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ORIGINAL ARTICLE

Genotoxicity and cytotoxicity evaluation of probiotic *Saccharomyces cerevisiae* RC016: a 60-day subchronic oral toxicity study in rats

M.L. González Pereyra[1,2], C. Dogi[1,2], A. Torres Lisa[1], P. Wittouck[3], M. Ortíz[4], F. Escobar[1,2], G. Bagnis[3], R. Yaciuk[3], L. Poloni[4], A. Torres[1,2], A.M. Dalcero[1,2] and L.R. Cavaglieri[1,2]

1 Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Río Cuarto, Córdoba, Argentina
2 Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina
3 Departamento de Patología animal, Facultad de Agronomía y Veterinaria, Universidad Nacional de Río Cuarto, Río Cuarto, Córdoba, Argentina
4 Departamento de Ciencias Naturales, Facultad de Ciencias Exactas, Fisico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Río Cuarto, Córdoba, Argentina

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Correspondence
Lilia R. Cavaglieri, Departamento de Microbiología e Inmunología, Universidad Nacional de Río Cuarto, Ruta 36 Km 601, 5800 Río Cuarto, Córdoba, Argentina.
E-mail: lcavaglieri@exa.unrc.edu.ar

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Abstract

Aims: To acquire data on the safety-in-use of the probiotic *Saccharomyces cerevisiae* RC016 and test its ability to reduce genotoxicity caused by dietary aflatoxins (AFs).

Methods and Results: The probiotic was orally administered to Wistar rats. Six groups ($n = 6$) were arranged: feed and probiotic controls, two levels of AFs-contaminated feed and two treatments including both the probiotic and the toxin. Genotoxiciy and cytotoxicity were evaluated with the bone marrow micronuclei assay and the comet assay and internal organs were macroscopically and microscopically examined. The tested *S. cerevisiae* strain did not cause genotoxicity or cytotoxicity *in vivo*, and it was able to attenuate AFs-caused genotoxicity. *Saccharomyces cerevisiae* RC016 did not cause any impairment on the rats' health and it showed no negative impact on the weight gain. Moreover, RC016 improved zootechnical parameters in AFs-treated animals. The beneficial effects were likely to be caused by adsorption of AFs to the yeast cell wall in the intestine and the consequent reduction in the toxin's bioavailability.

Conclusions: The dietary administration of RC016 does not induce genotoxicity or cytotoxicity to rats.

Significance and Impact of the Study: Incorporation of RC016 in the formulation of feed additives increases animal productivity. Similar effects may even occur in human food applications.

Introduction

Probiotics are commonly defined as live micro-organisms which when administered in adequate amounts confer a health benefit to the host (FAO/WHO Report 2002). During the past three decades probiotics have been increasingly included in various types of food products and also in pharmaceutical preparations. Some probiotics have also demonstrated antigenotoxic effects; species of *Lactobacillus*, *Streptococcus*, *Lactococcus* and *Bifidobacterium* have shown antimutagenicity in the Ames test, as well as an ability to decrease DNA damage in with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine-treated colon cells (Pool-Zobel *et al.* 1993, 1996). The development of new probiotic strains aims at more active beneficial organisms. Previous to incorporating new strains into products their efficacy and safety should be carefully assessed (Salminen *et al.* 1998). *Saccharomyces cerevisiae* is a yeast species

widely commercialized as zootechnical feed additives and digestibility enhancers. It is also considered a Generally Regarded as Safe (GRAS) micro-organism; therefore, its application as feed additive entails minimal risk. Mycotoxin adsorption by *S. cerevisiae* and lactic acid bacteria has been reviewed recently (Shetty and Jespersen 2006). There are studies that relate the consumption of *S. cerevisiae* or a fraction of its cell wall, with the reduction in aflatoxin-induced toxicity in animals (Stanley *et al.* 1993; Santin *et al.* 2003; Baptista *et al.* 2004; Madrigal-Santillan *et al.* 2006). *Saccharomyces cerevisiae* presents various proven beneficial effects that include stimulation of intestinal microbiota growth in mammals, pH modulation in the rumen, improvement of reproductive parameters in dairy cows, as well as reduction in the number of pathogenic micro-organisms in monogastric animals (Dawson 1993; Wallace 1994, 1998). In addition, the glucan of the *S. cerevisiae* cell wall was able to reduce the frequency of micronuclei induced by cyclophosphamide in an *in vivo* trial using mice (Chovatovicova and Mavarova 1992).

Aflatoxins (AFs) are a group of naturally occurring mycotoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus*, which grow in a wide variety of food commodities (Kurtzman *et al.* 1987). Contamination of feedstuffs with these toxins is a continuing worldwide problem as their effect on livestock productivity can lead to significant economic losses and public health issues (Whitlow and Hagler 2002). Aflatoxin B_1 is a potent carcinogen for many animal species, including rodents, fish, primates and humans, being classified as group 1 human carcinogen by the International Agency for Research on Cancer (IARC 2002). In all species, AFB_1's target organ is the liver, although tumours may also develop in organs such as lung, kidney and colon (Wang and Groopman 1999). Aflatoxin B_1 was shown to be genotoxic *in vitro* as well as *in vivo*, increasing the rate of DNA adducts, chromosomal aberrations and sister chromatid exchanges (el-Zawahri *et al.* 1990; Anwar *et al.* 1994; Neal 1995).

Aflatoxin contamination of agricultural commodities and its impact on animal and human health have led to the development of decontaminating techniques that include physical, chemical and biological methods. The reduction in AFs bioavailability using various inorganic adsorbents—like bentonite and zeolites—has been thoroughly studied. However, some of these adsorbents can reduce nutritional value of feeds, produce undesirable side effects, and they are not considered safe by the European Union (Kabak *et al.* 2006).

In recent years, much attention has been paid to the design of functional foods that contain probiotic microbial strains responsible for health benefits in the host (Kumura *et al.* 2004).

Saccharomyces cerevisiae RC016 was isolated from pig intestine in a previous study. It has demonstrated to efficiently bind several mycotoxins *in vitro*, including AFB_1, ochratoxin A and zearalenone. Simulated gastrointestinal tract (GIT) conditions did not interfere with the AFB_1 binding ability of *S. cerevisiae* RC016. Moreover, this strain is able to survive and grow in simulated GIT conditions and demonstrated to be a promising probiotic (Armando *et al.* 2011, 2012).

Even though the adsorption of AFB_1 has been proved for other *S. cerevisiae* strains, the binding and probiotic characteristics, described by Armando *et al.* (2011, 2012) and Dogi *et al.* (2011) indicate that strain RC016 is a promising candidate to be included in the formulation of feed additives to enhance animal performance. To complement *in vitro* experiments, an *in vivo* study to evaluate genotoxicity and cytotoxicity of *S. cerevisiae* RC016 was conducted. The objective of this study was to acquire data on the safety-in-use of the probiotic *S. cerevisiae* RC016. The bone marrow micronuclei assay and the comet assay were used to evaluate genotoxicity and cytotoxicity of dietary yeast administered to Wistar rats. In addition, its ability to reduce genotoxicity caused by dietary AFs was studied. Also, internal organs were macroscopically and microscopically examined to assess potential impairment in *S. cerevisiae* RC016- treated and control rats.

Material and methods

Animals

Inbred male Wistar rats (*Rattus norvegicus*; 8 weeks old, weight 173 ± 15 g, $n = 36$) were housed in stainless steel metabolic cages (three animals per cage) and kept in an environmentally controlled room with 12 h light/darkness cycles. Animals were housed in the animal facility centre of the National University of Río Cuarto in accordance with international sanitary and ethical guidelines.

Saccharomyces cerevisiae strain

Saccharomyces cerevisiae RC016 was isolated from pig intestine in a previous study (Armando *et al.* 2011). It was identified by molecular techniques through DNA extraction and 18S rRNA and 28S rRNA amplification and analysis, comparing sequences with the basic local alignment search tool (BLAST) within the NCBI database as described by Armando *et al.* (2011). The strain is currently deposited in the culture collection of the Universidad Nacional de Río Cuarto collection centre, located in Río Cuarto, Córdoba, Argentina.

Saccharomyces cerevisiae cultures

The inoculum of *S. cerevisiae* RC016 was prepared daily from a 30°C 24 h culture in yeast-peptone-dextrose (YPD) broth and harvested by centrifugation (20 min, 700 ***g***). The cells were resuspended in sterile phosphate buffer saline (PBS) solution and the concentration was adjusted to 10^9 cells ml^{-1}. Concentration of cell suspensions was determined using an haemocytometer. Cell viability was confirmed by standard plate count method using YPD agar. Animals were administered a daily dose of 10^8 viable cells or colony-forming units (CFU), as 0·2 ml of the 10^9 CFU ml^{-1} *S. cerevisiae* RC016 suspension in PBS was the maximum volume the animals would take orally. The concentration of the *S. cerevisiae* suspension was adjusted to the volume that was more easily incorporated by the rats (0·2 ml).

Aspergillus parasiticus culture material

Aspergillus parasiticus cultures were prepared to obtain aflatoxin concentrations enough to contaminate feed for the experiment. Seven-day culture plugs of reference strain *A. parasiticus* NRRL 2999 were inoculated in 250 ml Erlenmeyer flasks containing 25 g autoclaved rice and 10 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. The content of all flasks was placed in a metallic tray, covered with paper, let to dry at 60°C in a forced air oven and ground with a laboratory mill. Aflatoxin B_1 and aflatoxin G_1 (AFG_1) 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. This AFs concentrate was used to contaminate feed.

Diets

Commercial basal diet

A commercial basal mice/rat diet (GEPSA Feeds; Grupo Pilar S.A., Buenos Aires, Argentina) was used as basal feed 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 analyzed for AFs content by HPLC (Trucksess *et al.* 1994). Extraction and clean-up procedure was performed using Mycosep® Afla-Pat 228 columns (Romer Labs, Tulln, Austria), following methodology provided by the manufacturer. The basal feed did not show detectable levels of AFs.

Experimental control diet

The control diet was prepared by mixing 2·52 kg of finely ground commercial basal feed with 60 g agar dissolved in 2·5 l water. The mixture was homogenized manually 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 AFs-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 diet (1) 40 $\mu g\ kg^{-1}$ AFB_1 + 20 $\mu g\ kg^{-1}$ AFG_1 and diet (2) 100 $\mu g\ kg^{-1}$ AFB_1 + 50 $\mu g\ kg^{-1}$ AFG_1. 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 homogenized and added 60 g agar dissolved in 2·5 l water. After a second homogenization, 30 g pieces were moulded manually. After solidification, feeds were stored at −20°C until use. Aflatoxin B_1 and AFG_1 concentration of both experimental diets was confirmed by HPLC as described for the commercial basal diet.

Experimental model

Thirty-six male Wistar rats were kept in metabolic cages for 60 days (three rats per cage, two cages per experimental group, each group $n = 6$). Animals were divided into six experimental groups: (i) uncontaminated feed control, (ii) yeast control, (iii) diet 1, (iv) diet 2, (v) diet 1 + daily oral dose of 10^8 viable *S. cerevisiae* cells, and (vi) diet 2 + daily oral dose of 10^8 viable *S. cerevisiae* cells. Animals were fed 90 g feed per cage per day (estimating a daily intake of 30 g of per animal, as recommended by the feed manufacturer and the animal house's personnel) and consumed water *ad libitum*. Feed and water were replaced daily. Oral doses of 0·2 ml of a *S. cerevisiae* suspension (10^9 CFU ml^{-1}) were administered daily (everyday at the same time, 10:00 AM) to groups 2, 5 and 6 and 0·2 ml of saline solution (0·9% NaCl) was administered to groups 1, 3 and 4. Disposable syringes without needle were used. Animals were weighed three times a week. The general health status of rats was evaluated by recording any changes in behaviour, activity, posture, fur quality, feed and water intake, possible illness and deaths. Bone marrow samples were collected for the micronuclei assay. After 60 days, animals were sacrificed by decapitation. Blood, faeces, liver, lungs, intestines and testicle samples were also collected and reserved to further analyses. The

protocol for this study was approved by the Committee of Ethics and Biosecurity of the Universidad Nacional de Río Cuarto.

Genotoxicity and cytotoxicity evaluation

The effect of *S. cerevisiae* on genotoxicity in rat erythrocytes and lymphocytes was evaluated through the bone marrow rodent micronuclei assay and the comet assay, respectively (Singh *et al.* 1988; Tice *et al.* 2000; Ezekiel *et al.* 2011). For the micronuclei assay, the femurs were removed and the bone marrow flushed with foetal bovine serum (Sigma Aldrich Chemie Gmbh, Munich, Germany). Cells were centrifuged twice at 112 ***g*** for 10 min. Supernatants were discarded leaving only 0·1–0·2 ml to resuspend cell pellets. Slides were let to dry, fixed with absolute methanol and stained with 0·4% May-Grunwald and 5% Giemsa stains consecutively while rinsing and air drying after each staining. Duplicate slides were prepared from each animal, assigned a coded nomenclature and scored by three independent scorers who were each assigned one slide per animal. Two thousand erythrocytes per animal were scored to determine the rate of micronucleated normochromatic erythrocytes (MNNCE%: percentage of MNNCE over total erythrocytes) and micronucleated polychromatic erythrocytes (MNPCE%: percentage of MNPCE over total erythrocytes). Besides, the bone marrow cytotoxicity was determined by counting the frequency of polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) in 2000 erythrocytes per animal to obtain the PCE/NCE index. Data were analyzed by the general linear and mixed model (GLMM) using INFOSTAT (ver. 2.03 for Windows 2012; University of Cordoba, Argentina) software. The variation between means of each treatment was analyzed by Fisher's least significant difference (LSD) test ($P \leq 0{\cdot}05$).

The comet assay was performed as described by Singh *et al.* (1988) and Tice *et al.* (2000) with some modifications. Briefly, blood samples of *S. cerevisiae*-treated (daily oral dose of 10^8 viable *S. cerevisiae* cells) and control animals (0·9% NaCl solution) were diluted in 1 ml FCS/RPMI mixture (1 : 1 v/v), and the cells were precipitated by centrifugation (5 min, 112 ***g***). The pelleted cells were then resuspended in 100 μl of 0·75% low melting point agarose at 37°C. Immediately, 75 μl of this suspension were spread onto two microscope slides per concentration precoated with 0·75% normal melting point agarose. The slides were coverslipped and allowed to gel at 4°C for 20 min. The coverslips were gently removed and 75 μl of 0·75%, 37°C low melting point agarose were added. Again, the slides were coverslipped and allowed to gel at 4°C for 20 min. The coverslips were removed and the slides were immersed in cold, freshly prepared lysis solution (2·5 mol l^{-1} NaCl, 100 mmol l^{-1} EDTA, 10 mmol l^{-1} Tris (pH 10), supplemented with 1% Triton X-100 and 10% DMSO (Merck, Darmstadt, Germany). The slides were allowed to stand protected from light at 4°C for 1 h. They were placed in a gel box and immersed in an alkaline pH > 13 buffer (300 mmol l^{-1} NaOH, 1 mmol l^{-1} EDTA, prepared from a stock solution of 10 N NaOH and 200 mmol l^{-1} EDTA) for 20 min at 4°C before electrophoresis to allow the DNA to unwind. Electrophoresis was carried out in ice bath (4°C) for 20 min at 250 mA and 30 V (0·722 V cm^{-1}). The slides were immersed in neutralization buffer (0·4 mol l^{-1} Tris-HCl, pH 7·5) for 15 min, dried at room temperature and fixed in 100% ethanol for 10 min. The slides were dried and stored overnight or longer before staining. For staining, the slides were briefly rinsed in distilled water, covered with 25 μl 1× ethidium bromide staining solution (prepared from a 200 μg ml^{-1} 10× stock solution) and coverslipped. The material was evaluated immediately at 400× magnification using fluorescence microscope (Axiophot; Carl Zeiss, Göttingen, Germany) attached to an image-analysis system (Powershot G6, 7·1 megapixels; Canon Inc., Japan with software AXIOVISION RELEASE 4.6.3; Carl Zeiss), with 515–560 nm excitation filter and a 590 nm barrier filter. From each treatment, images from 100 'nucleoids' were captured with a camera attached to the fluorescent microscope and linked to COMETSCORE® 1.5 (Perceptive Instruments, Suffolk, UK) software. Highly damaged cells were not included in the scoring (clouds were not analyzed). Tail moment (TM) was used to estimate DNA damage (arbitrary units). TM data were transformed to $\log_{10}(x + 1)$ to obtain variance homogeneity and analyzed by ANOVA and LSD test ($P \leq 0{\cdot}05$).

Daily feed and aflatoxin intake, weight gain, feed efficiency and conversion and general health status

Estimated daily feed intake (DI) was 30 g per animal. Ninety grams was administered in each cage per day. Daily AFB_1 consumption was approximately 1·2 and 3·0 μg for animals receiving diets 1 and 2, respectively. Daily AFG_1 consumption for each diet was 0·6 and 1·5 μg, respectively. Total weight gain (TWG) was calculated as the difference between the weight of animals in the beginning and at the end of the experiment. Progressive weight gain (PWG) was calculated considering the PWG of animals (weighed three times a week) during the experiment. Feed efficiency (FE) was calculated as TWG/DI and feed conversion (FC) as DI/TWG. Each of these growth parameters was measured individually (per animal), per cage and per experimental group and statistically analyzed. Data were analyzed by the GLMM using INFOSTAT (ver. 2012) software, considering treatments as fixed effects and each animal, cage and weight registration as random effects.

First order continuous autoregressive correlations were considered, and the model was corrected to obtain the homogeneity of the variance. The variation between means of each treatment was analyzed by the LSD test ($P \leq 0{\cdot}05$). The general health status of rats was evaluated by recording any changes in behaviour, activity, posture, fur quality, feed and water intake, possible illness and mortality.

Organs and tissue examination

Macroscopic characteristics (weight, size and colour) of the organs collected during necropsy (liver, lungs, kidney and intestine) were registered. Tissue slices were fixed in 4% neutral buffered formalin (pH 7·4) for paraffin routine processing. These samples were cut at 4 μm thickness and stained with haematoxylin/eosin (H&E) stain and Masson's Trichrome stain to demonstrate the presence of connective tissue. Stained sections were examined by light microscopy. Photomicrographs were taken with a Zeiss Axiostar plus microscope using an Electronic Eyepiece camera and a Canon Power Shot G5 camera (Canon Inc.).

Results

Genotoxicity and cytotoxicity evaluation

The induction of micronuclei in each experimental group was reported as the mean occurrence of MNPCE and MNNCE. According to this test, the oral administration of the probiotic *S. cerevisiae* RC016 did not cause any genotoxicity or cytotoxicity by itself in group 2 showing no significant differences in MNPCE% compared with control animals of group 1. In contrast, the AFs-contaminated diets caused genotoxicity in groups 3, 4 and 5. However, the administration of a daily dose of *S. cerevisiae* RC016 was able to reduce the genotoxicity index in group 6 (0·0189) compared with group 4 (0·0376) within animals consuming the same level of toxin ($P \leq 0{\cdot}05$). The genotoxicity caused by AFs in group 6 was reduced to the level of the control group, showing no statistically difference when comparing MNPCE% with groups 1 and 2. In this study, AFs did not cause cytotoxicity at the levels tested (Table 1).

Genotoxicity of *S. cerevisiae* RC016-treated animals on lymphocytes was also assessed by the comet assay. Non-treated animals were used as negative controls. The results were expressed as data of TM. The mean TM $\pm$ SE (standard error) was 5·25 $\pm$ 0·22 for *S. cerevisiae*-treated animals and 4·79 $\pm$ 0·22 for negative controls, indicating no significant difference in cell migration between both treatments.

Daily feed and aflatoxin intake, weight gain, FE and conversion and general health status

Results showed that the administration of the oral dose of *S. cerevisiae* RC016 did not cause any adverse effects on weight gain (TWG and PWG), FC or FE during the experiment. Moreover, it was able to improve all zootechnical parameters in animals fed AFs-contaminated diets (groups 5 and 6) in comparison to AFs control groups 3 and 4. Mean TWG was higher ($P \leq 0{\cdot}05$) in groups 5 and 6. Compared with other groups, mean PWG was also higher in these two groups. FC and FE indexes followed the same tendency being significantly different in groups 5 and 6 (Table 2). Regarding the general health status of animals, rats from all groups appeared healthy, inquisitive and active throughout the experiment. The oral dose of *S. cerevisiae* RC016, did not

Table 1 Effect of *Saccharomyces cerevisiae* RC016 on the genotoxicity (MNPCE%) and cytotoxicity (PCE/NCE) indexes in rats consuming two different levels of aflatoxins and control rats

	Erythrocytes frequency*				Mean $\pm$ SE†	
Treatments	MNNCE	NCE	MNPCE	PCE	MNPCE%‡	PCE/NCE§
1. Uncontaminated feed control	9	1323	12	734	0·0151 $\pm$ 0·01^{b}	0·4747 $\pm$ 0·05
2. *S. cerevisiae* RC016 control	5	1293	6	697	0·0098 $\pm$ 0·01^{b}	0·4312 $\pm$ 0·04
3. 40 μg kg^{-1} AFB_1 + 20 μg kg^{-1} AFG_1 diet	17	1427	23	715	0·0297 $\pm$ 0·01^{a}	0·4828 $\pm$ 0·05
4. 100 μg kg^{-1} AFB_1 + 50 μg kg^{-1} AFG_1 diet	23	1436	29	871	0·0376 $\pm$ 0·01^{a}	0·5392 $\pm$ 0·05
5. 40 μg kg^{-1} AFB_1 diet + 20 μg kg^{-1} AFG_1 + *S. cerevisiae* RC016	29	1382	28	708	0·0355 $\pm$ 0·01^{a}	0·5270 $\pm$ 0·05
6. 100 μg kg^{-1} AFB_1 diet + 50 μg kg^{-1} AFG_1 + *S. cerevisiae* RC016	23	1487	19	945	0·0189 $\pm$ 0·01^{b}	0·6457 $\pm$ 0·07

MNNCE, micronucleated normochromatic erythrocytes; NCE, normochromatic erythrocytes; MNPCE, micronucleated polychromatic erythrocytes; PCE, polychromatic erythrocytes.

Values showing different superscript letters within the same column are significantly different.

*In 2000 cells (not mean values).

†Mean value $\pm$ standard error between slides counted by three different operators for each treatment.

‡$P \leq 0{\cdot}05$; §$P \leq 0{\cdot}01$.

Table 2 Effect of *Saccharomyces cerevisiae* RC016 on total weight gain (TWG: difference between initial and final weight of animals), progressive weight gain (PWG: weight changes calculated through the experiment, weighing animals three times a week), feed conversion (FC: TWG/daily feed intake) and feed efficiency (FE: daily feed intake/TWG) in rats consuming two different aflatoxin-contaminated diets and control rats

	Production parameters Mean ± SE			
Treatment	TWG (g)	PWG (g)	FC index	FE index
1. Uncontaminated feed control	79·17 ± 7·31[b]	3·22 ± 1·42[b]	0·41 ± 0·04[a]	2·64 ± 0·21[b]
2. *S. cerevisiae* RC016 control	83·33 ± 7·31[b]	3·58 ± 1·28[b]	0·37 ± 0·03[a]	2·78 ± 0·23[b]
3. 40 μg kg^{-1} AFB_1 + 20 μg kg^{-1} AFG_1 diet	86·60 ± 8·01[b]	3·65 ± 1·22[b]	0·35 ± 0·03[a]	2·89 ± 0·26[b]
4. 100 μg kg^{-1} AFB_1 + 50 μg kg^{-1} AFG_1 diet	89·50 ± 7·31[b]	3·77 ± 1·17[b]	0·35 ± 0·03[a]	2·99 ± 0·25[b]
5. 40 μg kg^{-1} AFB_1 + 20 μg kg^{-1} AFG_1 diet + *S. cerevisiae* RC016	101·17 ± 7·31[a]	4·05 ± 1·08[a]	0·30 ± 0·02[b]	3·21 ± 0·27[a]
6. 100 μg kg^{-1} AFB_1 diet + 50 μg kg^{-1} AFG_1 + *S. cerevisiae* RC016	103·50 ± 7·31[a]	4·17 ± 0·96[a]	0·30 ± 0·02[b]	3·39 ± 0·30[a]

SE, standard error.
Values showing different superscript letters within the same column are significantly different ($P \leq 0{\cdot}05$).

cause mortality, illness or significant changes in the general behaviour of animals. No significant difference in feed and water intake or fur quality between groups were observed either.

Organs and tissue examination

Gross examination of the internal organs of all rats revealed no detectable abnormalities, indicating that the probiotic yeast did not induce any organ damage. Macroscopic analyses of the different organs of *S. cerevisiae* RC016-treated rats did not show significant changes in colour and texture when compared with the control group. Representative microscopic findings in liver, lungs, kidneys and intestine of the *S. cerevisiae* RC016-treated and control rats are shown in Fig. 1. The histological examinations revealed that there were no differences between *S. cerevisiae* RC016-treated and control rats.

Discussion

In the present study, the genotoxicity and cytotoxicity of the probiotic *S. cerevisiae* RC016 in rats were tested to assess risk for its potential incorporation in the formulation of feed additives. In addition, the potential of this strain for reducing the MNNCE rate induced by dietary intake of AFs (a genotoxic group of mycotoxins) was studied. This strain had shown a remarkable mycotoxin-binding ability *in vitro* after simulated gastrointestinal passage that included AFB_1 adsorption (Armando *et al.* 2011; Dogi *et al.* 2011). However, *in vivo* studies to verify this ability in the animal's intestine were needed.

Previous *in vitro* studies indicated *S. cerevisiae* RC016 as the most suitable strain for a first *in vivo* trial (amongst other *S. cerevisiae* strains isolated from the animal environment) due to its probiotic and mycotoxin-binding properties (Dogi *et al.* 2011). Even though the reduction in AFs' effects by different *S. cerevisiae* strains has been thoroughly studied and some *S. cerevisiae*-based commercial products are already available in the market, it is the ability of this strain to act as a probiotic as well as an AFs binder what makes it an interesting candidate for the formulation of a new additive to improve animal performance. The daily oral dose of viable 10^8 CFU ml^{-1} to be administered to the animals was also selected according to previous *in vitro* studies. Armando *et al.* (2011) demonstrated that a 10^7 CFU ml^{-1} inoculum of *S. cerevisiae* RC016 was able to bind up to 82% of AFB_1 in PBS solution *in vitro*. As cell counts can be decreased by gastrointestinal passage (Dogi *et al.* 2011), a higher dose (10^8 CFU ml^{-1}) was used for the present *in vivo* study. The experimental model was designed following international guidelines for *in vivo* studies, and some parts were taken from other authors' models (Gelderblom *et al.*, 2002; Theumer *et al.* 2008). The AFs doses were chosen not to cause an acute intoxication but to simulate subchronic aflatoxicosis-causing doses found in naturally contaminated feeds (Gelderblom *et al.*, 2002; Theumer *et al.* 2008; Benford *et al.* 2010).

The rodent bone marrow micronuclei assay was applied on the one hand, to ensure the safety on the genotoxicity and cytotoxicity aspects, of the probiotic strain for animal use and on the other hand, to determine if *S. cerevisiae* RC016 could reduce the genotoxic effect of AFs due to its AFB_1-binding ability. Even though this assay is frequently performed on mice, it is demonstrated that it is equally effective when using Wistar rats (Hayes *et al.* 2009). The micronuclei assay is a successful method in the assessment of chromosome damage, genotoxicity and cytotoxicity (Krishna and Hayashi 2000; Grisolia *et al.* 2004; Resende *et al.* 2006; Mañas *et al.* 2009; Sabini *et al.* 2013). Neither *S. cerevisiae* nor AFs showed cytotoxic effect on bone marrow erythrocytes at the tested levels. As it was expected, the oral administration of the probiotic strain did not cause genotoxicity,

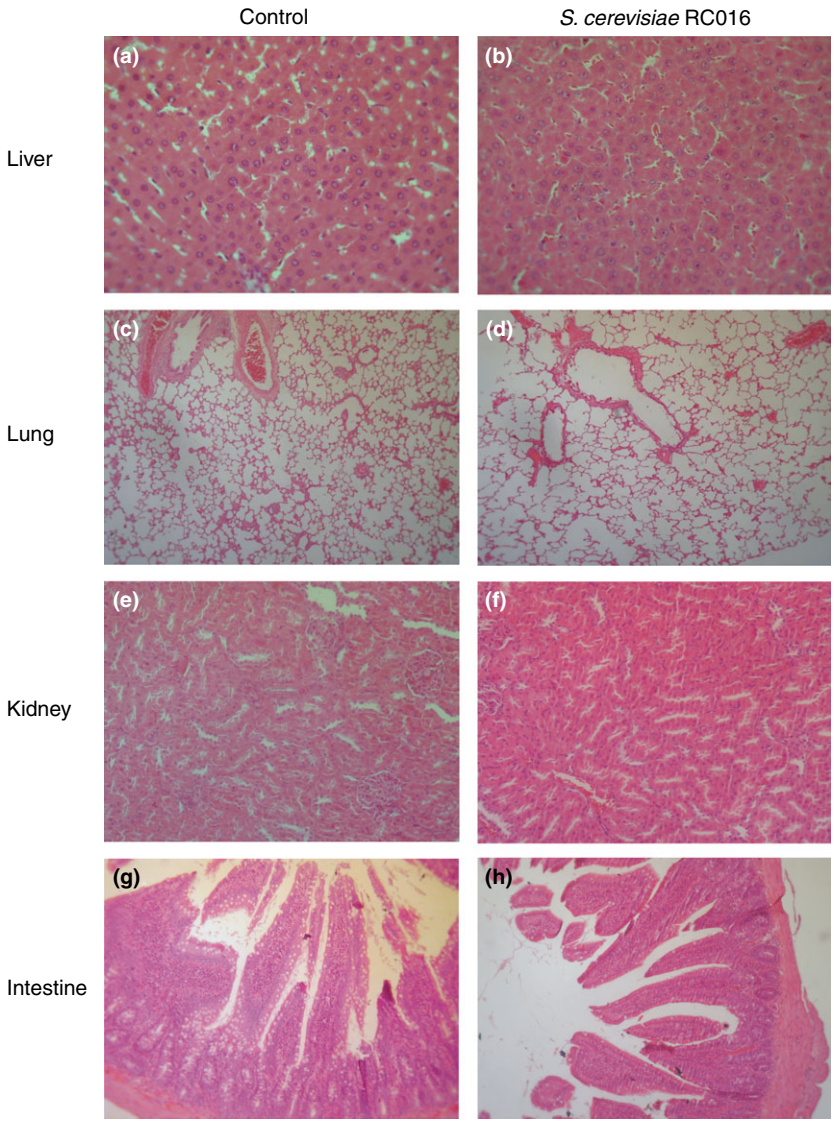

Figure 1 Histological examination of internal organs of *Saccharomyces cerevisiae* RC016-treated and nontreated (control) Wistar rats administered a daily oral dose of 10^8 viable yeast cells. (a) Liver ×400, control group; (b) Liver ×400, *S. cerevisiae* RC016-treated group; (c) Lung ×100, control group; (d) Lung ×100, *S. cerevisiae* RC016-treated group; (e) Kidney ×400, control group; (f) Kidney ×400, *S. cerevisiae* RC016-treated group; (g) Small intestine ×100; control group; (h) Small intestine ×100; *S. cerevisiae* RC016-treated group.

obtaining normal MNPCE rates in animals from group 2. In contrast, a higher MNPCE% was obtained in treatments consuming AFs, indicating genotoxicity induction. This finding is consistent with other reports that describe AFB_1 as a potent mutagen (Marquez-Marquez *et al.* 1993; Anwar *et al.* 1994; Madrigal-Santillan *et al.* 2006). However, MNPCE% in the yeast + AFs-treated group 6 was not significantly different from the control group 1 and group 2. Therefore, it was observed that, not only *S. cerevisiae* RC016 did not cause any increase in the genotoxicity index, but it was able to significantly reduce genotoxicity induced by the diet containing the highest amount of AFs. Armando *et al.* (2011) studied the adsorption isotherm for *S. cerevisiae* RC016 *in vitro* and demonstrated AFB_1 adsorption increased as the toxin's concentration increased as well. This could explain why the protective effect of *S. cerevisiae* could be evident in the treatment that received higher amount of AFs. Likewise, Madrigal-Santillan *et al.* (2006) observed an improvement in the micronucleated erythrocytes rate when administrating *S. cerevisiae* to mice consuming 0·4 and 0·8 mg kg^{-1} AFB_1-contaminated corn for 6 weeks. The results of the micronuclei assay were consistent with results of the comet assay demonstrating no induction of genotoxicity associated with the oral administration of *S. cerevisiae* RC016 to rats. The absence of cytotoxicity and genotoxicity associated with exposure to *S. cerevisiae* RC016 is consistent with the expected outcome for a probiotic organism, as well as the absence of illness, mortality and other health changes related to the intake of this strain. The examination of internal organs of *S. cerevisiae* RC016-treated and control rats revealed no macroscopic

or microscopic differences or lesions associated to the use of the probiotic. This is another promising result that suggests the strain is safe to use *in vivo*.

The selected yeast dose resulted effective to reduce genotoxicity caused by levels of AFs, similar to those found in naturally contaminated commodities from our region (below 40 μg kg^{-1}; González Pereyra *et al.* 2008a, b, 2009, 2011a,b, 2012a). As *S. cerevisiae* RC016 demonstrated to bind AFB_1 *in vitro,* we consider it is probable that this strain could be able to adsorb AFs *in vivo* in the rats' intestine reducing AFs bioavailability. Moreover, AFB_1 quantification in rat faeces obtained in this study indicated an increase of AFB_1 concentration in *S. cerevisiae* RC016-treated animals (González Pereyra *et al.* 2012b).

In our study, the application of a daily oral dose of *S. cerevisiae* RC016 did not cause any negative impact on the animals' weight gain, FC or FE. On the contrary, it was able to significantly improve weight gain and all the zootechnical parameters in rats consuming feed contaminated with two different levels of AFs compared with controls. Similar results were obtained in other *in vivo* studies performed by Madrigal-Santillan *et al.* (2006) and Çelýk *et al.* (2003) in mice and broiler chickens consuming AFB_1 and *S. cerevisiae.*

The beneficial effect of the administration of the yeast strain to animals was more evident in the groups consuming both, *S. cerevisiae* and the toxin, showing an improvement even when compared with the untreated controls or the yeast-only-treated animals. This could be explained by results obtained by Dogi *et al.* (2012) who demonstrated that, in the present *in vivo* study, the AFB_1-contaminated diets induced an increase in the depth of the intestinal crypt as well as in the length and width of the villi in the rat's intestine, compared with control group 1, which could enhance nutrient absorption. Moreover, Armando *et al.* (2012) suggested the exposure of *S. cerevisiae* RC016 to AFB_1 increases the yeast cell ratio growth, increasing also the cell surface and the number of exposed adsorbing sites. These effects combined could result in a beneficial effect on animals consuming both, the toxin and the yeast.

In conclusion, the notable absence of cytotoxicity and genotoxicity associated with exposure to *S. cerevisiae* RC016 and the ability of this strain to act as a probiotic as well as an AFs binder is what makes it an interesting candidate for the formulation of a new additive to improve animal performance. The use of additives based on beneficial micro-organisms instead of chemical products is a safer and eco-friendly option to increase animal productivity with a minimum environmental impact.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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