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Potentiality of yeasts obtained as beer fermentation residue to be used as probiotics

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Abstract

Beer is the most consumed alcoholic beverage worldwide and brewery is a growing industry. Biomass by-product of beer production is constituted by viable and non-viable flocculated yeasts which are discarded. To increase the value of this waste, the potential applications of the beer fermentation residue (BFR) as probiotic and bio-preservative were studied. Strains isolated from commercial brewing starters and BFRs were identified. The M6 BFR and its constituent strains, *Saccharomyces cerevisiae* CMUNLPY6.2 and *Pichia kudriavzevii* CMUNLPY6.1, proved to be the most resistant to gastrointestinal conditions *in vitro*. The cell-free supernatants obtained from micro-fermentations were capable to reduce *Aspergillus flavus* and *Aspergillus parasiticus* germination, two species well-known to produce the potent carcinogenic aflatoxin B_1 (AFB_1). A cytoprotective effect of the BFRs against AFB_1 on HepG2 cells was observed. Brewing yeasts bound AFB_1 *in vitro,* thus reducing the cell damage induced by the toxin. Throughout the study, yeasts grown in brewing wort showed better probiotic properties than the same yeasts grown in YPD broth. These results suggest that the wastes obtained from brewery would become a high-value probiotic product.

Keywords: Brewing yeast; beer fermentation residue; aflatoxin B_1 binding; probiotic.

1. Introduction

Beer is the most popular alcoholic beverages worldwide, and the third most consumed after water and tea. Global beer production has risen in the last decades, reaching 1.95 billion hectoliters in 2017 (Statista, 2018). Typically, the amount of brewing yeast biomass yield in lager fermentation is about 1.7 kg/m^3 - 2.3 kg/m^3 of final product (Ferreira, Pinho, Vieiraa &

Tavarela, 2010). This nutritive beer fermentation residue (BFR) is mostly discarded or utilized as feedstuff (Ferreira *et al*., 2010).

Growing efforts are aimed to search probiotics as a strategy for human health promotion and disease prevention. According to the Food and Agriculture Organization and the World Health Organization, a probiotic is "a live microorganism which, when administered in adequate amounts, confers a health benefit to the host" (FAO/WHO, 2002). Lyophilized *Saccharomyces cerevisiae var. boulardii* is a probiotic yeast used worldwide for the prevention and treatment of diarrheal diseases (Czerucka, Piche & Rampal, 2007). Brewing yeasts, specifically species belonging to the *Saccharomyces sensu stricto* complex, have morphological and physiological similarity with *S. boulardii* (van der Aa Kühle & Jespersen, 2003) and share cell wall compounds identified as possible responsible for *S. boulardii* probiotic effect (Ferreira *et al*., 2010). van der Aa Kühle, Skovgaard & Jespersen (2005) conclude that certain *S. cerevisiae* strains have potential as probiotics as they are able to tolerate low pH and bile and to reduce the intestinal pro-inflammatory response during bacterial infections. These reports reinforce our approach of studying brewing yeasts as potential probiotics.

On the other hand, there is a concern about the effect of mycotoxin consumption through contaminated food on human health. Mycotoxins are fungal carcinogenic metabolites produced mainly by *Aspergillus*, *Penicillium* and *Fusarium* genera (Pitt & Hocking, 2009). These fungi may develop in stored food and/or raw material, producing thermotolerant mycotoxins. As a strategy to face this problem, it was proposed that mycotoxins can be bound by certain yeasts, avoiding the toxin absorption in the gut and preventing disease (Fernandes Oliveira, Bovo, Corassin, Vincenzi Jager & Ravindranadha Reddy, 2013). The ability of dead brewing yeasts to bind mycotoxins such as aflatoxin B_1 (AFB_1), deoxynivalenol (DON), zearalenone (ZEA) and ochratoxin A (OTA) has been reported (Campagnolo *et al*., 2015;

Bovo, Franco, Rosim, Barbalho & Fernandes de Oliveira, 2015). As an alternative strategy to counteract mycotoxins, the development of new bio-preservative supplements which prevent fungal germination in raw material, stored food and feedstuff are desirable. Armando *et al*. (2013) conclude that the strains *S. cerevisiae* RC008 and RC016 can be considered effective biocontrol agents against *Aspergillus carbonarius* and *Fusarium graminearum*. Also, these strains reduce OTA, ZEA and DON production in environmental conditions related to feedstuff storage. Previous reports support the use of yeasts as biocontrol agents in food and beverage production (Shetty, Hald & Jespersen, 2007; Bleve, Grieco, Cozzi, Logrieco & Visconti, 2006).

Abovementioned mentioned reports on different *S. cerevisiae* strains suggest that brewing starters could be potential probiotics. The aim of this work was to study the potential AFB_1 binding capability of yeasts obtained from BFRs and their effect on AFB_1 cytotoxicity on a cell model. Additionally, antifungal effect of BFRs against aflatoxicogenic fungi was evaluated.

2 Materials and methods

2.1 *Strains: origin and culture conditions*

Four brewing yeast consortia and eight yeasts isolated from these consortia were studied. Starters M4 and M6 were kindly provided by regional home brewers as BFRs. Consortia Safbrew S-33 and Safbrew WB-06 (Fermentis, Lesaffre, Marcq-en-Baroeul, France) are commercial freeze-dried brewing yeasts which were reconstituted in YPD broth (yeast extract 10g/L, bacteriological peptone 20g/L, dextrose 20g/L).

The yeasts were grown in three different conditions: a) 10.0 ml YPD broth at 30°C for 48 h; b) Laboratory Scale Brewing Wort (LSBW) cultures of 10.0 ml sterilized brewing wort, original gravity (OG) of 1040 [equivalent to 9.98° Brix], at 30°C for 72h; c) 700.0 ml

sterilized brewing wort, OG of 1040, at 18°C until attenuation point, in order to harvest the yeast biomass residue, called BFR. The attenuation point was defined as the end of wort fermentation, obtaining the lowest sugar content for a specific yeast strain, measured by a hand-held refractometer Master 20T (Atago, Tokyo, Japan). The brewing wort was kindly provided by local home brewers and sterilized by autoclaving.

Aflatoxicogenic strains of *Aspergillus parasiticus* CMUNLP7 (Gamba *et al*., 2015) and *Aspergillus flavus* CMUNLPI5 (formerly called *A. flavus* PJA [unpublished], kindly provided by Professor Vero [Universidad de la República, Uruguay] and designed according to the instructions of the Cathedra of Microbiology´s collection), obtained from collection of Cathedra of Microbiology (UNLP, Argentina), were grown on Potato Dextrose Agar (PDA, Britania, Buenos Aires, Argentina) slants for 7 days at 30°C to induce sporulation.

2.2 *Cell Cultures*

The human hepatocellular carcinoma cell line HepG2 was obtained from the Multidisciplinary Institute of Cell Biology (IMBICE, Buenos Aires, Argentina). These cells have shown to keep many parenchymal cell functions (Gutierrez-Ruiz, 1999). HepG2 cells were routinely maintained according to Gamba *et al*. (2015). Monolayers were prepared in 48-well tissue culture plates (Greiner Bio One, Frickenhausen, Germany) by seeding with a solution of 10^6 CFU/mL (0.25 mL/well). Cells were used for bioassays according to the corresponding experimental protocol (Ou *et al*., 2012).

2.3 *Isolation and identification of yeasts strains*

Differentiated giant colonies were obtained as described by White & Zainasheff (2010), with minor modifications. An overnight YPD broth culture of each consortium was counted in Neubauer's chamber and diluted in sterile PBS buffer (phosphate-buffered saline solution) to obtain 50 cells/mL suspensions. 100 µL of the suspensions were plated in YGC agar (Biokar)

and incubated at 30 °C for 7 days. After incubation, colonies with different morphologies and textures were isolated in YGC agar until unique morphology was observed. The isolated yeasts were maintained in YPD agar slants (yeast extract 10g/L, bacteriological peptone 20g/L, dextrose 20g/L, 20g/L agar agar) at 4°C.

Yeast total DNA amplification from pure cultures was done by colony PCR (Mirhendi, Diba, Rezaei, Jalalizand, Hosseinpur & Khodadadi, 2006) using the primers ITS1 5'-TCCGTAGGTGAACCTGCGG-3'and ITS4 5'-TCCTCCGCTTATTGATATGC-3' (White, Bruns, Lee & Tailor, 1990), provided by Invitrogen company (Thermo Fisher Scientific Inc.®, MA USA). PCRs were carried out in a 20 µL final volume, using 1 µL of the DNA template, 200 µmol/L of each dNTP, 0.25 µmol/L of each primer, 2.5 mmol/L of $MgCl_2$, 10X buffer and 0.75 U of Taq DNA polymerase (Inbio Highway, Tandil, Argentina). PCR program consisted in a 4 min initial denaturalization step at 95°C, followed by 30 cycles of a denaturalization step at 95°C; an annealing step at 55°C for 30 s; an extension step at 72°C for 1 min; and a final extension step at 72°C for 5 min. The amplification products were analyzed by electrophoresis on 0.8% p/v agarose gels before they were submitted for sequencing (Macrogen, Seoul, Korea). Data analysis was performed using BioEdit Sequence Alignment Editor for Windows and BLAST algorithm from NCBI database.

2.4 *Resistance to simulated gastrointestinal (GI) conditions*

The procedure was performed according to Minekus *et al*. (2014). Briefly, consortia and strains YPD cultures were harvested, washed twice with physiologic solution (PS, NaCl 0.9 % p/v, pH 7.0), counted in Neubauer's chamber and re-suspended to a final concentration of 10^6 - 10^7 CFU/mL in Gastric Solution (3.0 g/L porcine pepsine [Sigma-Aldrich, St Louis, MO, USA] in sterile PS and pH adjusted to 2.5 with HCl 3 mol/L) pre-heated at 37°C and incubated for 2 h. Afterwards, yeasts were harvested, washed twice and re suspended in

Intestinal Solution (1 g/L porcine pancreatin [Sigma-Aldrich] and 70 g/L bile salts (Britania S.A., CABA, Argentina) in sterile PS and pH adjusted to 8.0 with NaOH 1 mol/L) pre-heated at 37°C and incubated for 2 h. Aliquots of each suspension were taken before incubation, after the simulated gastric digestion and after the simulated intestinal passage. Samples were enumerated in YPD agar.

2.5 *Fungal germination reduction by cell-free supernatants (CFS)*

CFS were obtained by centrifugation and sterile filtration of brewing yeasts grown in YPD broth and in brewing wort (micro-fermentations). *Aspergillus* sp. strains were cultured on sloped PDA and suspensions of 10^4 spores/mL were obtained with a "spore solution" of 0.01% w/v Sodium Lauryl Sulfate (SLS) and 1% w/w sodium chloride solution (Gamba *et al*., 2015). A 96-well sterile microplate was inoculated with 190 µL of CFS plus 10 µL of the spore suspensions. As a positive control of fungal germination, wells were seeded with 10 µL of the spore suspension plus 190 µL of sterile YPD broth or brewing wort. As negative control wells were plated with sterile YPD broth or sterile brewing wort plus 10 µL of the sterile "spore solution". The microplate was incubated at 30 °C for 48 h. The fungal germination was measured spectrophotometrically at 580 nm (Beckman DU 650, Palo Alto, USA). The rate of germination inhibition/reduction was calculated as follows:

$$A = [1 - (B\text{-}D/C\text{-}D)] * 100 \qquad [1]$$

Where A is the percentage of fungal germination reduction; B is the OD_{580} of the treatment; C and D are the OD_{580} of the positive and the negative controls, respectively.

2.6 *Aflatoxin B_1 (AFB_1) solution preparation*

Crystalline AFB_1 was purchased from Sigma Aldrich (St Louis, MO, USA). Stock solutions were prepared in acetonitrile/benzene (98/2). Methanolic working stocks were prepared by

evaporating the acetonitrile/benzene mixture and reconstituting in methanol. AFB_1 concentrations were determined spectrophotometrically at 354 nm (ε354= 19,800 mol/l·cm) and stocks were stored at -20°C. Aqueous work solutions were prepared in sterile PBS.

2.7 *HepG2 cell damage induced by AFB_1*

The cell damage induced by AFB_1 in HepG2 cell line was assessed according to Gamba *et al*. (2015). Briefly, HepG2 cells were incubated with 10^8 CFU/mL yeasts re-suspended in DMEM (Dulbecco's Modified Eagle Medium, Merck, Darmstadt, Germany) with added AFB_1 and incubated at 37 °C in 5% CO_2 atmosphere for 24 h. Positive (DMEM plus AFB_1) and negative (DMEM without AFB_1) controls were included. After incubation, cells supernatants were collected and lactate dehydrogenase (LDH) activity was quantified by LDH-P UV Unitest kit (Wiener Lab, Rosario, Argentina) using a spectrophotometer (Beckman DU 650). Data were analyzed according to the kit manufacturer instructions.

2.8 *AFB_1 binding assay*

The AFB_1 binding assay was performed according to Bueno, Casale, Pizzolitto, Salvano & Oliver (2007), with modifications. Yeasts were washed twice with sterile PBS, counted in Neubauer's chamber, re-suspended in AFB_1 solution to obtain suspensions containing 10^8 CFU/mL and incubated at 30°C for 30 min with agitation (300 rpm).

Then, cells were harvested by centrifugation and the supernatant containing unbound AFB_1 was collected and stored at -20◦C until quantification. Positive (PBS + mycotoxin) and negative (PBS + yeast) controls were included for all experiments. AFB_1 was quantified following the manufacturer recommendations of Aflatoxin competitive direct ELISA test Veratox® (Neogen Corporation, Lansing, USA).

166 The mycotoxin bound by yeasts was calculated according to Campagnolo *et al*. (2015) as
167 follows:

168 $$A = [B - (C - D)] / B * 100 \qquad [2]$$

169 Where A is the percentage of AFB_1 adsorbed by the yeasts, B is the concentration of AFB_1
170 added to buffer (300 ppb in PBS), C is AFB_1 concentration in supernatants after incubation
171 with the yeasts and D is the concentration of any interferences in the negative control.

172 2.9 *Simulated human GI digestion effect on AFB_1/yeasts complex*

173 After AFB_1 binding assay, yeasts were harvested by centrifugation and challenged to GI
174 passage as described in section 2.4. To prevent washing out of the adsorbed AFB_1, washes
175 with PS between gastric and intestinal incubations were avoided. Immediately after each
176 incubation, cells were centrifuged and aliquots of the supernatants were taken for
177 quantification of the released AFB_1. Controls were performed with yeasts incubated in PBS.
178 The percentage of released mycotoxin by yeasts in each incubation step was calculated as
179 follows:

180 $$A = (B/C) * 100 \qquad [3]$$

181 Where A is the percentage of AFB_1 released by yeasts, B is the concentration of AFB_1
182 quantified in the supernatant after the incubation, and C is AFB_1 concentration in PBS without
183 yeasts.

184 2.10 *Statistical analysis*

185 Results were graphed by Sigmaplot 10.0® software. The results of three independent assays
186 are presented as the mean values ± standard deviation. Differences in all parameters were

tested for significance by the analysis of variance (ANOVA) and Tukey test to determine significant effects at $P < 0.05$ by using Sigmaplot 10.0® software.

3 Results and Discussion

3.1 *Isolates identification and human GI resistance*

Yeast strains used in this study were obtained from local brewers or commercial starters bought in local markets. All isolates where identified by sequencing of ITS1/ITS2 region as *Saccharomyces cerevisiae*, except for the CMUNLPY6.1 strain isolated from M6 starter, identified as *Pichia kudriavzevii* (Table 1). This is to be expected, since *Saccharomyces* sp. is the traditional brewing yeast, being *S. cerevisiae* mainly used for ale beer production (White & Zainasheff, 2010). *Pichia kudriavzevii* strains are usually isolated from other fermented products such as Tanzanian *togwa* (Hellstrom, Almgren, Carlsson, Svanberg & Andlid, 2012); Ghanaian fermented milk *nunu* (Akabanda *et al*., 2013); and fermented cereal gruel *ogi* (Ogunremi, Sanni & Agrawal, 2015).

Survival through the gastrointestinal conditions is desirable in the selection of probiotics, since viability plays a significant role in some beneficial properties (Diosma, Romanin, Rey-Burusco, Londero & Garrote, 2013). Thus, the resistance of the microorganisms to the human gastrointestinal passage simulated *in vitro* was studied. As a standard method indicates (Minekus *et al*., 2014), we tested the yeasts grown in YPD broth. Table 1 shows that all the studied *S. cerevisiae* strains displayed a good resistance to GI conditions, with no significant reduction ($P>0.05$) in the counts for most strains, except for CMUNLPY4.1, CMUNLPY4.2 and CMUNLPY33.1 ($P<0.05$). Among the last four, reductions were between 58% and 79% regarding the initial viable counts, showing an overall good tolerance of *S. cerevisiae* strains to GI passage. Our results agreed with previous reports of high resistance to the GI passage of *Saccharomyces* sp. strains isolated from beer, wine and grape must (Gil-Rodríguez,

Carrascosa & Requena, 2015). *P. kudriavzevii* was the only strain fully capable to survive the GI passage, even increasing its colony counts. This behavior was previously reported for different *Pichia* strains (Greppi *et al*., 2017; Chelliah, Rani Ramakrishnan, Prabhu & Antony, 2016). Regarding consortia behavior, M6 showed the best resistance among the starters tested; and increased its counts after simulated passage to GI conditions. This could be explained by *P. kudriavzevii* presence in this starter.

S. cerevisiae strains, in particular *S. cerevisiae* var. *boulardii*, isolated from many fermented food and beverages, have been extendedly studied as potential probiotic yeasts (Tiago *et al*., 2012; Shetty *et al*., 2007; van der Aa Kuhle *et al*., 2005). *P. kudriavzevii*, which has got the GRAS status (Kurtzman, Fell & Boekhorst, 2011), has been isolated from different fermented and non-fermented beverages and foods, and identified as a potential probiotic (Greppi *et al*., 2017; Chelliah *et al*., 2016; Diosma *et al*., 2013; Akabanda *et al*., 2013). To the best of our knowledge, there are no reports of probiotic *Pichia* strains isolated from barley beer. In order to investigate both *Saccharomyces* and *Pichia* as potential probiotic yeasts, further studies were performed with the M6 starter and its strains (*P. kudriavzevii* CMUNLPY6.1 and *S. cerevisiae* CMUNLPY6.2) because of their good tolerance to human GI conditions. The same simulated human GI passage was performed with yeasts grown in brewing wort (at laboratory and micro-fermentation scale). BFR of *P. kudriavzevii* CMUNLPY6.1 displayed no significant reduction ($P>0.05$) in viable counts, showing a behavior similar to its YPD broth cultures at the end of GI passage (Table 2). All the yeasts cultured in LSBW showed a significant reduction ($P<0.05$) after GI passage compared to their initial counts, while YPD broth cultures did not ($P<0.05$). This could indicate a culture conditions dependence of the tolerance to GI passage, regardless of the yeast strain. However, M6 starter and *P. kudriavzevii* CMUNLPY6.1 showed no differences ($P>0.05$) in viable counts between the three culture conditions (YPD broth, LSBW and BFR) at the end of the assay, whereas BFR

of *S. cerevisiae* CMUNLPY6.2 significantly reduced ($P<0.05$) its counts compared to the other culture conditions.

BFRs of M6 starter and *S. cerevisiae* CMUNLPY6.2 significantly decreased ($P<0.05$) its viable counts after the GI passage. This increased sensitivity to GI conditions of brewing wort cultures compared to YPD broth cultures may be explained by extensive changes in the composition and structure of the cell wall induced by fermentation in a complex and sugar concentrated medium such as brewing wort (Boulton, 2017). The longer and stressful exposure to micro-fermentation conditions, which include depletion of O_2 (affecting sterols membrane composition) and lowering of pH, added to the osmotic stress of brewing wort (Boulton, 2017). The effects on the reduction of the viable counts after GI passage were no significant ($P>0.05$) for *P. kudriavzevii* CMUNLPY6.1 (Table 2). The ability of *P. kudriavzevii* CMUNLPY6.1 cultured in YPD broth to grow under the stressful GI conditions could explain this result as an overall higher resistance of this strain, regardless of the culture conditions.

3.2 *Fungal germination reduction by CFSs obtained from brewing yeasts*

A. parasiticus and *A. flavus* are two species capable to produce AFB_1, a mycotoxin with deleterious effects on human health including aflatoxicosis, immunosuppression and liver cancer (Kew, 2013; Pitt & Hocking, 2009). Fungal germination inhibition and the consequent prevention of the aflatoxin production is one possible strategy to reduce their impact. Thus, the capability of CFSs obtained from brewing yeasts to reduce these fungi germination was studied (Fig. 1). The CFS obtained from micro-fermentation culture conditions showed significant reduction ($P<0.05$) of the fungal germination, whereas the CFS obtained from the YPD showed no inhibitory effect. *A. flavus* CMUNLPI5 germination was reduced by all the

CFSs obtained by micro-fermentation. *A. parasiticus* CMUNLP7 was inhibited by M6 starter and *S. cerevisiae* CMUNLPY6.2 but not by *P. kudriavzevii* CMUNLPY6.1.

3.3 *Brewing yeasts effect on HepG2 cell damage induced by AFB_1*

Aflatoxin deleterious effects on health occur due to its accumulation in the liver. Thus, human hepatocarcinoma cell line HepG2 has been proposed as a model for aflatoxin studies (Mc Kean *et al*., 2006). Cell damage, associated with the level of lactate dehydrogenase released by eukaryotic cell wall permeabilization, can be indirectly quantified as LDH activity (Legrand et al., 1992). Gamba *et al*. (2015) reported that different amounts of AFB_1 induce dose-dependent damage in HepG2 cells. Brewing yeasts' protective effect upon HepG2 cells exposed to 500 ng/mL AFB_1 suspension was demonstrated. Moreover, the presence of brewing yeasts recovers the basal LDH activity of non-challenged HepG2 cells (Fig. 2). This is the first report about the protective effect of brewing yeasts on HepG2 cells against AFB_1 cytotoxic effect.

3.4 *AFB_1 binding by brewing yeasts*

The cytoprotective effect observed on HepG2 cells could be explained by reduction/elimination of the aflatoxin available to interact with cells. Our results indicate that BFRs (with no pre-treatment) and LSBW cultures bound between 80% and 90% of the AFB_1 present in the medium, while YPD broth-cultured yeasts barely attached 8% to 20% (Table 3). Previous reports support the key role of yeast cell wall in its detoxifying capability, since the mechanism involves the molecule adsorption on the yeast surface (Bueno *et al*., 2007; Yiannikouris *et al*., 2004). Consequently, differences in the structure and composition of the cell wall are related with yeasts competence to bind mycotoxins. Our hypothesis is that growing in a complex medium such as brewing wort induces an extensive rearrangement in the yeasts cell wall (Boulton, 2017), which enhances their mycotoxin binding capability. This fact reinforces the approach of using BFR instead of laboratory cultured yeasts as potential

detoxifying agents. Previous reports demonstrated that dried brewing yeasts and brewing yeasts-based products bind AFB_1 (Gonçalves, Rosim, Fernandes de Oliveira & Corassin, 2015; Campagnolo *et al*., 2015; Bovo *et al*., 2014). While these authors used dried yeasts, in this report BFRs without any pre-treatment were tested and found to bind AFB_1.

3.5 *BFR yeasts/AFB_1 complex stability through GI passage*

We evaluated the stability of the BFR/AFB_1 complex during the GI passage. According to Moslehi-Jenabian, Lindegaard Pedersen & Jespersen (2010), the *S. cerevisiae*-AFB_1 complex is stable during the passage through an *in vitro* GI model and the treatment enhanced yeast binding competence up to 78% of total added toxin. After gastric and intestinal incubations, remaining AFB_1 in supernatants (de-attached) was measured. Both set of conditions (gastric and intestinal) affected the yeast/mycotoxin complex. According to our results, *P. kudriavzevii* CMUNLPY6.1 and *S. cerevisiae* CMUNLPY6.2 lost about a 25% of the bound mycotoxin (Table 4), remaining 54% of the initial added AFB_1 strongly attached. For the M6 starter, this percentage significantly ($P<0.05$) increased to 56%. This suggests that most of the initial mycotoxin ingested would not be potentially absorbed in the gastrointestinal tract but excreted together with the yeasts in feces.

4. **Conclusions**

In order to improve BFR value, the potential applications of this waste as probiotic and bio-preservative agent were studied. We demonstrated that M6 brewing starter and *P. kudriavzevii* CMUNLPY6.1 and *S. cerevisiae* CMUNLPY6.2 isolated from this starter can tolerate gastrointestinal conditions simulated *in vitro*. The micro-fermentation supernatants showed fungal germination reduction of the aflatoxin producers' *A. parasiticus* and *A. flavus*. Moreover, BFRs were able to bind AFB_1 and decreased the cytotoxic effect of AFB_1 on HepG2 model. The stability of the AFB_1-yeast complex through the GI passage secures the

elimination of more than the 50% of the initial AFB_1 present in the medium. Further *in vivo* studies are required to corroborate these results. This is the first report of BFR (without any pre-treatment) with *in vitro* GI resistance and cytoprotective effect against AFB_1 on cell model. Food supplemented with BFR would be an interesting application, and these results reinforces this course of investigation.

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Table 4. Aflatoxin B_1 (AFB_1) desorption during *in vitro* simulated gastrointestinal passage.

BFR*	AFB_1 desorption**		
	After gastric digestion (%)[†]	After intestinal passage (%)[†]	Total (%)[†]
P. kudriavzevii CMUNLPY6.1	$12.9 \pm 0.1^{a,A}$	$12.2 \pm 0.3^{b,A}$	25.1 ± 0.4^{A}
S. cerevisiae CMUNLPY6.2	$13.1 \pm 0.1^{a,A}$	$12.8 \pm 0.2^{a,B}$	25.9 ± 0.3^{B}
M6 starter	$12.1 \pm 0.1^{a,B}$	$10.7 \pm 0.1^{b,C}$	22.8 ± 0.2^{C}

*BFR (beer fermentation residue). **AFB_1 was determined by ELISA kit Veratox® (Neogen Corporation, St. Louis, MO, USA), according to the manufacturer instructions. [†] Rate of de-attached aflatoxin was calculated with regard to the added AFB_1 in the binding assay buffer (300 ppb). Data are means ± standard deviations from three experiments in duplicate. Means within the same row with different lowercase letters are significantly different ($P < 0.05$). Means within the same column with different capital letters are significantly different ($P < 0.05$).

Table 1. Resistance of brewing starters and isolated yeasts to *in vitro* simulated human gastrointestinal (GI) passage.

Brewing starter/ isolated yeast	Initial count (log CFU/mL)	After simulated gastric digestion (log CFU/mL)	After simulated intestinal passage (log CFU/mL)	Identification by ITS1-ITS2 amplicon
M6 starter	7.12 ± 0.10 ^{a}	7.11 ± 0.07 ^{a}	7.24 ± 0.09 ^{a,A}	
M4 starter	7.71 ± 0.01 ^{a}	7.73 ± 0.05 ^{a}	7.19 ± 0.04 ^{a,ABC}	
Safbrew S-33	6.85 ± 0.07 ^{a}	7.27 ± 0.10 ^{b}	6.70 ± 0.22 ^{a,C}	
Safbrew WB-06	7.21 ± 0.11 ^{a}	7.26 ± 0.08 ^{a}	7.06 ± 0.08 ^{a,ABCD}	
CMUNLPY6.1	7.08 ± 0.05 ^{a}	6.97 ± 0.08 ^{a}	7.22 ± 0.01 ^{a,AB}	*P. kudriavzevii*
CMUNLPY6.2	7.21 ± 0.03 ^{ab}	7.41 ± 0.04 ^{a}	6.78 ± 0.09 ^{b,BCD}	*S. cerevisiae*
CMUNLPY4.1	7.91 ± 0.09 ^{a}	7.86 ± 0.01 ^{a}	7.23 ± 0.06 ^{b,AB}	*S. cerevisiae*
CMUNLPY 4.2	7.67 ± 0.02 ^{a}	7.75 ± 0.03 ^{a}	7.04 ± 0.03 ^{b,ABCD}	*S. cerevisiae*
CMUNLPY 33.1	7.29 ± 0.00 ^{a}	7.31 ± 0.01 ^{a}	6.90 ± 0.17 ^{b,ABCD}	*S. cerevisiae*
CMUNLPY 33.2	7.18 ± 0.07 ^{a}	7.26 ± 0.06 ^{a}	6.84 ± 0.08 ^{a,ABCD}	*S. cerevisiae*
CMUNLPY WB.1	7.08 ± 0.14 ^{a}	7.14 ± 0.04 ^{a}	6.52 ± 0.12 ^{a,D}	*S. cerevisiae*
CMUNLPY WB.2	7.09 ± 0.12 ^{a}	7.01 ± 0.04 ^{a}	6.85 ± 0.27 ^{a,ABCD}	*S. cerevisiae*

M6 and M4 consortia are harvested for re-use brewing yeasts kindly provided by local home brewers. Safbrew S-33 and Safbrew WB-06 (Fermentis, Lesaffre, France) consortia are commercial freeze-dried brewing yeasts which were reconstituted in YPD broth for the assay. CMUNLPY6.1 and CMUNLPY6.2 were isolated from M6 starter. CMUNLPY4.1 and CMUNLPY4.2 were isolated from M4 starter. CMUNLPY33.1 and CMUNLPY33.2 were isolated from Safbrew S-33 starter. CMUNLPYWB.1 and CMUNLPYWB.2 were isolated from Safbrew WB-06. Data are means ± standard deviations from three experiments in duplicate.

Data expressed as means ± standard deviations from three experiments in duplicate. Means within the same row with different lowercase letters are significantly different ($P < 0.05$). Means within the same column with different capital letters are significantly different ($P < 0.05$).

Table 2. Beer fermentation residue's (BRF) resistance to *in vitro* simulated human gastrointestinal passage.

Isolated yeast/ brewing starter	Initial count (log CFU/mL)	After simulated gastric digestion (log CFU/mL)	After simulated intestinal passage (log CFU/mL)
P. kudriavzevii LSBW*	7.69 ± 0.02 ^{a}	7.24 ± 0.01 ^{b}	6.88 ± 0.07 ^{b,AB}
P. kudriavzevii BFR**	7.15 ± 0.00 ^{a}	6.70 ± 0.06 ^{a}	6.29 ± 0.14 ^{a,AB}
P. kudriavzevii YPD†	7.08 ± 0.05 ^{a}	6.97 ± 0.08 ^{a}	7.22 ± 0.01 ^{a,A}
S. cerevisiae LSBW*	7.62 ± 0.01 ^{a}	7.19 ± 0.14 ^{b}	6.61 ± 0.12 ^{b,AB}
S. cerevisiae BFR**	8.05 ± 0.05 ^{a}	6.18 ± 0.14 ^{b}	4.64 ± 0.65 ^{b,B}
S. cerevisiae YPD†	7.21 ± 0.03 ^{ab}	7.41 ± 0.04 ^{a}	6.78 ± 0.09 ^{b,AB}
M6 starter LSBW*	7.59 ± 0.04 ^{a}	7.34 ± 0.09 ^{b}	7.18 ± 0.11 ^{b,AB}
M6 starter BFR**	7.37 ± 0.16 ^{a}	6.70 ± 0.36 ^{b}	6.41 ± 0.15 ^{b,AB}
M6 starter YPD†	7.12 ± 0.10 ^{a}	7.11 ± 0.07 ^{a}	7.24 ± 0.09 ^{a,A}

* LSBW, stands for Laboratory Scale Brewing Wort culture, 10.0 mL agitated brewing wort cultures at 30°C for 72 h, as described in section 2.1. ** BFR, stands for Brewing Fermentation Residue, 700.0 mL brewing wort cultures at 18°C without agitation, till attenuation point was reached (approximately 10 days), as described in section 2.1. † YPD broth culture, 10.0 mL agitated YPD broth cultures at 30°C for 48 h, as described in section 2.1.

Data expressed as means ± standard deviations from three experiments in duplicate. Means within the same row with different lowercase letters are significantly different ($P < 0.05$). Means within the same column with different capital letters are significantly different ($P < 0.05$).

Table 3. Aflatoxin B_1 (AFB_1) binding by brewing yeasts.

Microorganisms	AFB_1 binding[ŧ]		
	YPD* broth cultures (%)	LSBW cultures** (%)	BFR*** (%)
P. kudriavzevii CMUNLPY6.1	4.7 ± 2.4 [a]	83.8 ± 0.0 [b]	79.2 ± 1.4 [c]
S. cerevisiae CMUNLPY6.2	7.7 ± 0.9 [a]	83.8 ± 0.0 [b]	79.0 ± 0.0 [c]
M6 starter	7.8 ± 4.8 [a]	83.7 ± 0.1 [b]	79.1 ± 0.1 [c]

[ŧ] Aflatoxin B_1 was determined in supernatants by ELISA kit Veratox® (Neogen Corporation, St. Louis, MO, USA), according to the manufacturer instructions. Data are means ± standard deviations from three experiments in duplicate. Lowercase letters indicate statistically significant difference ($p < 0.05$) between different media for the same strain. * YPD (broth culture): 10 mL agitated YPD broth cultures at 30°C for 48 h, as described in section 2.1. ** LSBW (Laboratory Scale Brewing Wort culture): 10 mL agitated brewing wort cultures at 30°C for 72 h, as described in section 2.1. *** BFR (Brewing Fermentation Residue): 700 mL brewing wort cultures at 18°C without agitation, till attenuation point was reached (approximately 10 days), as described in section 2.1.

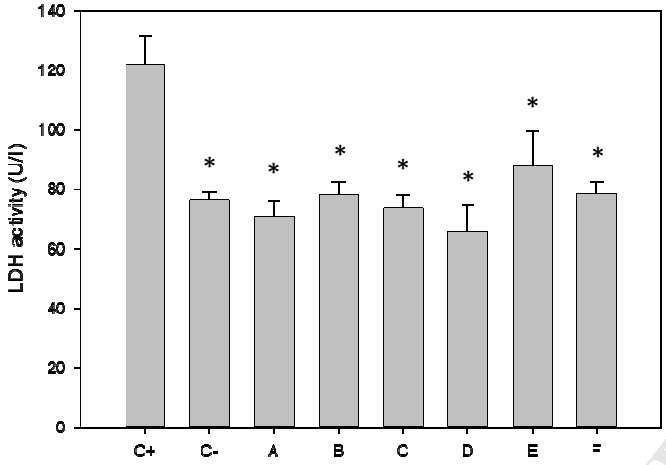

Fig 2. Protective effect of brewing yeasts against cytotoxicity induced by AFB_1 on HepG2 cells. LDH activity was determined by Wiener Lab® (Rosario, Argentina) according to the manufacturer instructions. A: *P. kudriavzevii* CMUNLPY6.1 YPD culture+ AFB_1 500 ppb. B: *S. cerevisiae* CMUNLPY6.2 YPD culture + AFB_1 500 ppb. C: M6 starter YPD culture + AFB_1 500 ppb. D: *P. kudriavzevii* CMUNLPY6.1 brewing wort culture+ AFB_1 500 ppb. E: *S. cerevisiae* CMUNLPY6.2 brewing wort culture + AFB_1 500 ppb. F: M6 starter brewing wort culture + AFB_1 500 ppb. C- (negative control): DMEM without AFB_1. C+ (positive control): AFB_1 500 ppb in DMEM. Bars are means ± standard deviations from three experiments in triplicate. * Mean values are significantly different ($P < 0.05$) compared to LDH activity induced by AFB_1 500ppb (C+).

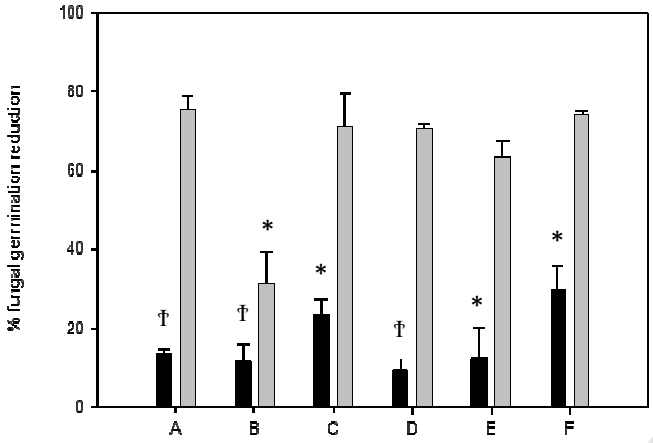

Fig 1. Fungal germination reduction by cell-free supernatants obtained from brewing yeasts. Grey bars: culture supernatants obtained from micro-fermentations in brewing wort. Black bars: culture supernatants obtained from YPD broth cultures. A: *A. parasiticus* CMUNLP7/M6 starter. B: *A. parasiticus* CMUNLP7/*P. kudriavzevii* CMUNLPY6.1. C: *A. parasiticus* CMUNLP7/*S. cerevisiae* CMUNLPY6.2. D: *A. flavus* CMUNLPI5/M6 starter. E: *A. flavus* CMUNLPI5/*P. kudriavzevii* CMUNLPY6.1. F: *A. flavus* CMUNLPI5/*S. cerevisiae* CMUNLPY6.2. Bars are means ± standard deviations from three experiments in quadruplicate. Symbols (*, ŧ) show significantly differences ($P < 0.05$).

Highlights

- Yeasts obtained as beer fermentation residue show potential probiotic activity.
- The beer fermentation residue protects HepG2 cells from aflatoxin B_1 cytotoxicity.
- Beer fermentation residue binds aflatoxin B_1 better than YPD cultured yeast.