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Wageningen Academic Publishers
P.O. Box 220
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Tel: +31 317 476516
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Aflatoxins and *Saccharomyces cerevisiae*: yeast modulates the intestinal effect of aflatoxins, while aflatoxin B_1 influences yeast ultrastructure

C. Dogi[1#], A. Cristofolini[2#], M.L. González Pereyra[1], G. García[1], A. Fochesato[1], C. Merkis[2], A.M. Dalcero[1] and L.R. Cavaglieri[1*]

[1]Departamento de Microbiología e Inmunología, Universidad Nacional de Río Cuarto, Ruta 36 km 601, 5800 Río Cuarto, Córdoba, Argentina; [2]Departamento de Microscopía Electrónica, Universidad Nacional de Río Cuarto, Ruta 36 km 601, 5800 Río Cuarto, Córdoba, Argentina; lcavaglieri@exa.unrc.edu.ar; #These authors contributed equally to the development of assays

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RESEARCH ARTICLE

Abstract

The gastrointestinal tract (GIT) is the main site where absorption of food components takes place and the first system coming into contact with mycotoxins of dietary origin. The aim of this work was to study the effect of probiotic *Saccharomyces cerevisiae* RC016 on intestinal villi of rats exposed to aflatoxins for 60 days. Moreover, the effect of *in vitro* aflatoxin B_1 (AFB_1) exposure on yeast cell ultrastructure was evaluated. Six treatments were applied (n=6) to inbred male Wistar rats: (1) uncontaminated feed control (F); (2) yeast control; (3) F + 40 µg/kg AFB_1 + 20 µg/kg aflatoxin G_1 (AFG_1); (4) F + 100 µg/kg AFB_1 + 50 µg/kg AFG_1; (5) F + 40 µg/kg AFB_1 + 20 µg/kg AFG_1 + daily oral dose 10^8 viable *S. cerevisiae* cells; and (6) F + 100 µg/kg AFB_1 + 50 µg/kg AFG_1 + daily oral dose 10^8 viable *S. cerevisiae* cells. Morphometric measurements (villus length and width, crypt depth, quantification of goblet cells) were assessed using image analysis. *S. cerevisiae* RC016 cells were exposed to 20 µg/ml of AFB_1 in intestinal solutions or in phosphate buffered saline and cells processed for transmission electron microscopy and high resolution light microscopy studies. Dietary exposure to the yeast did not induce significant differences in villus width but increased villus length and crypt depth. Aflatoxin-contaminated diets induced an increase in villus length, width and crypt depth and a significant decrease in the number of goblet cells which were improved by the addition of *S. cerevisiae* RC016. A significant increase in the yeast cell diameter was observed when RC016 was exposed to aflatoxins, suggesting this as an advantage since a larger cell would be able to adsorb mycotoxins more efficiently. The ability of this strain to act as probiotic and aflatoxin binder makes it a candidate for the formulation of new additives to improve animal performance.

Keywords: AFB_1, intestinal villi, probiotics, *Saccharomyces cerevisiae*

1. Introduction

The inclusion of probiotics, commonly defined as live microorganisms which confer a health benefit in the host, in various types of food and feed products has increased significantly during the past three decades (FAO/WHO, 2001). *Saccharomyces cerevisiae* has been supplied to food for centuries due to its huge profits providing vitamin B complex, minerals and proteins (Durdag and Karaoglu, 2005; Kogan and Kocher, 2007). Moreover, it has been commercialised in the form of zootechnical feed additives and digestibility enhancers. The consideration as Generally Regarded As Safe (GRAS) microorganism allows its application as feed additive with minimal risks. Previous *in vitro* studies demonstrated that a *S. cerevisiae* strain isolated from pig gut was able to adsorb aflatoxin B_1 (AFB_1), survive under gastrointestinal tract (GIT) conditions and promote beneficial properties to the host, demonstrating to be a potential probiotic (Armando *et al.,* 2011, 2012a; Dogi *et al.,* 2011).

Aflatoxins (AFs), a group of mycotoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus*, can contaminate a wide variety of foods and crops constituting a worldwide problem that compromises food and feed safety (CAST, 2003). Exposure of livestock to AFs results in an impairment of liver function, reduced feed intake and reduced milk production (Fink-Gremmels, 2008). In addition, AFs can reduce feed efficiency and productivity of livestock and their immunosuppressive properties can increase the occurrence of infectious diseases (Meissonnier *et al.,* 2008). In terms of toxic potency and occurrence, aflatoxin B_1 (AFB_1) is the most important of AFs that can be metabolised by ruminants and excreted as aflatoxin M_1 in milk (Alonso *et al.,* 2011; Prandini *et al.,* 2009). Low level chronic AFB_1 exposure is linked to the development of occult conditions such as impaired growth and immune function and chronic diseases (Yunus *et al.,* 2011a).

One available strategy for attenuating the effects of some groups of mycotoxins uses the unique adsorptive capacity of the carbohydrate complexes present in the yeast cell wall. Mycotoxin adsorption by *S. cerevisiae* has been reviewed by Shetty and Jespersen (2006). Several studies relate the consumption of *S. cerevisiae* (or a fraction of its cell wall) with the reduction of aflatoxin-induced toxicity in animals (Baptista *et al.,* 2004; Madrigal-Santillán *et al.,* 2006; Santin *et al.,* 2003). Although the effects of mycotoxins on cellular mechanisms, cellular toxicity, associated pathology and animal performance have been extensively reported, studies on the effect of these compounds on the GIT are limited. The rapid appearance of most mycotoxins in the circulation clearly indicates that the majority of the ingested toxin is absorbed in the proximal part of the GIT (Cavret and Lecoeur, 2006, Agence Française de Sécurité Sanitaire des Aliments, 2009). Therefore, intestinal cells and tissues can become a main target of mycotoxins as many of these metabolites are inhibitors of protein synthesis. It is widely known that the target organ of AFs is the liver (Wang *et al.,* 1999); however, there is little literature about the effects on AFs on the GIT, the main site where conversion and absorption of food components takes place, and it is also the first system coming into contact with mycotoxins of dietary origin. It is known that AFs is absorbed at high rates regardless of the species (Agence Française de Sécurité Sanitaire des Aliments, 2009). During the absorption, AFB_1 metabolism to its epoxide takes place in the small intestine tissue, indicating the intestinal tract as a major site of AFB_1 metabolism (Hsieh and Wong, 1994). The effect of AFs on dry matter and nitrogen digestibility and nitrogen-corrected apparent metabolisable energy has been documented, suggesting AFs increase the amino acid requirements and reduce energy utilisation (Applegate *et al.,* 2009; Kermanshahi *et al.,* 2007). However literature on the effects of AFB_1 on histology of GIT is scanty and non-conclusive, and these were some of the reasons for conducting the experiments, since the maintenance of a healthy GIT is crucial to maintain intestinal homeostasis.

The aim of the present study was to study the effect of probiotic *S. cerevisiae* RC016 on morphometric parameters (villus length, width and crypt depth) and goblet cells number in the intestine of rats exposed to AFs for 60-days. In addition, the effect of *in vitro* AFB_1 exposure on the yeast cell ultrastructure, diameter and cell wall thickness was also evaluated.

2. Materials and methods

Microorganisms, growth medium and culture conditions

S. cerevisiae RC016, isolated from pig intestine in a previous study (Armando *et al.,* 2011), was obtained from the collection centre at the Universidad Nacional de Rio Cuarto, Argentina. Stock cultures were maintained at -80 °C in 30% (v/v) glycerol. Working cultures were prepared from frozen stocks by two transfers in yeast extract-peptone-dextrose (YPD) broth (5 g yeast extract, 5 g peptone, 40 g dextrose, 1000 ml water) and incubated at 37 °C for 24 h. After that, yeast cells were harvested by centrifugation at 8,000×*g* for 10 min and washed twice in 0.85% NaCl.

Aflatoxigenic *A. parasiticus* NRRL2999 was used to produce AFs and was maintained at 4 °C on slants of malt extract agar (MEA) and at -80 °C in 15% glycerol.

Aflatoxins production

The reference strain *A. parasiticus* NRRL 2999 was cultured in order to obtain AF concentrations enough to contaminate feed for the experiment. *A. parasiticus* NRRL2999 was grown on MEA for 7 days at 25 °C to obtain heavily sporulating cultures. Seven-day culture plugs were inoculated in 250 ml Erlenmeyer flasks containing 25 g of autoclaved rice and 50 ml distilled water. Cultures were incubated in the dark, at 30 °C for 15 days, manually stirring the flasks vigorously, for 1 min, once a day during the first 5 days to enhance the dissemination of conidia in the rice. After incubation, the cultures were autoclaved (120 °C, 15 min) to inactivate mould but not AFs, which are stable up to their melting point of around 250 °C. The content of all flasks was placed in a metallic tray, covered with paper, dried in a forced air oven at 60 °C and ground with a laboratory mill. AFB_1, aflatoxin G_1 (AFG_1), aflatoxin B_2 (AFB_2) and aflatoxin G_2 (AFG_2) content of the resulting powder was quantified by high performance liquid chromatography (HPLC) according to the methodology described by Trucksess *et al.* (1994). The ratio of AFB_1 to AFG_1 concentration in the culture was 2:1. Levels of AFB_2 and AFG_2 were detectable but not quantifiable. This AF concentrate was used to contaminate feed.

Animals

An experimental model with rats was used. Male Wistar inbred rats (*Rattus norvegicus*) were chosen since they are proved to be less resistant to AF effects than mice and female rats (Steyn *et al.,* 1971). Animals (8 weeks old, weight 173±15 g, n=36) were obtained from the closed random bred colony maintained at the Universidad Nacional de Río Cuarto. After an acclimation period of 1 week, animals were divided into six groups (6 rats per group) and housed in stainless steel cages kept in a temperature-controlled (23±1 °C) and artificially illuminated (12 h dark/light cycle) room free from any source of chemical contamination, in accordance with international sanitary and ethical guidelines.

Diets

Commercial basal diet

Commercial basal mice-rat diet (GEPSA Feeds, Grupo Pilar S.A., Buenos Aires, Argentina) was purchased and used as the basis for formulating the different contaminated and control diets. Centesimal composition of this feed was >24% protein, <7% fibre, 1-1.2% calcium, >6% ether extract, 0.5-0.9% phosphorus, <8% total minerals and <13% moisture. The commercial feed was analysed by HPLC using Mycosep® AflaPat 228 columns (Romer Labs, Tulln, Austria) for clean-up and the method 99408-1994 of the AOAC described by Trucksess *et al.* (1994) for detection. Briefly, extraction was carried out using AflaPat Mycosep®228 clean-up columns (Romer Labs Inc., Union, MO, USA) following methodology supplied by the manufacturer. 25 g of rat feed were extracted with 100 ml acetonitrile:water (90:10, v/v) and shaken for 30 min. The mixture was filtered through no.4 Whatman paper and an 8 ml aliquot was cleaned up using a MycoSep® clean up column. The purified extract (4 ml) was collected in the column reservoir. Extracts were evaporated to dryness under N_2 flow and redissolved in 400 µl mobile phase acetonitrile:methanol:water (17:17:66, v/v/v). An aliquot (200 µl) was derivatised with 700 µl trifluoroacetic acid:acetic acid:water (20:10:70, v/v/v) and analysed by HPLC according to Trucksess *et al.*(1994) (Limit of detection (LOD): 3 µg/kg; limit of quantification (LOQ): 5 µg/kg). A calibration curve was constructed using standards solutions with concentrations equivalent to 2; 10; 20; 40 and 100 µg/kg AFB_1 and 2; 5; 10; 20 and 50 µg/kg AFG_1. Standard solutions for the calibration curve were prepared on the same day of the analysis. The basal feed did not show detectable levels of AFs.

Experimental control diet

Control diet was prepared by mixing 2.52 kg of finely ground commercial basal diet with 60 g agar dissolved in 2.5 l of water. The mixture was homogenised for 20 min in a big plastic container and 30 g pieces were moulded manually. After solidification, feed was stored at -20 °C until use.

Experimental aflatoxin B_1-contaminated diets

Two AF-contaminated diets were prepared weekly during the experiment in the same way as the control diet. They were prepared to have a final concentration of 40 µg/kg AFB_1 + 20 µg/kg AFG_1 for diet 1, and 100 µg/kg AFB_1 + 50 µg/kg AFG_1 for diet 2. The AFs levels used were chosen in order to simulate subchronic aflatoxicosis-causing doses naturally found in feeds (Benford *et al.,* 2010; Theumer *et al.,* 2008). Finely ground commercial basal feed (2.52 kg each) was added 2.1 or 5.25 g of the *A. parasiticus* culture powder to produce diets 1 and 2, respectively. Each feed was thoroughly homogenised and added 60 g agar dissolved in 2.5 l water. After a second homogenisation, 30 g pieces were moulded manually. After solidification, feeds were stored at -20 °C until use. AFB_1 and AFG_1 concentration of both experimental diets was confirmed by HPLC as described for the commercial basal diet.

Experimental model

A total of 36 male Wistar rats were divided into six different groups (n=6): (T1) control diet + 0.2 ml 0.85% NaCl orally administered; (T2) control diet + of *S. cerevisiae* (2×10^8 viable cells in 0.85% NaCl) orally administered; (T3) experimental diet 1 + 0.2 ml 0.85% NaCl orally administered; (T4) experimental diet 2 + 0.2 ml 0.85% NaCl orally administered; (T5) experimental diet 1 + *S. cerevisiae* (2×10^8 viable cells in 0.85% NaCl) orally administered; and (T6) experimental diet 2 + *S. cerevisiae* (2×10^8 viable cells in 0.85% NaCl). Yeast strain and NaCl were administered by gavage. Animals were fed 30 g of feed per day and consumed water *ad libitum*. Feed and water were replaced daily. The assay was carried out during 60 days in order to obtain a sub chronic effect and to resemble what really occurs when animals consume contaminated feed. At the end of the assayed period, rats were sacrificed by decapitation and the small intestines (duodenum) were recovered and prepared for histological studies. The protocol for this study was approved by the Committee of Ethics and Biosecurity of the Universidad Nacional de Río Cuarto.

Conventional histological technique

Portions of approximately 6 mm^2 of small intestine tissue samples (duodenum) were fixed in 4% (v/v) buffered-saline formaldehyde pH 7.2-7.4 at 4 °C, dehydrated in a graded series of ethanol (30, 50, 70, 80, 90, 95 and 100%) and xylene solutions (10 min each one), embedded in paraffin and cut in ±4 µm histological serial-sections (microtome Sorvall MT 1A – DuPont, Wilmington, DE, USA). The histological

sections were stained with haematoxylin/eosin (H/E) for microscopic analysis.

Histomorphometry

The morphometric measurements taken from the intestinal histological sections included villus length, villus width and intestinal crypt depth. Morphometric measurements of intestinal variables were carried out on two slides per animal/intestine, two sections per slide and five fields per section. Also, a quantification of goblet cells was performed. The goblet cells were counted at 40× only in villi axis. The quantification was performed on all the villi found in each section analysed (two slides per animal/intestine, two sections per slide). Digital images were captured with an Axiophot microscope (Carl Zeiss, Thornwood, NY, USA) fitted with high resolution Powershot G6 7.1 megapixelsdigital camera (Canon Inc., Tokyo, Japan). Digital image analysis and morphometric measurements were performed with Axiovision AxioVs40 V4.6.3.0. software (Carl Zeiss, Göttingen, Germany).

In vitro effect of aflatoxin B_1 on yeast cells

In order to study the *in vitro* effect of AFB_1 exposure on *S. cerevisiae* RC016 cells, four treatments were assayed: (T1) *S. cerevisiae* RC016 (1×10^8 cell/ml) in 1 ml phosphate buffered saline (PBS); (T2) *S. cerevisiae* RC016 (1×10^8 cell/ml) in 1 ml gastrointestinal (GI) solution; (T3) *S. cerevisiae* RC016 (1×10^8 cell/ml) in 1 ml PBS + 20 ×µg/ml AFB_1; (T4) *S. cerevisiae* RC016 (1×10^8 cell/ml) in 1 ml GI solution + 20 µg/ml AFB_1. Each treatment was incubated at 37 °C in constant agitation (100 rpm) for 1h. Afterwards, cells were washed twice with PBS and the pellets were processed for Transmission Electron Microscopy (TEM) and High Resolution Light Microscopy (HRLM) studies. Since intestine is the site where the yeast-mycotoxin interaction occurs, we decide to evaluate yeast ultrastructure in this matrix. The intestinal solution was prepared according to Armando *et al.* (2011): trypsin 1 mg/ml-Fluka 11,531 U/mg; chymiotrypsin 1 mg/ml-Fluka 80 U/mg; oxgall bile salts 0.3% (w/v) in water and adjusted to pH 8 with NaOH 5N.

Transmission electron microscopy

For TEM studies, yeast pellets were fixed in 2.5% glutaraldehyde 0.2 M pH 7.4 S-collidine buffer solution and post-fixed in 1% osmium tetroxide 0.2 M pH 7.4 S-collidine buffer solution. Cell pellets were then dehydrated in increasing concentration acetone solutions (1×50%, 1×70%, 1×90%, 3×100%), embedded in EMbed 812 resin and sectioned with an ultramicrotome. The ultrathin sections (±60 nm) were cut and placed on copper grids, counterstained with 50% saturated uranyl acetate during 8 min and aqueous lead citrate solutions during 30 min. The sections were examined in an Elmiskop 101 transmission electron microscope (Siemens, Munchen, Germany) and the cell wall thickness was measured as a morphometric variable with Digital Micrograph software (Gatan, Tokyo, Japan). In TEM the morphometric measurements were carried out by a single operator on five grids per treatment, counting a total of 200 randomly selected cells per treatment.

High resolution light microscopy

For HRLM studies, the *S. cerevisiae* cells processed for transmission electron microscopy technique were used. An ultramicrotome was used to obtain the semi thin sections (±0.25 µm). These sections were counterstained with toluidine blue and were cover-slipped in DPX (Merck, Darmstadt, Germany) embedding agent. They were then observed in an Axiophot light microscope (Carl Zeiss, USA) fitted with a Powershot G6 7.1 megapixels high resolution digital camera (Canon Inc.). Digital images analyses and morphometric measurements of cell diameter (Cd) were perfomed with Axiovision AxioVs40 V4.6.3.0. software (Carl Zeiss, Germany). In HRLM the morphometric measurements were carried out by a single operator on two slides per treatment, two sections per slide and five fields per section, counting a total of 1000 randomly selected cells per treatment

3. Results

Histomorphometry

The results obtained from the morphometric studies are presented in Table 1. The oral administration of *S. cerevisiae* RC016 alone (T2) showed similar values for villus width and an increase in villus length and crypt depth compared to the control group. Surprisingly, AFs-contaminated diets (T3 and T4) also induced an increase in villus length and width as well as in crypt depth compared to the control group (T1) ($P<0.05$). However, the effects of both, yeast and AFs seem not to be accumulative, since treatments receiving both did not show a significant increase in the values of morphometric variables compared with T3 and T4. Moreover, in T6 (yeast plus 100 µg/kg AFB_1 + 50 µg/kg AFG_1) it was observed that crypt depth was reduced by the addition of the probiotic, possibly due to AF-binding to the yeast cell wall. There was no significant difference in the number of goblet cells between the *S. cerevisiae* RC016 treated group (T2) and the control (T1), whereas AF-treated groups showed significantly lower cell counts ($P<0.05$). In T6, the probiotic increased the goblet cell count to the level of the control group (Table 1, Figure 1).

In vitro effect of aflatoxin B_1 on yeast cells

No significant differences in *S. cerevisiae* cell wall thickness were found by TEM between treatments (data not shown). The electron micrograph also showed that the architecture

Table 1. Morphometric measurements taken from intestinal histological sections of male Wistar rats from different treatments involving probiotic *Saccharomyces cerevisiae* RC016, two aflatoxin-contaminated feeds and controls during a 60-day aflatoxin exposure.

Treatments[2]	Morphometric measurement (µm) (mean ± standard deviation)[1]									
	Villus length		Villus width		Crypt depth		Goblet cell count		Villus:crypt ratio	
T1: Feed (F) control[3]	392.53±5.1	b	84.66±6.1	b	184.92±7.6	d	21.46±1.14	a	2.12	
T2: Yeast (Y) control[4]	507.84±7.2	a	82.80±8.3	b	247.84±7.8	b	21.66±1.12	a	2.05	
T3: F + 40 µg/kg AFB_1 + 20 µg/kg AFG_1	528.30±8.1	a	102.82±5.1	a	265.81±6.9	a	16.44±1.12	b	1.99	
T4: F + 100 µg/kg AFB_1 + 50 µg/kg AFG_1	528.62 ±10.3	a	100.60±8.2	a	293.06±16	a	16.20±1.12	b	1.80	
T5: F + 40 µg/kg AFB_1 + 20 µg/kg AFG_1 + Y	512.42±7.1	a	106.77±7.1	a	280.68±7.3	a	18.83±1.30	ab	1.82	
T6: F + 100 µg/kg AFB_1+ 50 µg/kg AFG_1 + Y	500.95±11.3	a	93.91±3.1	b	224.11±7.3	c	19.05±1.13	ab	2.23	

[1] Values with different letter within each column are significantly different according to Fisher's protected LSD test (P<0.05).
[2] AFB_1 = aflatoxin B_1; AFG_1 = aflatoxin G_1.
[3] Uncontaminated feed.
[4] Daily oral dose of 10^8 viable *S. cerevisiae* cells.

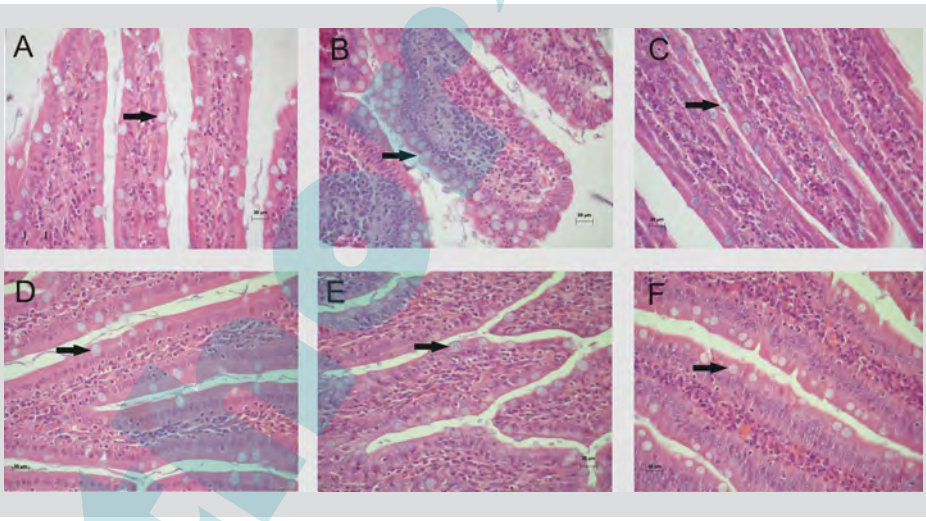

Figure 1. Goblet cells of intestinal histological sections exposed to different treatments. (A) control diet + 0.2 ml 0.85% NaCl; (B) control diet + *Saccharomyces cerevisiae* ($2×10^8$ viable cells in 0.85% NaCl); (C) experimental diet A + 0.2 ml 0.85% NaCl; (D) experimental diet B + 0.2 ml 0.85% NaCl; (E) experimental diet A + *S. cerevisiae* ($2×10^8$ viable cells in 0.85% NaCl); (F) experimental diet B + *S. cerevisiae* ($2×10^8$ viable cells in 0.85% NaCl).

of the cell wall consists of three layers: an electron dense outer layer, a middle layer and an inner layer, closer to the plasma membrane. The electron micrograph also showed cell ultrastructure highlighting the presence of size-variable vacuoles and the characteristic core crescent observed in some cells (Figure 2).

The results obtained by HRLM technique showed a statistically significant increase (P<0.0001) in *S. cerevisiae* RC016 cell diameter after T3 (yeast in PBS + 20 µg/ml AFB_1) and T4 (yeast in GI solution + 20 µg/ml AFB_1) compared with control treatments T1 (yeast in PBS) and T2 (yeast in GI solution) (Table 2). *S. cerevisiae* cell morphology and the determination of cell diameter are shown in Figure 3.

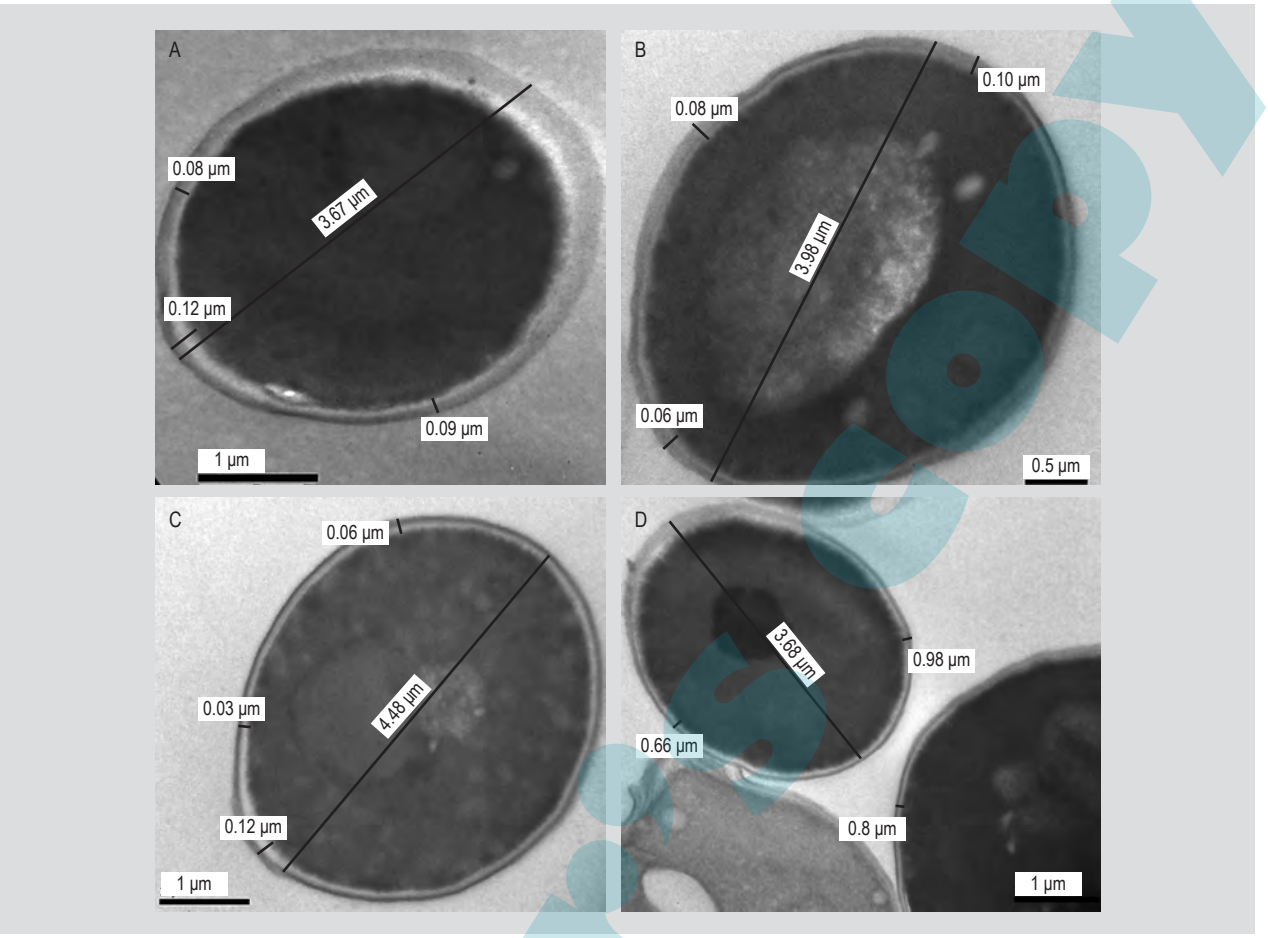

Figure 2. ***Saccharomyces cerevisiae*** **cell morphology by TEM. (A)** ***S. cerevisiae*** **RC016 (1×10^8 cells/ml) in phosphate buffered saline (PBS); (B)** ***S. cerevisiae*** **RC016 (1×10^8 cells/ml) in gastrointestinal solution (GI); (C)** ***S. cerevisiae*** **RC016 (1×10^8 cells/ml) in PBS + AFB_1; (D)** ***S. cerevisiae*** **RC016 (1×10^8 cells/ml) in GI + AFB_1.**

Table 2. ***Saccharomyces cerevisiae*** **RC016 cell diameter by high resolution light microscopy after exposure to different treatments** ***in vitro*****.**

Treatments[1]	Mean cell diameter ± standard deviation (µm)[2]
Yeast in PBS[3]	3.66±0.12 a
Yeast in GI	3.93±0.09 a
Yeast in PBS + AFB_1 20 µg/ml	4.52±0.10 b
Yeast in GI + AFB_1 20 µg/ml	4.59±0.11 b

[1] AFB_1 = aflatoxin B_1; GI = gastrointestinal solution; PBS = phosphate buffered saline.
[2] Values with different letter are significantly different according to Fisher's protected LSD test ($P<0.05$).
[3] Yeast = 1×10^8 *S. cerevisiae* RC016 cells/ml.

4. Discussion

The present study was carried out to study the effect of probiotic *S. cerevisiae* RC016 on morphometric parameters (villus length, width and crypt depth) and goblet cells number in the intestine of rats exposed to AFs for 60-days. In addition, the effect of *in vitro* AFB_1 exposure on the yeast cell ultrastructure, diameter and cell wall thickness was also evaluated. The effect of the oral administration of the yeast alone was also assessed by measuring changes in intestinal morphometric variables in order to evaluate its probiotic ability *in vivo.* Previous *in vitro* studies demonstrated that *S. cerevisiae* RC016 was able to bind different mycotoxins, being able to retain its binding ability under simulated GIT conditions and to have probiotic properties (Armando *et al.* 2011, 2012b; Dogi *et al.* 2011). In addition, this yeast strain showed biological control properties against *A. parasiticus*, *Fusarium graminearum* and *Aspergillus carbonarium* (Armando *et al.* 2012a, 2013). *S. cerevisiae* RC016 also demonstrated not to cause genotoxicity or cytotoxicity on rats' bone marrow erythrocytes *in vivo* (González Pereyra *et al.*, 2014).

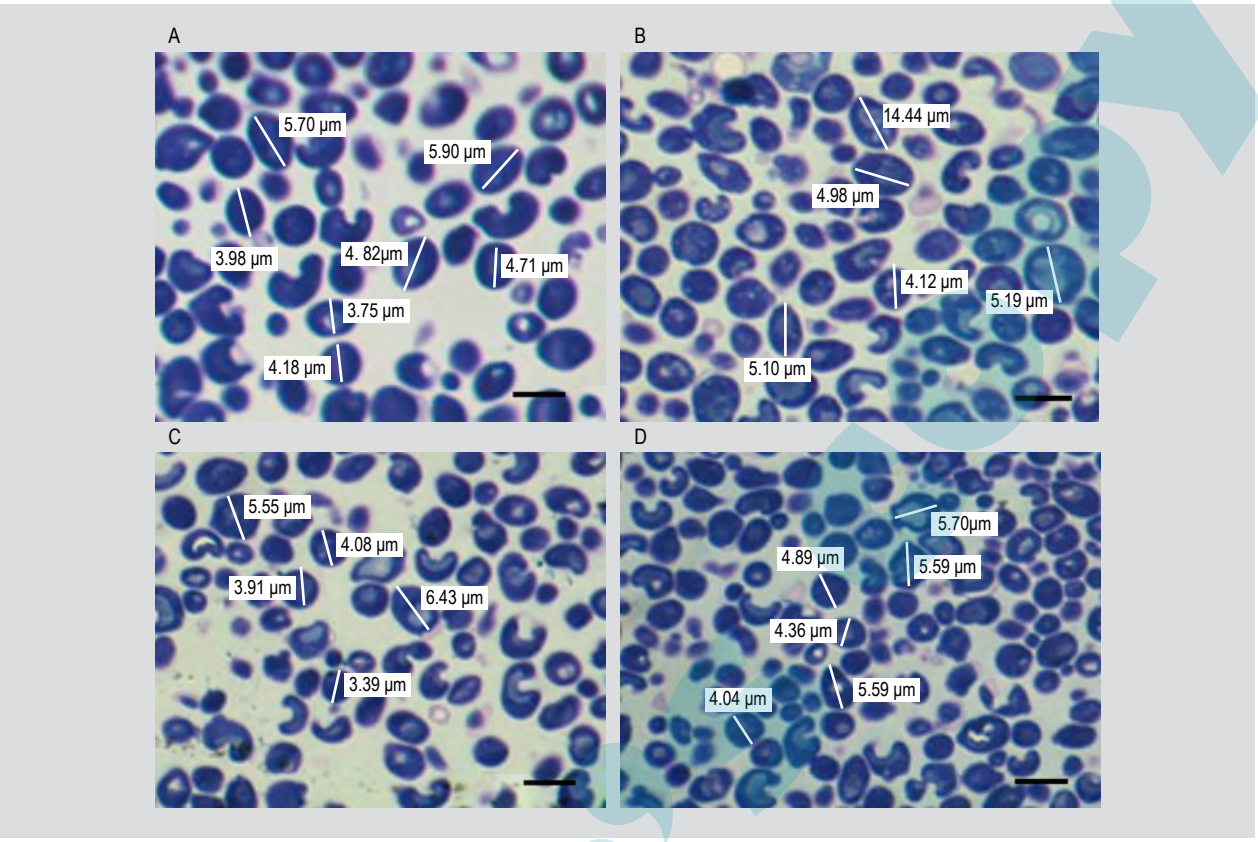

Figure 3. *Saccharomyces cerevisiae* cell morphology by HRLM technique and cell diameter. (A) *S. cerevisiae* RC016 (1×10^8 cell/ml) in PBS; (B) *S. cerevisiae* RC016 (1×10^8 cell/ml) in gastrointestinal solution (GI); (C) *S. cerevisiae* RC016 (1×10^8 cell/ml) in PBS + AFB_1; (D) *S. cerevisiae* RC016 (1×10^8 cell/ml) in GI + AFB_1.

Morphometric analysis is widely used in GIT research, since it is a quantitative assessment and thus more reliable and reproducible than a subjective assessment. Morphological changes of healthy mucosa occur under different experimental conditions, including drugs, special diets and the use of probiotic microorganisms. Moreover, morphometric analysis has been used to evaluate the condition of the intestinal mucosa after antibiotic treatment in patients with small intestine bacterial overgrowth (Haboubi *et al.,* 1991), the effectiveness of treatment, the prognosis in patients with coeliac disease (Cummins *et al.* 2001), and the influence of environmental factors on mucosal morphology (Kelly *et al.,* 2004).

Villus length and crypt depth are considered as the indicators of intestinal functions (Matur and Eraslan, 2012). In the present study, it was observed that the addition of *S. cerevisiae* RC016 to the animals' diet was able to induce an increase in the villus length and the intestinal crypt depth. This result is consistent with the expected outcome for a probiotic organism, as well as a normal goblet cell count. Several *in vivo* studies have reported an increase in villus length or height after the administration of probiotic microorganisms (Di Giancamillo *et al.,* 2008; Willing and Van Kessel, 2007; Yang *et al.,* 2009). Zhang *et al.* (2005) reported that *S. cerevisiae* was able to increase ileum villus height in birds. Data related to crypt depth reported that it was increased in piglets inoculated with *Lactobacillus fermentum* (Willing and Van Kessel, 2007). Similarly, an increase in crypt depth in duodenum, jejunum and ileum of chicks supplemented with *Bacillus subtilis* were informed (Pelicano *et al.,* 2005).

In this study, dietary exposure to AFs also induced an increase in small intestine villus length and width and in crypt depth, regardless of the level of AFs used. This result was not expected, however, it was consistent in all analysed samples. Up to date, the effect of AFs on intestinal mucosa morphology, especially on villi length or height, is not clear, since data reported by different authors are not consistent. Our results concur with those reported by Applegate *et al.* (2009) who observed an increase in intestinal crypt depth (that affected the villus:crypt ratio) and no changes in villus length or number of goblet cells in HyLine W36 hens fed diets containing a crude AFLA culture containing 0.6 to 2.5 mg/kg of AFB_1. However, these authors reported intestinal crypt depth increased linearly with increasing AFB_1 concentration. On the contrary, Yang *et al.* (2012) stated that villus height and the villus height:crypt depth ratio decreased significantly in broilers

fed diets contaminated with AFB_1 and AFB_2 affecting the morphology of the duodenum, and probably altering the absorption of nutrients.

Some aspects of aflatoxicosis, particularly effects on GIT, are not well documented, although GIT is the first organ coming into contact with mycotoxins of dietary origin. The increment of the villus length and crypt depth is a desirable characteristic on a probiotic because it enlarges the surface of the intestine favouring nutrient absorption. The changes in the morphology of villi and reduction in absorptive surface area due to the consumption of AFB_1 may reduce the nutrient absorption and hence lead to reduced production performance. According to Cera *et al.* (1988), maximal absorption and digestion capacity, provided by a large luminal area, with high villi is essential for the animals' development. However, in the case of AFs, the same effect could result in a problem since it could favour the mycotoxins' translocation and absorption affecting the animal's performance. These results could help enlighten some aspects of the effect of sub chronic aflatoxicosis on the intestinal morphology. In the present study, animals from T6 (receiving the higher level of AFs and the yeast) showed a remarkably significant reduction in crypt depth compared to animals receiving AF contaminated feed (T3 and T4) and with animals receiving the lower level of AFs and the yeast (T5). This could be due mainly to the binding of AFs to *S. cerevisiae*'s surface and the consequent reduction of its bioavailability reducing its effect on the intestine. In addition, no synergism or additive effect was observed in treatments receiving both, the toxin and the yeast (T5 and T6) for the enlargement of villus length. Literature available, regarding effects of the toxin on GIT, is particularly non-conclusive. The influence of AFB_1 on villus length or crypt depth cannot be explained by the tight junction proteins integrity alteration just Caloni *et al.* (2012) demonstrated that AFB_1 did not affect them. Yunus *et al.* (2011b) suggested that the absorptive surface of small intestine declines during a chronic exposure to low levels of AFB_1. In that study, broilers compensated for the reduced absorptive surface by increasing the length of the small intestine. In the current study, uncertainty remains as to why the villus length and crypt depth increased in the presence of AFB_1. There is evidence that GIT may adapt in some ways to a chronic aflatoxin challenge. Diaz *et al.* (2008) suggested that the opposite effects of increasing levels of AFB_1 on intestinal variables compared with birds' performance indicate that intestinal tissues may play an important role in adaptability toward AFB_1. As GIT is the first organ coming into contact with dietary aflatoxin challenge, its response toward the toxin may yield in the tissue adaptability during chronic aflatoxicosis.

The GIT epithelium is covered by protective mucus containing predominantly mucin glycoproteins that are synthesised and secreted by goblet cells. The concept of the mucus layer functioning as a dynamic defensive barrier is suggested by studies showing altered mucus-related indexes in germ-free animals (Gaskins *et al.,* 1998; Messlin *et al.,* 1999) and from consistent evidence of enhanced mucus secretion in response to intestinal microbes (Mack *et al.,* 1999). Because the mucus barrier is the first line of host defence against noxious agents and infections, we determined the effect of *S. cerevisiae* RC016 alone or in presence of AFs on the number of goblet cells. Aflatoxin-contaminated diets significantly decreased the number of goblet cells and the presence of *S. cerevisiae* RC016 was able to restore the number of these cells. It has been reported that some probiotics enhance mucin secretion and MUC2 gene expression in rat colon (Caballero-Franco *et al.,* 2007) or in HT-29 cell line (Duary *et al.*, 2014) and increase the number of goblet cells in mice duodenum (De Moreno de LeBlanc *et al.,* 2008).

In general, the protective effect of this yeast strain was more effective in the diet contaminated with the higher concentration of AFs (100 µg/kg AFB_1 + 50 µg/kg AFG_1). This result is in concordance with studies that report the beneficial effect of *S. cerevisiae* RC016 on rat performance when consuming the 100 µg/kg AFB_1 + 50 µg/kg AFG_1 diet, due to the mycotoxin adsorption by the yeast cell wall in the intestine and the reduction of its bioavailability (Gonzalez Pereyra *et al.,* 2014). This result was expected since, according to adsorption isotherms, mycotoxin adsorption by the yeast cell wall is a concentration dependent process. Bueno *et al.* (2007) proposed a physical adsorption model for the binding of AFB_1 to *S. cerevisiae.* The model permits the estimation of two parameters: the number of binding sites per microorganism (*M*) and the reaction equilibrium constant (Keq) involved, both of which are useful for estimating the adsorption efficiency (*M X*Keq) of a particular microorganism. El-Nezami *et al.* (1998) found that the amount of AFB_1 removed increased when increasing concentration of AFB_1. In concordance, *S. cerevisiae* RC016 demonstrated increased AFB_1 adsorption ability *in vitro* as AFB_1 concentration increased. The HRLM technique demonstrated a significant increase in *S. cerevisiae* RC016 cell diameter in the presence of AFB_1. This behaviour suggests an advantage, since a larger cell with a larger surface and a higher number of exposed binding sites would be able to act more efficiently as a mycotoxin adsorbent. In concordance, previous *in vitro* study demonstrated that *S. cerevisieae* RC016 increased its zearalenone binding ability under GI conditions (Armando *et al.,* 2012a). The TEM technique allowed a more accurate and precise morphometric analysis, by the examination of numerous ultrathin sections of different areas to corroborate results observed by HRLM. The observed results demonstrated ultrastructural changes were not related to cell wall thickness but with the increment in cell surface exposed to mycotoxin.

In conclusion, the probiotic strain *S. cerevisiae* RC016 demonstrated to have beneficial effects on the rat's small intestine morphology when viable cells were orally administered to healthy animals. In the case of animals receiving an AF-contaminated diet, it was estimated – according to these results and others obtained in a similar study (González Pereyra *et al.,* 2014) – that *S. cerevisiae* RC016 was able to bind AFs to its cell wall in the intestine, reducing its bioavailability. It was observed that *in vitro* exposure to AFB_1 induced an increase in the diameter of *S. cerevisiae* RC016, possibly exposing more binding sites and improving mycotoxin adsorption to the yeast cell wall. There are a lot of feed additives on the market with efficacy to bind AFs and alleviate their effects. However, the significance of this study lies in the ability of this yeast strain to act as a probiotic, as well as an AF-binder and makes it an interesting candidate for the formulation of a new additive to improve animal performance. The use of additives based on beneficial microorganisms instead of chemical products is a safer and eco-friendly option to increase animal productivity with a minimum environmental impact.

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