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ARTÍCULO DE INVESTIGACIÓN

Dynamics of the structure and composition of *Saccharomyces cerevisiae RC016* cell wall subjected to gastrointestinal conditions

Dinámica de la estructura y composición de la pared celular de *Saccharomyces cerevisiae* RC016 sometida a condiciones gastrointestinales

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ABSTRACT

The yeast *Saccharomyces cerevisiae* is a model organism, characterized by being a most valuable species for a variety of industrial applications. Between the applications of *S. cerevisiae* is its use as a livestock feed decontamination strategy, sequestering the mycotoxin molecule by adsorption, reducing the bioavailability from the digestive tract and preventing the AFB1 absorption through the intestine. The *S. cerevisiae* RC016 strain isolated from pig gut, showed biological control properties against *Aspergillus parasiticus*, *Fusarium graminearum* and *Aspergillus carbonarium*, was able to adsorb AFB_1, promoted beneficial properties to the host with the absence of cytotoxicity and genotoxicity and was able to survive under gastrointestinal tract (GIT) conditions. Is important to deepen the knowledge of the morphological and ultrastructural characteristics of the yeast cell wall that allow the adsorption of mycotoxin. The objective was to evaluate the dynamic of the *Saccharomyces cerevisiae* RC016 cell wall under gastrointestinal (GIT) through different microscopic techniques. Calcofluor white technique, transmission electron microscopy, scanning electron microscopy, morphometric analysis and fluorescence intensity expression analysis were used. The cell wall thickness as well as the β-glucan-chitin expression were significantly higher in the gastric solution. In previous studies, our research group has determined that *S. cerevisiae* RC016 adsorbs the higher levels of aflatoxin B_1 in gastric juice. In the present study the cells preserved the external and internal architecture in gastric solution while in the intestinal solution the three-dimensional morphology and ultrastructural conformation were notably altered. The yeast in gastric solution maintained its ultrastructural morphology, showed an important cell wall thickening and had high β-glucan-chitin levels. Calcofluor white staining, a specific and low-cost technique, allowed a later morphometric-densitometric analysis of the cell wall. It is important to have a technique that differentially determine the portion of the yeast cell wall that allows the adsorption. The use of *S. cerevisiae* RC016 as bio-detoxifier would be a promising and economical strategy to reduce the exposure of livestock to mycotoxins.

Key words: (yeast), (Calcofluor white), (transmission electron microscopy), (scanning electron microscopy)

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RESUMEN

La levadura *Saccharomyces cerevisiae* es un organismo modelo, caracterizado por ser una especie de gran valor para una variedad de aplicaciones industriales. Entre las aplicaciones de *S. cerevisiae* se encuentra su uso como estrategia de descontaminación de alimentos para ganado, secuestrando micotoxinas por adsorción, reduciendo la biodisponibilidad en el tracto digestivo y evitando la absorción de AFB_1 a través del intestino. La cepa S. *cerevisiae* RC016 aislada del intestino de cerdos, mostró propiedades de control biológico contra *Aspergillus parasiticus, Fusarium graminearum* y *Aspergillus carbonarium*, fue capaz de adsorber AFB_1, promovió propiedades benéficas para el huésped con ausencia de citotoxicidad y genotoxicidad y fue capaz de sobrevivir en condiciones del tracto gastrointestinal. El objetivo de este trabajo fue evaluar la dinámica de la pared celular de la cepa *Saccharomyces cerevisiae* RC016 bajo condiciones simuladas del tracto gastrointestinal, a través de diferentes técnicas microscópicas. Se utilizaron las técnicas de Calcofluor white, microscopía electrónica de transmisión, microscopía electrónica de barrido, análisis morfométrico y análisis de expresión de intensidad de fluorescencia. El espesor de la pared celular, así como la expresión de β-glucano-quitina fueron significativamente mayores en la solución gástrica. En estudios previos, nuestro grupo de investigación ha determinado que *S. cerevisiae* RC016 presentó los mayores niveles de adsorción de aflatoxina B1 en solución gástrica simulada. En el presente estudio las células en solución gástrica, conservaron su arquitectura externa e interna; mientras que en solución intestinal la morfología tridimensional y la conformación ultraestructural se alteraron notablemente. La levadura en solución gástrica mantuvo su morfología ultraestructural, mostró un importante engrosamiento de la pared celular y presentó niveles elevados de β-glucano-quitina. La aplicación de la tinción de Calcofluor white, técnica específica y de bajo costo, permitió un posterior análisis morfométrico-densitométrico de la pared celular. Resulta importante contar con una técnica que permita determinar diferencialmente la porción de la pared celular de la levadura involucrada en la adsorción. El uso de *S. cerevisiae* RC016 como biodetoxificante sería una estrategia prometedora y económica para reducir la exposición del ganado a las micotoxinas.

Palabras clave: (levadura), (Calcofluor white), (microscopía electrónica de transmisión), (microscopía electrónica de barrido)

INTRODUCTION

The livestock food contaminated with mycotoxins is a global problem related to the quality of animal products and the possible impacts on human health associated with the chain production. The mycotoxins can contaminate a wide variety of foods and crops constituting a worldwide problem that compromises food and feed safety[20, 21]. Several studies have demonstrated that more than 25 % world's cereals, like other food products, are contaminated by different mycotoxins, among them aflatoxins (AFs)[11, 14]. The aflatoxin B_1 (AFB_1) reduces feed efficiency and productivity and its immunosuppressive properties can increase the occurrence of infectious diseases[19]. Livestock exposure to AFs results in an impairment of liver function, reduced feed intake and reduced meat and milk production[8].

A promissory livestock feed decontamination strategy is the use of biological agents that capture the mycotoxin molecules by adsorption, reducing the bioavailability from the digestive tract and preventing the AFB_1 absorption through the intestine. The yeast *Saccharomyces cerevisiae* is a model organism, characterized by being a most valuable species for a variety of industrial applications[25]. The yeast cell wall is a dynamic structure that can adapt to physiological and morphological changes. It is composed largely of polysaccharides (β-glucans, mannans, and chitin) and proteins[16, 18]. The yeast cell wall inner layer is comprised of glucan polymers and chitin. The β-1,3 glucan forms a backbone with β-1,6 glucan side chains, which is in turn attached to highly glycosylated mannoproteins[26]. Binding of different mycotoxins such as AF, ochratoxin and zearalenone to yeast cell surface has been reported and the binding has been attributed to cell wall glucans[26, 27]. Previous *in vitro* studies demonstrated that *S. cerevisiae RC016* strain isolated from pig gut[2], showed biological control properties against *Aspergillus parasiticus*, *Fusarium graminearum* and *Aspergillus carbonarium*[3, 5]. Also, was able to adsorb AFB_1, promote beneficial properties to the host[2, 4, 7] with the absence of cytotoxicity and genotoxicity[5, 13]. Also, *S. cerevisiae RC016* was able to survive under gastrointestinal tract (GIT) conditions in agreement with similar works[17, 21, 24, 25] demonstrating to be a potential probiotic.

The objective of this work was to evaluate morphological and ultrastructural changes in the cell wall of *S. cerevisiae* RC016, subjected to GIT simulated conditions. The β-glucan-chitin portion present in the cell wall was measured by binding to Calcofluor white, and ultrastructural analysis and three-dimensional morphology analysis were performed. Also, morphometric alterations and fluorescence intensity expression alterations were evaluated.

MATERIALS AND METHODS

Yeast strain and culture conditions

Saccharomyces cerevisiae strain RC016 was isolated from pig intestine, and deposited in National University of Rio Cuarto, Cordoba, Argentina, collection center. Stock cultures were maintained at - 80 °C in 30 % (v/v) glycerol. Working cultures were prepared from frozen stocks by two transfers on Yeast extract – Peptone – Dextrose-modified (YPD-modified) broth (5 g yeast extract, 5 g peptone, 40 g dextrose, 1000 mL water) and incubate at 28 °C for 24 h on an orbital shaker (180 rpm). Morphological and molecular characteristics were previously informed[2].

Simulated gastrointestinal tract

Simulated gastrointestinal tract conditions were prepared as follows: Artificial salivary solution: the cells were added to lysozyme 2 mg/mL (Sigma 47700 U/mg) in saline solution pH 6.5 and incubated at 37 °C for 5 min under agitation in an orbital shaker (100 rev min 1) to simulate the mastication process. Simulated gastric juice: the cells were added to NaCl 125 mmol, KCl 7 mmol, NaHCO3 45 mmol, pepsin 3 g/L (Sigma 427 U/mg) adjusted to pH 3 with HCl and incubated at 37 °C for 45 min under agitation in an orbital shaker (50 rev min 1) simulating the peristalsis process. Artificial intestinal fluid: the cells were added to trypsin 1 mg/mL (Fluka 11531 U/mg), chymotrypsin 1 mg/mL (Fluka 80 U/mg), bile salts 0.3 % (w/v) adjusted to pH 8 with NaOH 5 mmol/L and incubated at 37 °C for 30 min under agitation in an orbital shaker (100 rev min 1)[10]. Yeast pellet after the passage through the GIT solutions were obtained by centrifugation following the methodology proposed by Armando *et al*[3].

Calcofluor white (CFW) staining

After the passage through the GIT solutions *S. cerevisiae* RC016 pellets were washed twice with 200 µl of Sorensen buffer phosphate (PBS) for 5 min, centrifuged at 5000 g 10 min after each wash. The cells were resuspended in 50 µl PBS and incubated, at room temperature and protected from light for 40 min, with 50 µl of Calcofluor white stain (work dilution 1/10 aqueous solution 18909 CFW Stain, Fluka Analytical, Sigma Aldrich, USA). Cellular suspensions were collected by centrifugation at 5000 g 10 min and washed twice with PBS. A 30 µl suspension of each treatment was applied to glass slides and observed in an epifluorescent microscope Axiophot (Carl Zeiss, Thornwood, NY, USA). Then, the yeast cells were subjected to a morphometric analysis and to a fluorescence intensity quantification.

Morphometric analysis

Then Calcofluor White (CFW) staining the samples were observed to an Axiophot epifluorescence microscope (Carl Zeiss, Thornwood, NY, USA). Twenty images per treatment were acquired through the Powershot G6 7.1 megapixel digital camera (Canon Inc., Tokyo, Japan). The images with a 1000x magnification were processed in TIFF format. The morphometric digital analysis (cell wall thickness) was performed through AxioVision 4.6.3 software (Carl Zeiss, Göttingen, Germany). The cell wall thickness measurement was carried out on three hundred *Saccharomyces cerevisiae* RC016 cells per treatment. The results were analyzed with InfoStat Version 2020e software[6].

β-glucan-chitin quantification

The same way, on these twenty images per treatment acquired through the Powershot G6 7.1 megapixel digital camera (Canon Inc., Tokyo, Japan), to 1000x magnification in TIFF format, was performed the β-glucan-chitin quantification. The fluorescence intensity expression was analyzed through the Image-Pro Plus 6.0® software (Media Cybernetics, USA). The digital analysis was carried out on cells 300 per treatment. The image in general was stabilized applied filters and the background fluorescence was subtracted. The cells were selected one to one, the intensity values of each cell were loaded, in addition were discarded the aggregated cells. The results were analyzed with InfoStat Version 2020e software[6].

Scanning electron microscopy

The samples were fixed in a 2.5 % glutaraldehyde solution, pH 7.4 phosphate buffer for 72 h, and dehydrated in a graded series of alcohol. After fixation and dehydration, samples were critically dried with carbon dioxide (Denton Vacuum DCP-1). Finally, the samples were placed in aluminum sample holders with double-sided graphite adhesive tape and coated with gold by spraying (20-24 nm) on a Denton Vacuum Desk IV. Observations and imaging were made with a ML Leica DM500 with built-in camera and SEM JEOL Model JSM 6480 LV (Japan), from the Laboratory of Scanning Electron Microscopy (LASEM-UNSa).

Transmission electron microscopy

Yeast pellet were fixed in 2.5 % glutaraldehyde in 0.2 M S-collidine pH 7.4; a post fixation was performed in 1 % osmium tetroxide in 0.2 M S-collidine pH 7.4. Then they were dehydrated under increasing concentrations of acetone, embedded in epoxy resin EMbed 812 and sectioned in an ultramicrotome (Sorvall MT-1A, DuPont, USA). These ultrathin sections (60-80 ηm) were cut and placed in copper grids, contrasted with saturated uranyl acetate and aqueous lead citrate. The sections were examined with a transmission electron microscope JEM 1200 ExII (JEOL, Japan), using a Digital Micrograph TM (Gatan, Inc., Japan) software.

Statistical analyses

Data were analyzed with InfoStat Version 2020e software[6]. An ANOVA test and a posteriori LSD-Fisher test were performed in every case. When a parametric ANOVA test could not be accomplished, even with transformations of the variable, a nonparametric ANOVA by ranks (Kruskal-Wallis test) was used. Data are expressed as mean ± SEM, and means were considered to be statistically different at $P < 0.05$.

RESULTS

Figure 1 shows the Calcofluor white (CFW) images obtained after the passage of yeast through GIT conditions. This technique allowed the specific staining of the β-glucan-chitin portion of the cell wall, favoring the acquisition and digital analysis by morphometry and of fluorescence intensity. The Table 1 shows the morphometry of *S. cerevisiae* RC016 cell wall under GIT conditions. The morphometric analysis revealed that the cell wall thickness was significantly higher ($P < 0.001$) in gastric juice (1.26 µm) that in salivary solution and intestinal fluid. Whereas the cell wall thickness was significantly lowest ($P < 0.001$) in the intestinal fluid (1.12 µm) with respect to other treatments (Figure 2).

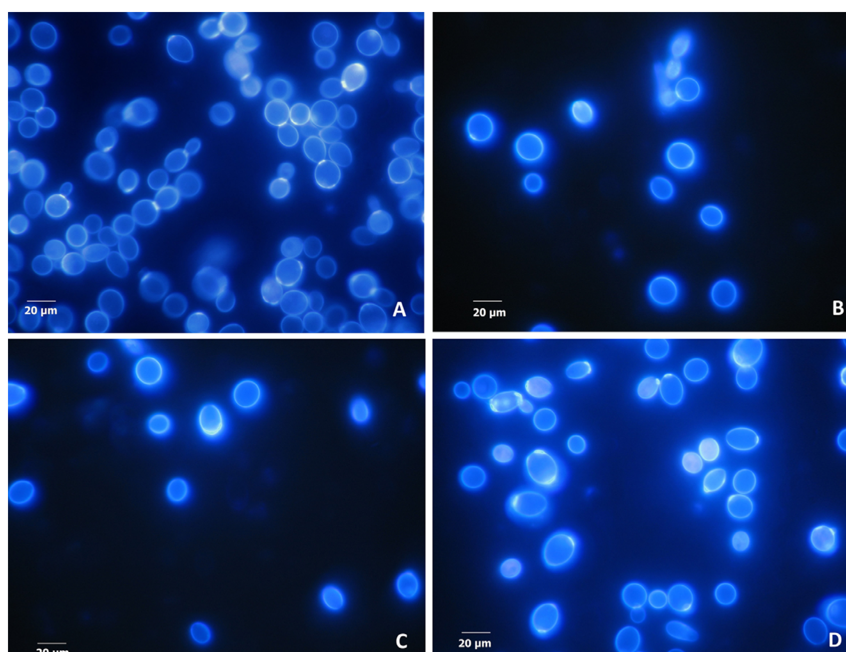

Figure 1. Calcofluor white technique images after the passage through GIT solutions. **A-** Control; **B-** Salivary solution; **C-** Gastric juice; **D-** Intestinal fluid. Scale bar: 20 µm.

Table 1. Morphometric analysis of *Saccharomyces cerevisiae* RC016 cell wall thickness in simulated gastrointestinal conditions

Simulate gastrointestinal tract	Cell wall thickness (µm)
Control	1.16 ± 0.02 [a b]
Salivary solution	1.21 ± 0.02 [b c]
Gastric juice	1.26 ± 0.02 [c]
Intestinal fluid	1.12 ± 0.02 [a]

Different letters indicate significant differences ($P < 0.001$) according to least significant difference Fisher's protected least significance tests.

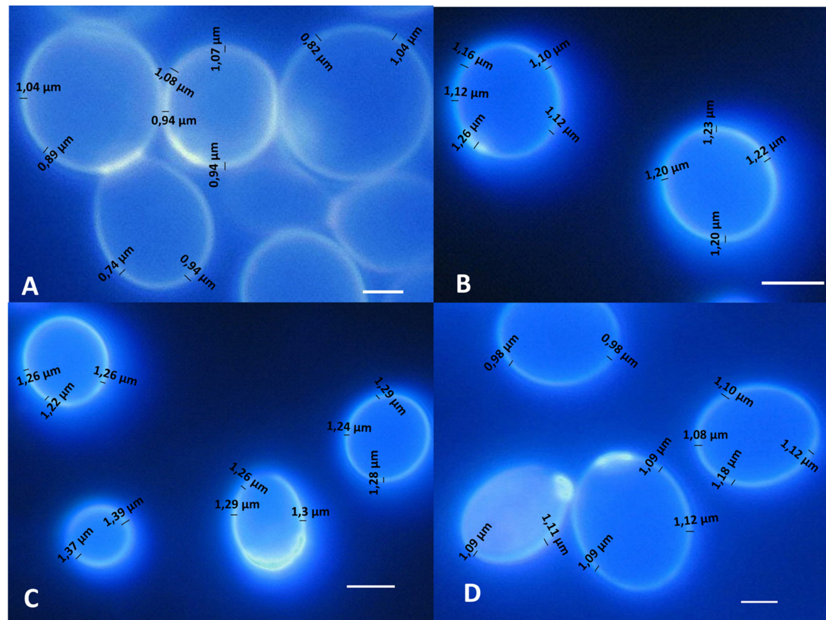

Figure 2. *Saccharomyces cerevisiae* RC016 cell wall thickness measurements under GIT conditions. **A-** Control; **B-** Salivary solution; **C-** Gastric juice; **D-** Intestinal fluid. Scale bar: A and D: 5 µm; B and C: 10 µm.

The Figure 3 shows the β-glucan-chitin quantification of the yeast cell wall through the analysis of the fluorescence intensity expression. Significant differences were found in all treatments ($P < 0.05$). An increased significantly was detected during the passage through salivary and gastric solutions. The fluorescence intensity expression had an average value of 140.36 in gastric juice, while it decreased significantly in intestinal fluid.

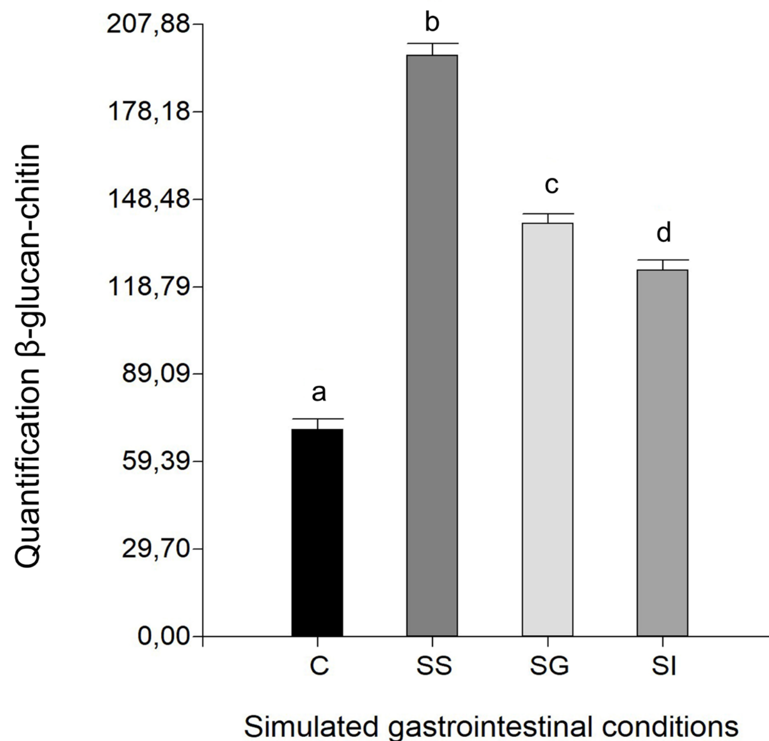

Figure 3. Quantification of β-glucan-chitin of *Saccharomyces cerevisiae* RC016 cell wall under GIT conditions. C- Control; SS- Salivary solution; SG- Gastric juice; SI- Intestinal fluid. Different letters indicate differences ($P < 0.05$) according to least significant difference Fisher's protected least significance tests.

The ultrastructural and three-dimensional morphology of the yeast cell wall determined by scanning electron microscopy and transmission electron microscopy are shown in Figure 4. The scanning electron microscopy showed that in simulated gastric juice the *S. cerevisiae* RC016 cells preserved the external architecture and cellular morphological alterations were not observed (Figure 4 A). Whereas in simulated intestinal fluid the three-dimensional morphology was notably altered, the cells showed external alterations and a highly rough morpho-architecture (Figure 4 B).

On the other hand, the *S. cerevisiae* RC016 cell ultrastructure determined by transmission electron microscopy was unchanged in simulated gastric juice. The cell walls preserved the ultrastructural conformation and highlighted the electrolucent middle layer corresponding to β-glucans (Figure 4 C). While, in simulated intestinal fluid were observed a heterogeneous cell size, a generalized ultrastructural alteration, greater cytoplasmic vacuolization and loss cellular turgor. Also, notable changes were observed in the cell wall, such as thinning of the middle layer and detachments of external layer (Figure 4 D).

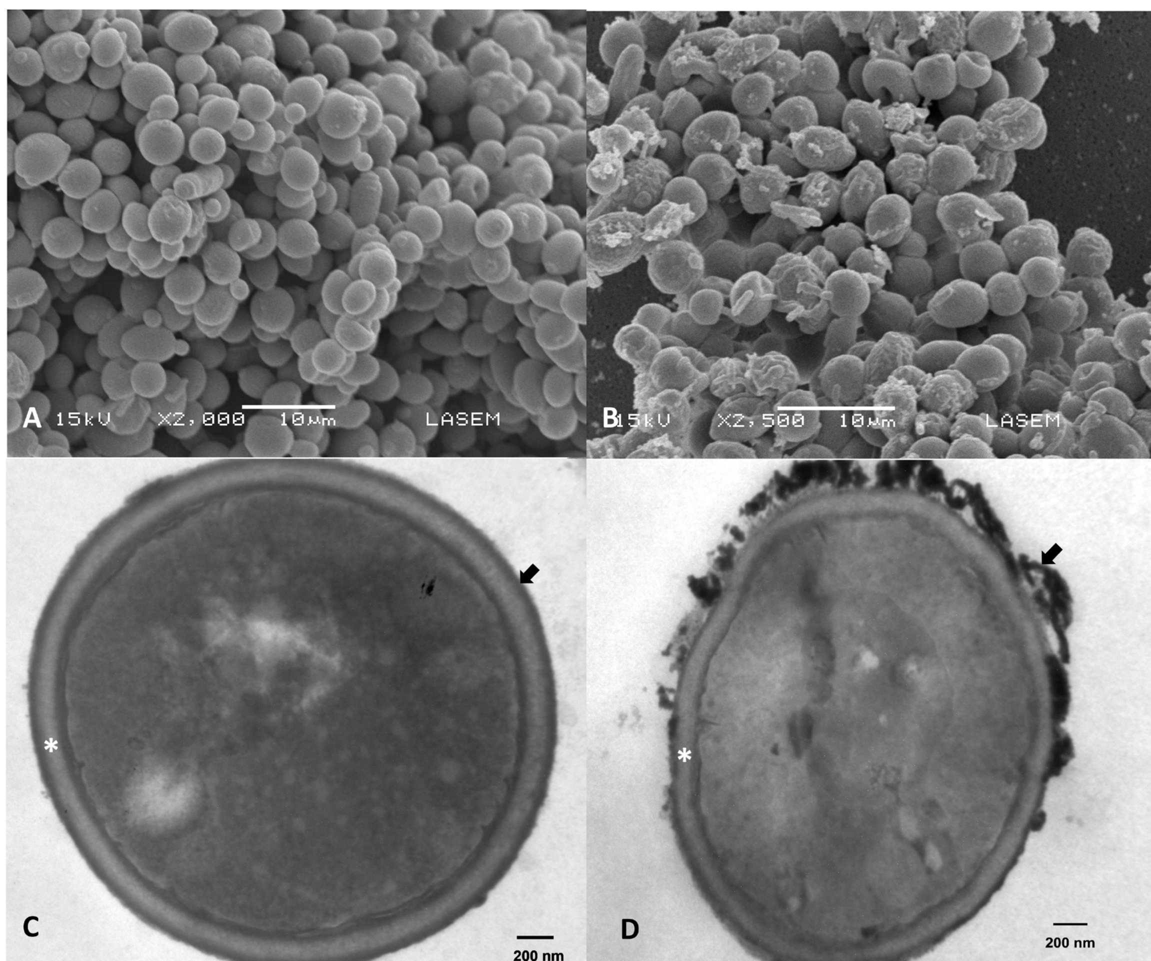

Figure 4. Scanning electron microscopy images (A and B) and transmission electron microscopy images (C and D). **A** and **C**: yeast cells treated with gastric juice. **B** and **D**: yeast cells treated with intestinal fluid. **Asterisk**: Middle cell layer. **Black arrow**: External cell layer. Scale bar: A-B: 10 µm; C and D: 200 nm.

DISCUSSION AND CONCLUSIONS

In the present work the CFW technique was fine-tuned to evaluate the layer of chitin-beta glucans in the yeast cell wall. CFW staining is a low-cost and specific technique that allow a morphometric and densitometric analysis and has been used for the confirmation of pathogenic parasites in certain clinical studies[1, 12].

The yeast cell wall structure is highly dynamic and can vary according to the yeast strain[21]. Environment conditions strongly modulate the quantity and structural properties of glucans, mannans and chitin in cell walls[15]. The yeast cell wall responds quickly to changes in the environment and stress. The ability of yeast to bind mycotoxins and the type of binding strongly depend on the molecular structure of the yeast cell wall components and the kind of mycotoxin[28]. In this study the thickness and the fluorescence intensity expression of *S. cerevisiae* RC016 cell wall were statistically evaluated, using a low-cost and specific technique, to determine the influence of the GIT conditions on the cell wall ultrastructural and morphological characteristics.

In the present study were found differences in *S. cerevisiae* RC016 cell wall thickness and composition according to simulated GIT conditions. These finding should be evaluated in future studies to determine its relationship with the yeast adsorption capacity. In previous research Fochesato[10] evaluated the AFB_1 adsorption level in simulated GIT and found that AFB_1 adsorption values ranged from 8.8 % in intestinal fluid to 95.3 % in gastric juice

The transmission and scanning electron microscopy demonstrated changes in the morpho-structural conformation when the yeast passed through the simulated GIT. It is important to highlight that the cell wall maintained its ultrastructural characteristics and its three-dimensional morphology in gastric solution.

Saccharomyces cerevisiae RC016 is a strain isolated from the animal environment, it can support the GIT simulated conditions of monogastric animal, and it does not produce cytotoxicity nor genotoxicity[2, 3, 4, 13]. Also, our finding indicates that in gastric solution this strain maintains its ultrastructural morphology, shown an important cell wall thickening had the greater quantification of β-glucan-chitin. Other studies indicate that also under these conditions it is capable to adsorb the AFB_1 highest level[9, 10] so that the *S. cerevisiae* RC016 strain could be used as an excellent bio-detoxifier. Future studies should be conducted to determine the relationship between chitin-beta glucans responsible for the mycotoxin adsorption.

This study highlights the influence gastrointestinal conditions on *S. cerevisiae* RC016 cell wall dynamics. Also, this study determined that CFW-technique is a low-cost microscopic technique that allows evaluate the portion of the yeast cell wall involved in the mycotoxin binding process. In addition, CFW-technique allowed the posterior application of morphometric and densitometric analysis. The use of *S. cerevisiae* RC016 cell walls as bio-detoxifier is a promising and economical strategy to reduce the exposure of livestock to mycotoxins.

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