# THE MAIZE TRUST

# FINAL REPORT FOR COMPLETED MYCOTOXIN RESEARCH PROJECTS

## Project no 16/05

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| **Closing date :** | **29 May 2020** |

***1. Title of research project***

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| **Enzymatic degradation of the fumonisin mycotoxins during commercial dry milling of maize under experimental conditions.** |

***2. Personal details*** *(refer to application)*

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|---|---|
| **Title of applicant:** | Dr |
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| **Citizenship:** | South Africa |

***3. Contact details of applicant*** *(refer to application)*

| | |
|---|---|
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***4. Project duration and funding overview*** *(refer to application)*

| | | |
|---|---|---|
| **Duration of project:** | 2017-2019 | |
| **Total annual budget of project:** | R 250 401 | |
| **Annual amount requested from Maize Trust:** | R 250 401 | |
| **Other sources of funding for this project:** | **Contributor** | **Amount requested / agreed** |
| | - | - |

***5. Summary*** *(description of the project, capturing the main findings; maximum 250 words)*

The present study evaluated fumonisin esterase FumD (EC 3.1.1.87) (FUM*zyme*®; BIOMIN, Austria) for detoxification of $FB_1$ in a commercial dry milling experimental plant utilising whole maize. The enzyme was introduced to maize during conditioning, involving softening of the pericarp by increasing the % moisture content followed by 4 h 10 min rolling and standing. The efficacy of enzymatic detoxification of $FB_1$ through the complete milling process, as well as the distribution of $FB_1$ and $HFB_1$ in maize milling products intended for human food and animal feed production, was determined by utilizing a validated LC-MS/MS method. During the water control treatment (0 U/kg maize), $FB_1$ was ± 3-fold concentrated in Total hominy feed resulting in unacceptable high levels (4081 µg/kg). $FB_1$ levels were low in Super (193 µg/kg) and Special maize meal (646 µg/kg), and Semolina (447 µg/kg), intended for human consumption. Introduction of FumD (40 U/kg) impacted mainly Total hominy feed, which constitutes up to 30% of the total milling fraction and is currently primarily used for incorporation into animal feed. The enzyme treatment resulted in 99% reduction of $FB_1$ in Total hominy feed, Super (0% reduction) and Special maize meal (15% reduction), and Semolina (37%), with an approximate 1:1 µmole conversion ratio of $FB_1$ into the formation of $HFB_1$. The FumD treatment had no significant effect on the nutritional composition of maize. Greatly reduced $FB_1$ levels in Total hominy feed following enzymatic dry milling could open up new applications, i.e. incorporation as a source of fibre in maize-based food intended for human consumption, extended use in the animal feed industry and applications in subsistence farming communities using rudimentary milling processes.

***6. Objectives*** *(refer to application)*

***6.1 Strategic objectives*** *(alignment with Maize Trust objectives)*

| **Strategic objectives** | Yes/No |
|---|---|
| To support the establishment of the magnitude of mycotoxin contamination of maize during the stages of its production, storage, and processing in South Africa. | **NO** |
| To support the regular monitoring of the occurrence of the fumonisins, aflatoxins, zearalenone, and trichothecenes (DON and NIV) in locally produced and imported maize. | **NO** |
| To support the determination of the factors which contribute to mycotoxin contamination during the production (pre-harvest), storage (post-harvest) and processing of maize. | **NO** |

| Strategic objectives | Yes/No |
|---|---|
| To support the development of practical, affordable and environmentally sound methods to manage toxigenic fungi in maize, with particular emphasis on the introduction of resistance in local maize cultivars. | **NO** |
| To support the development of sound mycotoxin risk management practices in the maize supply chain to ensure the delivery of safe products to the consumer. | **YES** |

***6.2 Project objectives*** *(list main objectives)*

1. Collection of a bulk commercial maize sample (120 kg) for the project:

   The sample should present commercial maize suitable for milling [Total fumonisin $B_1$ and $B_2$ concentration below the RSA regulatory level of 4000 µg/kg (DOH, 2016)].

2. Preliminary laboratory experiments: Determination of the optimal FumD activity to use during the conditioning step in subsequent milling experiments.

3. FumD dry milling of maize, collection of milling fractions and combining milling products.

4. Reconstruction of whole maize, degermed maize and Total hominy feed.

5. Determination of $FB_1$, $FB_2$, $FB_3$ (FB) and hydrolysed $FB_1$ ($HFB_1$) concentrations in the original maize sample, conditioning samples and milling products.

6. Determination of the effect of the enzyme treatment on the nutritional composition of maize.

7. Determination of FumD residues in milling products.

***7. Work plan*** *(refer to application)*

***7.1 Work plan as stated in the application*** *(list main tasks)*

1. Compilation of Material Transfer and Non-Disclosure Agreements between CPUT and BIOMIN, Austria.

   The association with BIOMIN is purely on a scientific level and a clause has been included stating that the results from this study will only be used for publication purposes.

2. Maize samples [CPUT; Pioneer Foods; Mass Spectrometry Unit, Central Analytical Facility (CAF), Stellenbosch University (SU)].

   An experimental bulk commercial maize sample (120 kg) was obtained, representing commercial maize suitable for milling [Total fumonisin $B_1$ and $B_2$ concentration below RSA regulatory levels (DOH, 2016)].

3. Preliminary laboratory experiments (CPUT; Pioneer Foods; CAF, SU).

   Determination of the optimum enzyme activity to be used during the conditioning step of commercial dry milling of maize. This experiment was performed in the pilot experimental milling plant of Pioneer Foods. The optimal enzyme activity was used in subsequent milling experiments.

4. Enzymatic dry milling of maize (CPUT; Pioneer Foods).

   The experiments were performed in the experimental pilot plant of Pioneer Foods.
   - Addition of FumD during the conditioning step.
   - Dry milling: Conditioning; Degerming; Experimental dry milling.

5. Reconstruction of whole maize, degermed maize and Total hominy feed (CPUT; Pioneer Foods).

6. Determination of FB and hydrolysed $FB_1$ ($HFB_1$) concentrations in the original maize sample, milling products and reconstructed maize samples (CPUT; CAF, SU).
   - Extraction and quantification of FB concentrations in milling products utilizing analytical standards produced by CPUT and a validated LC-MS/MS analytical technique.

7. Determination of the effect of enzyme treatment on the nutritional composition of maize [CPUT; Southern African Grain Laboratory (SAGL)].
   - Total starch content; Total protein content; Amino acid profile: alanine, arginine, aspartic acid; cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophane, tyrosine, valine; Total dietary fibre; Total fat (crude); fatty acid content.

8. Determination of enzyme residues in maize milling products (BIOMIN).

***7.2. Achieved tasks according to the stated work plan*** *(list measurable units as milestones and provide an indication of progress made, e.g. tasks achieved or not [add additional rows if necessary])*

| **Milestones** | **Achievements** |
|---|---|
| Material Transfer and Non-Disclosure Agreements between CPUT and BIOMIN, Austria. | Task achieved. |
| Acquisition of a bulk commercial maize sample (120 kg) suitable for milling [Total $FB_1$ and $FB_2$ concentration below the RSA regulatory level of 4000 µg/kg (DOH, 2016)]. | Task achieved (CPUT; Pioneer Foods). |
| Determination of FB and $HFB_1$ concentrations in the original commercial maize sample. | Task achieved.<br>FB and $HFB_1$ extraction and LC-MS/MS quantification; data analyses (CPUT; CAF, SU). |
| Evaluation of different FumD activities during the conditioning step of dry milling of maize. | Task achieved:<br>1. FumD enzyme activities incorporated: 0, 1, 2, 4, 8, 16 and 32 U per 100 g maize kernels.<br>2. Conditioning experiments performed (CPUT; Pioneer Foods).<br>3. FB and $HFB_1$ extraction and LC-MS/MS quantification (CPUT; CAF, SU); data analyses.<br><br>An enzyme activity of 4 U/100 maize (= 40 U/kg maize) was selected for the complete dry milling experiments. |

| Milestones | Achievements |
|---|---|
| Complete dry milling of maize utilizing a FumD activity of 40 U/kg maize kernels: Two independent runs including each a control and an enzyme run. | Task achieved:<br>1. Complete enzymatic dry milling of maize (Two independent runs, each including an enzyme and a control run (CPUT; Pioneer Foods).<br>2. Collection of milling fractions (control and enzyme runs).<br>3. Combination of fractions to obtain the four milling products (Special and Super maize meal; Semolina and Total hominy feed). |
| Preparation of reconstructed whole maize, degermed maize and Total hominy feed (control and enzyme runs). | Task achieved (CPUT; Pioneer Foods):<br>1. Determination of the percentage of each milling fraction in relation to whole maize.<br>2. Combination of calculated percentages for each milling product, thereby providing the finest representatives of the original maize fractions. |
| Determination of FB and $HFB_1$ concentrations in milling products and reconstructed maize samples (control and enzyme runs). | Task achieved:<br>FB and $HFB_1$ extraction and LC-MS/MS quantification; data analyses (CPUT; CAF, SU). |
| Determination of the effect of the FumD treatment on the nutritional composition of maize. | Task achieved:<br>Determinations of moisture content, % starch content, % crude protein, total dietary fibre, amino acid content and fatty acid content in the original composite maize, control reconstructed maize and the reconstructed enzyme treated maize (CPUT; SAGL). |
| Determination of FumD residues and activity in maize milling products. | Task achieved (BIOMIN). |

***8. Detailed report covering the research during the full grant period*** *(introduction, methods, results, tables, figures and discussion)*

### 8.1. Introduction

Maize is an important staple food commodity in southern Africa and frequently contaminated with unacceptable levels of the fumonisin B (FB) mycotoxins, specifically in certain subsistence farming communities where regulations are either lacking or not enforced (Alberts et al. 2019a; 2019b). Although commercial maize is contaminated with lower levels, chronic exposure could be a risk factor for disease development in impoverished communities (Burger et al., 2010; Tshalibe et al., 2020).

Milling of maize is a physical process regarded as the first step in the production of maize-based products by removing the outer structures, the hull, pericarp (bran), germ and tip cap

to expose the endosperm, which is then utilised to produce various milling products such as the grits, germ, meal and flour (Burger et al., 2013). In South Africa, dry milling comprises three main steps, i.e. conditioning and degerming, to remove the outer kernel structures and milling, during which various milling products are produced. These include Special maize meal, Super maize meal, Semolina and the Milling hominy feed (large pieces of pericarp and hull with some endosperm attached and clean medium sized pieces of bran and hull as well as the remnants of the tip cap). The milling products mostly utilised as human foodstuffs are the flaking grits and flour (Special maize meal), whereas the bran and germ milling products (Total hominy feed) are mainly used for animal feed (Sharma et al., 2008) and as a source of fibre in human foods (Ai and Jane, 2016). Maize bran produced during dry milling is preferred by the food industry above other sources of bran, being easier to process and incorporate into extruded food products and bakery goods.

Due to the complexity of the milling process mycotoxins are distributed, yielding higher or lower levels in the various milled products (Burger et al., 2013; Vanara et al., 2018). Fumonisin mycotoxins are known to be mainly concentrated in the surface layers of the maize kernel and the resultant milling products such as the germ, hull, pericarp and tip cap product (Total hominy feed) contain high levels due to fungal colonisation of mainly the outer layers of the kernel with contamination progressing into the inner layer as a function of time (Brera et al., 2004; Kent and Evers, 1994). During dry milling, the degerming process concentrate the fumonisins mainly in the Hominy fraction as reported previously (Burger et al., 2013). Therefore, the efficiency of degerming also affects the yield and composition of the grits whilst impacting on the levels of fumonisins in milling products. During subsequent milling of degermed maize, total fumonisins were more associated with Special maize meal known to contain less endosperm and some of the pericarp, hull, germ and tip cap. The lowest percentage total fumonisin distribution was located in Super maize meal consisting of the coarse granulated endosperm. The level of mycotoxin contamination in whole maize and the distribution thereof between milling products is a food safety challenge. Studies investigating the effect of commercial milling on $FB_1$ confirmed a reduction in contamination levels in the resultant dry milling products intended for human consumption.

The lack of effective and environmentally safe chemical control methods for fumonisin B (FB) in maize has led to investigations into biologically safe alternatives to prevent these contaminants from entering the food chain (Alberts et al., 2016; 2019b). Enzymatic detoxification of FB has become a promising approach, with a focus on targeted modification of the chemical structures of the fumonisins by enzymatic cleavage or conversion of chemical bonds/groups that play a key role during cytotoxicity (Heinl et al., 2010; Grenier et

al., 2017; Alberts et al., 2019b). The discovery of a fumonisin esterase FumD (EC 3.1.1.87), FUM*zyme*® (BIOMIN, Austria) capable of effectively detoxifying the fumonisin mycotoxins by de-esterification and formation of hydrolysed $FB_1$ ($HFB_1$), resulted in the development of a FumD FB reduction method in a maize kernel enzyme incubation mixture (Alberts et al., 2019b). FumD detoxification resulted in significant reduction (≥80%) in FB contaminated maize kernels with an approximate 1:1 µmole conversion ratio of $FB_1$:$HFB_1$. It has been suggested, based on the outcomes of the FumD FB reduction method, that it could find broader application, i.e. commercial maize-based milling practices.

The present study evaluated fumonisin esterase FumD for detoxification of $FB_1$ in a commercial dry milling pilot plant under experimental conditions. The enzyme was introduced during the initial conditioning step, prior to the degerming step of the milling process. The study evaluated the efficacy of FumD detoxification of FB as well as the distribution of $FB_1$ and $HFB_1$ in milling products intended for human food and animal feed production.

## 8.2. Materials and methods

### *8.2.1. Chemicals*

Methanol, acetonitrile, formic acid (HPLC grade) and Whatman filter paper were obtained from Merck (Darmstadt, Germany). Water for all experiments was successively purified by reverse osmosis followed by Milli-Q water purification (Millipore, Massachusetts, USA).

### *8.2.2. Experimental maize sample*

Commercial whole white maize (120 kg) was obtained from a prominent South African grain-based manufacturing company. The bulk was thoroughly mixed and divided into six subsamples. Four incremental samples (250-300 g each) were taken from each subsample, pooled and thoroughly mixed. Each sample was further divided into two additional subsamples where after one (3.3 kg) was randomly selected for FB analyses. Samples were ground in a laboratory mill (Falling Number AB, Stockholm, Sweden) and kept in airtight containers at -20˚C until analysed. The remaining pooled samples were kept at 20˚C until used in the milling experiments. Control maize containing no FB was obtained from the Southern African Grain Laboratory (SAGL) (Pretoria, South Africa) and used for the preparation of a maize extract used for matrix-matched LC-MS/MS calibration curves for FB analyses.

### *8.2.3. FumD enzyme preparation*

A fumonisin esterase, designated FumD (EC 3.1.1.87; FUM*zyme*®), was obtained from BIOMIN (Tulln, Austria) with a specific activity of 10 000 U/g. One unit is the enzymatic activity defined to release 1 µmol tricarballylic acid per minute from 100 µM $FB_1$ in 20 mM Tris-HCl buffer pH 8.0 containing 0.1 mg/ml bovine serum albumin at 30°C. A stock FumD solution (20 U/ml) was prepared in distilled water.

### *8.2.4. Preliminary experiment for selection of optimal FumD activity for $FB_1$ hydrolysis during conditioning phase*

Whole maize was screened and cleaned from non-kernel impurities by hand and the % moisture content determined with a Grain Analysis Computer (Dickey-John GAC 2100, USA) (five replicates). Maize (100 g) was weighed out in containers (300 ml) with tight fitting lids. Conditioning was conducted by adding water to increase the moisture content of maize in order to create differential swelling resulting from the higher absorbing moisture of germ and pericarp, that lead to a loss of tissue connection between the germ and the endosperm and a faster removal of these fractions (Eckhoff et al., 2004; Vanara et al., 2018). It is important to note that each commercial mill is likely to have its own particular conditioning procedure. Conditioning of samples was performed in two stages, i.e. (i) softening and (ii) loosening of the pericarp in order to facilitate the degerming process (Burger et al., 2013). The following formula was used to calculate the volume of water to be added to obtain a % maize moisture content of 14.5% in the samples.

Mass x (Target moisture % - Actual moisture %) / (100-Target moisture %).

FumD solutions, 2, 4, 8, 16 and 32 U per 100 g maize, were prepared in distilled water and added (± 2 ml) during the first conditioning phase to facilitate a moisture content of 14.5%. Five replicates per treatment and reference water controls were included. The containers were transferred to a sealed bucket and rolled horizontally on an in-house rolling device at ambient temperature for 1 h and left standing for an additional 3 h. For the second conditioning stage, distilled water (± 2 ml) was added to each sample to 16.5% moisture content and rolled horizontally for 10 min. The maize was ground in a laboratory mill (Falling Number AB, Stockholm, Sweden) to a fine meal and kept in airtight containers at -20°C until FB and $HFB_1$ analyses.

*8.2.5. FB degradation during dry milling of maize*

Whole maize was weighed out (3 x 1.5 kg) in buckets (5 L) with tight fitting lids. FumD solution was added to a final concentration of 40 U/kg based on the optimal $FB_1$ conversion obtained during the preliminary experiment and conditioning conducted as described above. Degerming was performed as described by Burger et al. (2013) producing two products, i.e. (i) germ (a mixture of the germ hull, pericarp and tip cap) and (ii) degermed maize (endosperm and remnants of the germ). The resultant germ and degermed maize were weighed and the germ stored at -20°C in sealed containers. The degermed maize was further processed in an experimental dry milling plant (Buhler MIJJ-202 laboratory mill, Buhler, Switzerland), producing nine milling fractions from each sample (B1-3; S1-3; H1-2 and Semolina) (Burger et al., 2013). The fractions were combined into four main milling products, i.e. Special and Super maize meal; Semolina and Milling hominy feed (the latter containing kernel surface layers, i.e. pericarp, hull, bran and tip cap). Two independent experiments were performed and reference water controls were included.

*8.2.6. Reconstruction of whole maize, degermed maize and Total hominy feed*

The percentage of each milling product produced during dry milling was recorded (Table 1), ensuring that the correct ratios are used during reconstruction of maize fractions (Table 2). Reconstructed milling products were prepared by combining calculated percentages of each fraction, thereby providing the finest representatives of the original maize fractions (Burger et al., 2013). Such samples are homogenous and LC-MS/MS analysis results in accurate mycotoxin quantification.

Table 1. Percentages (%) of milling products obtained during dry milling.

| **Maize sample** | **Special maize meal (B1 - B3) (%)** | **Super maize meal (S1 - S3) (%)** | **Semolina (%)** | **Germ (%)** | **Milling hominy feed (H1 - H2) (%)** |
|---|---|---|---|---|---|
| Water control | 11.2 | 44.8 | 15.3 | 23.7 | 5.0 |
| FumD | 11.3 | 43.2 | 16.4 | 22.8 | 6.4 |

Table 2. Percentages (%) of milling fractions used to reconstruct whole maize, degermed maize and Total hominy feed.

| Maize | Special maize meal (%) | Super maize meal (%) | Semolina (%) | *Reconstructed Total hominy feed | | |
|---|---|---|---|---|---|---|
| | | | | Germ (%) | Milling hominy feed H1 (%) | Milling hominy feed H2 (%) |
| **Reconstructed whole maize** | | | | | | |
| Water control | 11.2 | 44.8 | 15.3 | 23.7 | 2.8 | 2.2 |
| FumD | 11.3 | 43.2 | 16.4 | 22.8 | 3.2 | 3.1 |
| **Reconstructed degermed maize** | | | | | | |
| Water control | 14.7 | 58.6 | 20.0 | - | 3.7 | 2.9 |
| FumD | 14.6 | 56.0 | 21.2 | - | 4.2 | 4.0 |

*Reconstructed Total hominy feed: germ + milling hominy feed fractions (H1 and H2).

### *8.2.7. FB and $HFB_1$ analyses*

$FB_1$, $FB_2$, $FB_3$ and $HFB_1$ concentrations were determined in the original maize sample (five replicates), the water control and FumD treated samples obtained from conditioning, as well as in milling products and reconstructed whole maize (three to five replicates).

Analytical standards of $FB_1$, $FB_2$, $FB_3$ and $HFB_1$ (purity >97%) were prepared at the Cape Peninsula University of Technology, South Africa, according to the methods of Cawood et al. (1991) and Gelderblom et al. (1993). Stock solutions of the individual purified fumonisin standards were prepared [1 mg/ml in acetonitrile-$H_2O$ (1:1)] and aliquots used to prepare an evaporated working solution containing the fumonisin standards at individual concentrations of 5 µg/ml (Alberts et al., 2019b). For compiling matrix-matched calibration curves, five working standard dilutions were prepared with blank maize matrix extract as solvent, as described below.

Validated extraction and LC-MS/MS quantification methods were used (Tables 3 and 4) (Alberts et al., 2019b). Briefly, 100 ml of extraction solvent [methanol: acetonitrile: water (25:25:50; v/v/v)] was added to ground maize (10 g) and placed on a shaker (80 rpm) for 20 min. The extracts were subsequently centrifuged (4000 x g) in a refrigerated Sorvall RC-3B centrifuge (DuPont, Norwalk, Connecticut, USA) at 4$^{o}$C for 10 min. The supernatant was

diluted (1:1) with methanol:water (25:75), filtered (Whatman No 4 filter paper) and filtrates analyzed by direct injection into the LC-MS/MS. FAPAS (London, England) quality control reference maize samples (Cat no T22123QC), containing the mycotoxins in known concentration ranges, were included. Matrix-matched standard solutions for calibration curves were prepared utilising an extract prepared from control maize.

Quantification of FB and $HFB_1$ in the original maize sample and milling products was performed by the Mass Spectrometry Unit of the Central Analytical Facility of Stellenbosch University, South Africa. FB and $HFB_1$ levels in the original maize sample were confirmed by SAGL. The mycotoxins were separated on a reversed-phase BEH $C_{18}$ column (2.1x100 mm; particle size 1.7 µm) (Waters, Milford, Massachusetts, USA) and analysed with positive electrospray ionisation (EI) in the multiple reaction monitoring (MRM) mode in a Waters Acquity Ultra high performance Liquid Chromatograph (UPLC) coupled to a Tandem Quadrupole Mass spectrometer (Waters Xevo TQ MS, Milford, Massachusetts, USA). Eluent A was water and eluent B was methanol, both containing 0.1% formic acid. The chromatographic method held the initial mobile phase composition (15% B) constant for 2 min, followed by a linear gradient to 100% B within 3 min. This final condition was held for 3 min, followed by 8 min of column re-equilibration at 15% B. The flow rate of the mobile phase was 0.35 ml/min. For each compound, one precursor and two product ions were monitored, one product ion for quantification and one for confirmation. A calibration curve consisting of five matrix-matched standards for each mycotoxin was used for quantification.

Table 3. LC-MS/MS conditions for quantification of fumonisins and hydrolysed fumonisin $B_1$ by positive ESI at capillary voltage 3.5 kV.

| **Analyte** | **Cone voltage** | **Precursor** | **Quantifier (Collision energy)** | **Qualifier (Collision energy)** |
|---|---|---|---|---|
| Fumonisin $B_1$ | 50 | 722.3 | 334.3 (40) | 352.3 (38) |
| Fumonisins $B_2$ and $B_3$ | 50 | 706.3 | 318.3 (40) | 336.3 (40) |
| Hydrolysed fumonisin $B_1$ | 25 | 406.6 | 334.3 (25) | 352.4 (20) |

Table 4. Validation of the analytical method for fumonisin analyses in maize.

| **Analyte** | **LOQ (µg/kg)** | **Spike level (µg/kg)** | **Recovery (%)** | **RSDr (%)** |
|---|---|---|---|---|
| Fumonisin $B_1$ | 3.5 | 1060 | 84 | 2 |
| Fumonisin $B_2$ | 2.8 | 925 | 66 | 4 |
| Fumonisin $B_3$ | 2.8 | 520 | 79 | 1 |

| Analyte | LOQ (µg/kg) | Spike level (µg/kg) | Recovery (%) | RSDr (%) |
|---|---|---|---|---|
| Hydrolysed fumonisin $B_1$ | 2.8 | 800 | 80 | 2 |

LOQ, Lower limit of quantification; RSDr, Relative standard deviation for repeatability.

*8.2.8. Determination of the effect of FumD dry milling on the nutritional composition of maize (% crude protein, % crude fat, total dietary fibre, % starch content, total amino acid content)*

The following protocols were used:

- Percentage moisture content [SAGL: 130ºC; 1 h; AACI 44-15.02, South African National Accreditation System (SANAS) accredited method].
- Percentage crude protein (SAGL: Dumas method; AACI 46-30.01; Soxhlet method; SANAS accredited method).
- Percentage crude fat (SAGL: Petroleum ether extraction; SANAS accredited method).
- Percentage total dietary fibre (SAGL: In-house method 102; SANAS accredited method).
- Percentage starch content (SAGL: Polarimeter method; In-house method 019).
- Total Amino acids (SAGL: In-house methods 007, 015 and 028).

*8.2.9. Determination of the effect of FumD dry milling on the fatty acid content of maize.*

Separate extraction and methylation methods (Folch and Stanley, 1957; Smuts et al., 1994) and a direct methylation method (Ulberth and Henninger, 1992; Suykhija and Palmquist, 1988; Whitney et al., 1999) were evaluated. Samples were eventually analysed with the separate extraction and methylation method, which produced more consistent results and is regarded a benchmark assay for % total fatty acids.

*8.2.10. Determination of FumD residues and enzyme activity in milling products.*

Residual FumD concentrations (FumD protein and peptide fragments) in milling products were determined by ELISA (Figure 2), and residual FumD activity with a tricarballylic acid assay (Figure 3) (BIOMIN, Austria).

*8.2.11. Statistical analyses*

The NCSS Version 11 software (2016) was used for statistical analysis. Data were subjected to natural log (ln) transformation of all variables and analysed within a generalised linear model ANOVA. Multiple comparisons were analysed using the Tukey-Kramer's multiple comparison procedure. This method provides joint simultaneous confidence intervals for all

pairwise differences between the means; and also provides the multiple comparison P-value. Generally, P<0.05 was used as statistical significance. In addition, the size of the F-ratios was used to measure relative sizes of differences.

Kindly note that parts of the statistical analyses are in progress.

## 8.3. Results

### *8.3.1. FB and $HFB_1$ concentrations and % moisture content in maize*

FB and $HFB_1$ concentrations (µg/kg) in the original maize sample (mean values with standard deviation values in brackets): $FB_1$, 1614 (129); $FB_2$, 361 (23); $FB_3$, 137 (13); $HFB_1$, 2 (1). Mean moisture content (%) of samples: 12.53 (0.12).

### *8.3.2. Percentage recovery during degerming and dry milling*

The % loss of maize during degerming and dry milling was determined by the combined mass of the degermed maize and germ obtained after degerming in relation to the weight of the reconstructed whole maize. An average % sample loss of 2.20 (0.01) was recorded for the water control maize and 2.23 (0.09) for the FumD treated maize, respectively.

### *8.3.3. Optimal FumD activity and conversion ratios during conditioning prior to degerming and dry milling*

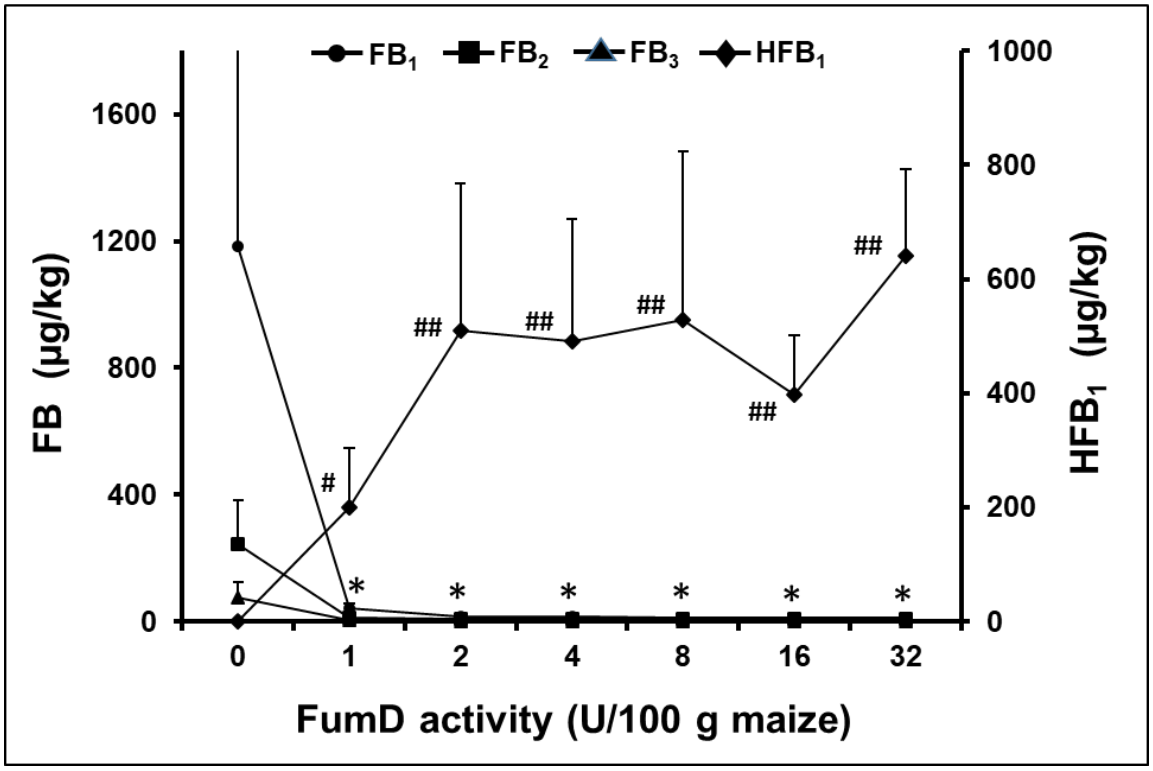
#### *8.3.3.1. $FB_1$ hydrolysis and formation of $HFB_1$ in maize as a function of FumD activity*

FB and $HFB_1$ concentrations in maize as a function of FumD activity are presented in Figure 1. FB concentrations were significantly (P<0.05) reduced as a function of an increased enzyme activity. Treatment with ≥1 U/100 g maize resulted in ≥96% reduction in total FB ($FB_T$) concentrations when compared to the water control (Table insert). No significant (P>0.05) differences existed in FB reduction between the different FumD activities utilised. The reduction in $FB_1$ concentrations coincided with a significant (P<0.05 increase in $HFB_1$ concentrations from 1 to 32 U/100 g maize.

#### *8.3.3.2. Comparative enzyme kinetics of $FB_1$ conversion*

Maximum $FB_1$ was converted in the maize samples in the presence of FumD activity between 2 and 4 U/100 g maize (Table 5). This also became evident when considering the amount of $HFB_1$ liberated, which was slightly lower although the decrease was not significant. Of interest is that the $FB_1$:$HFB_1$ ratios indicated that the conversion at the two lowest enzyme activities was delayed. Enzyme kinetics indicated that the maximum conversion rate was obtained with the 1 U/100 g maize, where after increased enzyme

activity resulted in substrate depletion and therefore a decreased conversion rate. A similar pattern became evident when considering $HFB_1$ formation kinetics, which was the highest at the two lowest enzyme activities.




| FumD activity (U/100 g maize) | 0 (Control) | 1 | 2 | 4 | 8 | 16 | 32 |
|---|---|---|---|---|---|---|---|
| Total FB (µg/kg) | 1505 (1052) | 58 (18) | 26 (8) | 26 (3) | 23 (3) | 19 (3) | 21 (4) |
| % Reduction | - | 96 (1) | 98 (1) | 98 (0) | 98 (0) | 99 (0) | 99 (0) |

Figure 1. Fumonisin B ($FB_1$, $FB_2$ and $FB_3$) and hydrolysed fumonisin $B_1$ ($HFB_1$) concentrations (µg/kg) as a function of FumD activity (0, 1, 2, 4, 8, 16, and 32 U/100 g maize) after 4 h 10 min conditioning prior to degerming. Values represent means of five replications of experiments and error bars indicate standard deviations. The statistical analyses are based on natural log (ln) transformations. The *($FB_1$), ($FB_2$), ($FB_3$) and #($HFB_1$) indicate significant ($p<0.05$) differences of means (all enzyme activities) from the water control (0 U/L) treatments. Table insert: % reduction of the total FB as a function of FumD activity after 4 h 10 min. Standard deviations are in brackets.

*8.3.4. Distribution pattern of $FB_T$, $FB_1$ and $HFB_1$ in milling products*

The degerming step resulted in an almost 70% reduction in $FB_T$ with the remainder associated with the pericarp and germ (Figure 2A). When compared to reconstructed whole maize, the bulk of $FB_T$ (72%) was associated with Total hominy feed (milling hominy + degermed hominy) following dry milling in the absence of FumD (Figure 2B). $FB_T$ was the lowest in the Super maize meal (5%) followed by Semolina (9%) and Special maize meal (15%). Milling in the presence of FumD resulted in an almost complete loss (99%) of the $FB_T$ in Total hominy feed contributing only 3% of the $FB_T$ compared to the 72% in the untreated FumD maize (Figure 2; Table insert). The Special maize meal contained the bulk $FB_T$ making up 55% with the Super maize meal and Semolina products containing in the order of 20%. The respective reduction of $FB_T$ in the Special maize meal and Semolina milling products were in the order of 7 and 50% while the content in Super maize meal increased by 10%.

The conversion of $FB_1$ into $HFB_1$ during dry milling followed a similar pattern with more than 80% and almost 100% converted in the reconstructed whole maize and Total hominy feed, respectively (Table 6). Very little conversion was noticed in Super maize meal, while 15 and 50% were converted in Special maize meal and Semolina, respectively. The $HFB_1$ formation followed a similar pattern with the highest level recorded in the Total hominy feed, however no significant difference was noticed between the $FB_1$:$HFB_1$ ratios recorded following the conditioning and milling procedure approaching the 1:1 ratio conversion.

*8.3.5. The effect of FumD dry milling on the nutritional composition of maize*

The results indicated no significant ($p>0.05$) differences between the original composite maize, the reconstructed control maize and the reconstructed FumD maize for the listed nutritional factors and amino acids (Table 7).

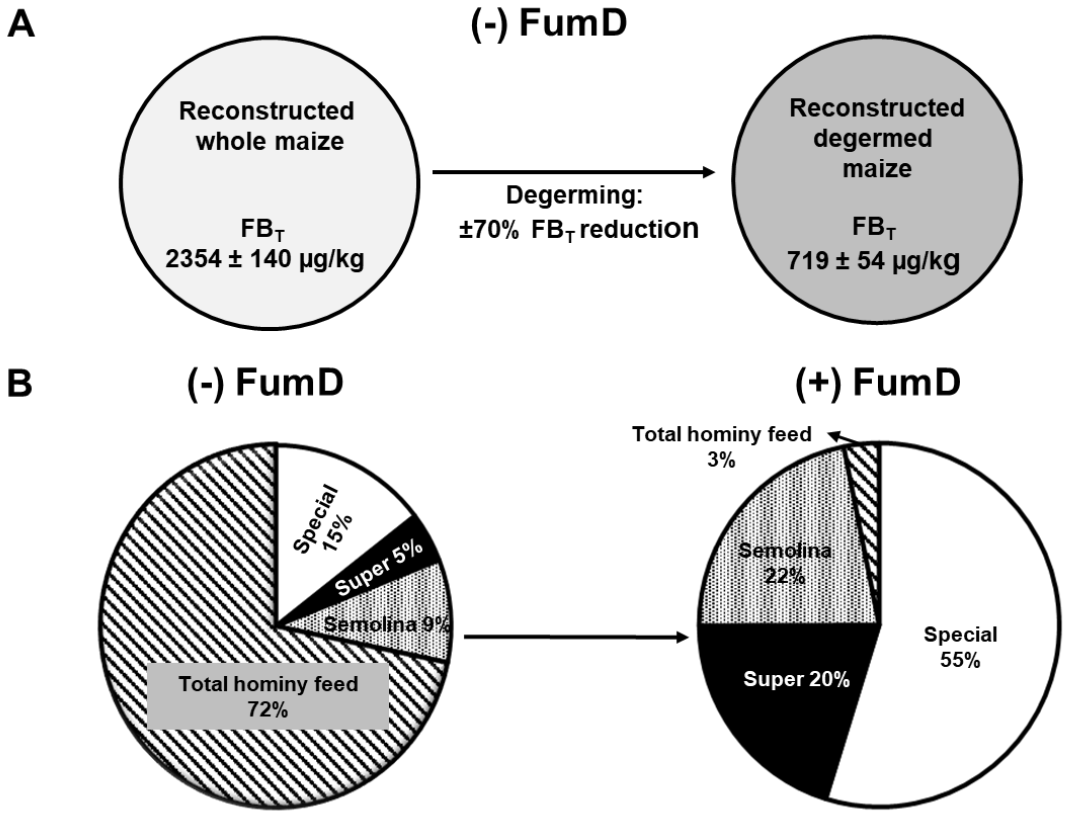
*8.3.6. The effect of FumD dry milling on the fatty acid content of maize*

No significant ($p>0.05$) differences in fatty acid content were observed between the original composite maize, reconstructed control maize and reconstructed FumD treated maize (Table 8).

Table 5. Enzyme kinetic parameters regarding rates and ratios of $FB_1$ conversion relative to $HFB_1$ formation of different FumD activities during 4 h 10 min conditioning prior to degerming.

| **FumD activity (U/100 g maize)** | **$FB_1$ (nmole/100 g maize)** | **$FB_1$ converted (nmole/100 g maize)** | **$FB_1$ hydrolysis (nmole/min/mg enzyme)** | **$HFB_1$ (nmole/100 g maize)** | **$HFB_1$ formation** (nmole/min/mg enzyme)** | **$FB_1$:$HFB_1$ nmole conversion ratio** |
|---|---|---|---|---|---|---|
| 0* | 164.22 (121.66)a | - | - | 0.25 (0.17)a | - | - |
| 1 | 5.80 (2.18)b | 158.42 (2.10)a | 6.34 (0.09)a | 48.68 (25.60)a | 1.95 (1.02)a | 4.17 (2.27)a |
| 2 | 2.50 (0.00)c | 161.72 (0.00)a | 3.24 (0.00)b | 103.06 (47.01)b | 2.06 (0.94)a | 2.13 (1.68)a |
| 4 | 2.02 (0.36)c | 162.19 (0.36)a | 1.63 (0.00)c | 120.26 (52.33)b | 1.21 (0.52)a | 1.54 (0.69)a |
| 8 | 1.80 (0.24)c | 162.41 (0.24)a | 0.81 (0.00)d | 129.36 (72.21)b | 0.65 (0.36)b | 1.72 (1.08)a |
| 16 | 1.47 (0.27)c | 162.75 (0.27)a | 0.41 (0.00)e | 97.10 (25.29)b | 0.24 (0.06)b | 1.76 (0.45)a |
| 32 | 1.55 (0.27)c | 162.66 (0.27)a | 0.20 (0.00)f | 156.62 (37.22)b | 0.20 (0.05)b | 1.08 (0.26)b |

The statistical analyses are based on natural log (ln) transformations. Values represent means with standard deviations of five replications of experiments in brackets. Statistical significant ($p<0.05$) differences in a column for FumD activities are indicated with different letters. *Shaded areas represent the water control treatment (0 U/100 g maize). **$HFB_1$ levels were corrected accordingly in the presence of the enzyme.




| Milling products( | FB$_T$ (µg/kg) | | Mean % reduction/ Increase (-)/(+) |
|---|---|---|---|
| | (-) FumD | (+) FumD | |
| Special maize meal | 1213 (8) | 1127 (109) | (-) 7 |
| Super maize meal | 374 (48) | 414 (48) | (+) 10 |
| Semolina | 765 (100) | 395 (20) | (-) 48 |
| Total hominy feed | 5979 (158) | 65 (14) | (-) 99 |
| Reconstructed degermed maize | 719 (54) | 568 (36) | (-) 21 |
| Reconstructed whole maize | 2354 (140) | 445 (39) | (-) 81 |

Figure 2. 2A, Reduction of total FB (FB$_T$) during degerming of whole maize in the absence of FumD; 2B, Distribution of FB$_T$ in dry milling products as a percentage of the total, in the absence and presence of FumD; and Table insert, FB$_T$ levels and percentage FumD-induced reduction in the resultant milling fractions and reconstructed degermed and whole maize. Values represent means with standard deviations of three replicates in brackets.

Table 6. FumD (40 U/kg maize) conversion of $FB_1$ into $HFB_1$ during the full duration of the dry milling protocol as a function of $FB_1$ to $HFB_1$ conversion ratios in milling products.

| Milling products | $FB_1$ (µmole/kg) | | % $FB_1$ loss | $HFB_1$ formation* (µmole/kg) | $FB_1$:$HFB_1$ µmole ratio |
|---|---|---|---|---|---|
| | (-) FumD | (+) FumD | | | |
| Special maize meal | 0.90 (0.03)a | 0.76 (0.04)a | 14.86 (0.99) | 0.10 (0.02) | 1.30 (0.34) |
| Super maize meal | 0.27 (0.04)b | 0.29 (0.05)b | n/a | 0.03 (0.00) | n/a |
| Semolina | 0.68 (0.12)c | 0.34 (0.11)b | 50.59 (15.56) | 0.17 (0.02) | 2.01 (0.44) |
| Reconstructed whole maize | 1.93 (0.13)d | 0.34 (0.07)b | 82.68 (3.44) | 1.22 (0.10) | 1.32 (0.0) |
| Reconstructed degermed maize | 0.55 (0.02)c | 0.41 (0.03)b | 26.17 (5.82) | 0.09 (0.00) | 1.58 (0.37) |
| Total hominy feed | 5.66 (0.19)e | 0.06 (0.02)c | 98.86 (0.33) | 4.31 (0.15) | 1.30 (0.04) |

Values represent means with standard deviations of three replicates in brackets. The statistical analyses are based on natural log (ln) transformations. Statistical significant ($p<0.05$) differences in a column between the control and FumD treatments are indicated with different letters. *$HFB_1$ levels were corrected accordingly in the presence of the enzyme. n/a, not applicable.

Table 7. The nutritional content of the (i) original maize sample, (ii) reconstructed control whole maize, and (iii) reconstructed FumD whole maize.

| Nutritional compound | Original maize sample | Reconstructed control whole maize | Reconstructed FumD whole maize |
|---|---|---|---|
| **General (%)** | | | |
| Moisture | 13.40 ± 0.00 | 15.20 ± 0.00 | 14.10 ± 00 |
| Crude protein | 7.85 ± 1.20 | 8.83 ± 0.04 | 8.87 ± 0.01 |
| Crude fat | 4.10 ± 0.00 | 4.30 ± 0.00 | 4.10 ± 0.00 |
| Total dietary fibre | 9.35 ± 0.07 | 10.50 ± 0.14 | 10.00 ± 0.28 |
| Starch content | 74.45 ± 0.92 | 71.45 ± 0.35 | 72.30 ± 1.56 |
| **Amino acid content (g/100 g maize)** | | | |
| Tryptophane | 0.05 ± 0.00 | 0.05 ± 0.00 | 0.05 ± 0.00 |
| Methionine | 0.25 ± 0.00 | 0.26 ± 0.01 | 0.18 ± 0.00 |
| Cystine | 0.25 ± 0.01 | 0.26 ± 0.02 | 0.24 ± 0.03 |
| Hystidine | 0.21 ± 0.00 | 0.20 ± 0.00 | 0.22 ± 0.00 |
| Serine | 0.38 ± 0.00 | 0.38 ± 0.00 | 0.40 ± 0.00 |
| Arginine | 0.36 ± 0.01 | 0.35 ± 0.00 | 0.39 ± 0.00 |
| Glycine | 0.30 ± 0.00 | 0.31 ± 0.02 | 0.32 ± 0.01 |
| Aspartic acid | 0.51 ± 0.00 | 0.49 ± 0.01 | 0.53 ± 0.01 |
| Glutamic acid | 1.41 ± 0.01 | 1.39 ± 0.01 | 1.45 ± 0.00 |

| Nutritional compound | Original maize sample | Reconstructed control whole maize | Reconstructed FumD whole maize |
|---|---|---|---|
| Threonine | 0.27 ± 0.00 | 0.27 ± 0.00 | 0.28 ± 0.00 |
| Alanine | 0.56 ± 0.00 | 0.54 ± 0.00 | 0.59 ± 0.00 |
| Proline | 0.68 ± 0.00 | 0.67 ± 0.01 | 0.72 ± 0.01 |
| Lysine | 0.25 ± 0.00 | 0.23 ± 0.01 | 0.24 ± 0.00 |
| Tyrosine | 0.20 ± 0.01 | 0.22 ± 0.02 | 0.21 ± 0.01 |
| Valine | 0.37 ± 0.01 | 0.35 ± 0.00 | 0.37 ± 0.00 |
| Isoleucine | 0.26 ± 0.01 | 0.24 ± 0.00 | 0.27 ± 0.01 |
| Leucine | 0.92 ± 0.01 | 0.88 ± 0.01 | 0.97 ± 0.01 |
| Phenylalanine | 0.38 ± 0.00 | 0.36 ± 0.01 | 0.39 ± 0.00 |
| Total amino acid content | 7.59 ± 0.04 | 7.41 ± 0.07 | 7.80 ± 0.07 |

Table 8. The percentage total fatty acid content of the (i) original maize sample, (ii) reconstructed control whole maize, and (iii) reconstructed FumD whole maize.

| Fatty acid | Original composite maize | Reconstructed control maize | Reconstructed FumD maize |
|---|---|---|---|
| | % Total Fatty acids | | |
| C14:0 | 0.08 ± 0.01 | 0.1 ± 0.03 | 0.08 ± 0.01 |
| C16:0 | 15.24 ± 0.16 | 16.06 ± 0.89 | 15.85 ± 0.56 |
| C16:1n-7 | 0.19 ± 0.02 | 0.20 ± 0.03 | 0.19 ± 0.01 |
| C18:0 | 2.23 ± 0.18 | 2.11 ± 0.18 | 2.18 ± 0.11 |
| C18:1n-9 | 24.84 ± 0.96 | 22.98 ± 1.72 | 24.06 ± 2.18 |
| C18:1n-7 | 0.72 ± 0.02 | 0.79 ± 0.05 | 0.76 ± 0.05 |
| C18:2n-6 | 54.20 ± 1.10 | 55.04 ± 0.95 | 54.26 ± 1.57 |
| C18:3n-3 | 1.34 ± 0.09 | 1.51 ± 0.17 | 1.43 ± 0.22 |
| C20:0 | 0.42 ± 0,02 | 0.40 ± 0.04 | 0.42 ± 0.04 |
| C20:1n-9 | 0.23 ± 0.01 | 0.24 ± 0.01 | 0.24 ± 0.03 |
| Total N-6 | 1892.06 ± 191.60 | 1407.52 ± 310.31 | 1607.46 ± 184.83 |
| Total N-3 | 47.35 ± 4.02 | 38.63 ± 6.13 | 43.00 ± 6.84 |
| Total PUFA | 1939.41 ± 194.57 | 1446.16 ± 315.86 | 1650.47 ± 188.56 |
| Total SATS | 639.03 ± 59.83 | 481.96 ± 79.04 | 559.05 ± 59.34 |
| Total MONO | 907.61 ± 89.50 | 625.26 ± 165.37 | 748.73 ± 104.62 |

*8.3.7. Determination of FumD residues and enzyme activity in milling products*

Enzyme residues: Minor signals were observed with the ELISA for control samples (Figure 3). These signals were attributed to non-specific antibody binding. The results indicated FumD residues (± 0.007-0.01 mg/100 g maize) in Total Hominy feed, with lower levels in reconstructed maize (± 0.005 mg/100 g maize) and nominal levels in Special and Super maize meal, and Semolina.

Enzyme activity: Samples from the control milling experiment showed no activity (Figure 4). FumD activity was detectable in products following enzymatic milling, i.e. Total Hominy feed (± 1.45-2.45 U/100 g maize), reconstructed FumD whole maize (±1.18 U/100 g maize) and nominal activity in Special and Super maize meal, and Semolina (± 0.25-0.4 U/100 g maize). The reconstructed FumD whole maize represents the original maize subjected to enzymatic milling (40 U FumD/kg maize), and resulted in 30% FumD activity remaining after milling. The results indicated that a proportion of FumD endured the milling process, with activity remaining in products. $FB_1$ levels (Progress Reports Project 16/05, 2017 and 2018) and FumD residues and activity levels indicated that the enzyme activity was more concentrated in the surface layers of the maize kernel (Total Hominy feed), though a portion also penetrated the endosperm (Special and Super maize meal, and Semolina products).

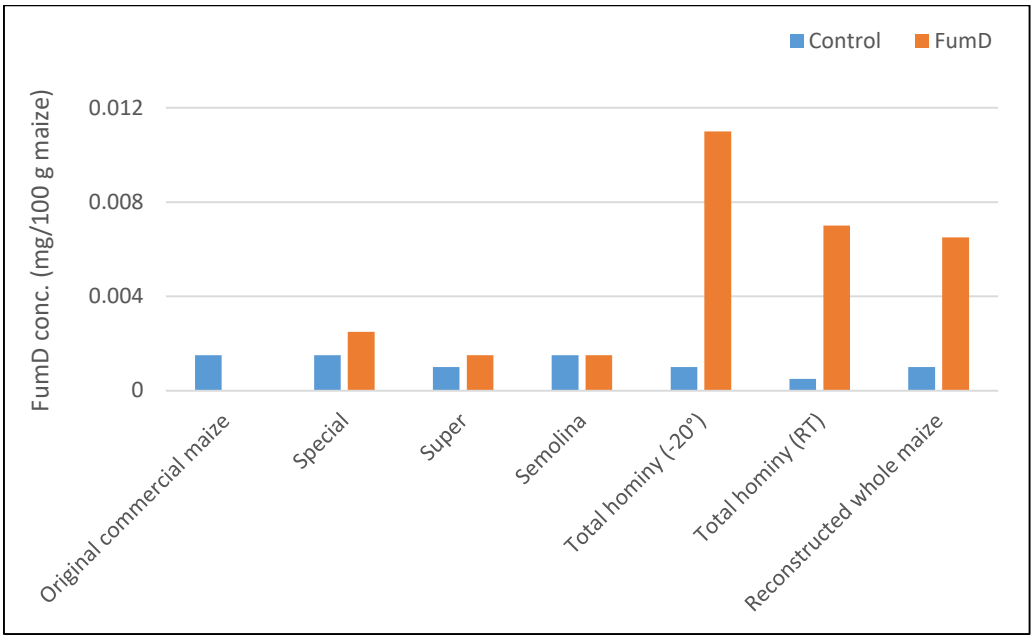



Figure 3: FumD (protein residues) concentration (mg/100 g maize) in milling products without (control) and with enzyme (FumD) treatment.

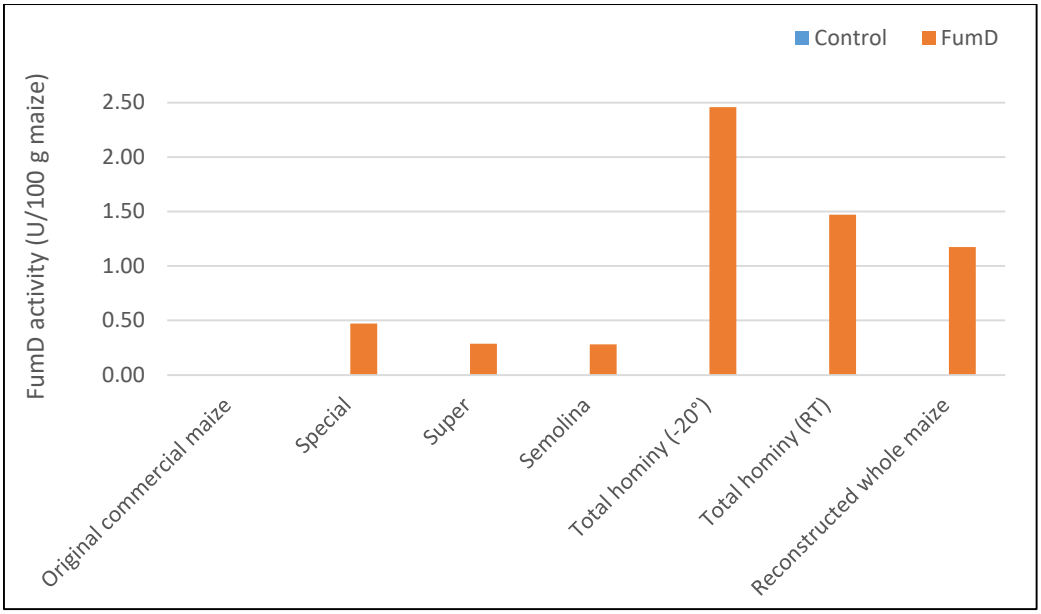



Figure 4: FumD activity (U/100 g maize) in milling products without (control) and with enzyme treatment (FumD).

### 8.4. Discussion

Reduction methods to eradicate mycotoxins in food have become important as technological advances provided opportunities to safeguard human populations to their adverse biological effects. Recently, community and technological based methods have been highlighted and their application under different scenarios, underlying the risk to different populations, critically evaluated (Alberts et al. 2016; 2017). Although different models have been proposed, very few has find application to effectively reduce the presence of mycotoxins in feed and food and strict regulations were set in place to control the levels and safeguard humans to the adverse effects.

Fumonisin esterase FumD effectively catalyses the degradation of FB into hydrolysed FB products, which have been shown to correlate with the reduction of toxicity in pigs and poultry (Grenier et al., 2012; Grenier et al., 2017; Masching et al., 2016). This implies that FumD could find commercial application in animal feed to effectively reduce FB exposure with beneficial economic implications. Very few studies have been conducted to utilise FumD to directly benefit humans to the extent that it could be used as a food additive. One such approach has recently been developed whereby a FumD FB reduction method eradicated the mycotoxins in “low” and “high” FB contaminated home-grown maize (Alberts et al., 2019b). The method forms part of community based approaches suggested to find application in subsistence farming communities utilising home-grown maize as main dietary staple and known to be exposed above the tolerable daily intake levels of 2 µg/kg body

weight/day set by the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) (Burger et al., 2014; Alberts et al., 2017; Shephard et al., 2019; Tshalibe et al., 2020). The treatment regimen indicated that FumD resulted in up to 80% reduction of $FB_T$ levels in "low" and "high" FB contaminated maize and could especially be promising as additive during the customary washing of maize prior to cooking in these community settings and should be further investigated.

At present it is not known whether the FumD FB reduction method could be also introduced during commercial milling practices of maize to facilitate FB degradation and reduce the levels in the products intended for human and animal food and feed. The current study evaluated the efficacy of FumD FB reduction method during commercial dry milling of maize and whether it could modulate the distribution of FB and $HFB_1$ in milling products. Differences in milling practices, kernel characteristics and the large quantities of maize utilised complicates the accurate assessment of distribution patterns of mycotoxin into the different products (Burger et al., 2013). Experimental milling under controlled conditions, however, provide more accurate distribution patterns of the mycotoxins between the different milling products, which could provide a basis for application in commercial settings. A recent study indicated that milling utilising an experimental dry milling plant, reduced mycotoxins FB, deoxynivalenol (DON) and zearalenone (ZEA) in milling fractions, whilst it was concentrated in "Total hominy Feed" generally intended for animal feed (Burger et al., 2013). The present study outlay provides a unique opportunity to investigate the role of the FumD on the reduction and distribution of FB and $HFB_1$ in different milling products.

In the current study, effective de-esterification of FB resulted in ≥96% reduction in the presence of FumD (1 U/L) during the conditioning phase prior to degerming. A lower enzyme activity (1-4 U/100 g maize) suspended in a small volume of water (± 4 ml/100 g maize), was used during the conditioning step, while a far higher enzyme activity (20 and 200 U/100 g maize) in a total volume of 200 ml was used during the FB FumD reduction method to obtain ≥80% FB reduction (Alberts et al., 2019). Comparing the $FB_1$ conversion kinetics between the two methods after a period of 4 h 10 min, a rate of between 1 to 5 nmole/min/mg protein was obtained for "low" and "high" FB contaminated maize when using 20 U FumD/100 g maize with the FB FumD reduction method versus that of 0.4 and 0.2 nmole/min/mg protein for 16 and 32 U FumD/100 g maize during dry milling, respectively. For the "low" FB contaminated maize (Alberts et al., 2019b), which was more aligned to the current study, an approximately 2.5 difference existed in the conversion rate. Therefore, it seems that the enzyme was more active in the presence of an increased level of water, i.e.

a higher humidity when compared to the optimal humidity that prevail during dry milling, which is restricted to between 14.5 and 16.5%.

The FB conversion kinetics seems to be determined by softening of the pericarp that facilitate the diffusion rate and availability of FB to the enzyme at the kernel/enzyme aqueous interphase as a function of the time, which occur much readily in the presence of water. When considering the $FB_1$ to $HFB_1$ conversion the extent of fungal damage and FB contamination to the maize pericarp/endosperm is a determining factor for the amount of enzyme required for maximum $FB_1$ hydrolysis, yielding a 1:1 ratio (Alberts et al., 2019b). The addition of a small volume enzyme solution, which is fully absorbed during conditioning and results in softening and loosening of the pericarp enhances contact with the enzyme. In the current experimental outlay, the optimal conversion was obtained in the presence of 4 U/100g maize and above when considering the $FB_1$ conversion rate (1.63 nmole/min/mg protein) and $FB_1$:$HFB_1$ ratio approaching 1:1. However, it should be recognised that partial hydrolysed forms is likely to exist prohibiting accurate conversion kinetics. FB in commercial maize are likely to occur mainly on the pericarp of kernels and is therefore easily accessible to the enzyme hence facilitates enzyme efficacy. The decreased conversion rates as a function of increased FumD enzyme activity could therefore be related to substrate limitation/accessibility when utilising excess enzyme activities as suggested previously (Alberts et al., 2019b).

The efficacy of FumD during the conditioning phase of maize could be related, as mentioned above, to the accessibility of FB which is mainly located in the outer pericarp layer of the maize kernel. This became evident that most prominent reduction of FB was observed in Total hominy feed representing ±30% of maize, while Super maize meal, equivalent to maize grits, and Special maize meal, similar to maize flour, (Burger et al., 2013) products showed very little reduction in the presence of FumD. The slight reduction and/or increase in $FB_T$ levels in Special and Super maize meal could also be related to the large variation in FB analyses between maize batches selected for the current study, which is known to prevail due to the random distribution of infected kernels throughout a specific sample within a single season and growing region (Saunders et al., 2001; Janse van Rensburg et al., 2011; Mogensen et al, 2011). Therefore, FumD treatment during dry milling did not affect $FB_T$ levels in these products, while a different reduction efficacy is noticed when considering the Semolina milling product known to contain more hominy feed (Burger et al., 2013). With regards to the effect on the nutritional composition of maize, FumD treatment had no significant ($p>0.05$) effect on the amino acid, dietary fibre, starch, crude fat and total fatty acid composition. Determination of the levels of enzyme residues and enzyme activity

provided information on the penetration of the enzyme into kernel layers and enzyme remaining in products. FumD residues were present in Total Hominy feed (± 0.007-0.01 mg/100 g maize), with lower levels in reconstructed maize (± 0.005 mg/100 g maize) and nominal levels in Special and Super maize meal, and Semolina. FumD activity was detectable in products following enzymatic milling, i.e. Total Hominy feed (± 1.45-2.45 U/100 g maize), reconstructed FumD maize (±1.18 U/100 g maize) and nominal activity in Special and Super maize meal, and Semolina (± 0.25-0.4 U/100 g maize).

The current study suggests that incorporation of FumD during the dry milling process could be beneficial not only for the animal feed industry but could also have important implications for human nutrition. Maize bran is considered one of the most valuable feed ingredients (KBAF, 2020). It contains minerals, crude proteins, crude fat, crude fibers lignin and starch as well as insoluble ash and fatty acids. It is also considered an excellent source of energy for livestock. When mixed with other feed ingredients, it produces best results in the quality of meat and eggs in birds, increased milk production in dairy cattle, as well the quality of fish meat. The co-products of ethanol production from maize, i.e. wet and dry distillers' grains and solubles (WDG and DDGS), are increasingly being marketed as protein-rich and cost-saving inclusion in livestock and poultry feed (Wu and Munkfold, 2008). As low grade maize is generally used for ethanol production, high concentrations of fumonisins (>4 mg/kg) are measured in WDG and DDGS following the fermentation and distillation processes during which the fumonisins are ± 2-fold concentrated. This could result in economic losses of millions of dollars in the USA, specifically in the pig industry. FumD could significantly reduce the level of $FB_T$ in WDG and DDGS, thereby provide support for the increasing demand for incorporation into animal feed. With respect to health food products, the extended use of the bran component (germ, hull and pericarp) of Total hominy feed could have significant economic implications. Total hominy feed, although it is mainly utilised for animal foods, contain bran which is considered a popular health food as it contains a wide range of phytochemicals (Ai and Jane, 2016). In addition, maize flour could be enriched with these constituents to provide a product with higher bioactive compounds than the more refined products such as the Special and Super maize meal to enhance its health beneficial properties. Studies indicated that the regular consumption of whole grain maize lowers the risk of developing chronic diseases such as cardiovascular disease, type 2 diabetes, and obesity and improves digestive health (Sheng et al., 2018).

The prominent reduction of FB levels in Total hominy feed following FumD dry milling could open up new applications for this product: (i) incorporation as a source of maize bran in maize-based foods intended for human consumption (Ai and Jane, 2016), and (ii) extended

use in the animal feed industry providing a far safer product. The adaption of enzymatic detoxification of FB could provide an alternative approach to reduce exposure specifically when considering the animal feed industry. In summary, the current study demonstrated effective FumD detoxification in a commercial experimental pilot milling plant utilizing commercial maize. Cost effectiveness of upscaling the method to an industrial level, requiring up to 40 000 U FumD/ton maize will depend on the commercial value of the Total hominy feed lacking $FB_T$ before such an expenditure could be considered.

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## *9. Scientific outputs*

### *9.1 Outputs of project as stated in the application*

| **Expected outputs** | **Numbers** | **Achieved (yes/no or n.a.)** | **Nature of output e.g. title of papers, description, etc.** |
|---|---|---|---|
| Scientific papers: | 2 | Yes | 1. Tshalibe RS**,** Rheeder JP, **Alberts JF**, Taljaard-Krugell C, Gelderblom WCA, Shephard GS, Lombard MJ, Burger H-M. **2020**. Multi-mycotoxin exposure of children (0 - 24 months) in rural maize-subsistence farming areas of the Eastern Cape Province, South Africa. World Mycotoxin Journal. ISSN 1875-0796 online, DOI 10.3920/WMJ2019.2439. (Impact factor: 2.406)<br><br>2. **Alberts JF**, Schatzmayr G, Moll W-D, Davids I, Rheeder JP, Burger H-M, Shephard GS, Gelderblom WCA. **2019**. Detoxification of the fumonisin mycotoxins in maize: an enzymatic approach. Toxins 11, 523, doi:10.3390/toxins11090523 (Impact factor 3.895) |

| Expected outputs | Numbers | Achieved (yes/no or n.a.) | Nature of output e.g. title of papers, description, etc. |
|---|---|---|---|
| | | | 3. **Alberts J,** Rheeder J, Gelderblom W, Shephard G, Burger H-M. **2019**. Rural Subsistence Maize Farming in South Africa: Risk assessment and intervention models for reduction of exposure to mycotoxins. Toxins. doi:10.3390/toxins11060334. (Impact factor 3.895).<br><br>4. Shephard GS, Burger H-M, Rheeder JP, **Alberts JF,** Gelderblom WCA. **2019**. The effectiveness of regulatory maximum levels for fumonisin mycotoxins in commercial and subsistence maize crops in South Africa. Food Control 97, 77-80. (Impact factor 4.248)<br><br>5. **Alberts JF**, Lilly M, Rheeder JP, Burger HM, Shephard GS, Gelderblom WCA. **2017**. Technological and community-based methods to reduce mycotoxin exposure. Food Control 73, 101-109. (Impact factor 4.248)<br><br>6. O'Donnell K, McCormick SP, Busman M, Proctor RH, Ward TJ, Doehring G, Geiser DM, **Alberts JF,** Rheeder JP. **2018.** Marasas et al.: Toxigenic *Fusarium* Species: Identity and Mycotoxicology Revisited. Mycologia. https://doi.ord/101080/00275514.2018.151.9773. (Impact factor 2.861)<br><br><u>Popular articles in agriculture and industry related magazines</u>:<br><br>**JF Alberts**. Biocontrol: promising weapon in fight against maize ear rot. Farmers Weekly, 19 May 2017, p. 76-78.<br><br>**JF Alberts**. Reducing fungal toxins in maize: Biological approaches. Food Science and Technology (FST) magazine, July 2017, p. 10-11. |
| Book Chapters: | n/a | n/a | 1. **Alberts JF**, Burger H-M, Rheeder JP, Shephard GS, Gelderblom WCA. **2018**. Enzymatic decontamination as an approach for reduction of human exposure to mycotoxins in rural subsistence farming communities in Africa. In: *World Nutrition Forum: Scientific challenges* |

| Expected outputs | Numbers | Achieved (yes/no or n.a.) | Nature of output e.g. title of papers, description, etc. |
|---|---|---|---|
| | | | *and opportunities in the protein economy.* Binder, E.M. (Ed.); Anytime Publishing, Leicestershire, UK; pp. 305-312; ISBN 978-3-200-05831-6. |
| Scientific conferences: | n/a | Yes | 1. **Alberts JF**, Moll W-D, Schatzmayr G, Davids I, Rheeder JP, Burger H-M, Shephard GS, Gelderblom WCA. Enzymatic detoxification of the fumonisin mycotoxins during dry milling of maize. Gordon Research Conference. 16-21 June 2019. Stonehill College, Boston, Massachusetts, USA. (Poster presentation)<br><br>2. **Alberts JF**, G Schatzmayr, W-D Moll, I Davids, JP Rheeder, GS Shephard, WCA Gelderblom. Enzymatic approaches for detoxification of the fumonisin mycotoxins in maize: rural community-based methods and possible technological applications. International Conference of the World Nutrition Forum. 3-5 October 2018, CTICC, Cape Town. (Invited speaker)<br><br>3. **Alberts JF**, Schatzmayr G, Moll W-D, Davids I, Rheeder JP, Gelderblom WCA. A novel enzymatic maize kernel wash method for reduction of the fumonisin mycotoxins in rural home-grown maize. International Conference of the African Society of Mycotoxicology 2018. Mombasa, Kenya, 25-28 June 2018. (Oral presentation)<br><br>4. **Alberts JF**, van Zyl WH, Gelderblom WCA. Biological approaches for reducing fumonisin mycotoxins in maize. International conference of the Pan African Environmental Mutagen Society (PAEMS), Cairo, Egypt, 20-21 June 2017. (Invited speaker) |
| Technical reports: | 3 | Yes | 3 Annual progress reports (2017-2019) and a final report (2020). |
| Databases: | n/a | n/a | n/a |
| Procedures/methods: | Yes | | • Development of an enzymatic method to reduce fumonisin levels during dry milling of maize;<br>• Technological methods to reduce mycotoxin exposure in humans and animals. |

| Expected outputs | Numbers | Achieved (yes/no or n.a.) | Nature of output e.g. title of papers, description, etc. |
|---|---|---|---|
| Human capacity development: | | Yes | Inter-institutional collaboration nationally and internationally between partners of the industry and academia;<br><br>Training of MSc student:<br>1. Training on extraction methods of fumonisins from maize (CPUT; February 2017).<br>2. Training on LC-MS for small molecules (Dr M Stander, CAF training initiative, SU; June 2017).<br>3. Training on extraction methods and GC quantification of fatty acids in maize (Dr S Abel, CPUT; March 2017).<br>4. Training on laboratory techniques with regards to enzymatic conditioning during dry milling of maize (Pioneer Foods, Paarl; February - July 2017). |
| Technology transfer: | n/a | Yes | • Development of an enzymatic method to reduce fumonisin levels during dry milling of maize;<br>• Publications in peer-reviewed scientific journals;<br>• Publications in journals focused on the South African maize farmers and the general public (Journal of the South African Association of Food Science and Technology (SAAFost; Farmers' Weekly);<br>• Presentations at international conferences (oral and poster presentations). |
| Other outputs: | • South African Maize Trust Mycotoxin Research Review Panel: Panel member.<br><br>• African Society of Mycotoxicology (ASM): Member of the management committee (Treasurer).<br><br>• Oral presentations at industry related meetings:<br>1. Alberts JF. Enzymatic approaches for detoxification of the fumonisin mycotoxins in maize. Meeting with BIOMIN on enzymatic reduction methods, CPUT, 1 October 2018. (Oral presentation) | | |

| Expected outputs | Numbers | Achieved (yes/no or n.a.) | Nature of output e.g. title of papers, description, etc. |
|---|---|---|---|
| | 2. Alberts JF. Enzymatic reduction of the fumonisin mycotoxins during commercial dry milling of maize. SASKO, Pioneer Foods, Paarl, 15 September 2018. (Oral presentation)<br><br>3. Alberts JF. Fumonisin esterase FumD maize kernel wash method for reduction of the fumonisin mycotoxins in rural home-grown maize. BIOMIN, Tulln, Austria, 4 September 2017. (Oral presentation) | | |

***9.2 Reasons for outputs not achieved***

| |
|---|
| n/a |

***10. Successful institutional and inter-institutional collaboration***

| Researcher | Institution | Role |
|---|---|---|
| Ms I Davids | MSc student (UWC) | Enzymatic Maize milling project |
| Dr G Schatzmayr | BIOMIN, Austria | Collaborator (FumD enzyme; enzyme residues and activities) |
| Dr W-D Moll | BIOMIN, Austria | Collaborator (FumD enzyme; enzyme residues and activities) |
| Prof WCA Gelderblom | IBMB, CPUT | Collaborator (Biochemistry) |
| Dr S Abel | IBMB, CPUT | Collaborator (Fatty acid analyses) |
| Ms H Meyer | SAGL | Nutritional analyses |
| Mr A Wessels | Pioneer Foods | Infrastructure: experimental maize milling pilot plant |
| Ms K O'Kennedy | Pioneer Foods | Assistance with planning of maize milling experiments |
| Mr C Carolissen | Pioneer Foods | Assistance with conditioning experiments |
| Dr M Stander | Central Analytical Facility (CAF), Stellenbosch University | LC-MS analyses: fumonisin levels in maize |

***11. Benefit of the outputs to the maize industry***

This study provides an innovative technological approach to develop a new tool for successful mycotoxin risk management and the delivery of safe maize products to the consumer. It provides highly important insights into the broad scale applicability of FumD enzymes in industrial milling processes. Reduction of fumonisin levels in Total Hominy feed containing the hull, pericarp, germ and tip cap of maize, is relevant. During normal dry milling, $FB_1$ levels in Whole maize is ± 3-fold concentrated in Total Hominy feed resulting in $FB_1$+$FB_2$ levels far above (5061 µg/kg) South African regulations (2000 µg/kg $FB_1$+$FB_2$). Incorporation of the enzyme (40 U/kg maize kernels) during dry milling resulted in considerable reduction of fumonisin levels in Total Hominy Feed and whole maize, to levels far below the South African regulatory levels. Total Hominy feed comprises ± 30% of whole maize, presently has low economical value and is mainly used for incorporation into animal feed. Enzymatic milling, impacting on Total Hominy feed and Whole maize, could have an important economic impact on the maize milling industry. Greatly reduced fumonisin levels could open up new applications: (i) incorporation of Total Hominy feed as a source of fibre in maize-based foods intended for human consumption, (ii) extended use of Total Hominy feed in the animal feed industry and (iii) applications in subsistence farming communities using rudimentary milling processes. This technology could have a major impact on health, food safety and security of the population heavily reliant on maize as a staple foodstuff.

***12. Progress with regards to human resource development*** *(e.g. training of post-graduate students in mycotoxin research)*

Ms I Davids (MSc student, UWC):

Ms Davids worked on this project as part of her MSc degree.
She has completed the experimental part of her MSc degree and is currently in the process of writing up her thesis.

***13. Statement whether funds were adequate to complete the project***

The funds are adequate, thank you.

***14. Comments*** *(discuss anything you wish to share with The Maize Trust)*

The Project leader wants to thank the South African Maize Trust for their financial support of research on enzymatic methods for reduction of mycotoxin concentrations in maize.

***15. Signature of the Project Leader***

| Bellville | 29 May 2020 |
|---|---|
| **Project Leader**<br>**Dr JF Alberts** | **Place** | **Date** |

***16. Signature of Responsible Authority***

| **BELLVILLE** | **29 May 2020** |
|---|---|
| **Name / Institution** | **Place** | **Date** |