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Efficiency of *Saccharomyces cerevisiae* and lactic acid bacteria strains to bind aflatoxin M_1 in UHT skim milk

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ABSTRACT

The purpose of this study was to evaluate the ability of a *Saccharomyces cerevisiae* strain and a pool of three lactic acid bacteria (LAB) strains (*Lactobacillus rhamnosus*, *Lactobacillus delbrueckii* spp. *bulgaricus* and *Bifidobacterium lactis*), alone or in combination, to bind aflatoxin M_1 (AFM_1) in UHT (ultra-high-temperature) skim milk spiked with 0.5 ng AFM_1 mL^{-1}. All the LAB pool (10^{10} cells mL^{-1}) and *S. cerevisiae* (10^9 cells mL^{-1}) cells were heat-killed (100 °C, 1 h) and then used for checking the effect of contact time (30 min or 60 min) on toxin binding in skim milk at 37 °C. The mean percentages of AFM_1 bound by the LAB pool in milk were 11.5 ± 2.3% and 11.7 ± 4.4% for 30 min and 60 min, respectively. Compared to the LAB pool, *S. cerevisiae* cells had higher ($P < 0.05$) capability to bind AFM_1 in milk (90.3 ± 0.3% and 92.7 ± 0.7% for 30 min and 60 min, respectively), although there were not significant differences between the contact times evaluated. When using *S. cerevisiae* + LAB pool, a significant increase ($P < 0.05$) was observed in the percentage of AFM_1 bound (100.0%) during 60 min. Results of this trial indicate that heat-killed *S. cerevisiae* cells, alone or in combination with the LAB pool used, has a potential application to reduce the concentration of AFM_1 in milk.

1. Introduction

Aflatoxins are secondary metabolites of low molecular weight produced by filamentous fungi, particularly *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*, distinguished by their wide distribution in food and pronounced toxic properties (Moss, 1998). There are currently 18 similar compounds described by the term aflatoxin, but the most prevalent and toxic is aflatoxin B_1 (AFB_1) (Murphy, Hendrich, Landgren, & Bryant, 2006). When AFB_1 in contaminated feed is ingested by livestock, including dairy cattle, it is biotransformed in the liver to aflatoxin M_1 (AFM_1), a hydroxylated metabolite which is excreted in milk, tissues and biological fluids of animals (Prandini et al., 2009). Although AFM_1 is about 10 times less toxigenic than AFB_1, its cytotoxic, genotoxic and carcinogenic effects have been demonstrated in several species (Murphy et al., 2006). The International Agency for Research on Cancer (2002) has classified AFM_1 as belonging to Group 1, a human carcinogen.

Milk is the main nutrient for the development of children, whose sensitivity to aflatoxins is remarkable and potentially greater than adults (Galvano, Galofaro, & Galvano, 1996). Taking into account the health risks associated to the human exposure to dietary aflatoxin levels, several countries have adopted tolerance limits for AFM_1 in milk (Prandini et al., 2009), but the regulatory limits differ widely (Van Egmond & Jonker, 2004). While in Brazil and United States the maximum allowable level of AFM_1 in fluid milk is 0.5 μg L^{-1} (Agência Nacional de Vigilância Sanitária, 2011, pp. 72–73), the European Union has established a ten-fold lower limit (0.05 μg L^{-1}) for AFM_1 in raw milk, heat-treated milk and milk for the manufacture of dairy products (European Commission, 2006).

Ideally, the best way to prevent aflatoxin contamination in the food chain is the adoption of improved agricultural practices and control of storage conditions of products. However, practical difficulties to effectively prevent contamination, along with the stability of aflatoxins under normal food processing conditions, have led to investigation on decontamination methods for food products that could be safe, effective, environmentally friendly and presenting a cost-benefit (Wu et al., 2009). The use of microorganisms offers an attractive alternative for the control or elimination of aflatoxins in foodstuffs (Alberts, Gelderblomb, Botha, & Van Zyl, 2009). *Saccharomyces cerevisiae* is the most effective for binding AFB_1 (Shetty & Jespersen, 2006), although several lactic acid bacteria (LAB) strains have shown different capabilities for binding AFM_1 in phosphate buffer solutions and in milk (Bovo, Corassin, Rosim, & Oliveira, 2012; El-Nezami, Kankaanpää, Salminen, & Ahokas, 1998; Haskard, El-Nezami, Kankaanpää, Salminen, & Ahokas, 2001; Kabak

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& Var, 2008; Pierides, El-Nezami, Peltonem, Salminen, & Ahokas, 2000). There is no previous report on the use of *S. cerevisiae* for decontamination of milk containing AFM_1. Therefore, the aim of the present study was to evaluate the ability of a *S. cerevisiae* strain, alone or in combination with a pool of three commercially available LAB strains, to bind AFM_1 in UHT (ultra-high-temperature) skim milk spiked with 0.5 μg AFM_1 L^{-1}, during contact times of 30 min and 60 min.

2. Material and methods

2.1. S. cerevisiae and LAB strains

Commercially available dry lager yeast strain (*S. cerevisiae*, SAFLAGER W37/70, Fermentis Ltd., France) and three LAB strains (*Lactobacillus delbrueckii* spp. *bulgaricus* LB340, *Lactobacillus rhamnosus* HOWARU® and *Bifidobacterium lactis* FLORA-FIT BI07 donated by Danisco Ltd., Brazil) were used in the experiment. LAB strains have been previously evaluated and showed individual percentages of AFM_1 removal in UHT milk at 37 °C ranging from 24.5 to 33.5% (Bovo et al., 2012).

The lyophilized yeasts were reactivated in sterile water and cultivated at 23 °C, according to recommendations of Fermentis Ltd. The number of yeast cells in the suspension was determined by light microscopy using a modified Neubauer chamber. The suspension was diluted with sterile water until reaching a cell concentration of 1.0×10^9 cells mL^{-1}.

Individual LAB lyophilized strains were reactivated in MRS (de Man, Rogosa and Sharpe) broth (Acumedia®, Lansing, MI, USA) and incubated at 37 °C until reaching at least 1.0×10^9 colony forming units (CFU) mL^{-1}, as determined by serial dilution and pour plate counting (Wehr & Frank, 2004) after incubation at 37 °C for 24 h under anaerobic condition. Convenient volumes of each cultured broth were mixed to achieve a pool of the three LAB strains containing a total cell concentration of 1.0×10^{10} cells mL^{-1}. All *S. cerevisiae* and LAB cells were heat-killed, being inactivated by boiling at 100 °C for 1 h before the binding assays, to avoid any possible milk fermentation during the contact time.

2.2. Aflatoxin M_1 binding assays

The ability of *S. cerevisiae* and LAB strains to bind AFM_1 was evaluated using commercial UHT skim milk samples previously evaluated for AFM_1 to confirm levels below the detection limit of the method (0.01 ng mL^{-1}), spiked with 0.5 ng mL^{-1}. AFM_1 standard solution (Supelco™, Bellefonte, PA, USA) was diluted in acetonitrile in order to obtain a 2.5 μg mL^{-1} working solution, which was calibrated according to Scott (1990). Two-hundred microliters of the working solution were transferred to an Erlenmeyer, evaporated to dryness under N_2 and then 1 L of UHT skim milk was added to the flask. Spiked milk samples were stirred, kept at 37 °C for 15 min, and immediately used in the binding assays with *S. cerevisiae* and LAB strains.

The AFM_1 binding assays were performed in triplicate as described by Pierides et al. (2000) with some modifications. Convenient volumes of culture broths containing 10^9 cells of *S. cerevisiae*, 10^{10} cells of LAB pool, or 10^9 cells of *S. cerevisiae* + 10^{10} cells of LAB pool were transferred to Eppendorf tubes and centrifuged (Microcentrifuge CT-14000, Cientec, Piracicaba, SP, Brazil) at 1800 g for 15 min. The supernatant was discharged and the bacterial or yeast pellets were washed twice with sterile ultrapure water (Milli-Q, Millipore, Bedford, MA, USA). After, the pellets were resuspended in 1.0 mL of UHT skim milk containing AFM_1, vortexed for 3 min and incubated at 37 °C during 30 min or 60 min. Following the contact times, the tubes were centrifuged again at 1800 g for 15 min, being the supernatant (milk layer) removed for analysis of AFM_1. The same procedures as described above were performed in triplicate positive controls (only spiked skim milk containing 0.5 AFM_1 μg L^{-1}), negative controls (only *S. cerevisiae*, LAB pool or *S. cerevisiae* + LAB pool) and non-spiked skim milk controls.

2.3. Analysis of aflatoxin M_1 in milk

Extraction and purification of the supernatant from the binding assays for AFM_1 determination were performed as described by Fernandes, Correa, Rosim, Kobashigawa, and Oliveira (2012), with some adaptations proposed by the manufacturer of the immunoaffinity columns (NeoColumn®, Neogen, Neogen Europe Ltd., Scotland, UK). Identification and quantification of the AFM_1 residues was achieved by injecting 20 μl of sample extracts in a high performance liquid chromatography (HPLC), using a Shimadzu (Kyoto, Japan) 10VP liquid chromatograph with a 10 AXL fluorescence detector (excitation at 366 nm and emission above 428 nm). A Synergy Fusion column (4.6 × 150 mm, 4 μm, Phenomenex, Torrance, CA, USA) and a Shim-Pack pre-column (4 × 10 mm, 5 μm CLC G-ODS) were used. The system was stabilized for one hour at a flow rate of 1 mL/min at room temperature with an isocratic mobile phase containing water, acetonitrile and methanol (60:20:20). Under these conditions, the retention time for AFM_1 was approximately 5.7 min. Calibration curve of AFM_1 was prepared using standard solutions of AFM_1 (Sigma, St Louis, MO, USA) previously evaluated according to Scott (1990), at concentrations of 0.5, 1.0, 2.5, 5.0 and 10.0 ng mL^{-1}. The determination limit of the analytical method was 0.01 ng mL^{-1}, considered the minimum amount of AFM_1 that could generate a chromatographic peak three times over the baseline standard deviation.

The equation below was used to determine the percentage of AFM_1 bound by the microorganisms tested in each assay. Letters *B*, *C*, *D* and *E* are the mean areas of chromatographic peaks of positive controls, non-spiked skim milk controls, sample analyzed and negative controls, respectively.

$$A = \left\{\frac{[(B-C)-(D-E)]}{B-C}\right\}*100$$

2.4. Statistical analysis

Statistical analysis of AFM_1 binding assays was carried out in the General Linear Model of SAS® (SAS, 2004) by using the Tukey Test for significant differences between the microorganisms tested (*S. cerevisiae*, LAB and *S. cerevisiae* + LAB) and contact time at $P < 0.05$.

3. Results and discussion

Table 1 shows the AFM_1 levels in UHT skim milk samples in the binding assays with heat-killed *S. cerevisiae* cells, alone or in combination with LAB pool cells. AFM_1 levels in spiked skim milk samples (0.5 ng AFM_1 mL^{-1}) treated with LAB pool cells (10^{10} cells mL^{-1}) for 30 and 60 min ranged from 0.442 ± 0.022 to 0.443 ± 0.011 ng mL^{-1}. For milks treated with *S. cerevisiae* cells (10^9 cells mL^{-1}) during the same contact times, or *S. cerevisiae* + LAB cells during 30 min, AFM_1 mean concentrations were 0.037 ± 0.004 to 0.048 ± 0.002 ng mL^{-1} and 0.042 ± 0.003 ng mL^{-1}, respectively. Milk samples treated with *S. cerevisiae* + LAB pool cells for 60 min had no detectable levels of AFM_1.

The percentages of AFM_1 bound in UHT skim milk by the microorganism studied after different contact times are presented in Table 2. LAB pool showed mean percentages of AFM_1 bound of 11.5 ± 2.3% and 11.7 ± 4.4% for 30 min and 60 min, respectively.

Table 1
Aflatoxin M_1 levels in UHT skim milk samples in the binding assays with heat-killed *Saccharomyces cerevisiae* and lactic acid bacteria pool cells.

AFM_1 added to milk (ng mL^{-1})	LAB pool[a] (cells mL^{-1})	*S. cerevisiae* (cells mL^{-1})	Contact time (min)	AFM_1 in milk[b] (ng mL^{-1})
0	0	0	(Control)	<0.010[c]
0	10^{10}	0	30	<0.010
0	10^{10}	0	60	<0.010
0	0	10^9	30	<0.010
0	0	10^9	60	<0.010
0	10^{10}	10^9	30	<0.010
0	10^{10}	10^9	60	<0.010
0.5	10^{10}	0	30	0.443 ± 0.011
0.5	10^{10}	0	60	0.442 ± 0.022
0.5	0	10^9	30	0.048 ± 0.002
0.5	0	10^9	60	0.037 ± 0.004
0.5	10^{10}	10^9	30	0.042 ± 0.003
0.5	10^{10}	10^9	60	<0.010

[a] Pool of lactic acid bacteria (LAB) strains: *Lactobacillus delbrueckii* spp. *Bulgaricus*, *Lactobacillus rhamnosus* and *Bifidobacterium lactis*.
[b] Values expressed as mean ± standard deviation of samples analyzed in triplicate.
[c] Determination limit of the analytical method: 0.010 ng mL^{-1}.

Compared to the LAB pool, *S. cerevisiae* cells had higher ($P < 0.05$) capability to bind AFM_1 in milk (90.3 ± 0.3% and 92.7 ± 0.7% for 30 min and 60 min, respectively), although there were no differences ($P > 0.05$) between the contact times evaluated. When using *S. cerevisiae* + LAB pool, a significant increase ($P < 0.05$) was observed in the percentage of AFM_1 bound in the contact times, which values were 91.7 ± 0.5% (30 min) and 100.0% (60 min).

The percentages of AFM_1 bound by the LAB pool obtained in the present study are in agreement with those reported by Pierides et al. (2000), who observed that heat-killed cells of *L. rhamnosus* bound to 18.8% of AFM_1 in reconstituted skim milk powder and to 26.0% of toxin in reconstituted whole milk powder after 16 h of contact. Peltonem, El-Nezami, Haskard, Ahokas, and Salminen (2001) also found similar binding percentages (5.6–25.7%) when using 15 LAB strains for AFB_1 removal from phosphate buffer solutions. Accordingly, Kabak and Var (2008) observed differences in the binding percentages to AFM_1 by viable (7.85–25.94%) and non-viable cells (12.85–27.31%) of *Lactobacillus* and *Bifidobacterium* strains during 4 h.

By the findings of this study and others cited, it is apparent that bacterial viability is not a prerequisite for removal of AFM_1 by LAB. Although the mechanism of action of these microorganisms on aflatoxin has not been clarified yet, it has been hypothesized the occurrence of a physical union with the bacterial cell wall components, mainly to polysaccharides and peptidoglycans, instead through a covalent binding or degradation by the microorganisms metabolism (Lahtinen, Haskard, Ouwehand, Salminen, & Ahokas, 2004; Shetty & Jespersen, 2006). However, both polysaccharides and peptidoglycans of bacterial cell wall are expected to be greatly affected by the heat treatment, leading to denaturation of proteins and increasing the hydrophobic nature of its surface. It is considered that such disturbances still allow aflatoxin to bind to bacterial cell wall, and also to components of plasmatic membrane which were not available when cell wall was intact (Haskard et al., 2001). Thus the integrity of bacterial cell wall components is important in the process of aflatoxin removal. Hernandez-Mendoza, Guzman-de-Peña, and Garcia (2009) concluded that both bacterial cell wall and their purified fragments were able to remove the AFB_1 from the medium, but when the loss or destruction of cell wall (total or partial) occurred in response to enzymatic treatments, a significant decrease in removal capacity was observed.

Table 2
Percentages of aflatoxin M_1 bound in UHT skim milk by heat-killed *Saccharomyces cerevisiae* and lactic acid bacteria pool cells after different contact times.

Microorganism	% Binding of AFM_1 (mean ± SD)[a]	
	30 min	60 min
LAB pool[b]	11.5 ± 2.3^{bA}	11.7 ± 4.4^{cA}
S. cerevisiae	90.3 ± 0.3^{aA}	92.7 ± 0.7^{bA}
LAB pool[b] + *S. cerevisiae*	91.7 ± 0.5^{aB}	100.0 ± 0.0^{aA}

[a–c] In the same column, means followed by different letters differ significantly ($P < 0.05$).
[A–B] In the same line, means followed by different letters differ significantly ($P < 0.05$).
[a] Percentage of bound AFM_1 from UHT skim milk spiked with 0.5 ng mL^{-1} AFM_1.
[b] Pool of lactic acid bacteria (LAB) strains: *Lactobacillus delbrueckii* spp. *bulgaricus*, *Lactobacillus rhamnosus* and *Bifidobacterium lactis*.

In our study, *S. cerevisiae* cells bound to 90.3 ± 0.3% and 92.7 ± 0.7% of AFM_1 content in UHT skim milk for 30 min and 60 min, respectively. There is no previous report on the use of *S. cerevisiae* for decontamination of milk containing AFM_1, although removal rates of AFB_1 from feeds nearly to 90% were obtained by other authors (Devegowda, Arvind, & Morton, 1996; Santin et al., 2003). The mechanism involved in *S. cerevisiae* ability to bind aflatoxins remains unclear. It is currently accepted that yeast cell wall has the ability to adsorb the toxin (Bueno, Casale, Pizzolitto, Salano, & Olivier, 2006; Parlat, Ozcan, & Oguz, 2001; Raju & Devegowda, 2000). Bueno et al. (2006) and Lee et al. (2003) concluded that both viable and non-viable *S. cerevisiae* cells have the same adsorbent ability to bind AFB_1, which is in accordance with data on removal of AFM_1 by *S. cerevisiae* in milk as reported in present study.

When heat-killed cells of *S. cerevisiae* was used with LAB, the removal efficiency of AFM_1 slightly increased in the 30 min contact time, and was fully effective (100%) when incubated for 60 min. There are no previous studies evaluating the concomitant use of *S. cerevisiae* and LAB for removal of AFM_1. The increase in the binding percentages may be explained by an additive effect between *S. cerevisiae* and LAB cells, due the presence of a greater number of cells available for the sequestration of AFM_1. Although low levels of AFM_1 in milk can be achieved by prevention through controlling contamination levels of AFB_1 in feed, our results indicate that non-viable cells of *S. cerevisiae* and LAB strains may be useful for completely removing AFM_1 from milk containing up to 0.5 ng mL^{-1}, without any changes in the flavor or acidity of milk by fermentation. However, not only the strains, the contact time and the viability of the cells can influence on the formation and stability of the *S. cerevisiae* and/or LAB − aflatoxin complex. Other factors such as the concentration of microorganisms in milk, AFM_1 levels, pH and temperature of incubation may change the efficiency of microorganisms to remove aflatoxins from food products (Bovo et al., 2012; El-Nezami, Mykkänen, Haskard, Salminen, & Salminen, 2004; Lee et al., 2003). Thus further studies are necessary to investigate the influence of those variables in the ability of *S. cerevisiae* or LAB cells to bind to AFM_1 in milk.

4. Conclusion

Heat-killed *S. cerevisiae* cells, alone or in combination with the LAB pool used, has a high efficiency (>90%) to bind AFM_1 in UHT milks in a relatively short period, as there were no differences in the toxin binding between the contact times of 30 or 60 min. Therefore the methods of aflatoxin removal employing *S. cerevisiae*, LAB and *S. cerevisiae* + LAB, mainly those strains that are already currently used in food products, have a potential application for reducing the levels of AFM_1 in milk. However, additional studies are needed to investigate the mechanisms involved in the removal process of toxin by *S. cerevisiae* and/or LAB and the factors that affect the stability of the toxin sequestration aiming the commercial application in the dairy industry.

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