of bound FB_1 by *P. pentosaceus* (D39) was the lowest (23% at pH 6 and 43% at pH 4), *L. delbrueckii* (CIP 57.8 T) (51% at pH 6 and 69% at pH 4) and *L. plantarum* (FS2) (52% at pH 6 and 73% at pH 4) were similar and *L. plantarum* (R1096) was the highest (71% at pH 6 and 86% at pH 4). At 30 °C, the percentage of bound FB_2 by *L. plantarum* (FS2) (83% at pH 6 and 92% at pH 4) was similar to *L. plantarum* (R1096) (83% at pH 6 and 93% at pH 4), but higher than *L. delbrueckii* (CIP 57.8 T) (62% at pH6 and 85% at pH 4) and *P. pentosaceus* (D39) (39% at pH 6 and 82% at pH 4). At 37 °C, the percentage of bound FB_2 by *P. pentosaceus* (D39) (42% at pH 6 and 89% at pH 4) was also the lowest, then *L. delbrueckii* (CIP 57.8 T) (64% at pH6 and 95% at pH 4), then *L. plantarum* (FS2) (87% at pH 6 and 95% at pH 4), and *L. plantarum* (R1096) (85% at pH6 and 98% at pH 4) highest.

Figure 3.2 compares the percentage of fumonisins (FB_1 and FB_2) bound by the 4 LAB strains under baseline parameters (pH 4, 30 °C at the end of ogi fermentation) against lower pH of 2 and incubation temperature of 37 °C found during human gastric digestion. For all 4 LAB strains and both FB_1 and FB_2, the binding ratio was higher at pH 2, 37 °C than at pH 4, 30 °C (all P-values <0.0001), ranging from 38-57% to 80-98% for FB_1 and from 82-93% to 97-100% for FB_2 at pH 4, 30 °C and pH 2, 37 °C, respectively. The percentage of bound FB_1 by *L. plantarum* (R1096) was the highest at 46%, followed by *P. pentosaceus* (D39) at 42%, with *L. plantarum* (FS2) and *L. delbrueckii* (CIP 57.8 T), both at 28%. The percentage of bound FB_2 by *P. pentosaceus* (D39) was the highest at 17%, followed by *L. delbrueckii* (CIP 57.8 T) at 14%, *L. plantarum* (FS2) at 7% and *L. plantarum* (R1096) at 5%. However, amongst the 4 LAB strains, ANOVA indicated significant differences for FB_1 (P=0.0438), but not for FB_2 (P=0.1126). Tukey's HSD test applied to the strains for FB_1 indicated that *P. pentosaceus* (D39) was significantly lower (38% at pH 4 and 80% at pH 2) than the other

three strains *L. plantarum* (FS2) (57% at pH 4 and 86% at pH 2), *L. plantarum* (R1096) (51% at pH 4 and 97% at pH 2) and *L. delbrueckii* (CIP 57.8 T) (54% at pH 4 and 82% at pH 2). These results indicate not only the stability of the LAB-fumonisin (FB_1 and FB_2) complex under lower pH and incubation temperature of 37 °C, but also an increase in the amount of fumonisins bound by all 4 LAB strains under such conditions, particularly for FB_1.

3.4.3. Effect of simulated ogi storage conditions *in vitro* on binding stability

The stability of the LAB-fumonisin (FB_1 and FB_2) complex was evaluated during simulated storage conditions (6 days at 30 °C) of fermented ogi (pH 4), against baseline parameters (pH 4, 30 °C at the end of ogi fermentation) (Figure 3.3). The ANOVA indicated significant differences for both FB_1 and FB_2 amongst storage times ($P<0.0001$) and among the 4 LAB strains ($P=0.0005$ for FB_1; $P<0.0001$ for FB_2). The percentage of FB_1 and FB_2 bound decreased after 6 days of storage for all LAB strains, except for *L. plantarum* (R1096) for which it increased (from 51% to 88% for FB_1 and from 93% to 100% for FB_2).The highest decrease in percentage bound for FB_1 was *L. delbrueckii* (CIP 57.8T) at 29%, followed by *P. pentosaceus* (D39) at 19% and *L. plantarum* (FS2) at 8% whereas the highest decrease in percentage bound for FB_2 was *P. pentosaceus* (D39) at 67%, followed by *L. plantarum* (FS2) at 21% and *L. delbrueckii* (CIP 57.8T) at 6%.

3.5. Discussion

The HPLC method used for the analysis of FB_1 and FB_2 was validated. Successful binding of FB_1 and FB_2 was observed for all LAB strains tested, with FB_2 being more bound than FB_1. This study also showed that binding of fumonisins increased with a decrease in pH from 6 to 4 (observed during ogi fermentation process) and from 4 to 2 (acidic pH in the stomach), and an increase in temperature (from 30 to 37 °C). However, as storage time increased (up to 6 days at pH 4, 30 °C, simulating ogi storage conditions), binding stability decreased for *L. plantarum* (FS2), *P. pentosaceus* (D39) and *L. delbrueckii* (CIP 57.8 T) and increased for *L. plantarum* (R1096).This work revealed that efficiency in binding FB_1 and FB_2 varied between strains with *Lactobacillus* species (*L. plantarum* and *L. delbrueckii*) being the most efficient and *Pediococcus pentosaceus* the least efficient.

Various authors have reported the binding affinity of LAB strains for mycotoxins, such as aflatoxin B_1, zearalenone, α-zearalenol, deoxynivalenol, nivalenol, patulin, and FB_1 and FB_2 (El-Nezami *et al.,* 1998, 2002; Niderkorn *et al.,* 2006a; Dalié *et al.,* 2010; Topcu *et al.,* 2010;

Ahlberg *et al.,* 2015; Zhao *et al.,* 2016), with an effect of bacterial concentration, pH, temperature and incubation time on such binding ability. Niderkorn *et al.* (2006a), who were the first to evaluate in vitro the binding ability of various LAB strains for FB_1 and FB_2, obtained 74 and 97% binding of FB_1 and FB_2, respectively, with 10^{10} cfu/ml of *L. plantarum* (R1096). Niderkorn's protocols with few modifications were used in the present study and resulted in 51 and 94% binding of FB_1 and FB_2, respectively, for the same LAB strain with a bacterial concentration of 10^{13} cfu/ml. Zhao *et al.* (2016) obtained 53% binding of FB_1 and 85% binding of FB_2 with 10^9 cfu/ml of *L. plantarum* B7. Thus, these previous studies, as in the present work, showed a higher binding for FB_2 than FB_1, but also an increase in fumonisin binding with decreasing pH and rising temperature, as well as differences in binding efficiency among LAB strains.

Studies have also been conducted to elucidate the mechanism of mycotoxin removal by LAB (Dalié *et al.,* 2010). It was demonstrated that binding is the main mechanism involved in fumonisin removal, with adhesion to LAB cell wall components occurring rather than covalent binding or metabolism (Niderkorn *et al.,* 2009; Dalié *et al.,* 2010; Zhao *et al.,* 2016). Our preliminary results were in agreement with a binding mechanism, since fumonisin removal was observed in presence of both live and dead LAB (unpublished data). LAB strains are Gram positive and so their cell wall mainly consists of peptidoglycans, teichoic acids, proteins and polysaccharides (Delcour *et al.,* 1999; Chapot-Chartier and Kulakauskas, 2014;). It was reported that peptidoglycans of LAB cell wall are the main binding sites for fumonisins; their structural integrity is essential and their amino acid sequence plays an important role in the efficiency of the binding mechanism. TCA chains in fumonisins also are important components in the binding process (Niderkorn *et al.,* 2009; Dalié *et al.,* 2010; Zhao *et al.,* 2016).

Various factors can contribute to and explain the differences observed in the percentage of FB_1 and FB_2 bound by the four LAB strains used in this study under the parameters tested (pH, temperature, incubation time): the shape, size and surface area of LAB cells, the composition, structure and surface charge of LAB cell walls, and the conformation and electrostatic potential of each fumonisin molecule.

As can be seen from the results, theory vs practical quantification indicates a different hypothesis as to the binding reaction in that theoretically, it was expected that the binding of

FB_1 and FB_2 would be the same due to their similarity in chemical structure (Beier *et al.,* 1995; Beier and Stanker, 1997), as previously reported for zearalenone and α-zearalenol by El-Nezami *et al.* (2002), where both toxins competed for the same binding site. Practical results obtained in the present study and by other researchers (Niderkorn *et al.,* 2006a,b; Zhao *et al.,* 2016) have indicated otherwise, i.e. higher binding for FB_2 than FB_1. The only structural variation between FB_1 and FB_2 consists of an additional hydroxyl group in C_{10} for FB_1. Niderkorn *et al.* (2006a, 2009) suggested that the spatial conformation resulting from the hydrogen bond between this extra hydroxyl group and a carboxyl group may limit the accessibility of FB_1 to the binding sites of LAB cell walls.

Statistical analyses have indicated that binding affinity of LAB strains for FB_1 and FB_2 is significantly affected by temperature ($P < 0.001$) and pH ($P < 0.0001$). The combination of higher incubation temperature (37 °C) and low pH (4) produced the highest binding of FB_1 and FB_2 by all LAB strains tested ($P < 0.0001$). The increase in incubation temperature from 30 °C to 37 °C is the factor that illustrates a direct relationship to the increase in fumonisin binding. LAB grows optimally at 37 °C where the cells prepare for growth either by 'make before break' or 'inside to outside' principle, as explained by Delcour *et al.* (1999). In both principles, more peptidoglycans are added to the cell wall (Delcour *et al.,* 1999) providing more favourable binding sites for FB_1 and FB_2 (Delcour *et al.,* 1999; Niderkorn *et al.,* 2009; Zhao *et al.,* 2016), and hence enhanced binding.

The increase in fumonisin binding rate as the pH decreases from 6 to 2 could be attributed to the change in pH affecting the surface charge in the constituents of the LAB cell wall providing favourable binding sites for each of the fumonisins (Schar-Zammaretti and Ubbink, 2003; Luxbacher, 2014). Zhao *et al.* (2016) gave two explanations for this result. The first one is that acidic pH would break bonds among certain constituents of the LAB cell wall altering its original structure in a way that would provide more favourable binding sites for FB_1 and FB_2. The second one is that under acidic conditions hydrolysis of TCA structure in fumonisins may be inhibited, permitting increased binding with LAB cell wall as TCA structure plays an important role in the binding process.

The variation in fumonisin binding efficiency observed among the four LAB strains could be explained by the difference in shape, size and surface area of LAB cells, i.e. bacilli rods vs cocci. It is well known that bacilli rods tend to occur singularly with a cell size of 0.19-1.2

µm×3- 8 µm (Pederson, 1935), whereas cocci tend to occur in tetrads with a single cocci cell size of 0.5-1 µm (Semjonovs, 2010). Due to the preference of cocci to occur in tetrads, this arrangement drastically reduces the surface area of binding sites for FB_1 and FB_2. *P. pentosaceus* (D39) had the lowest binding affinity for FB_1 and FB_2, whereas the *Lactobacillus* species showed the most binding affinity. Over time the tetrads auto-aggregate as per their natural preference (Holzapfel *et al.,* 2006) and this characteristic drastically reduces the binding. Jankovic *et al.* (2012) demonstrated that *L. plantarum* also possessed the ability to auto-aggregate; after 24 h at least 80% of the bacterial cells were aggregated. The decrease of binding ratio over time (i.e. under simulated ogi storage conditions) for the three LAB strains, *P. pentosaceus* (D39), *L. plantarum* (FS2) and *L. delbrueckii* (CIP 57.8T), could also be an indication that during storage these strains have a tendency to autoaggregate, which is one of the desirable attributes for a probiotic strain (Jankovic *et al.,* 2012).

Differences in binding efficiency among LAB strains could also be explained by the fact that the genetic differences between each strain would produce different components in the cell wall (i.e. peptidoglycans, teichoic acids, polysaccharides and proteins) that could or could not enhance binding affinity for FB_1 and FB_2 (Schar-Zammaretti and Ubbink, 2003). In particular, the molecular structure of peptidoglycans which are the main binding sites for FB_1 and FB_2, varies with bacterial species (Niderkorn *et al.,* 2009; Zhao *et al.,* 2016).

3.6. Conclusion

Three LAB species (*L. plantarum, L. delbrueckii* ssp. *delbrueckii* and *Pediococcus pentosaceus*) that are predominant in African traditional fermented maize based foods (ogi and mahewu) were successfully evaluated in binding FB_1 and FB_2. Assessment of the binding ratio and complex stability under varying parameters of pH, temperature and incubation time indicated that the four LAB strains used in this study have the ability to bind FB_1 and FB_2, with all the strains exhibiting higher binding affinity for FB_2 than FB_1. Binding ratio increased with a decline in pH from 6 to 2 and an increase in incubation temperature from 30 to 37 °C. Nevertheless, the percentage of FB_1 and FB_2 bound to LAB at pH 4, 30 °C decreased after 6 days of storage for all tested LAB strains, except for the positive control strain, *L. plantarum* (R1096), for which it increased. These results indicate the stability of the LAB-fumonisin (FB_1 and FB_2) complex under the pH and temperature conditions observed during ogi fermentation and in gastric conditions (showing even an increase in the percentage of fumonisins bound by the LAB strains under such conditions), but not after 6 days under ogi

post fermentation storage conditions. In addition, FB_1 and FB_2 binding efficiency varied between LAB strains, with *Lactobacillus* species being the most efficient in binding fumonisins, whereas *Pediococcus sp*. was the least efficient. These results show the potential for the *Lactobacillus* species to be used as main starter cultures in African traditional fermented maize based foods, specifically ogi and mahewu, with a two-fold advantage of being probiotics coupled with the ability to reduce the fumonisin contamination. However, this requires that the efficiency of *L. plantarum* and *L. delbrueckii* strains in binding fumonisins need to be evaluated in the full matrix, i.e. during ogi and mahewu fermentation, with the analysis of both hidden and bound fumonisins, as well as the effect of these strains on the physicochemical and sensory properties of the final fermented maize products to establish consumer acceptability. In addition, the binding ratio and complex stability under proper simulated human gastric digestion and the toxicity of the complex LAB-fumonisin (FB_1 and FB_2) should be tested.

3.7. References

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Table 3.1: Performance parameters of the HPLC method for fumonisin analysis

	Linearity R^2	Recovery %	LOD ng/ml	LOQ ng/ml	RSD_r %	RSD_R %
FB_1	0.9970	75-117*	0.3	1	3-9*	9-29*
FB_2	0.9976	63-71*	0.3	1	2-4*	6-32*

LOD: Limit of detection
LOQ: Limit of quantification
RSD_r: Relative standard deviation calculated from repeatability data
RSD_R: Relative standard deviation calculated data
* Range of values obtained at different tested pH (2, 4, 5 and 6)

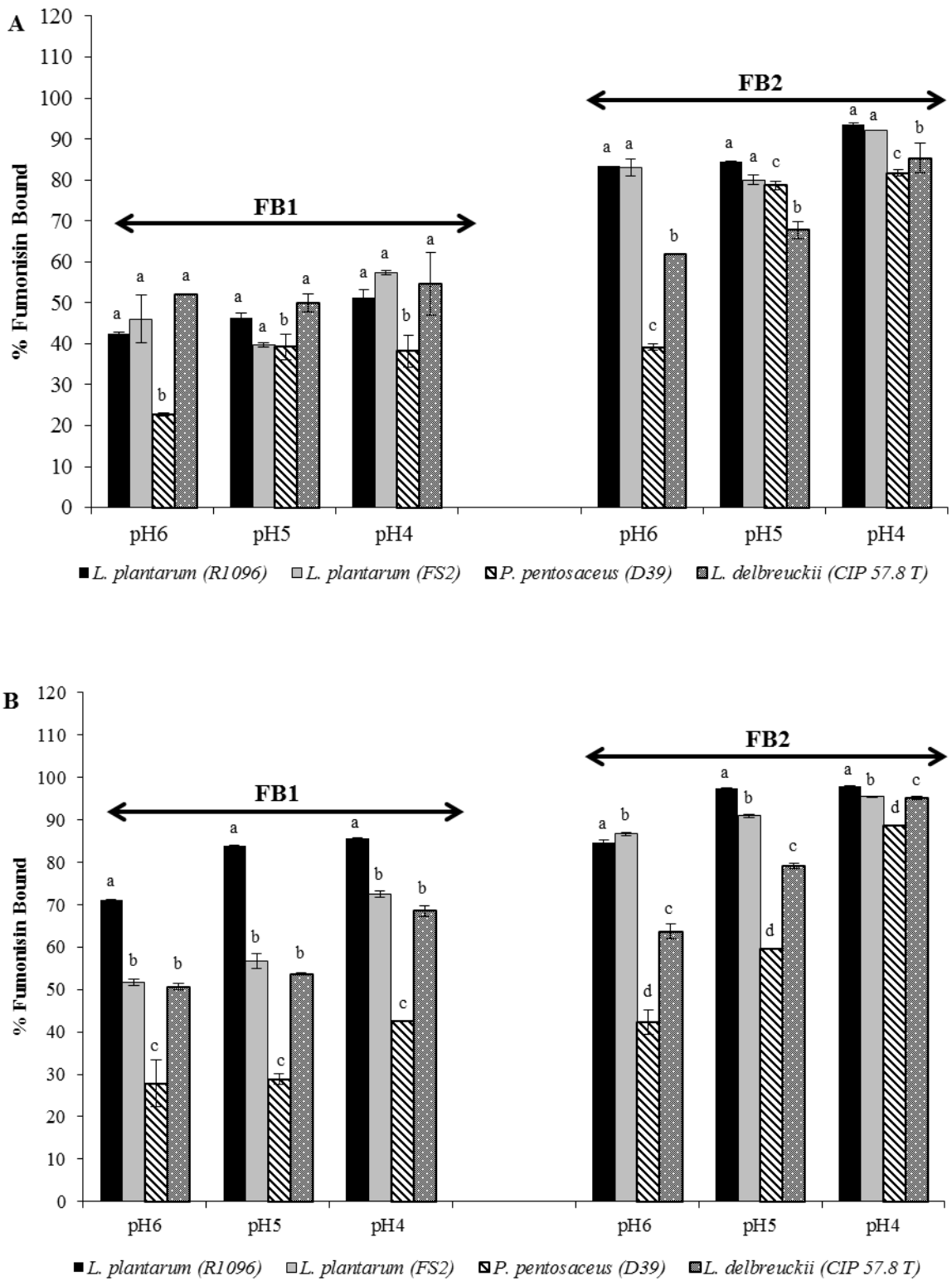

Figure 3.1: Effect of pH and temperature on the percentage of fumonisins (B_1 and B_2) bound by LAB strains: A) at 30 °C and B) at 37 °C. For each combination of fermentation condition, the letters indicate the results of the Tukey HSD test on the strains. Strains found to be **not different** at 95% significance level are labelled with the same letter

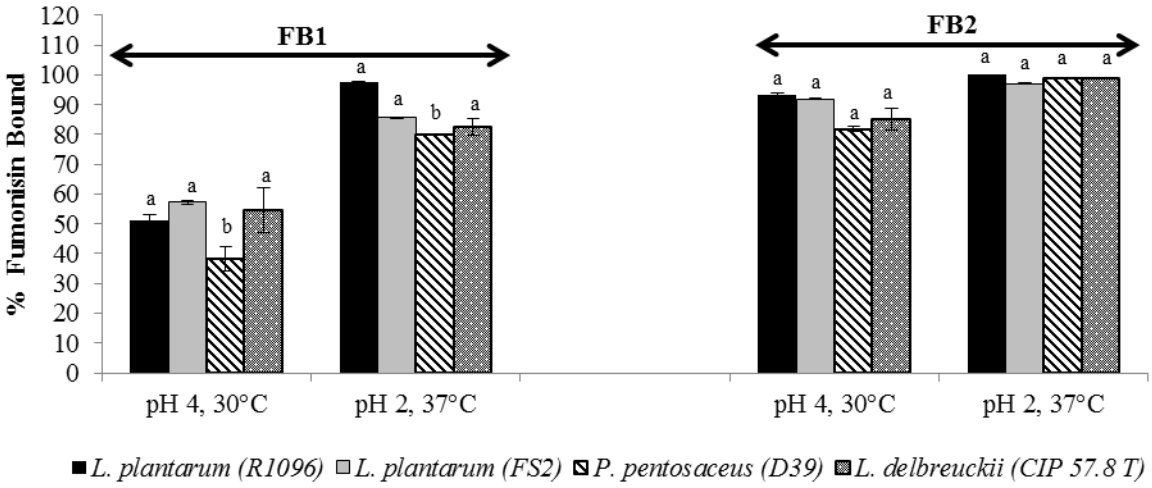

Figure 3.2: Effect of lower pH (2) and incubation temperature of 37 °C *in vitro* on the stability of LAB - fumonisin (B_1 and B_2) complex by comparison of the binding ratio under baseline parameters (pH 4, 30 °C at the end of ogi fermentation). For each combination of fermentation condition, the letters indicate the results of the Tukey HSD test on the strains.

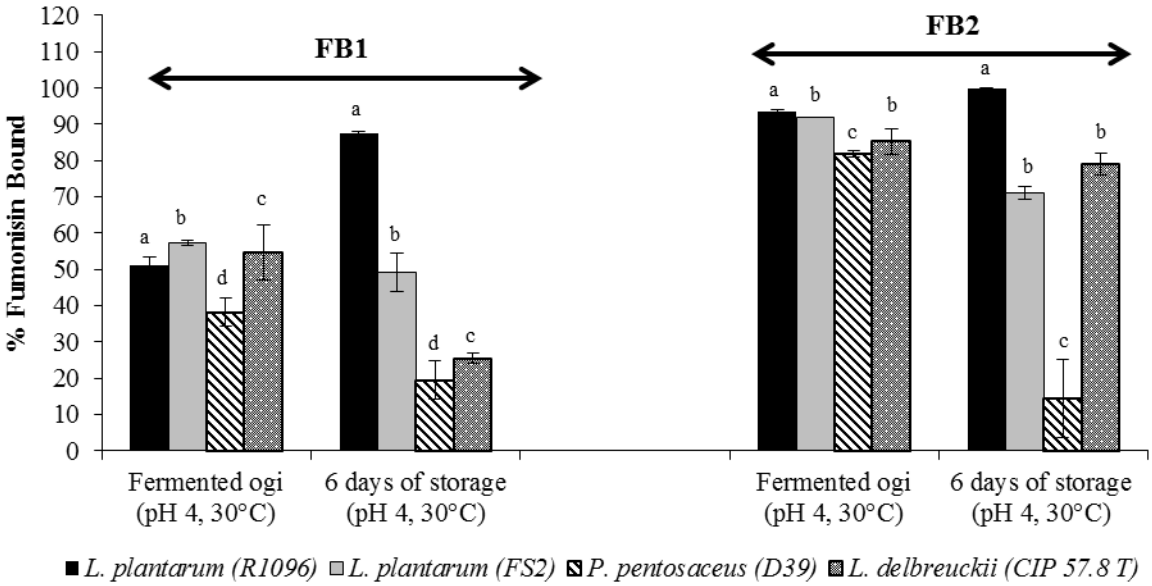

Figure 3.3: Effect of simulated storage conditions (6 days at 30 °C) of fermented ogi (pH 4) *in vitro* on the stability of LAB - fumonisin (B_1 and B_2) complex. For each combination of fermentation condition, the letters indicate the results of the Tukey HSD test on the strains.

Chapter Four

Lactic acid bacteria from ogi, a fermented indigenous African beverage, reduce toxicity of fumonisins (B_1 and B_2)

4.1. Abstract

Fumonisin (B_1 and B_2) are mycotoxins that are widely prevalent in maize that forms the base substrate of fermented cereal products such as ogi and mahewu. Consumption of fumonisin contaminated foods can result in liver carcinogenicity due to their role in inhibiting ceramide synthase causing a build-up of sphinganine in tissues. Reduction of fumonisin levels in food therefore can potentially be a strategy for the control of fumonisin associated carcinogenicity. This study therefore aimed to investigate the effect of lactic acid bacteria (LAB) namely, *Lactobacillus plantarum* R1096, *L. plantarum* FS2, *L. delbrueckii* CIP 57.8T and *Pediococcus pentosaceus* D39, isolated from fermented maize products, on reducing the exposure of fumonisins (B_1 and B_2) to HepG2 cells *in vitro.* Toxicity of fumonisins (B_1 and B_2) to HepG2 cells in presence and absence of LAB was measured using the sulforhodamine B (SRB) assay. No aggregation, binding or interaction could be detected between the LAB and HepG2 cells in during this preliminary test, indicating the singular effect of fumonisins (B_1 and B_2) on the HepG2 cells. Results indicated the ability of the LAB strains to "protect" the HepG2 cells by binding fumonisins (B_1 and B_2). *L. plantarum* which was the most efficient of all the strains tested, increased HepG2 cell viability from 20 to 66% when exposed to fumonisins at a concentration of 600 µg/ml. *P. pentosaceus*, the less efficient, increased HepG2 cell viability up to 37% only. The IC_{50} values for fumonisins against HepG2 cells were increased in the presence of all the LAB strains, indicating a protective ability of the probiotic strains towards liver cells. In conclusion, the probiotic LAB strains isolated from indigenous fermented cereal products can be utilised for their potential hepatoprotective potential against fumonisins.

4.2. Introduction

The use of LAB has emerged as a promising approach in the reduction and detoxification of mycotoxins in foods and feeds (Dalié *et al.*. 2010; Karlovsky *et al.,* 2016; Dawlal *et al.,* 2017). Mycotoxins are secondary, toxic fungal metabolites with diverse chemical structures that account for different biological properties and effects, which can be carcinogenic, tetratogenic, mutagenic, oestrogenic, neurotoxic or immunotoxic (Ferrigo *et al.,* 2016).

The discovery and structural elucidation of fumonisins were first reported by South African researchers (Bezuidenhout *et al.,*1988; Gelderblom *et al.,*1988). Fumonisins are a class of mycotoxins divided into 4 categories: A (A_1, A_2 and A_3), B (B_1, B_2 and B_3), C (C_1, C_3 and C_4) and P (P_1, P_2 and P_3) with Groups A and B presumed to be the most important. They are non-fluorescent, water-soluble and polar (Ahangarkani *et al.,* 2014). Fumonisins are produced primarily by *Fusarium verticillioides* (formerly known as *Fusarium moniliforme*) and other *Fusarium* species such as *F. graminearum, F. napiforme, F. dlamini* and *F. nygami* (Ahangarkani *et al.*, 2014).

A very large area of concern is mycotoxins in any food product that can adversely affect humans. Researchers elsewhere have reported the ability of LAB to bind fumonisins (Niderkorn *et al.*, 2006a, b). Investigations of the interaction between LAB and fumonisins (B1 and B2) *in vitro* were undertaken by Niderkorn *et al.* (2006a, b) who reported that the LAB were able to bind FB_1 and FB_2. Further research conducted by Dawlal *et al.* (2017) investigated the stability of the LAB – fumonisin (B_1 and B_2) complex through decreasing pH and increasing incubation temperature. Results indicated stability of the bound complex and increased binding of fumonisins (B_1 and B_2) with decreasing pH.

Fumonisin B_1 is the most copiously produced fumonisin (Abrunhosa *et al.*, 2016; Ferrigo *et al.,*, 2016) and is linked to areas of high oesophageal cancer in Eastern Cape, South Africa, formerly known as Transkei (Sydenham *et al.*, 1990) and other parts of Africa (Zain, 2011). The occurrence of fumonisins at high contamination rates and levels in maize has been reported in several studies in Africa (Dawlal *et al.*, 2010; Vismer *et al.* 2015; Mngqawa *et al.,* 2016; Udomkun *et al.*, 2017) with levels that exceed 10 µg/g (Okeke *et al.,* 2015; Chilaka *et al.,*2016). Fumonisins B_1 (FB_1) and B_2 (FB_2) are the most commonly found in food and feed (Abrunhosa *et al.*, 2016; Ferrigo *et al.*, 2016). These compounds are

structurally related and consist of a long hydroxylated hydrocarbon chains (20 carbon atoms) containing methyl and amino groups in which the hydroxyl groups on C_{14} and C_{15} are esterified with tricarboxylic acid (TCA) (Abrunhosa *et al.*, 2016).

Fumonisin mode of action is the interference with *de novo* synthesis of complex glycol-sphingolipids by inhibition of ceramide synthase (Wang *et al.,* 1991). This inhibition leads to lipid mediated alteration in signalling and metabolic pathways crucial to cell growth, apoptosis, differentiation, morphology and endothelial cell permeability observed in both *in vivo* and *in vitro* relating to the development of cancer (Riley *et al.,* 2001; Gelderblom and Marasas, 2012; Voss and Riley, 2013). Following digestion, the main site of absorption is the small intestine (Voss and Riley, 2013; Chuturgoon, 2015;) from where it is transported to the liver where it initiates the accumulation of sphinganine in the tissue that could cause toxic effects to the liver such as carcinogenicity, and nephrotoxicity in the kidneys (Nour *et al.,* 2007; Dheeb *et al.,* 2013).

The objective of this study therefore was to investigate the effect of LAB isolates from traditionally fermented cereals, on reducing exposure of fumonisins (B_1 and B_2) to HepG2 cells *in vitro* using the SRB assay.

4.3. Materials and Methods

4.3.1. LAB strains and media

In this study, 3 LAB strains predominant in African traditional fermented maize based foods (ogi and mahewu) were selected as test strains, two LAB strains, *L. plantarum* FS2 and *Pediococcus pentosaceus* D39, with probiotic characteristics, were isolated from Nigerian ogi (Fayemi and Buys, 2017; Fayemi *et al.*, 2017), and *L. delbrueckii* ssp. *delbrueckii* CIP 57.8T was isolated from sour grain mash and sourced from the Pasteur Institute, France.. They were tested against *Lactobacillus plantarum* R1096 sourced from Lallemand SAS, France, as a positive control strain as it was previously shown to have the ability to bind high levels of FB_1 and FB_2 (Niderkom *et al.*, 2006a).

MRS broth and MRS agar (de Man *et al.,* 1960) were used to culture the LAB strains, which were subsequently stored on MRS agar slants at 4 ^{o}C for 3 months. For long term conservation, strains were cryopreserved at -80 ^{o}C in 12.5 % glycerol (Jacobs, 1991) and on

microbeads (MAST Diagnostics, France).

4.3.2. Preparation of fumonisins B_1 and B_2 solutions

Fumonisins B_1 and B_2 (Sigma Aldrich, South Africa) were dissolved in dimethyl sulfoxide (DMSO) to final concentrations of 600, 300,150, 75 and 37.5 µg/ml before addition to the microtitre plates.

4.3.3. Preparation and determination of LAB cell concentration for use in SRB assay

Once grown, the LAB cells were harvested by centrifugation at 3000 g for 3 min. The supernatant was removed and cells re-suspended in 1 ml of Ultrapure (UHQ) water, washed by centrifugation at 3000 g for 3 min and re-suspended in UHQ water. LAB cell concentration was then adjusted to 0.5 McFarland standard (1.5 x 10^8 cfu/ml), cell counts determined with a hemocytometer and then diluted in UHQ water to obtain a cell concentration of 10^4 cfu in 50 µl. A standardized concentration of LAB cells (10^4 cfu in 50 µl) was systematically obtained and used for all strains throughout the experiment.

4.3.4. Sulforhodamine B (SRB) assay

The cytotoxic effect of fumonisins B_1 and B_2 on HepG2 cells was determined using the SRB assay according to Skehan *et al.* (1990). The HepG2 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 10% fetal bovine serum and incubated in a 5% CO_2-buffered and humidified incubator at 37 ^oC until the cells reached confluency. A 100 µl of cell suspension was inoculated into each well of a 96 well microtiter plate to give a concentration of 10^4 cells per well, and then incubated at 37 ^oC for 24 h in 5% CO_2 and 100% humidified incubator. After 24 h, the fumonisin B_1 and B_2 toxin solution in DMSO was added at various concentrations along with LAB cell concentration of 10^4 cells per well, incubated at 37 ^oC for a further 24 h. After incubation, 50 µl of cold 50% trichloroacetic acid (TCA) was added to fix the cells. The microtiter plate was then stored at 4 ^oC for 1-2 hours. The supernatant was removed, the well on the plates gently washed with tap water and air-dried. One hundred microliters of 4% sulforhodamine B (SRB) solution in 1% acetic acid was added to each well and incubated at room temperature for 10 min. The wells on the plates were then washed 5 times with 1% acetic acid and air-dried. Then 10 mM of trizma base (pH 10.5) was used to solubilize the stains; the intensity of the absorbed radiation was read on an automated plate reader (Tecan Infinite 200, Austria) at a wavelength of 540 nm.

For the effect of LAB on viability of HepG2 cells, the SRB assay was similarly carried out as in Section 4.3.4 as there were wells with HepG2 cells and LAB, only HepG2 cells and only LAB cells without fumonisin B1 and B2. The same concentration of cells was used for HepG2 and LAB cells: 10^4 cfu per well (in duplicate). The blanks contained complete medium without any cells.

The SRB Assay is performed at Biosciences, CSIR, in accordance with the protocol of the Drug Evaluation Branch, NCI, and the assay has been adopted for these types of screens therefore the assay which requires a Z' factor between 0.5 and 1.0 which quantifies the quality of the assay has already been achieved and is maintained rigorously.

4.3.5. Statistical analysis

All experiments were performed in duplicates and data analysis was performed using GraphPad Prism software (GraphPad Software, 7825 Fay Avenue, USA). Cell growth inhibition (IC_{50}) was determined by non-linear regression and results were analysed using a one-way ANOVA to determine significant differences among the strains, using SAS statistical software (Version 9.4) (SAS Institute Inc., 100 SAS Campus Drive, USA) at a significance level of 95%.

4.4. Results

4.4.1. Effect of LAB on viability of HepG2 cells

Data is reported on the effect of LAB on viability of HepG2 cells as discussed in Section 4.3.4. Absorbance readings obtained from each well (Table 4.1) indicate that the initial concentration of HepG2 cells added individually to the wells did not influence the protein content assayed in the SRB assay. Further, in comparison to the absorbance readings obtained from the wells containing the LAB cells only, the results indicated that the LAB cells did not aggregate nor attach to the microtiter plate and therefore did not affect the experiment adversely. Finally, *L. plantarum* R1096, *L. plantarum* FS2, *L. delbrueckii* CIP57.8T and *P. pentosaceus* D39 were tested individually with HepG2 cells to ascertain their possible aggregation, binding and interaction with the HepG2 cells. The absorbance readings recorded after the addition of LAB cells indicated no increase/decrease the protein content assayed. This is a strong indication that the results obtained during the toxicity testing were solely

from the effects of fumonisins on the HepG2 cells. This indicates that the change in toxicity in the presence of the LAB strains were as a result of the interaction of the LAB strains with the fumonisins as LAB strains have a higher affinity for fumonisins than for HepG2 cells.

Table 4.1: Viability of HepG2 cells alone and in the presence of lactic acid bacterial isolates from fermented maize products (ogi and mahewu) based on Sulforhodamine B assay

	Viability of LAB cells only, HepG2 cells only and HepG2 cells with LAB cells		
Lactic Acid Bacteria Strain	LAB cells ONLY	HepG2 cells ONLY	HepG2 cells + LAB cells
L. plantarum R1096	0	1.62 ± 0.00	1.63 ± 0.01
L. plantarum FS2	0	1.62 ± 0.00	1.56 ± 0.02
L. delbrueckii subsp. *delbrueckii* CIP57.8T	0	1.76 ± 0.01	1.81 ± 0.03
P. pentosaceus D39	0	1.54 ± 0.01	1.55 ± 0.01

4.4.2. Viability of HepG2 cells when exposed to fumonisins

In order to determine the IC_{50} value of the fumonisins to HepG2 cells, these cells were treated with various concentrations of fumonisins (B_1 and B_2) solution in DMSO. Viability of the HepG2 cells after incubation with the fumonisins was assessed using the SRB assay. The concentration of 308.6 µg/ml with an assay quality of 0.92 (Z' factor) was found to be the IC_{50} value for the fumonisin (B_1 and B_2) solution (Figure 4.1). The IC_{50} value indicates the concentration of the inhibitory compound, in this case fumonisins, at which 50% of the biological function or process, in this case viability of HepG2 cells, will be inhibited/reduced.

4.4.3 Viability of HepG2 cells when exposed to fumonisins in the presence of LAB

HepG2 cells were exposed to fumonisin (B_1 and B_2) in conjunction with selected probiotic LAB strains to determine the effect of LAB on the reduction of the fumonisins to the HepG2 cells. As already mentioned in section 4.4.3, the IC_{50} value of fumonisins alone with HepG2 cells was 308.6 µg/ml as interestingly, the IC_{50} value for fumonisins was much higher (doubled) in the presence of LAB for all the strains tested. In the presence of *L. plantarum* R1096, positive control strain, the IC_{50} value was the highest being at 903.1 µg/ml in comparison with the test strains in decreasing value *L. delbrueckii* CIP 57.8T (857.3 µg/ml) < *L. plantarum* FS2 (856.5 µg/ml) < *P. pentosaceus* D39 (701.4 µg/ml) (Table 4.2). The qualities of the assays, the Z' factor in decreasing values in the presence of the LAB strains

were as follows: *L. plantarum* FS2 = *P. pentosaceus* D39 < *L. plantarum* R1096 < *L. delbrueckii* CIP 57.8T (Table 4.2) (Figure 4.1).

Table 4.2: The IC_{50} values of fumonisins (FB_1 and FB_2) on the human liver cell line (HepG2) in the presence of different lactic acid bacterial strains from ogi and mahewu based on the Sulforhodamine B assay

Lactic Acid Bacteria Strain	IC_{50}(µg/ml)*	Z' Factor
L. plantarum R1096	903.1	0.84
*L. delbrueckii*subsp.*delbrueckii*CIP57.8T	857.3	0.75
L. plantarum FS2	856.5	0.99
P. pentosaceus D39	701.4	0.99

*Each value is average of n = 8

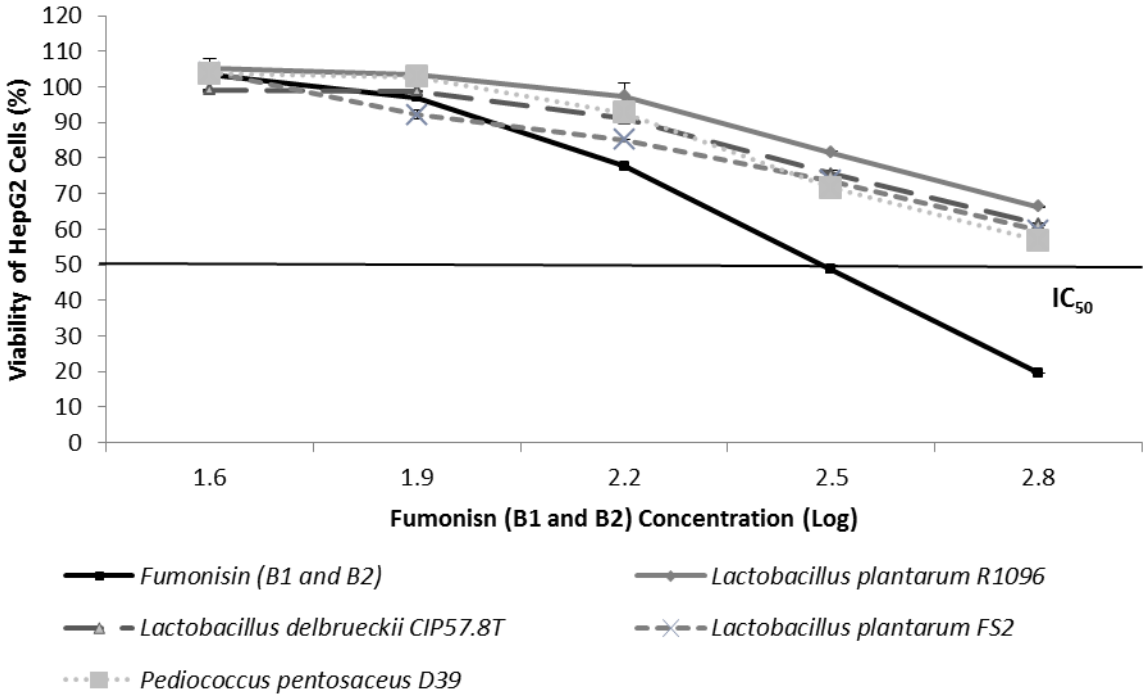

Figure 4.1: The effect of fumonisin (B_1 and B_2) on cell viability (HepG2) in the presence of LAB strains isolated from fermented cereal products (ogi and mahewu), demonstrating the IC_{50} value, based on the Sulforhodamine B assay

4.5. Discussion

In this study, to assist in developing a natural means of reducing toxicity in food products, LAB strains isolated from traditional fermented foods, ogi and mageu, were studied in HepG2 cells. This study has shown that the selected LAB strains provided limited protection

and reduced toxicity of fumonisins (B_1 and B_2) to HepG2 cells *in vitro*. Preliminary test on the individual effect of the LAB cells on the HepG2 cells indicated no aggregation, binding nor interaction between the two as indicated by similar absorbance readings obtained from the protein binding assay. Toxicity test of fumonisins (B_1 and B_2) on the HepG2 cells indicated cytotoxicity occurred at >308.6 µg/ml indicating the IC_{50} value.

In cytotoxicity screening, biological issues affect the choice of assay, either one of 2 major techniques are used: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and sulforhodamine B (SRB) assay. The MTT assay is based on the conversion of the yellow water soluble substrate into an insoluble dark blue formazan product by viable cells, which then gives the number of viable cells (Fotakis and Timbrell, 2006). The SRB assay is based on the measurement of whole culture protein content (Skehan *et al.,* 1990; Rubinstein *et al.,* 1990). Both assays have advantages and disadvantages but in the evaluation of compound toxicity the SRB assay has better linearity with cell number, higher sensitivity, staining is not cell line dependent, ability to stain recently lysed cells and the cell debris is not stained, therefore drug sensitivity data is not affected (Keepers *et al.,* 1991). The only disadvantage of the assay is the production of fluid shearing forces when pipetting the trichloro acetic acid used to fix the cells on the plate, which could result in dislocation of cells (Papazisis *et al.,* 1997).

The advantages and sensitivity required for accurate, true results needed in the study prompted the use of the SRB assay. HepG2 cells were seeded onto the microtitre plates and allowed to grow until confluency (~24 h period). Confluency refers to ~80% of the base of the well, being covered with a single layer of cells. Probiotics have the ability to autoaggregate within a 24 h period (Jankovic *et al.,* 2012). Due to the nutrient rich medium and optimal growth temperature (37 ^{o}C) in which the HepG2 cells grow, it was expected that the LAB cells would as well grow, increase in cell count to the point of aggregating with the possibility of displacing the HepG2 cells and adhering to the well of the plate. The other possibility was that the LAB cells would attach/adhere to the HepG2 cells. To dispel these possibilities in the experiments, the effect of LAB cells for each strain onto the HepG2 cells was tested. Absorbency results for the experiment and the controls indicate that all LAB strains tested did not affect the HepG2 cells by increasing protein content as assayed in the SRB assay (similar absorbency results obtained for wells with LAB cells and HepG2 cells only and for wells with HepG2 cells only). The results were comparable to those of

researchers elsewhere. These researchers reported that the LAB strains to have a protective ability towards liver cells, a human colon adenocarcinoma cell line (Han *et al*., 2005), human gastric cancer cell line (Kim *et al.,* 2002) and human hepatocellular carcinoma cell line (Ou *et al.* 2012).

A previous study conducted by Dawlal *et al.* (2017), established that LAB bind to and form a stable complex with FB_1 and FB_2 naturally. Fumonisin (B_1 and B_2) were exposed to HepG2 at varying concentrations, along with a fixed concentration of LAB cells for each strain, to test the reduction of fumonisin toxicity in the presence of LAB cells. This was incubated for 15min to allow the cells to acclimatize. Hypothetically, fumonisin (B_1 and B_2) should have bound to and killed HepG2 cells in the 15 min window as indicated by the IC_{50} value but as the LAB cells were added, the fumonisin (B_1 and B_2) bound with the LAB cells rather than the HepG2 cells thereby providing a protective function towards the HepG2 cells as the IC_{50} values doubled for all the strains. This indicates that fumonisin (B_1 and B_2) binding to the LAB cell is more preferable than the liver cells thereby reducing fumonisin toxicity (Kim *et al.,* 2002; Ou *et al.* 2012). Different researchers have demonstrated the same successful ability of LAB to bind and detoxify aflatoxins (Haskard *et al.*, 2001; Salim *et al.*, 2011) patulin and ochratoxin A (Fuchs *et al.*, 2008), zearalenone (El-Nezami *et al.,* 2002) and fumonisins (Niderkorn *et al.,* 2006a, b).

Fumonisins (B_1 and B_2) have a similar structure to cellular sphingolipids specifically sphinganine and sphingosine (Stockmann-Juvala and Savolainen, 2008), which are important precursors in sphingolipid biosynthesis. Fumonisin exhibits a toxic effect by competing with the sphingoid bases and causing inhibition of the enzyme ceramide synthase (Stockmann-Juvala and Savolainen, 2008). This disrupts the sphingolipid biosynthesis (Soriano *et al.,* 2005; Escriva *et al.,* 2015) and increases accumulation of sphinganine, which controls apoptosis and cell proliferation pathways connected to the development of cancer (Gelderblom and Marasas, 2012; Chuturgoon 2015). However as addressed by many researchers (Stockmann-Juvala and Savolainen, 2008; Van der Westhuizen *et al.,* 2010; Dheeb *et al.,* 2013; Escriva *et al.,* 2015; Chuturgoon 2015; Wentzel *et al.,* 2016), it is difficult to identify one single mechanism to explain the types and mechanisms of cytotoxicity.

The International Programme on Chemical Safety (IPCS, 2011) states that European fumonisin daily intake is 0.2 µg/kg body weight whereas African fumonisin daily intake is

200 µg/kg body weight (Domijan and Abramov, 2011) which exceeds the Provisional Maximum Tolerable Daily Intake (PMTDI) of 2 µg/kg bw/day (JECFA, 2001). The IC_{50} value obtained in this study was higher than the African fumonisin daily intake, which critically analysed would mean that liver cells would lose almost 50% viability when fumonisin contaminated food is ingested. Dawlal *et al.* (2017), have indicated that the 4 LAB strains bind to FB_1 and FB_2 forming a stable complex. This binding alters the FB_1 and FB_2 structure from inhibiting the ceramide synthase pathway thereby reducing the toxicity. Further testing of the LAB – fumonisin complex is required against the perceived mechanisms of toxicity to determine true detoxification. However, this study indicates a good reduction in FB_1 and FB_2 toxicity on liver cell line in the presence of LAB strains, thus indicating that LAB cells provide a protective effect.

4.6. Conclusion

Predominant LAB strains from traditional African fermented foods (ogi and mahewu) were successfully evaluated in reducing toxicity of fumonisin (B_1 and B_2) in HepG2 cells *in vitro.* The IC_{50} values of fumonisins against the HepG2 were higher in the presence of the LAB cells with a concomitant increased viability of HepG2 cells in the presence than in the absence of these bacteria. However, reduction of fumonisins toxicity against the HepG2 cells varied among different LAB with *L. plantarum* R1096 conferring the best protection while *P. pentosaceus* D39 was the least effective. Based on these findings, it can be suggested that these LAB strains be used for their hepatoprotective effect against fumonisins. Therefore, the probiotic LAB strains predominant in fermented maize beverages have the potential to be used for reduction or prevention of fumonisins related hepatotoxic effects, especially in rural communities where such products are consumed almost on daily basis.

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Chapter Five

General discussion, General conclusion and Recommendations

5.1. General discussion

This chapter serves to discuss and critically analyse the methodologies employed and conclude on the objectives achieved in each chapter to provide a complete and holistic view of the research undertaken. Lastly, possible avenues for future work in the reduction of fumonisin exposure in African fermented maize-based foods with LAB strains will be proposed.

5.1.1. Visualization and quantification of fumonisins bound by viable and non-viable lactic acid bacteria isolated from traditional fermented cereal, ogi and mahewu

The first aim of the study was to visualize the binding interaction between fumonisins (B_1 and B_2) and 4 selected LAB strains namely, *L. plantarum* FS2, *L. delbrueckii* subsp. *delbrueckii* CIP 57.8T and *P. pentosaceus* D39 tested against a positive control strain *L. plantarum* R1096. The approach was to fluorescently tag (derivatize) the fumonisin molecules and then allow for the interaction with the non-fluorescent viable and non-viable LAB cells. Controls were in place to assure the presence of non-aggregated LAB cells observed under white light microscopy and natural, non-fluorescent LAB cells with confocal laser scanning microscopy (CLSM). Similarly, the derivatized fumonisin molecules were viewed individually with CLSM to assure derivatized fluorescence. Both components (LAB cells and derivatized fumonisins) were then allowed to react on a microscope slide and viewed under CLSM. This provided physical evidence that the fluorescent fumonisins (B_1 and B_2) bound to the LAB cells externally as the binding occurred with non-viable cells as well and both the viable and non-viable cells fluoresced green under CLSM. Unfortunately, neither difference in emission intensity nor any difference in binding was seen between the viable and non-viable cells. However, to the best of our knowledge, this is the first study to visualize the interaction between LAB and fumonisins with CLSM.

Due to the difficulty in using dual fluorescent probes simultaneously (Zotta *et al.,* 2012), conventional microbiological methods (viable vs non-viable cells) in combination with derivatized fumonisins achieved the means with simple, unique efficiency; unfortunately clear definition of the binding site between the fumonisins and LAB cells could not be definitively visualized and identified with CLSM. Even though, good results were achieved, in hindsight, better results might have been achieved with the use of atomic force microscopy.

Atomic force microscopy (AFM) as the name suggests employs the use of microscopy that can provide images of atoms on or in surfaces of objects at the atomic level in an operational environment of air or liquid (Burgain *et al.,* 2014a,b). With microscopy, traditional methods utilised in the identification of functional membrane proteins or cell wall components, usually entail the presence of the membrane that interferes with the study; the biological membrane has an important role to play in the protein structure and function and it may be uncertain as to whether the data obtained from the extracted protein is a true reflection of its function; the fluorescently labelled proteins to aid detection can also deter the protein from its natural state of function whereas with AFM none of these is required and it allows for the study of the proteins and membrane structures within its natural state and native conditions during AFM with the additional advantage of detecting individual structure and behaviour of single molecules (Whited and Park, 2014).

In the application of AFM to visualize the binding interaction between LAB cells and fumonisins, the structures in the Gram positive cell wall would need to be mapped first. Umeda *et al.* (1998) have indicated that AFM is able to show the differences between a Gram negative and Gram positive cell wall. As explained in the thesis (Chapter Two and Three), fumonisin has an affinity to bind to peptidoglycan in the cell wall. Andre *et al.* (2010) demonstrated the visualization of peptidoglycan insertion into cell wall of *Lactococcus lactis* by utilizing a tip modified with a Lysin motif to detect localized peptidoglycan molecules, a similar method can be employed to detect peptidoglycan on the 4 LAB strains used in this study. In the same way, with affinity imaging mode, the first proof of concept has been provided by imaging microscale streptavidin patterns using a biotinylated tip (Ludwig *et al.,* 1997); lectin modified tip for mapping wall polysaccharides on living yeasts (Gad *et al.,* 1997); red blood cells (Grandbois *et al.,* 2000); endothelial cells (Almqvist *et al.,* 2004) and mycobacteria (Dupres *et al.,* 2005). Chen *et al.* (2015) produced an aptamer based microcantilever biosensor array for detection of FB_1, the array is proposed to use this cantilever for affinity imaging mode for FB_1 mapping. The same method can be employed for FB_2. Once, affinity images have been obtained for peptidoglycan, FB_1 and FB_2 per strain, images can be overlayed and a binding affinity map can be produced per strain which would provide a much clearer, defined, binding affinity image.

5.1.2. Potential of lactic acid bacteria for the reduction of fumonisin exposure in African fermented maize based foods

As reported in Chapter Four, a binding strength had been established at pH 4 and temperature of 30 ^{o}C that the stability studies could be based on. Analysing the fermentation process of ogi, the main parameters on were selected to base the stability of the complex on were the incubation temperature of 30 ^{o}C and decreasing pH from 6 to 4 that occurs in a natural ogi fermentation process. For the additional experiments, parameters chosen included the same decreasing pH value and the optimal growth temperature of the LAB strains (which is 37 ^{o}C), due to countries in Northern Africa experiencing room temperatures of 37 ^{o}C as well. These results indicated that whether ogi fermentation process occurs at 30 ^{o}C or at 37 ^{o}C (Northern Africa), the binding strength for both fumonisins was still very high. In addition, as the pH decreased it could be seen that the binding for both fumonisins increased.

Once the ogi was ready for consumption, it was also decided to evaluate a simulated gastric condition of a lower pH of 2 and temperature of 37 ^{o}C. Binding strength for FB_1 and FB_2 indicated that the binding affinity of LAB was quite high and the complex formed was still stable under these conditions. As ogi is stored, we also evaluated the stability of the complex over a storage period of 7 days at pH 4 at 30 ^{o}C. *L. plantarum* R1096 was the only strain that increased in binding quantification through the storage period whereas the binding strength towards fumonisins of other strains decreased over time.

Binding strength results throughout the fermentation process conditions indicated that the LAB-fumonisin complex is quite stable. Results indicated that, as pH decreased, the binding increased. However, across all strains, FB_2 was bound more than FB_1. Different aspects can add to and describe the variation seen between the binding strength: the shape, size and surface area of LAB cells, the composition, structure and surface charge of LAB cell walls, and the conformation and electrostatic potential of each fumonisin molecule, as discussed in the relevant chapter. This can assist in resolving the discrepancy regarding the difference in binding affinity of LAB for FB_1 and that of FB_2, as well as the difference seen in the binding strength between the strains.

In Chapter Three, we were able to establish a clear, baseline stability of the LAB – fumonisin complex in a simple solution using the fermentation conditions that were of interest to us. Better understanding of the stability of the complex would have been achieved with the use

of a zetasizer as this would have evaluated the zeta potential aka. eletrokinetic potential (Luxbacher, 2014). The zeta potential is often used to determine the stability of complexes in colloidal suspensions. Stability results from physicochemical interactions from surface characteristics of LAB that are directly associated with the chemical composition of the cellular surface with a special account to proteins, polypeptides and polysaccharides (Ly *et al.*, 2006).

It has been established that LAB generally are negatively charged (Boonaert and Rouxhhet, 2000). Studies have indicated the ability of LAB to adsorb heavy metals such as cadmium, arsenic and lead from water (Halttunen *et al.*, 2008; Zoghi *et al.,* 2014) and by use of the zeta potential it was possible to demonstrate that binding is dependent on bacterial strain and pH, although the binding did occur by electrostatic interactions to the cell wall components. Wang *et al.* (2015) and Ge *et al.* (2017) investigated the adsorption of patulin and tenuazonic acid on heat inactivated LAB cells, respectively. The researchers also investigated the stability of the bound complexes by the zeta potential and came to the conclusion that when the pH is low the surface groups adsorb H^+ in solutions and the surface has a positive charge which enhances the effect adsorption for the toxins but the reverse was noted when pH increased (Wang *et al.,* 2015; Ge *et al.,* 2017). It would be interesting to use the zetasizer to investigate if a similar result could be seen for fumonisins with our 4 LAB strains in this project.

In one of the experimental parameters, a lower pH of 2 and a higher incubation temperature of 37 ^{o}C was used to simulate post fermentation conditions of consumption. Even though higher binding percentages were seen at pH 2 and 37 ^{o}C, it is not a true reflection of digestion or consumption conditions but it is a good baseline to work from. For a better understanding of the stability and reaction of the complex, the binding ratio should be quantified under proper simulated human gastric digestion as per the Minekus Protocol to take into account the possible reaction with gastric enzymes and salts (Minekus *et al.*, 2014).

Even though stability of the LAB – fumonisin complex has been established through simulated parameters of ogi fermentation and baseline results recorded, further results obtained could be justified by evaluating the complex in the actual ogi fermentation process. Fayemi and Buys (2017) and Fayemi *et al.* (2017) designed an ogi fermentation processing method which can be followed with points of inoculation for LAB strains and removal of

samples for analysis to determine binding strength, following similar research (Okeke *et al.*, 2015). Dall'Asta *et al.* (2009) developed a verified method for the analysis of masked, hidden and bound fumonisins in a cereal product with minimal sample clean-up to follow.

5.1.3. Lactic acid bacteria from ogi, a fermented indigenous African beverage, reduce toxicity of fumonisins (B_1 and B_2)

In Chapter Three, the stability of LAB – fumonisin complex was established leading to Chapter Four where the aim was to investigate the effect of LAB isolates on reducing the toxicity of fumonisins (B_1 and B_2) to HepG2 cells *in vitro*. Toxicity of fumonisins to HepG2 cells was measured using the sulforhodamine B (SRB) assay in the presence and absence of LAB cells. Firstly, the viability of the HepG2 cells when exposed to LAB cells and fumonisins was assessed, individually. The results indicated that the LAB cells did not attach, aggregate or disrupt the HepG2 cells as the absorbance readings did not increase nor decrease throughout the 24-48 h incubation period. Secondly, the toxicity of the fumonisin alone in the HepG2 cells gave the IC_{50} value on which to base the comparison of the reduction of toxicity of the fumonisins in the presence of the LAB strains. The comparative results indicated that LAB cells 'protected' the HepG2 cells by binding to the fumonisin and not allowing the fumonisin to bind to the HepG2 cells thereby decreasing the toxicity and increasing the IC_{50}values. All LAB strains had more than doubled the IC_{50} value by being present and bound to fumonisins.

Fumonisins do not cross the intestinal epithelium barrier in large doses but the liver and kidney are the main target organs (Ahangarkani *et al.,* 2014). For a further justification of the work presented here, the *in vitro* experiments should be supported with *in vivo* experiments. The most similar GIT system to a human is that of the pig (Almond, 1996). Oswald *et al.* (2003) evaluated fumonisin B_1 along with *Escherichia coli* on intestinal colonization in pigs and concluded that fumonisin B1 is a predisposing factor to infectious diseases. The same design can be used to determine the effect of the reduction of toxicity by the LAB strains.

5.2. General conclusion

We have demonstrated that the selected LAB strains have a high binding affinity for both fumonisin B_1 and B_2 with LAB strain was able to bind relatively both fumonisin molecules repeatedly. This indicated that the amount of bound fumonisins was fixed per viable LAB cell under normal fermentation conditions for ogi (pH 4 and 30 ^{o}C). By proof of binding occurring visually, physically and quantitatively with non-viable LAB cells, the mechanism of toxin removal has been established to be that of adsorption rather than biotransformation. Through various conditions of incubation temperature and pH, it has been established that the LAB-fumonisin complex to be relatively stable as well. Finally, it has been proven that the application of LAB strains reduces the toxicity of fumonisin (B_1 and B_2) in HepG2 cell line whereby the LAB strains "protect" the HepG2 cells by binding to the fumonisins.

The visualization of the binding interaction that occurs between the LAB cell and the fumonisin molecule would enable an understanding of how the reduction of the toxicity would occur with the configuration of the binding that occurs i.e. whether or not the binding blocks the part of the fumonisin molecule that induces the toxicity. From this study, there is an established indication of the binding strength (Chapter Three) which indicates a known amount of LAB cells can bind a known amount of fumonisin. This formulation can be applied to base cereal product that is contaminated with a known amount of fumonisins. A comparative amount of LAB cells will need to be added to the base cereal which can then bind to fumonisins. Further testing will be needed regarding masked and hidden fumonisins, but these fixed quantitative amounts indicate that further usage in detoxifying ventures can be used in fermented products.

L. plantarum FS2, *L. delbrueckii* CIP 57.8 T and *P. pentosaceus* D39 test strains isolated from African traditional fermented foods, were successfully tested against the positive control strain *L. plantarum* R 1096, in reducing the toxicity of fumonisin B_1 and B_2. It is proposed that these LAB strains be utilised in traditional fermented products for their ability in reducing / detoxifying fumonisins.

5.3. Recommendations

The scope and aims of the project provided a view from which to operate the project, having achieved the various aims, suggesting different interesting avenues that could be explored in the future.

In this study, it has been physically shown that derivatized fumonisin bind externally to LAB cell walls. Atomic force microscopy has the ability to study the microscopic focus further by actually being able to physically observe binding at the atomic level. As soon as a baseline of binding forces has been established per strain and for each fumonisin molecule, it would be interesting to combine the two molecules and be able to physically observe and document the actual binding interaction that occurs. This will also enable the identification of the cell wall structure that is directly involved in binding interaction. It could provide interesting studies into various binding interactions and forces between the fumonisins and LAB strains.

While this study has focused on the stability of the LAB-fumonisin complex *in vitro*, the use of a zetasizer would be able to evaluate the stability of the complex by establishing the zeta potential. It could be recommended to also look at binding complex formed during the fermentation process; to evaluate the stability of the complex in contact with other materials such as starch; and to evaluate the effect of hidden and masked fumonisins in the fermentation process as well. Additionally, it would also be valuable to evaluate the stability of the complex in a simulated gastro intestinal system, to evaluate if the complex retains stability through the various digestive processes and pH, or if the complex disintegrates and the fumonisin regains its toxicity.

The data acquired provides a good quantitative indication for these LAB strains should they be used in future as fumonisin detoxifying agents, in that they can give a predictable indication of their binding capacity if the level of fumonisin contamination in the fermented product is known. In the long term, there are many more studies and questions that need to be answered and accounted for. More binding strength studies for each mycotoxin with LAB strains as well as studies consisting of multiple mycotoxins also need to be conducted. (Dellafiora and Dall'Asta, 2017). But the data gathered and put forward in this study provides a good baseline from which to work on.

5.4. References

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