Visualization and quantification of fumonisins bound by lactic acid bacteria isolates from traditional African maize-based fermented cereals, ogi and mahewu

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Abstract:

Consumption of fumonisin contaminated foods 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 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) using confocal laser scanning microscopy (CLSM) and to then quantify the LAB-bound fumonisin using high performance liquid chromatography (HPLC). The objective was to obtain a physically visible and quantifiable binding interaction between fumonisins and LAB strains (viable and non-viable cells) with the aim of utilising LAB as a possible detoxifying agent. Fumonisins were derivatized using naphthalene-2, 3-dicarboxaldehyde (NDA) and then combined with non-fluorescent LAB cells (viable and non-viable). For the quantification of bound fumonisins, viable and non-viable cells were incubated in presence of predetermined concentrations of fumonisins and the level of fumonisin in the suspension was determined. Confocal scanning microscopy showed the derivatized green fluorescent fumonisins binding to the surface of each of the LAB cells. For viable cells, *L. plantarum* FS2 bound FB_1 most effectively while *P. pentosaceus* D39 bound the least level of FB_1. The highest levels of FB_2 were bound by *L. plantarum* R 1096 and the least by *L. delbrueckii* CIP 57.8 T. For non-viable cells *L. plantarum* FS2 was also the most effective for binding both fumonisins with *P. pentosaceus* D39 and *L. delbrueckii* CIP 57.8 T being the least effective for FB_1 and FB_2, respectively. To our knowledge, this is the

first study to visualize the interaction between LAB and fumonisins. We demonstrate that LAB isolates from indigenous fermented maize based beverages bind fumonisins and thus present a potential strategy for their reduction in these traditional foods.

Keywords: mycotoxins, fumonisins, fermented maize, lactic acid bacteria, non-viable cells

Introduction

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 makes their decontamination/detoxification complicated and difficult. Lactic acid bacteria (LAB) have a natural affinity to bind mycotoxins and are the most dominant microorganisms present in traditional fermented cereal products. Most LAB strains have been used for the binding of aflatoxin (Haskard *et al.*, 2001; Salim *et al.*, 2011); ochratoxin A (Fuchs *et al.*, 2008) and zearalenone (El-Nezami *et al.*, 2002). Recently, Niderkorn *et al.* (2006a) and Dalié *et al.* (2010) have illustrated that LAB also bind to and deactivates 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, the binding interaction between these two components has not been visualized due to the difficulty in finding and selecting appropriate fluorescent probes that could allow simultaneous detection of mycotoxin and LAB (Zotta *et al.*, 2012). The added difficulty is the inability to prove 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). The current study aimed 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.

Researchers elsewhere have hypothesized that the main mechanism of fumonisin removal could involve their adhesion to cell wall components rather than by covalent binding or metabolism (Niderkorn *et al.*, 2009; Dalié *et al.*, 2010; Zhao *et al.*, 2016). 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.

Therefore, 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), using confocal laser scanning microscopy (CLSM) and high performance liquid chromatography (HPLC). Furthermore the study quantitatively compared binding of fumonisins by viable and non-viable LAB cells to deduce the mechanism of interaction between LAB and fumonisins.

Materials and Methods

LAB cultures

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

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

1.1. 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.

1.2. 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 heat treated in a water bath at 80 ^{o}C for 10 min and immediately cooled in ice. The cells were harvested and treated similar to

the viable cells. 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.

1.3. 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.

1.4. 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 in 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).

1.5. Visualization of interaction between LAB cells and fumonisins

To visualize the interaction, 0.5 µl of LAB cells, both viable and non-viable, were separately 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 CLSM at an excitation of 488 nm and emission of 505 nm. For control samples, LAB cells or derivatized fumonisin alone were spotted onto a clean microscope slide and visualized with CLSM.

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

2.1. Culture and harvest of LAB cells

Culturing and harvesting of LAB cells was 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 was verified as stated above (Section 1.2).

2.2. Determination of LAB cell concentration

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

2.3. 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 a final concentration of 250 µg/ml, aliquoted into 40 amber vials and stored at -20^{o}C. A standardized concentration of 10 µg/ml for each fumonisin was used across all analysis according to Niderkorn *et al.* (2006a).

2.4. Quantification of fumonisins (B_1 and B_2) bound by LAB

Exposure of fumonisins to LAB

Subsequent to quantification of LAB cell concentration, 250 ml of each culture was centrifuged at 3000 x g for 10 min at <10 ^{o}C. The supernatant was discarded and the bacterial cells re-suspended and washed thrice in 100 ml of 0.01 M phosphate buffer solution (PBS) 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 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 1 h with agitation (480 rpm) and for 23 h without agitation. The samples were then centrifuged at 3000 x g for 10 min 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).

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 incubated 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:

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

Statistical analysis

Statistical analyses were carried out on the data obtained for the baseline binding quantification, 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: 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.

Results

1. Visualization of binding interaction between fumonisins and LAB cells

1.1. Visualization of derivatized fumonisins

Both fumonisins did not show any fluorescence under conventional white light (Fig 1 A and B). However, upon visualization under CLSM they both fluoresced green (Fig 1 C and D).

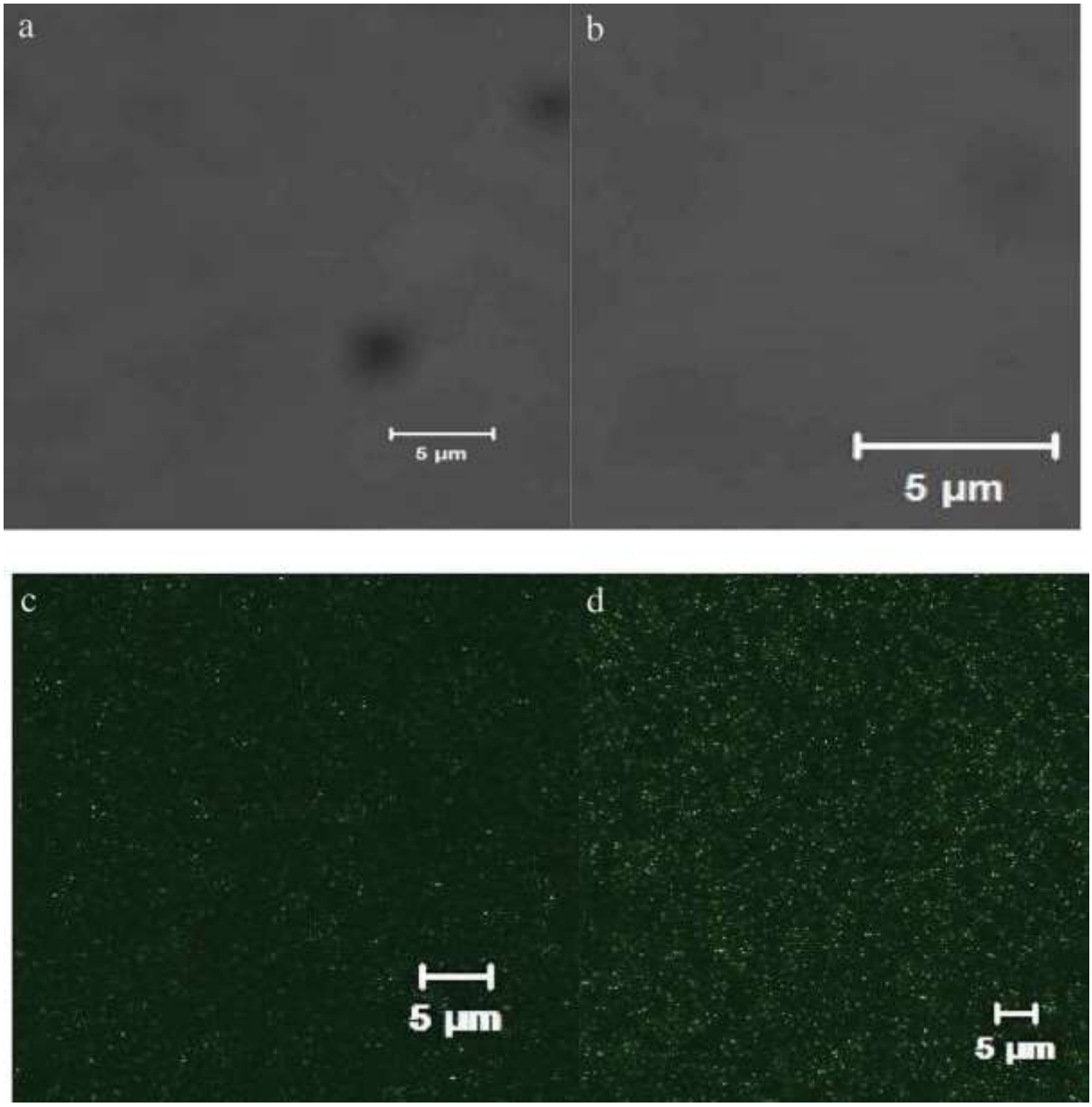

Figure 1. Derivatised fumonisins under conventional white light: (a): FB_1, (b) FB_2 and CLSM: (c): FB_1, (d) FB_2.

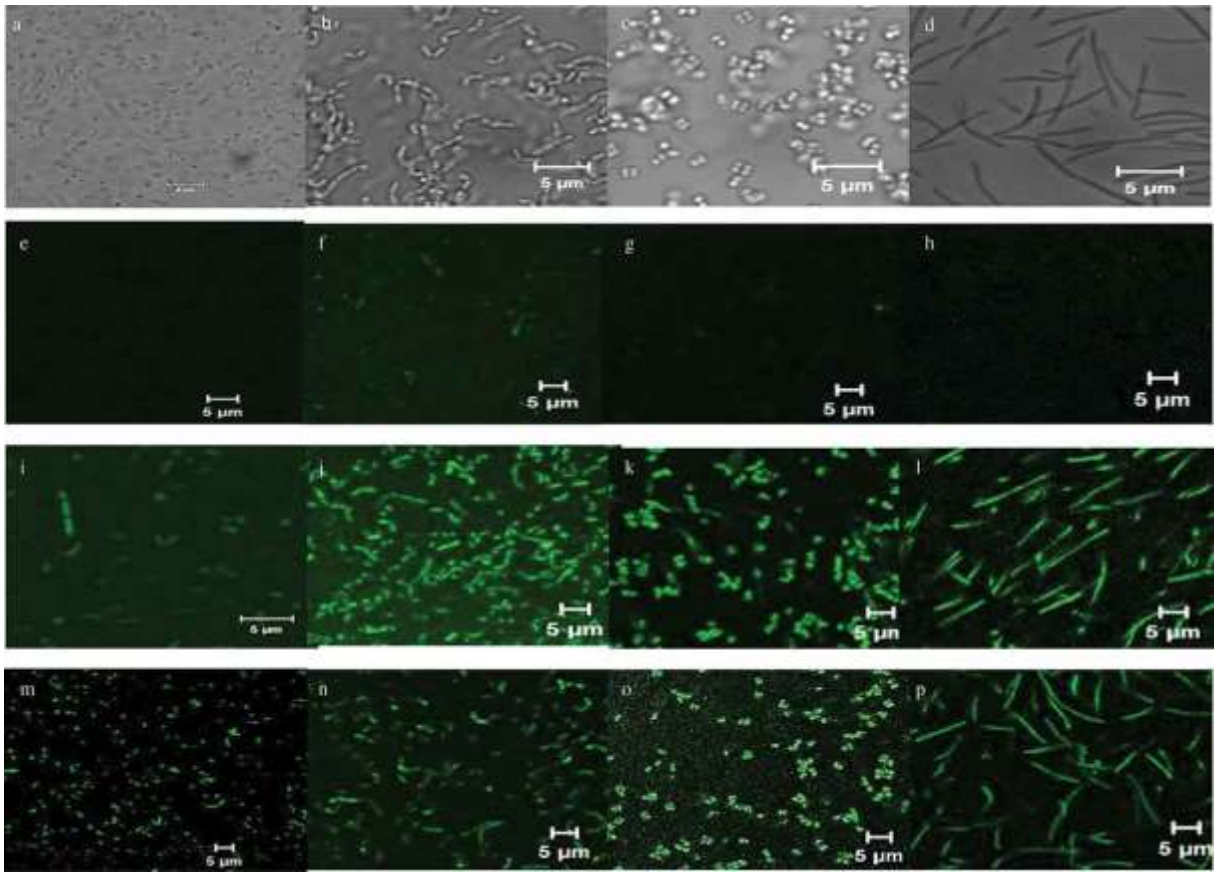

Figure 2. Visualisation of interaction between viable LAB cells and fumonisins. Viable cells visualised under white light: *L. plantarum* R1096 (a), *L. plantarum* FS2 (b), *P. pentosaceus* D39 (c) and *L. delbrueckii* CIP 57.8T (d). Viable cells only, viewed under CLSM: *L. plantarum* R1096 (e), *L. plantarum* FS2 (f), *P. pentosaceus* D39 (g) and *L. delbrueckii* CIP 57.8T (h). Viable cells mixed with derivatised 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 derivatised FB_2 viewed under CLSM: *L. plantarum* R1096 (m), *L. plantarum* FS2 (n), *P. pentosaceus* D39 (o) and *L. delbrueckii* CIP 57.8T (p).

1.2. Visualization of interaction between viable LAB cells and fumonisins

L. plantarum R1096 cells viewed under white light indicated were non-aggregated (Fig. 2 A). When the same cells were viewed under CLSM it was observed they were non-fluorescent (Fig. 2 E). However, subsequent to their mixing with derivatized fumonisins and visualization under CLSM, these cells appeared fluorescent green (Fig. 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 B) and non-fluorescent (Fig. 2 F). These cells were observed to be "coated" with the fluorescing FB_1 and FB_2 (Fig. 2 J and N) under CLSM indicating binding of the fumonisins to the surface of the LAB cell wall. *P. pentosaceus* D39 cells were non-fluorescent and arranged in tetrads under white light (Fig. 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 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 D) and non-fluorescent (Fig. 2 H). Once mixed with the derivatized fumonisins they also fluoresced green under CLSM (Fig. 2 L and P).

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. 3 A) for *L. plantarum* R1096, *L. plantarum* FS2 (Fig. 3. B), *P. pentosaceus* D39 (Fig. 3. C) and *L. delbrueckii* CIP57.8T (Fig. 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. 3 F). Even in cell death *L. plantarum* R1096 (Fig. 3 I) and *L. plantarum* FS2 (Fig. 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. 3 M) and *L. plantarum* FS2 (Fig. 3 N) and for FB_2, Fig 3 Q for *L. plantarum* R1096 and Fig. 3 R for *L. plantarum* FS2. Similarly, *P. pentosaceus* D39, when viewed under white light (Fig. 3 G) indicated a similar degree of aggregation of tetrad formation as to viable cells and also indicated no natural fluorescence under CLSM (Fig. 3 K). However, when combined with derivatized fumonisins, the tetrad cells fluoresced green with the FB_1 (Fig. 3 O) and FB2 (Fig. 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. 3 H), including no natural fluorescence (Fig. 3 L). Consistent to viable cells mixed with derivatized fumonisins caused the elongated cells to be coated and fluoresced green under CLSM (Fig. 3 P and T).

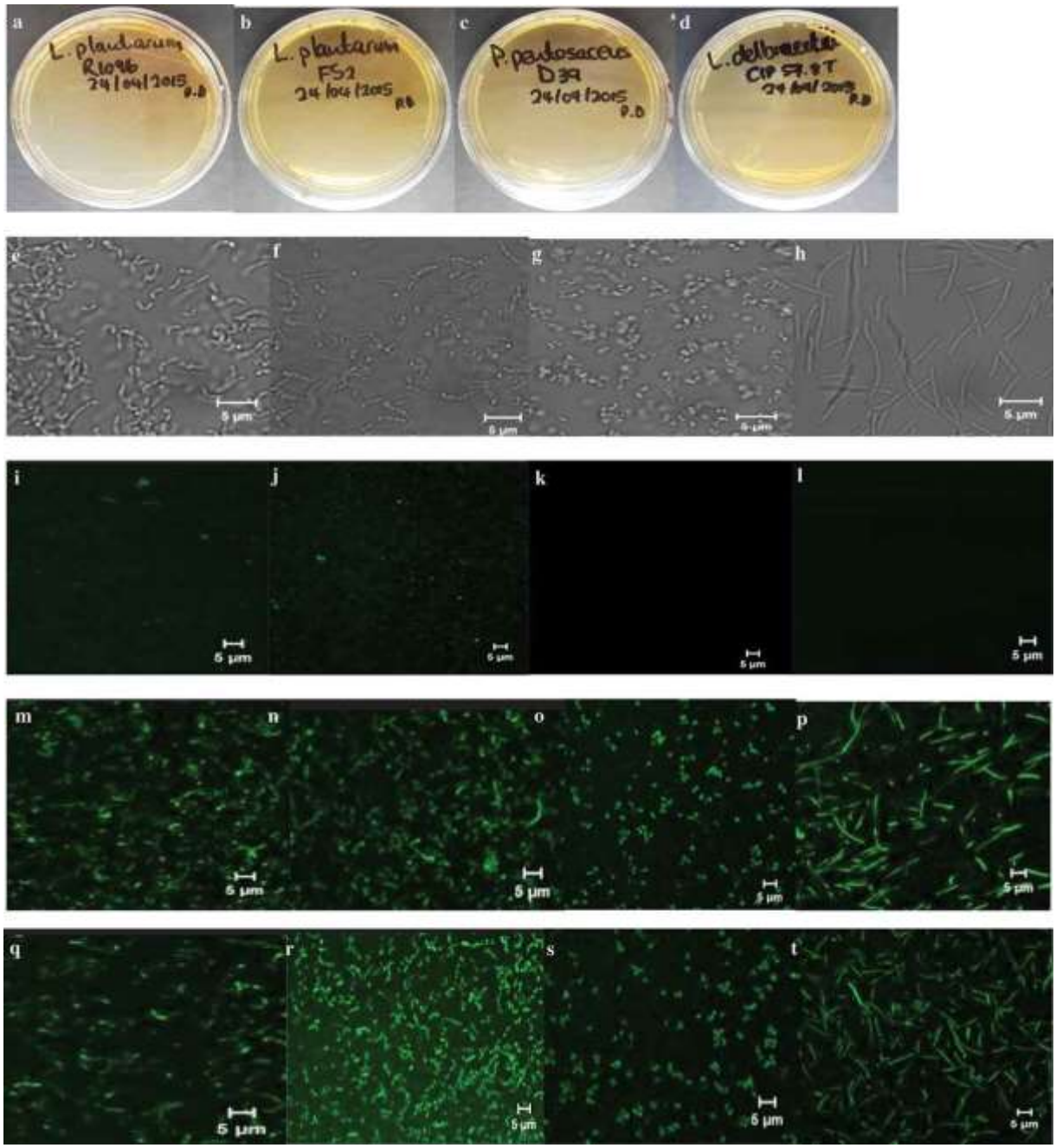

Figure 3. Visualisation of interaction between non-viable LAB cells and fumonisins. Agar plates of non-viable cells: *L. plantarum* R1096 (a), *L. plantarum* FS2 (b), *P. pentosaceus* D39 (c) and *L. delbrueckii* CIP 57.8T (d). Non-viable cells visualised under white light: *L. plantarum* R1096 (e), *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 derivatised 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 derivatised 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. Quantification of fumonisins

2.1. Quantification of fumonisins bound by viable LAB cells

During fermentation of ogi, the baseline parameter of pH is 4 and temperature is 30 ^{o}C, hence these parameters were used to obtain baseline quantification of binding between the LAB strains and fumonisins (B_1 and B_2). Figure 4 illustrates the quantified FB_1 and FB_2 bound by LAB as well as the difference in levels of fumonisins bound by different LAB strains. Binding 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 measured (P = 0.0009), with the *L. plantarum* (R 1096) binding 93%, *L. plantarum* FS2, 92%, *P. pentosaceus,* 85% and *L. delbrueckii* CIP 57.8T binding the least at 81%. The greatest difference in binding of the fumonisin B_1 and B_2 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 highest amounts of fumonisins while *P. pentosaceus* D39 bound the least.

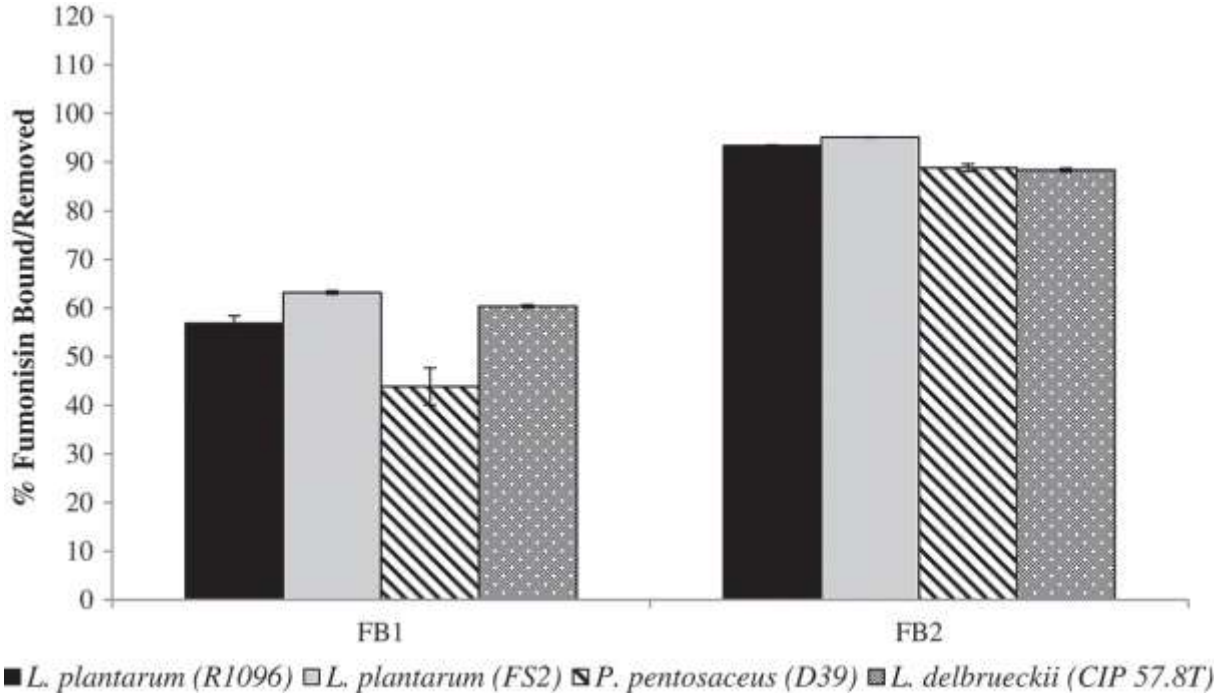

Figure 4. Effect of pH of 4 and incubation temperature of 30°C on the percentage of fumonisins (B_1 and B_2) bound by viable cells of LAB strains.

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

Binding of fumonisins by viable cells was compared to that bound by non-viable LAB cells as a means of differentiating the mechanism of binding i.e. adsorption vs biotransformation (Fig. 5). The ANOVA test for an overall difference between strains for viable and non-viable LAB cells 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 observed for FB_1 (P <0.0001) and FB2 (P = 0.0083) when comparing non-viable cells of all strains, 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 only indicated a significant statistical difference between the binding for the viable and non-viable cells for FB_1 (P = 0.0375), FB_2 binding levels were similar

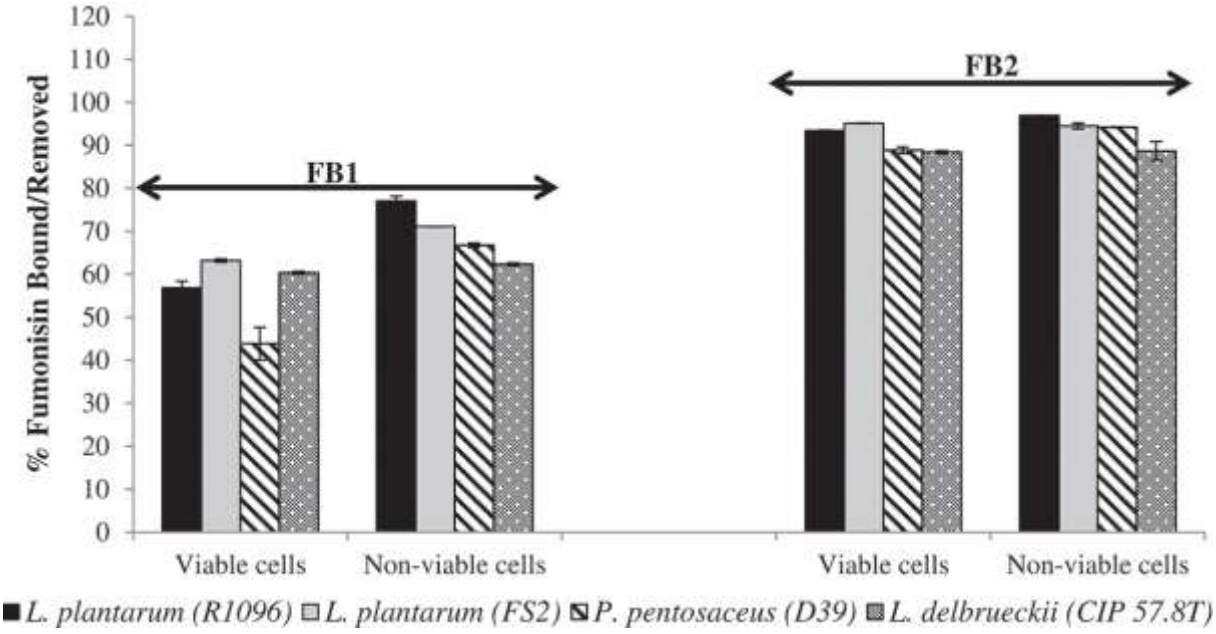

Figure 5. Comparison of fumonisin (B_1 and B_2) levels bound by viable and non-viable LAB cells at pH 4 and incubation temperature of 30°C.

2.3. Reproducibility of fumonisin binding capacity by viable LAB cells

Figure 6 compares the percentage of 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 bound FB_2. 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 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. There were no differences between levels of FB_1 and FB_2 bound by each LAB strain in each trial ($P > 0.05$), indicating reproducibility and reliability of the results obtained.

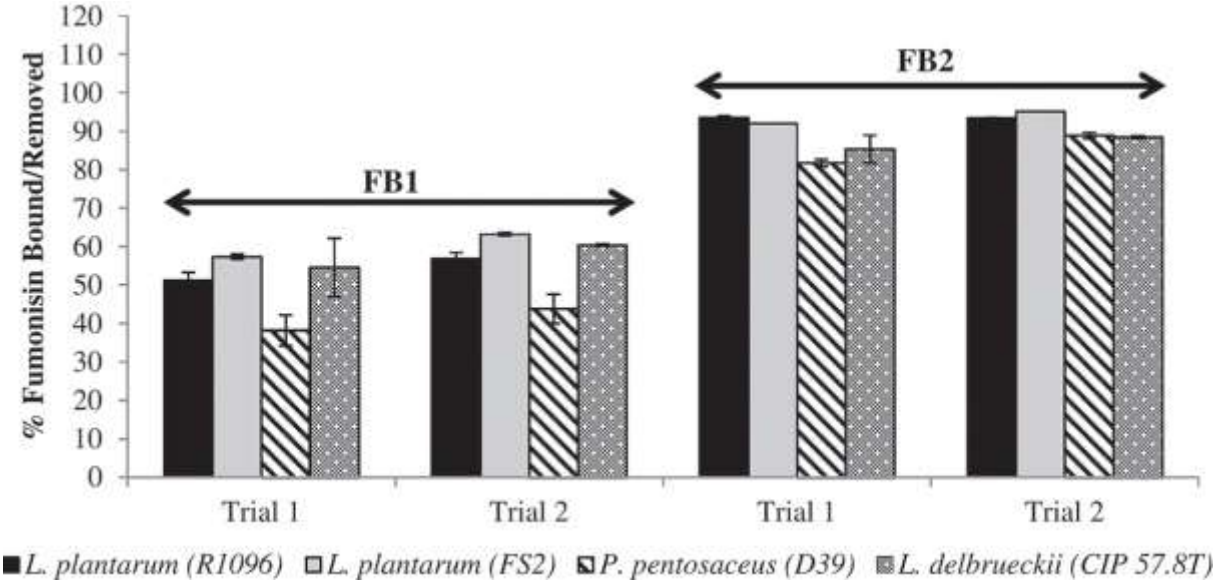

Figure 6. Comparison of fumonisin (B_1 and B_2) levels bound by viable LAB strains at pH 4 and incubation temperature of 30°C in independent experiments (Trial 1 and Trial 2).

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 CLSM and 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. Comparison of the various images recorded under white light and CLSM for each of the strains and combined with derivatized FB_1 and FB_2, indicated successful binding interaction.

Different methods were investigated for their ability to fluorescently tag both the LAB cells and the fumonisins with opposing/dual fluorescence in order to visualise the fluorescent binding interaction using 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 confirmed that the LAB cells were non-viable after the heat shock treatment by their inability to form colonies on agar plates, showing that they had not just sustained damage to the membrane, as discussed by Zotta *et al.* (2012). 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 interaction with controls in place.

Clarification of the mechanism of toxin removal, i.e. adsorption vs biotransformation, has been the subject of many experiments. 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 confirmed that fumonisins bind to the LAB cells and that it was 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 the derivatized fumonisins can be seen binding externally to the cell walls of the LAB strains. Our results were in correlation with previous studies which 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 its structural organization, chemical properties of the surface constituents and conformation of the surface macromolecules (Schar-Zammaretti and Ubbink, 2003). Due to the differences 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 FB1 and FB2 (Delcour *et al.*, 1999) explaining the higher binding ratio seen for the non-viable LAB cells. Observation of the cell wall surface area would lead to the conclusion 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 2.0-9.0µm) (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 that 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. This indicates 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 suggest that when under heat stress, the cell wall structure of *L. plantarum* cells is easily disrupted/disintegrated and therefore provide more favourable binding sites than *L. delbrueckii*. The differences between the LAB strains such as varying numbers of peptidoglycans, teichoic acids, proteins and polysaccharides, which allow for strain identification (Niderkorn *et al.*, 2009; Zhao *et al.*, 2016), could also contribute to differences in amounts of fumonisin binding between the strains (Schar-Zammaretti and Ubbink, 2003). More research needs to be undertaken relating to cell wall structure and the manner in which binding occurs in viable cells. Additionally, the studies can determine how cell wall disruption/disintegration occurs, which allows non-viable cells to bind fumonisins.

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). The fumonisins molecules carry different surface electrostatic potentials, which indicates that each molecule would have preferential, different binding sites on the LAB cell wall, demonstrating the misconception that they will compete for the same binding sites on the LAB cell wall. It was expected that the fumonisins will have the same binding site and therefore would compete for binding as reported by El-Nezami *et al.* (2002) for zearalenone and α-zearalenol, where both these 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 FB1 to the binding sites of LAB cell walls (Niderkorn *et al.* 2006a; 2009) and thereby decreased its binding affinity. The other reason could be that FB2 has a "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 biospecific 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). However, physical photographic observation of the binding (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 showed the entire cell to be fluorescing green.

We indicated 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

Conclusion

Indigenous LAB from traditional African fermented maize-based products (ogi and mahewu) bind fumonisins (B_1 and B_2). When present in these fermented maize-based products, these LAB strains can serve as detoxifying agents to reduce toxicity of fumonisins and, since they are also probiotics, additionally confer beneficial effects to the consumers, especially those in poor rural communities who consume these products on a daily basis. Thus, these LAB strains have the potential ability to improve the safety of traditional fermented cereal products. The main limitation of this study was that no visual discrepancies, which could serve as an indication for differences in binding, could be observed between the strains, due to both fumonisins fluorescing the same. 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 visualization of binding interaction between LAB and fumonisins, the use of atomic force microscopy is recommended as it could potentially also show specificity per site and per toxin,.

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