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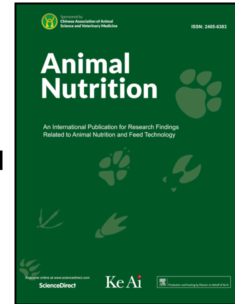

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Saccharomyces cerevisiae RC016-based feed additive reduces liver toxicity, residual aflatoxin B_1 levels and positively influences intestinal structure in broiler chickens fed on chronic aflatoxin B_1 levels-contaminated diets

Valeria Poloni [a,b], Alejandra Magnoli [a,b], Analía Fochesato [a,c], Andrea Cristofolini [a,d], Matías Caverzan [d], Cecilia Merkis [d], Mariana Montenegro [b,e], Lilia Cavaglieri [a,b] *

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1 **_Saccharomyces cerevisiae_ RC016-based feed additive reduces liver toxicity, residual**
2 **aflatoxin B_1 levels and positively influences intestinal morphology in broiler chickens fed**
3 **chronic aflatoxin B_1-contaminated diets**

4

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22 **Abstract**

23 The present study was conducted to investigate the ability of *Saccharomyces cerevisiae*
24 RC016 (Sc)-based feed additive to reduce liver toxicity, residual aflatoxin B_1 (AFB_1) levels
25 and influence intestinal structure in broiler chickens fed chronic aflatoxin B_1-contaminated
26 diets. A total of 100 one-day-old male commercial line (Ross) broiler chickens were divided
27 into 4 treatments, with 5 pens per treatment and 5 broiler chickens per pen. Birds were
28 randomly assigned to 4 treatments, which were namely treatment 1 (T_1), control diet (CD); T_2,
29 CD + Sc at 1 g/kg; T_3, CD + AFB_1 at 100 µg/kg; T_4, CD + Sc at 1 g/kg + AFB_1 at 100 µg/kg.
30 The liver histopathology of broiler chickens fed diets with AFB_1 showed diffused
31 microvacuolar fatty degeneration. The addition of Sc showed normal hepatocytes similar to
32 the control. The small intestine villi from AFB_1 group showed atrophy, hyperplasia of goblet
33 cells, prominent inflammatory infiltrate and oedema. In contrast, the small intestine villi from
34 birds that received the yeast plus AFB_1 showed an absence of inflammatory infiltrate, and
35 atrophy; moreover, a lower number of goblet cells compared to the groups with AFB_1 was
36 observed. The morphometric intestine studies showed that a significant decrease ($P < 0.05$) in
37 the crypt depth values when Sc was applied to AFB_1-contaminated diets. Although the
38 intestinal villus height and apparent adsorption area did not show significant differences ($P >$
39 0.05), there was a tendency to improve these parameters. The residual levels of AFB_1 in livers
40 were significantly reduced ($P < 0.05$) in the presence of the yeast. The present work
41 demonstrated that the addition of Sc alone or in combination with AFB_1 in the broiler chicken
42 diets had a beneficial effect in counteracting the toxic effects of AFB_1 in livers besides
43 improving the histomorphometric parameters and modulating the toxic effect of AFB_1 in the
44 intestine.

45

46 **Keywords:** Aflatoxin B_1; Broiler chickens; Histomorphometry; Histopathology toxic effects.

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47 **1. Introduction**

48

49 Mycotoxins are secondary metabolites produced by *Aspergillus*, *Penicillium* and *Fusarium*
50 fungal species. Frequently, mycotoxins are found in very low concentrations in plants, which
51 is impossible to control by chemical or biological methods and tend to accumulate causing a
52 negative impact on animals (Speijers and Speijers, 2004). Aflatoxin B_1 (AFB_1) is one of the
53 most toxic mycotoxins classified by the International Agency for Research on Cancer as
54 group 1 carcinogens (IARC, 2012). The effect of AFB_1 in broiler chickens mainly depends on
55 the time and dose of exposure (Oguz et al., 2003). Moreover, it has been widely known as
56 hepatotoxic and hepatocarcinogenic agent, causing immunosuppression during chronic
57 intoxications due to its ability to bind DNA, affecting protein synthesis. Aflatoxin B_1 has been
58 shown to produce morphological alterations of the intestinal epithelium by increasing the
59 depth of the crypts and decreasing the height of the villi, mainly at the level of the small
60 intestine (duodenum and jejunum). This alteration results in a modification of the
61 functionality of the small intestine, affecting the absorption of the nutrients and consequently
62 the productive parameters (Yunus et al., 2010).
63 Adsorbent agents incorporated in diets sequester the toxins in the gastrointestinal tract
64 forming insoluble complexes that are eliminated in the faeces (Yiannikouris et al., 2006).
65 Thus, reducing the bioavailability of mycotoxins decreases their toxic effects. Several
66 zeolites, bentonites and clinoptilolite, which are natural adsorbents, have been evaluated *in*
67 *vitro* and *in vivo* for their ability to adsorb **a**flatoxins (Magnoli et al., 2011; Nemati et al.,
68 2014). Although these products are widely available as commercial feed additives, they have
69 negative effects reducing nutritional value of feeds or producing undesirable side effects
70 (Zain, 2011). Organic compounds such as *Pichia* sp. yeast based product and yeast cell wall-
71 based products have also been suggested to reduce *in vivo* the negative effects produced by

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72 mycotoxins (Roto et al., 2015; Magnoli et al., 2017). While *in vitro* studies are extensive, few
73 *in vivo* studies have demonstrated the effectiveness of biological adsorbents by evaluating the
74 effects on intestinal integrity and liver toxicity with chronic experimental levels of AFB_1.
75 Thus, the aim of this study was to investigated the ability of *Saccharomyces cerevisiae*
76 RC016 (Sc)-based feed additive to reduce liver toxicity, residual AFB_1 levels and to influence
77 on intestinal morphology in broiler chickens fed chronic AFB_1-contaminated diets.
78
79
80 **2. Materials and methods**
81
82 The working protocol and the used techniques comply with the regulations of the
83 Subcommittee on Animal Bioethics under the Ethics Committee of Scientific Research, as
84 established in Resolution 253/10 of the Superior Council of the National University of Rio
85 Cuarto.
86
87 *2.1. Yeast biomass production*
88 *Saccharomyces cerevisiae* RC016 isolated from animal ecosystem was identified by
89 molecular techniques through DNA extraction and 18S rRNA and 28S rRNA amplification
90 and analysis, comparing sequences with the Basic Local Alignment Search Tool (BLAST)
91 within the National Centre for Biotechnology Information (NCBI) database (Armando et al.,
92 2012). The strain is currently deposited in the culture collection of the Universidad Nacional
93 de Río Cuarto collection centre, located in Río Cuarto, Córdoba, Argentina.
94 *Saccharomyces cerevisiae* RC016 biomass was obtained from 24-h culture in Yeast-Peptone-
95 Dextrose broth added 1 g of PO_4H_2K per litre in a BioFlo 2000 fermentor (New Brunswick
96 Scientific Co., Inc., Enfield, CT, USA) operated at 28ºC, at 3.6223 g and aeration 1.5 vessel

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97 volume per minute during 8 h. The pH value was adjusted to 5 with 6 mol/L sodium
98 hydroxide (NaOH). The working volume was 2 L. The fermenter was first inoculated with 4.5
99 $\times 10^6$ cells/mL and samples were taken every hour during 10 h. The optical density at 640 nm
100 was measured and the number of viable cells was counted in a haemocytometer by the trypan
101 blue exclusion assay. After biomass was produced, cells were harvested and concentrated by
102 centrifugation (20 min, 698.75 $\times$ *g*) at room temperature, and the pellets were lyophilized and
103 homogenized to be incorporated into the control diet in order to provide a concentration of 1 g
104 yeast/kg feed (0.1%). The levels of inclusion of Sc were selected according to bibliographic
105 references (Seidavi et al., 2017).

106

107

108 *2.2. Aflatoxin B_1 production*

109

110 Enough AFB_1 concentration to contaminate feed for the experiment was produced. Seven-day
111 culture plugs from a reference strain *Aspergillus parasiticus* NRRL2999 were inoculated in
112 250-mL Erlenmeyer flasks containing 25 g autoclaved rice and 10-mL distilled water.
113 Cultures were incubated in the dark at 28 °C for 7 d, manually stirring the flasks vigorously
114 for 1 min once a day during the first 5 d to enhance the dissemination of conidia in the rice.
115 After incubation, the cultures were autoclaved. The content of flasks was placed in a metallic
116 tray, covered with paper, let dry at 60 °C in a forced air oven and ground with a laboratory
117 mill. Aflatoxin B_1 content of the resulting powder was quantified by High-Performance
118 Liquid Chromatography (HPLC) according to the methodology described by Trucksess et al.
119 (1994). The ratio of AFB_1 to AFG_1 concentration in the culture was 2:1. Aflatoxin B_1 is the
120 most abundant in food and contaminated feed, toxic and carcinogenic to human beings and
121 animals. Therefore, the AFB_1 effect was only tested. The analyses were performed in

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122 triplicate. The milled contaminated substrate (60.0 ± 1.1 μg/g) was added to the control diet
123 pre-premixing to provide a concentration of about 100 μg of AFB_1/kg of feed.
124
125 *2.3. Aflatoxin B_1 determination in feed*
126
127 Feed sampling for AFB_1 analysis was carried out following the recommendations of the
128 European Union (Regulation 401/2006 and its modification by Regulation 178/2010). Food
129 samples (1 kg) were homogenized and quartered to obtain a laboratory sample. Twenty-five
130 grams of ground feed were extracted with 125 mL of methanol/water (60:40, vol/vol), 80 mL
131 hexane, and 2 g NaCl and shaken 30 min in an orbital shaker. The mixture was filtered using
132 Whatman No 4 filter paper (Whatman, Inc., Clifton, NJ, USA) and 25 mL of the
133 methanol/water phase of the filtrate was extracted twice with 25 and 15 mL of chloroform,
134 respectively. The chloroform phase was vacuum-dried using a rotatory evaporator and the
135 extract was redissolved in 200 μL of mobile phase. The concentration of AFB_1 in each diet
136 was estimated by HPLC-fluorescence according to Trucksess et al. (1994). The mobile phase
137 was methanol–acetonitrile–water (1:1:4, vol/vol/vol), pumped at a flow rate of 1.5 mL/min.
138 For derivatization, aliquots (200 μL) were mixed with 700 μL of acetic acid–trifluoroacetic
139 acid–water (20:10:70) solution and allowed to stand for 9 min at 65 °C in the dark (AOAC,
140 1994). An HPLC Waters Alliance 2695 system coupled to a fluorescence detector (Waters
141 2487) was used. Excitation and emission wavelengths were set on of 395 and 470 nm,
142 respectively. Separation was carried out in a C18 Luna Phenomenex column (150 × 4.6 mm, 5
143 μm). Standards for the calibration curve were prepared by dilution of a stock solution of AFB_1
144 2.06 μg/mL. The concentrations of chromatographic standards were 0.005, 0.010, and 0.015
145 μg/mL of AFB_1. Standard solutions for the calibration curves were prepared daily. Although
146 the levels of other mycotoxins were not determined, the animals did not present

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147 symptomatology associated with deoxynivalenol, nivalenol, zearalenone, ochratoxin A,
148 fumonisin B_1 + B_2 and T-2 and HT-2 mycotoxins. The levels of AFB_1 in the broilers control
149 diet were 3 ± 1 μg/kg (natural contamination) and 100 ± 6 μg/kg for the contaminated diets of
150 AFB_1. The analysis was developed in triplicates.
151

152 *2.4. Experimental design*

153 A total of 100 one-day-old male broiler chickens vaccinated against Mareck disease were
154 divided into 4 treatments with 25 broiler chickens each. The broiler chickens were fed *ad*
155 *libitum* with each of the experimental diets from d 1 to 22 of age. On d 1, birds were weighed
156 individually (BW ± SD) and were allocated randomly into treatments. The birds were
157 provided continuous fluorescent lighting with feed and water available *ad libitum* until they
158 were 22 d old. During the experimental period, broiler chickens received the diet
159 corresponding to each treatment. A standard corn-soybean meal starter diet commercial (basal
160 diet) that met of Ross 308 Guidelines (Aviagen. Ross 308 Broiler Management Manual,
161 2014) requirements was used to formulate the different experimental diets (Table 1).

162 The experimental diets for each treatment were as follows: treatment 1 (T_1), control diet (CD);
163 T_2, CD + Sc at 1 g/kg; T_3, CD + AFB_1 at 100 μg/kg; T_4, CD + Sc at 1 g/kg + AFB_1 at 100
164 μg/kg.

165 Broiler chickens were monitored daily for signs of morbidity and mortality. At the end of the
166 feeding trial, residual levels of AFB_1, macroscopic (colour and size) and microscopic changes
167 in the liver, histopathological changes and morphometric parameters (villus length and width,
168 and crypt depth) in the intestines of the broiler chickens were evaluated.

169 When broiler chickens reached 22 d old, the feeding trial was terminated, and 5 broiler
170 chickens from each treatment were selected randomly and sacrificed by cervical dislocation.
171 The livers and duodenal loops were removed and fixed in 10% neutral buffered formalin. The

172 fixed tissues were trimmed, embedded in paraffin, and stained with hematoxylin–eosin for
173 histopathological examination by optical microscopy studies. Part of the liver was conserved
174 a -20ºC for residual AFB_1 levels analysis.

175

176 *2.5. Liver and intestinal histopathology*

177

178 Portions of approximately 6 mm^2 of liver and duodenal tissue samples
179 were fixed in 4 % (vol/vol) buffered-saline formaldehyde pH 7.2 to 7.4 at 4 °C, dehydrated in
180 a graded series of ethanol (30%, 50%, 70%, 80%, 90%, 95% and 100%) and xylene solutions,
181 embedded in paraffin and cut in ± 4 µm histological serial-sections. The histological sections
182 were stained with hematoxylin–eosin for microscopic analysis. Liver slides were examined
183 for characteristic intoxication signs and hepatocellular degeneration of livers was evaluated
184 according to Magnoli et al. (2011). Intestine were examined for damage and inflammation
185 using a standard histopathological grading system described by Del Carmen et al. (2013),
186 histological findings identical to normal mice (grade 0); mild mucosal and/or submucosal
187 inflammatory infiltrate (admixture of neutrophils) and oedema, punctate mucosal erosions
188 often associated with capillary proliferation, muscularis mucosae intact (grade 1); grade 1
189 changes involving 50% of the specimen (grade 2); prominent inflammatory infiltrate and
190 oedema (neutrophils usually predominating) frequently with deeper areas of ulceration
191 extending through the muscularis mucosae into the submucosa; rare inflammatory cells
192 invading the muscularis propria but without muscle necrosis (grade 3); grade 3 changes
193 involving 50% of the specimen (grade 4); extensive ulceration with coagulative necrosis
194 bordered inferiorly by numerous neutrophils and lesser numbers of mononuclear cells;
195 necrosis extends deeply into the muscularis propria (grade 5); grade 5 changes involving 50%
196 of the specimen (grade 6). High histological scores indicate increased damage in the

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197 intestines. Digital images were captured with an Axiophot microscope (Carl Zeiss,
198 Thornwood, NY) fitted with high-resolution Power shot G6 7.1 megapixels digital camera
199 (Canon INC, Japan). Digital image analysis and morphometric measurements were performed
200 with Axiovision AxioVs40 V4.6.3.0 software (Carl Zeiss, Göttingen, Germany).
201

202 *2.6. Intestinal morphology*
203

204 Morphometric measurements of intestinal variables were carried out on 2 slides per animal's
205 intestine, 2 sections per slide and 5 fields per section. The morphometric measurements taken
206 from the intestinal histological sections included villus length and width, intestinal crypt
207 depth and quantification of goblet cells. Digital images were captured with an Axiophot
208 microscope (Carl Zeiss, Thornwood, NY) fitted with high resolution Power shot G6 7.1
209 megapixels digital camera (Canon INC, Japan). Digital image analysis and morphometric
210 measurements were performed with Axio vision AxioVs40 V4.6.3.0. software (Carl Zeiss,
211 Göttingen, Germany). Later, apparent absorptive surface area was calculated using the
212 following formula according to Iji et al. (2001).
213 Apparent absorptive surface area = (3.1 × Villi width + 3.2 × Villi height) × 1 - (2 × Villi
214 height).
215

216 *2.7. Residual levels of aflatoxin B_1 in livers*
217

218 A total of 20 livers (n = 20) were selected, i.e. 5 livers from each treatment. Aflatoxin B_1 in
219 the liver tissue was extracted according to AOAC (1995) as described by Tavčar-Kalcher et
220 al. (2007) with some modifications. Briefly, the ground liver sample (50 g) was
221 mixed thoroughly with 5 mL of a 20% aqueous citric acid solution and diatomaceous earth

222 (10 g). The toxin was extracted with 100 mL dichloromethane by stirring for 30 min at room
223 temperature. The organic phase was filtered through Whatman No 4 filter paper (Whatman
224 International Ltd., Maidstone, UK). The water was removed by the addition of 5 g of
225 anhydrous sodium sulphate and the extract was filtered a second time. Twenty milliliters of
226 the filtrate was evaporated to dryness at 60 °C. The residue was redissolved in 20 mL of
227 acetonitrile–H_2O (75:25, vol/vol) and extracted with 10 mL of hexane for the removal of fat.
228 The mixture was thoroughly mixed, centrifuged and 10 mL of the aqueous phase were
229 evaporated to dryness. For cleaning, the dry extract was redissolved in 10 mL of methanol–
230 H_2O (80:20, vol/vol), 90 mL of distilled water was added and passed through a
231 preconditioned OASIS, HLB, 6 mL (200 mg) SPE cartridges (Waters Corporation, Milford,
232 MA, USA) according to the methodology described by Sørensen and Elbaek (2005). Solid
233 phase extractions were performed on a Vac Elut 20 position Manifold SPE (Agilent
234 Technologies Inc., Santa Clara, CA, USA). The toxin was eluted with 7 mL of methanol,
235 evaporated to dryness and stored at -20 °C until analysis. The extracts were redissolved in 500
236 mL of methanol–H_2O (20:80 vol/vol) and AFB_1 was quantified by HPLC according to
237 Magnoli et al. (2016).

238

239 *2.8. Statistical analysis*

240

241 Data were analysed by general linear and mixed model (GLMM) using InfoStat (version 2.03
242 for Windows 2012; University of Cordoba, Argentina) software 2008 (Di Rienzo et al., 2008).
243 The parameters data were analysed by analysis of variance (ANOVA). Means and standard
244 error (SEM) were compared using the Fisher's protected least significant test ($P < 0.05$ and P
245 < 0.0001).

246

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247 **3. Results**

248

249 *3.1. Liver and intestine histopathology*

250

251 During the experimental period no signs of morbidity or mortality were observed.
252 Macroscopic changes in the colour, size, weight, consistency, and shape the livers from
253 broiler chickens fed the different diets were not observed (Fig. 1).
254 Figures 2 shows the photomicrographs of haematoxylin and eosin-stained liver sections of
255 chickens in different dietary treatments. Livers from control and *S. cerevisiae* groups did not
256 show histopathological alterations (Figs. 2A and 2B). In contrast, histological analysis results
257 revealed significant damage ($P < 0.05$) in the liver tissue of broiler chickens that consumed
258 100 µg/g AFB_1 alone (Fig. 2C) showing diffuse microvacuolar fatty degeneration throughout
259 the organ. These effects were prevented in livers from broiler chickens fed diets with AFB_1
260 plus addition of Sc, showing normal hepatocytes similar to the control (Fig. 2D). Liver tissues
261 had moderate hydropic and an unmarked peripheral degeneration. Also, there was no
262 proliferation of bile ducts. Hepatocytes from 3 hepatic lobules showed generalized vacuolar
263 type cytoplasm. Moreover, they showed a marked decrease in the fat microvacuoles being
264 similar in appearance to the livers of broiler chickens fed with yeast alone (Fig. 2B) and the
265 livers of broiler chickens fed control diet (Fig. 2A).

266

267 *3.2. Intestinal morphology*

268 Figure 3 shows the representative microstructure of intestinal villi of broilers at 22 d of age
269 from each treatment. Non histopathological alterations were observed in the small intestine of
270 control broiler chickens (grade 0) (Figs. 3A and 4A). The villi in the small intestine from the
271 yeast group showed long villi with slight atrophy, absence of hyperplasia of the goblet cells,

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272 hyperemia and inflammatory infiltrate (grade 1) (Figs. 3B and 4B). The small intestine villi
273 from the AFB_1 group showed an important atrophy, hyperplasia of goblet cells, prominent
274 inflammatory infiltrate and oedema (neutrophils usually predominating) (grade 3) (Figs. 3C
275 and 4C). In the group with yeast plus AFB_1, the absence of hyperemia, normal villi, lower
276 number of goblet cells and atrophy compared to the group with AFB_1 was observed (grade 1)
277 (Figs. 3D and 4D).
278
279 Table 2 shows the results obtained from the morphometric measurements, villus height, villus
280 width, crypt depth, the goblet cells number and apparent adsorption area for the different
281 treatments assayed. The morphometric intestinal studies showed that AFB_1 had significant
282 toxic effect on crypt depth, adsorption area and villus height compared to the control ($P <$
283 0.05). In broiler chickens fed diets with AFB_1 the villus height and the apparent absorption
284 area showed the lowest values in relation to the control. The crypt depth was inversely
285 affected in relation to the other parameters mentioned above. On other hand, when yeast was
286 applied in the diets with the toxin, a significantly decrease ($P < 0.05$) in the values of the crypt
287 depth, were observed while height intestinal villus and apparent adsorption area did not show
288 significant differences, however a tendency to improve these parameters was observed.
289 There was no significant difference in the number of goblet cells among the assayed
290 treatments.
291
292 *3.3. Residual levels of aflatoxin B_1 in livers*
293
294 Table 3 shows the residual levels of AFB_1 in livers of broiler chickens in different treatments.
295 The livers of treatment control (T_1) and treatment with yeast (T_2) did not show detectable
296 residual levels of AFB_1. Livers from animals fed diets with 100 µg/kg of AFB_1 (T_3) showed

297 the presence of AFB_1 in livers (1.26 µg/g). The AFB_1 residual levels in broiler chickens livers
298 fed diets with Sc plus AFB_1 (T_4) were significantly lower (1.01 µg/g) than those receiving
299 AFB_1 alone ($P < 0.05$).
300
301 **4. Discussion**
302
303 Aflatoxins contamination is a constant hazard to the poultry industry that results in substantial
304 economic losses to producers due to sub-lethal but toxic effects of AFB_1. In the present study,
305 the liver histopathology of broiler chickens fed diets with Sc did not show the typical pattern
306 of subclinical aflatoxicosis demonstrated with AFB_1; the macroscopic and microscopic
307 alterations of the tissue were not observed, highlighting its beneficial effect. The effects of
308 AFB_1 in of broiler chickens are well known; other researchers reported microscopic lesions of
309 livers as target organs in broiler chickens fed dietary whit levels 50 and 100 µg/kg AFB_1
310 (Magnoli et al., 2011). Azizpour and Moghadam (2015) reported that the addition of yeast cell
311 wall (0.05% and 0.1%) mitigated the negative effects of AFB_1 on the liver histopathology in
312 broiler chickens. Also, Magnoli et al. (2017) using *Pichia kudriavsevii* (0.1%) demonstrated
313 the effectiveness to prevent the toxic effects of AFB_1 in the liver macroscopy and
314 histopathology of broiler chickens fed diets with 100 µg/kg of AFB_1 at 22 d of age.
315 Morphological parameters such the length of the villi, depth of the crypt, villus to crypts ratio
316 and surface area of the villi are usually used to investigate the effects of microorganisms on
317 intestinal morphology and cell proliferation. These parameters but especially the area was
318 positively related with the absorptive efficiency of the small intestine in broiler chickens
319 considered indicators of intestinal functions (Matur and Eraslan, 2012). The present study
320 showed long villi only with slight atrophy when Sc was added demonstrating a preventive
321 effect on the histomorphological damage in the intestine caused by AFB_1 that decreased

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322 height of the intestinal villi, number of goblet cells small intestine's surface area for
323 absorption. These results were similar to those demonstrated by Zhang et al. (2005) who
324 observed that supplementation of diets with yeast cell wall showed longer and intact villi in
325 relation to controls without cell wall. Liew et al. (2018) and Wang et al. (2018) evaluated the
326 damage of the small intestine induced by AFB_1 in broiler chickens and found similar AFB_1
327 lesions than those in the present study.
328 The microorganisms used as probiotics affect the functions and counts of the goblet cells in
329 the intestinal mucosa. The mucus secreted by these cells is one of the factors that make up the
330 intestinal barrier preventing the invasion of pathogens in the digestive tract (Matur and
331 Eraslan 2012). In the present study, the number of goblet cells in the small intestine of the
332 broiler chickens was similar among all treatments. However, when histological evaluation
333 was performed goblet cells hyperplasia and inflammatory infiltrate was observed in broiler
334 chickens fed diets with AFB_1, similar to results reported by Liew et al. (2018) who
335 demonstrated an accumulation of lymphocytes in intestine indicating the occurrence of
336 inflammation in rats feed with AFB_1. However, the presence of Sc showed normal and
337 reduced atrophy compared to the AFB_1 treatment.
338 The liver is the target organ where AFB_1 is metabolized, detoxified and/or conjugated with
339 nucleic acids and proteins (Liew et al., 2018). There is a percentage of **a**flatoxin that can
340 accumulate without changes in the liver, muscle and other edible animal tissues (Magnoli et
341 al., 2011). In the present study, no detectable residual AFB_1 levels were found both in control
342 livers and those from animals receiving dietary yeasts. Chicks fed diets with AFB_1 (100
343 μg/kg) showed the presence of residual levels in livers, whereas Sc addition (1 g/kg)
344 significantly reduced them in livers. Similar results were reported by Magnoli et al. (2017)
345 who observed a decrease in AFB_1 residual levels in broiler chickens liver fed diets with AFB_1
346 (100 μg/kg) and the yeast *Pichia kudriavzevii* (0.1%).

347

348 **5. Conclusion**

349

350 In conclusion, the probiotic Sc administration was effective in counteracting the toxic effects
351 caused by low levels of AFB_1 in broiler chicken livers and gut histomorphometry. Moreover,
352 residual levels of AFB_1 were prevented in livers. These results are promising for the
353 development of future feed additives that provide benefits for both food safety and consumer
354 health. More experiments are needed to optimize the way to incorporate these additives in
355 feed and to evaluate their viability over time.

356

357 **Conflict of interest statement**

358

359 None of the authors has any financial or personal relationships that could inappropriately
360 influence or bias the content of the paper.

361

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363

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366

367 **References**

368

369 Armando MR, Pizzolitto RP, Dogi CA, Cristofolini A, Merkis C, Poloni VL, Dalcero AM,
370 Cavaglieri LR. Adsorption of ochratoxin A and zearalenone by potential probiotic

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371 *Saccharomyces cerevisiae* strains and its relation with cell wall thickness. J Appl
372 Microbiology 2012;113(2):256–264.
373 AOAC. Official methods of analysis 14th Ed. Association of Official Agricultural Chemists,
374 Gaithersburg. 979.22;1994.
375 AOAC. Official methods of analysis, 16th Ed. Association of Official Agricultural Chemists,
376 Gaithersburg. 990.33;1995.
377
378 Aviagen. Ross 308 broiler: nutrition specification. Newbridge, Midlothian, Scotland,
379 UK: Ross Breeders Limited; 2014
380 Azizpour A, Moghadam N. Effects of yeast glucomannan and sodium bentonite on the toxicity
381 of aflatoxin in broilers. Rev Bras Cienc Avic 2015;17:7–13.
382 Del Carmen S, Zurita-Turk SM, Alvarenga-Lima F, Coelho Dos Santos RS, Leclercq SY,
383 Chatel JM, Azevedo V, De Moreno de Leblanc A, Miyoshi A, Leblanc JG. A novel
384 interleukin-10 DNA mucosal delivery system attenuates intestinal inflammation in a mouse
385 model. Eur J Inflamm 2013;3:641–654.
386 Di Rienzo JA, Casanoves F, Balzarini MG, Gonzalez L, Tablada M, Robledo CW. InfoStat,
387 versión 2008, Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina.
388 Iji PA, Saki A, Tivey DR. Body and intestinal growth of broiler chicks on a commercial starter
389 diet. 1. Intestinal weight and mucosal development. Br Poult Sci 2001;42:505–513.
390 Liew WPP, Mohd-Redzwan S. Mycotoxin: Its Impact on Gut Health and Microbiota. Front.
391 Cell. Infect. Microbiol (2018) 8:60. doi: 10.3389/fcimb.2018.00060
392 Magnoli AP, Monge MP, Miazzo RD, Cavaglieri LR, Magnoli CE, Merkis CI, Cristofolini AL,
393 Dalcero AM, Chiacchiera SM. Effect of low levels of aflatoxin B_1 on performance,
394 biochemical parameters, and aflatoxin B_1 in broiler liver tissues in the presence of monensin
395 and sodium bentonite. Poult Sci 2011;90:48–58.

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396 Magnoli AP, Rodriguez MC, Poloni VL, Rojo MC, Combina M, Chiacchiera SM, Dalcero AM,
397 Cavaglieri LR. Novel yeast isolated from broilers' feedstuff, gut and faeces as aflatoxin B_1
398 adsorbents. J Appl Microbiology 2016;121:1766–1776.
399 Magnoli AP, Rodriguez MC, González Pereyra ML, Poloni VL, Peralta MF, Nilson AJ, Miazzo
400 RD, Bagnis G, Chiacchiera SM, Cavaglieri LR. Use of yeast (*Pichia kudriavzevii*) as a novel
401 feed additive to ameliorate the effects of aflatoxin B_1 on broiler chicken performance.
402 Mycotoxin Res 2017. ISSN 0178-7888. DOI 10.1007/s12550-017-0285-y.
403 Matur E, Eraslan E. The impact of probiotics on the gastrointestinal physiology in New
404 Advances in the Basic and Clinical Gastroenterology (ed. Brzozowski, R.). 2012;51–74,
405 https://doi.org/10.5772/34067.
406 Nemati Z, Janmohammadi H, Taghizadeh A, Moghaddam GH, Maleki Nejad H. Effect of
407 bentonite supplementation to the contaminated diets with aflatoxin B_1 on broiler
408 performance. Presented at the 6th Iranian congress on animal science, Tabriz. 2014.
409 Oguz H, Hadimli HH, Kurtoglu V, Erganis O. Evaluation of humoral immunity of broilers
410 during chronic aflatoxin (50 and 100 ppb) and clinoptilolite exposure. Rev Med Vet
411 2003;154:483–486.
412 Roto SM, Rubinelli PM, Ricke SC. An introduction to the avian gut microbiota and the effects
413 of yeast based prebiotic-type compounds as potential feed additives. Frontiers in Vet Sci
414 2015;2:28.
415 Seidavi A, Dadashbeiki M, Alimohammadi-Saraei MH, van den Hoven R, Payan-Carreira R,
416 Laudadio V, Tufarelli V. Effects of dietary inclusion level of a mixture of probiotic cultures
417 and enzymes on broiler chickens immunity response. Environ. Sci Pollut Res 2017;
418 24: 4637-4644.

Journal Pre-proof

419 Sørensen LK, Elbæk TH. Determination of mycotoxins in bovine milk by liquid
420 chromatography tandem mass spectrometry. J Chromatog B Analytical Technologies in the
421 Biomedical and Life Sciences 2005;820:183–196.
422 Speijers GJA, Speijers MHM. Combined toxic effects of mycotoxins, J Els Toxicol Lett
423 2004;153:91–98.
424 Tavčar-Kalcher GK, Vrtač U, Pestevšek A, Vengust. Validation of the procedure for the
425 determination of aflatoxin B_1 in animal liver using immunoaffinity columns and liquid
426 chromatography with post-column derivatisation and fluorescence detection. Food Control
427 2007;18:333–337.
428 Trucksess MW, Stack ME, Nesheim S, Albert R, Romer T. Multifunctional column coupled
429 with liquid chromatography for determination of aflatoxins B_1, B_2, G_1 and G_2 in corn,
430 almonds. Brazil nuts, peanuts, and pistachio nuts: collaborative study. J AOAC Int.
431 1994;77:1512–1521.
432 Wang F, Zuo Z, Chen K, Gao C, Yang Z, Zhao SD, Li J, Song H, Peng X, Fang J, Cui H,
433 Ouyang P, Zhou Y, Shu G, Jing B. Histopathological Injuries, Ultrastructural Changes, and
434 Depressed TLR Expression in the Small Intestine of Broiler Chickens with Aflatoxin B_1.
435 Toxins 2018;10: 131; doi:10.3390/toxins10040131.
436 Yiannikouris A, André G, Poughon L, Francois J, Dussap C, Jeminet G, Bertin G, Jouany JP.
437 Chemical and conformational study of the interactions involved in mycotoxin complexation
438 with beta-D-Glucans. Biomacromol 2006;7: 1147–1155.
439 Yunus M, Moingeon B, Lehmann-Ortega L. Building social business models: lessons from the
440 Grameen experience Long Range Plann 2010;43: 308–325.
441 Zain ME. Impact of mycotoxins on humans and animals. J Saudi Chem Soc 2011;15:129–144.

442 Zhang AW, Lee BD, Lee SK, Lee KW, An GH, Song KB, Lee CH. Effects of yeast
443 (*Saccharomyces cerevisiae*) cell components on growth performance, meat quality, and ileal
444 mucosa development of broiler chicks. Poult Sci 2005;84:1015–1021.

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Table 1. Ingredients of the experimental diet (g/kg, as fed basis).

Item	Content
Macro ingredients	
Milled corn CP 8.0%	641.7
Soybean meal	289.0
Meat meal 40%	53.3
Sodium chloride	2.6
Calcite 38%	4.3
Micro ingredients	
Premix of vitamin and mineral[1]	4.0
Baking soda	1.4
DL- methionine	1.1
L- lysine	2.6
Total (Macro + Micro)	1,000

[1] Premix contains the following per 2.5 kg powder: calcium 27.0%, starch 0.04%, crude fibre 0.03%, vitamin A 4,000,000 IU, vitamin D_3 800,000 IU, vitamin E 12,000 IU, vitamin B_1 800 mg, vitamin B_2 2,000 mg, vitamin B_6 1600 mg, vitamin B_{12} 8,000 µg, vitamin K_3 800 mg, pantothenic acid 4,000 mg, niacin 16,000 mg, biotin 60,000 µg, folic acid 400 mg, choline chloride 60,000 mg, iron 16,000 mg, iodine 400 mg, copper 4,000 mg, manganese 32,000 mg, zinc 24,000 mg, selenium 60 mg.

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Table 2. Length of intestinal villi, depth of crypts, adsorption area, villus width and goblet cells determinations of broilers in different treatments.

Item[1]	Intestinal villus height, µm	Villus width, µm	Crypt depth, µm	Apparent adsorption area, µm^2	Goblet cells, Nº cells per villus
T_1	3012.59 ± 535.98[b]	2123.71±1055.88[c]	250.22 ± 190.94[a]	6519.9 ±1206.6[b]	205.8 ± 71.33
T_2	1891.53 ± 521.38[a]	630.46±454.53[bc]	291.59 ± 7.31[b]	6231.5 ±411.63[b]	196.4 ± 47.90
T_3	1733.67 ± 72.23[a]	1317.73±257.42[ab]	283.92 ± 17.94[b]	2557.1 ±912.63[a]	203.1 ± 76.20
T_4	1840.39 ± 42.76[a]	390.62±114.72[a]	254.29 ± 28.42[a]	2590.8 ±392.55[a]	208.9 ± 87.43

[1] Treatment 1 (T_1), control diet (CD); T_2, CD + *Saccharomyces cerevisiae* RC016 (Sc) at 1 g/kg; T_3, CD + aflatoxin B_1 (AFB_1) at 100 µg/kg; T_4, CD + Sc at 1 g/kg + AFB_1 at 100 µg/kg.

[a, b, c] With in a column, means without common superscripts are significantly different ($P < 0.05$) according to the Fisher´s protected least significant test.

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Table 3. Residual levels of aflatoxin B_1 (AFB_1) in livers of broilers fed different diets.

Item[1]	AFB_1, µg/g
T_1	nd
T_2	nd
T_3	1.26 ± 0.04 [a]
T_4	1.01 ± 0.03 [b]

nd = not detected.

[1] Treatment 1 (T_1), control diet (CD); T_2, CD + *Saccharomyces cerevisiae* RC016 (Sc) at 1 g/kg; T_3, CD + aflatoxin B_1 (AFB_1) at 100 µg/kg; T_4, CD + Sc at 1 g/kg + AFB_1 at 100 µg/kg.

[a, b] With in a column, means without common superscripts are significantly different ($P < 0.05$) according to the Fisher´s protected least significant test.

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T2

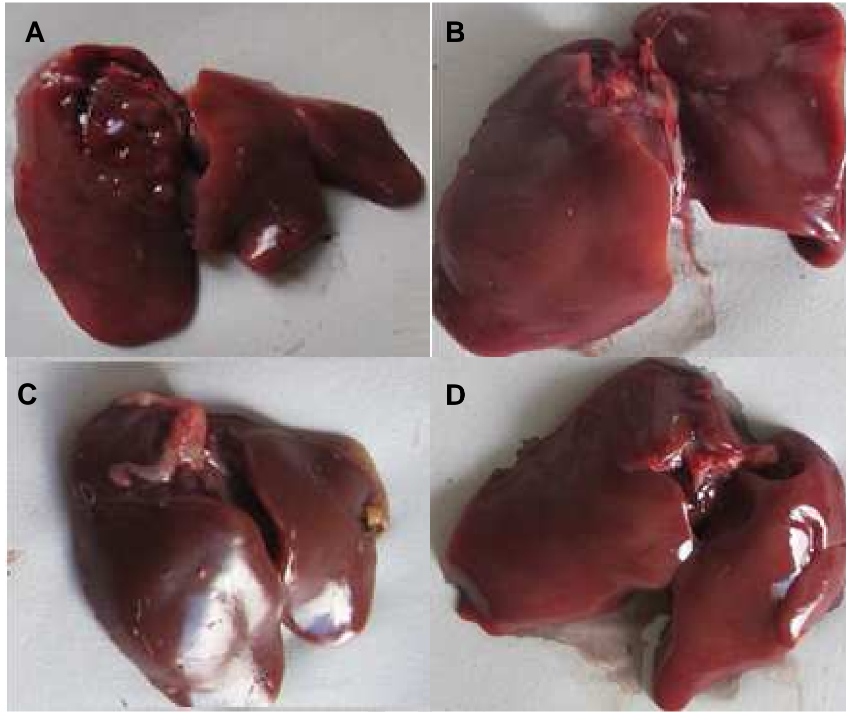

Fig. 1. Representative livers from broilers (22 d old) in different treatments. (A) Treatment 1 (T_1): control diet (CD); (B) T_2: CD + *Saccharomyces cerevisiae* RC016 (Sc) at 1 g/kg; (C) T_3: CD + aflatoxin B1 (AFB1) at 100 µg/kg; (D) T_4: CD + Sc at 1 g/kg + AFB1 at 100 µg/kg.

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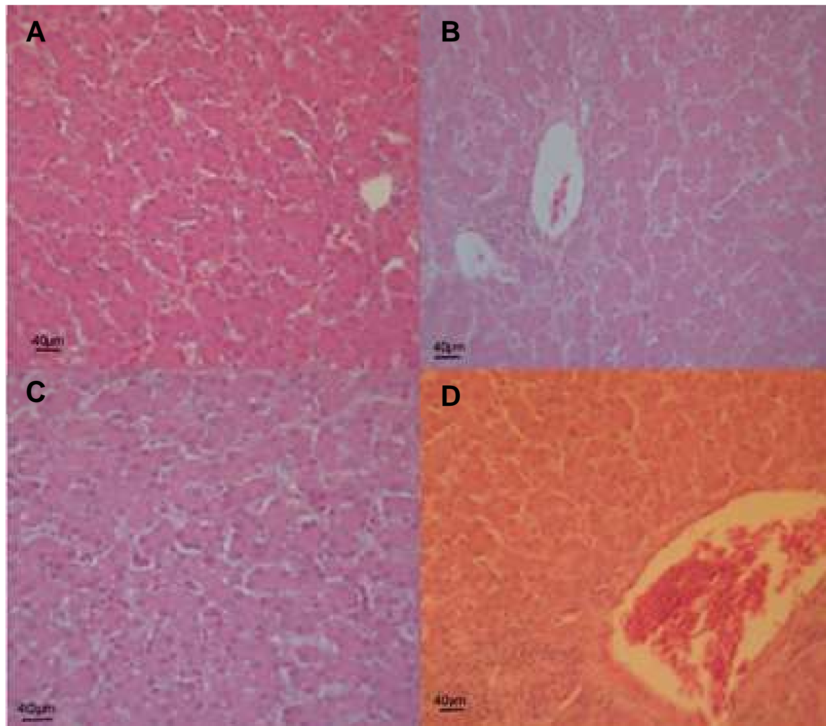

Fig. 2 Photomicrographs (optical microscopy) of haematoxylin and eosin-stained broiler liver sections in 4 treatments, 40×. (A) Treatment 1 (T_1): control diet (CD); (B) T_2: CD + *Saccharomyces cerevisiae* RC016 (Sc) at 1 g/kg; (C) T_3: CD + aflatoxin B1 (AFB1) at 100 µg/kg; (D) T_4: CD + Sc at 1 g/kg + AFB1 at 100 µg/kg.

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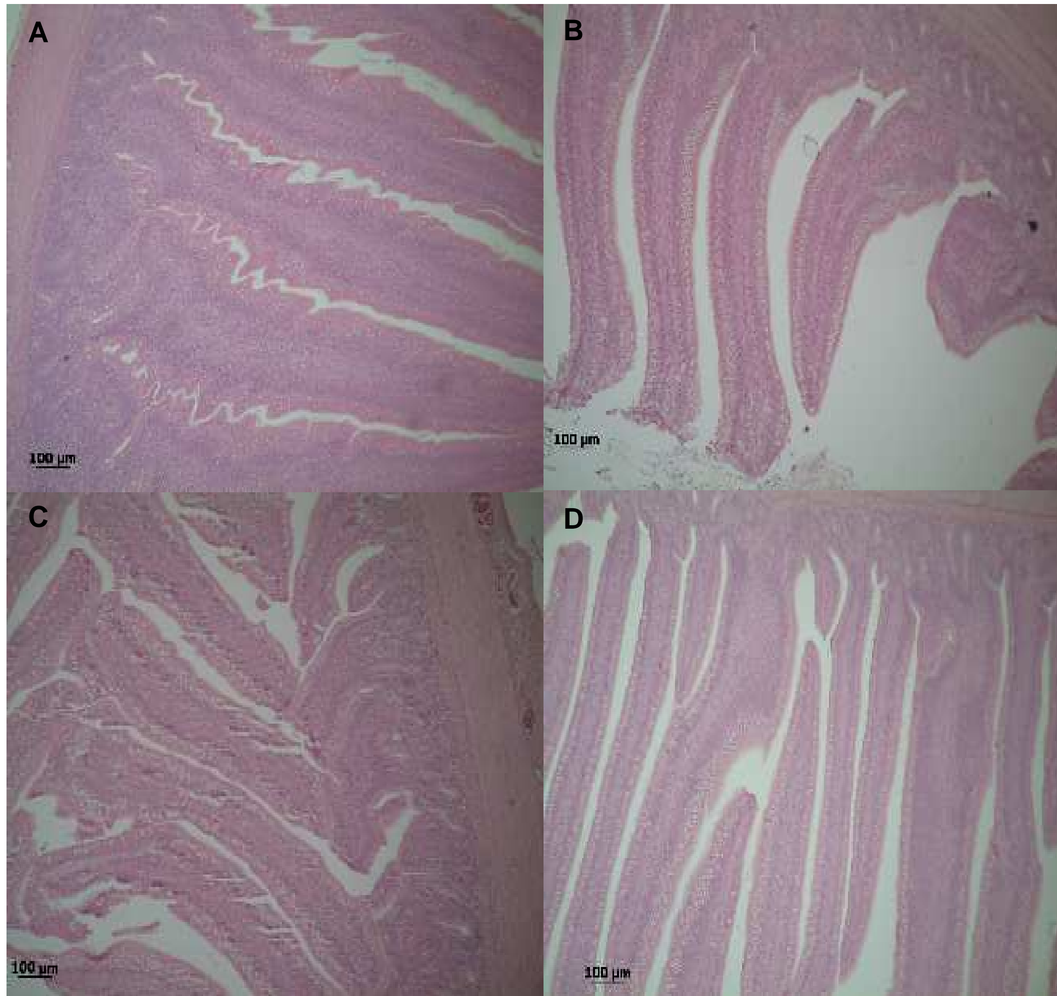

Fig. 3 Photomicrographs (optical microscopy) of representative microstructure of intestinal villi of broilers at 22 d of age in 4 treatments, 10×. (A) Treatment 1 (T_1): control diet (CD); (B) T_2: CD + *Saccharomyces cerevisiae* RC016 (Sc) at 1 g/kg; (C) T_3: CD + aflatoxin B_1 (AFB_1) at 100 µg/kg; (D) T_4: CD + Sc at 1 g/kg + AFB_1 at 100 µg/kg. Scale bar = 100 µm.

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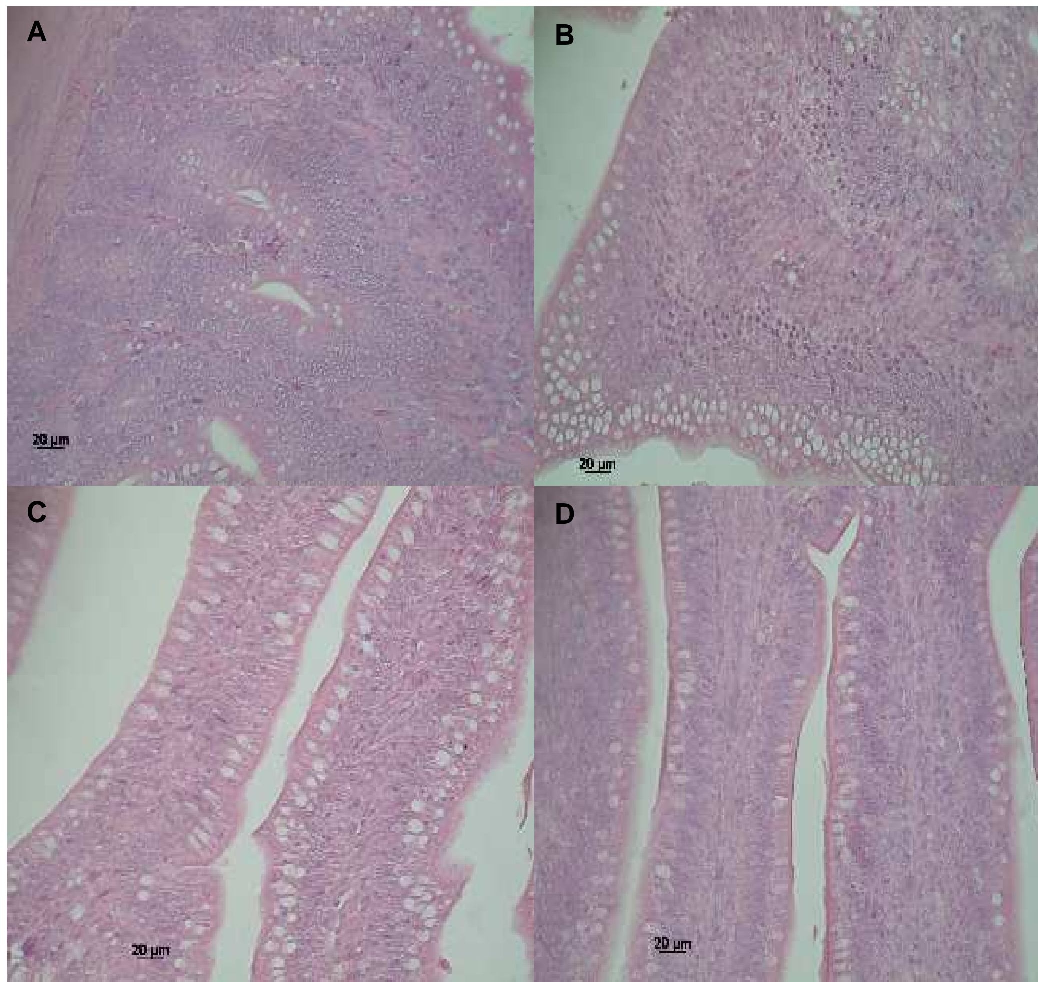

Fig. 4 Photomicrographs (optical microscopy), 40×. The representative microstructure of intestinal villi at 22 d of age from each treatment. (A) Treatment 1 (T_1): control diet (CD); (B) T_2: CD + *Saccharomyces cerevisiae* RC016 (Sc) at 1 g/kg; (C) T_3: CD + aflatoxin B_1 (AFB_1) at 100 µg/kg; (D) T_4: CD + Sc at 1 g/kg + AFB_1 at 100 µg/kg. Haematoxylin and eosin stain. Scale bar = 20 µm.

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Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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