Evaluation of Anti-Aflatoxin M₁ effects of heat-killed cells of *Saccharomyces cerevisiae* in Brazilian commercial yogurts

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**Abstract**

This study aimed to assess the aflatoxin M₁ (AFM₁) levels in 72 samples of yogurt from eight processing plants in São Paulo, Brazil, and the ability of heat-killed cells of *Saccharomyces cerevisiae* (10¹⁰ yeast cells/g) to reduce AFM₁ (0.5 µg/kg) in experimental yogurts (*n* = 3). Analyses were conducted by high performance liquid chromatography (HPLC). Only seven samples (9.8%) had AFM₁ at a mean level of 0.071 ± 0.08 µg/kg. *S. cerevisiae* efficiently reduced (P < 0.05) the AFM₁ levels in spiked yogurts, with a maximum reduction of 46% after 30 days of storage. Further studies should investigate potential effects of *S. cerevisiae* on the sensory properties of yogurts.

**Keywords**: AFM₁; decontamination; *Saccharomyces cerevisiae*; yeasts; yogurts

**Introduction**

Aflatoxins are the most known and vastly distributed mycotoxins in food and feed products, being synthesized by fungi species from the genus *Aspergillus*, especially *A. flavus*, *A. parasiticus*, and *A. nomius* (Wochner *et al.*, 2018). Although more than 20 types of aflatoxin have been identified, aflatoxin B₁ (AFB₁) is accounted as the main toxic metabolite produced by fungi in naturally contaminated cereals and other food products, as well as in animal feed. AFB₁ is classified as a Group 1 carcinogen by the International Agency for Research on Cancer (2002). Feeding dairy cows with any ingredient contaminated with AFB₁ can result in the further conversion of the parent composite into aflatoxin M₁ (AFM₁), which is excreted in urine and milk (Gonçalves *et al.*, 2015). In milk, AFM₁ is associated with casein, which persists bound to the toxin during the production of dairy products, including powdered milk, cheese, and yogurt (Campagnollo *et al.*, 2016; Kuharic *et al.*, 2018; Makhdoumi *et al.*, 2021). Besides, AFM₁ in milk or dairy products cannot be completely removed by regular heat treatments, like pasteurization or sterilization (Assaf *et al.*, 2019; Campagnollo *et al.*, 2016; Muaz *et al.*, 2021; Ondiek *et al.*, 2022). However, previous studies indicate that AFM₁ levels in milk can be reduced by the addition of yeast cells of *Saccharomyces cerevisiae*, in view of the ability of this yeast species to absorb and/or inactivate AFM₁ (Corassin *et al.*, 2013).

*S. cerevisiae* is one of the most important yeasts used in the food industry, also being considered a GRAS (“generally recognized as safe”) organism (Van der Hoek *et al.*, 2019). Thus, a biological approach for reducing aflatoxin based on *S. cerevisiae* strains that are already used in food products is an attractive alternative to reduce the AFM₁.
levels in yogurt and other fermented dairy products. The incorporation of nonviable cells of *S. cerevisiae* in Minas Frescal cheese, alone or in combination with lactic acid bacteria, resulted in up to 100% reduction of AFM, in this type of cheese after 20 days of storage (Gonçalves et al., 2020). Furthermore, some yeast species have probiotic properties, including resistance to the acidified medium of stomach and ability to improve the gut microbiota (Souza et al., 2021). *S. boulardii* and *Pichia kudriavzevii* have been added to beverages (Paula et al., 2019) and cereal-based fermented foods (Greppi et al., 2017), respectively, to provide beneficial effects to the human host, thus opening new perspectives for the development of innovative yeast-based functional food products.

Milk and dairy products are essential segments of the human diet, being largely consumed by people of different age groups, especially the elderly and children (Campagnollo et al., 2016). Therefore, the occurrence of AFM in milk and milk products represents a notable hazard to human health (Gonçalves et al., 2020; Souza et al., 2020; Sumon et al., 2021). In this context, several studies revealed that human exposure to the aflatoxins may be increased through consumption of AFM- contaminated milk and dairy products (Campagnollo et al., 2016; Gonçalves et al., 2021; Hassan and Kassaify, 2014; Makhdoumi et al., 2021; Womack et al., 2016). In Brazil, some studies regarding the occurrence of AFM showed high incidence of contaminated samples, ranging from 63 to 100%, and levels ranging from 0.0002 to 0.106 µg/L among different yogurt and other milk products (Gonçalves et al., 2021; Iha et al., 2011; Picinin et al., 2013). Despite these limited occurrence data, there is no information on the frequency and levels of AFM, in yogurt collected directly from Brazilian dairy producers.

Yogurt is obtained by natural fermentation of whole or standardized milk with *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (Cruz et al., 2013). In addition, yogurt is one of the most consumed fermented milks in Brazil (Iha et al., 2011), and it is also an excellent vehicle for delivering probiotics (Cruz et al., 2013) and prebiotics (Muaz et al., 2021). Therefore, it can be hypothesized that the addition of yeasts in the manufacture of yogurts may reduce the AFM levels in the contaminated product. This is in accordance with the need for safe and practical decontamination methods that are acceptable to consumers and can be applied during biotechnological processes of fermented foods such as yogurts (Piotrowska et al., 2021). However, the addition of *S. cerevisiae* cells into yogurts to decontaminate AFM, in the final product has never been explored. In this context, the present study aimed to determine the occurrence of AFM, in yogurt samples collected from eight different dairy processing plants in São Paulo state, Brazil, and to evaluate the ability of *S. cerevisiae* to reduce the AFM levels in spiked yogurt with or without the addition of yeast.

### Material and Methods

#### Assessment of aflatoxin M₁ in yogurt manufactured in dairy processing plants

Sampling procedures were carried out in eight yogurt processing plants located in the northeastern region of the state of São Paulo, Brazil. A total of 72 yogurt samples were collected (n = 9, for each plant). In each factory, nine batches of yogurt production were sampled, totaling 72 batches of yogurt evaluated in the study. All collected samples were transported to the laboratory in a thermal box with dry ice and stored at 4°C until AFM₁ determination analysis.

#### Assessment of the ability of *S. cerevisiae* to reduce aflatoxin M₁ in yogurt

Twelve yogurt samples (1-L bottles) from the same lot and the same manufacturer were purchased from a local supermarket and used to evaluate the ability of *S. cerevisiae* to reduce AFM₁ in the product. All yogurt samples were formerly analyzed and considered free of AFM₁ (below the detection limit of the analytical method: 0.017 µg/kg). Each yogurt sample was assigned to one treatment in a completely randomized study using a factorial arrangement of 2 × 2, corresponding to two levels of *S. cerevisiae* (0 and 10⁸ yeast cells/kg yogurt) and two levels of AFM₁ (0 and 0.5 µg/kg yogurt), totaling four treatments with three repetitions per treatment. The two levels of *S. cerevisiae* (0 and 10⁸ yeast cells/kg yogurt) were selected based on previous studies on the application of this yeast for AFM₁ decontamination in milk (Corassin et al., 2013) and cheese (Gonçalves et al., 2021).

The *S. cerevisiae* strain (categorized as a GRAS organism) used for incorporation into the yogurts was a commercially available brewer’s biological dry yeast (Fermentis K-97, SafAle, Bruggeman, Belgium) containing 1.0 × 10¹⁰ cells/g. Prior to the addition to yogurts, the cells of *S. cerevisiae* were submitted for inactivation in an autoclave at 121°C for 10 min, to avoid any effect on the fermentation of yogurt. The AFM₁ used (Sigma-Aldrich, USA) was previously diluted in acetonitrile at 0.5 µg/mL. An aliquot of 0.5 mL of this solution was evaporated in a flask under nitrogen flow, then 0.5 kg of yogurt and 0.5 g of the heat-killed yeast cells biomass were added in the flask and mixed thoroughly for 15 min, to obtain the required levels of AFM₁ and yeast in the prepared yogurts. The prepared yogurts were stored at 4°C for 30 days, and samples were collected immediately and after preparation (day 0) and at 10-day intervals.
Determination of aflatoxin M₁ in yogurt

AFLM₁ was extracted and purified from all yogurt samples (collected in dairy plants and artificially spiked with AFLM₁ and/or yeast cell biomass) using immunoaffinity columns (Aflatest WB, Vicam, Watertown, MA, USA), exactly as described by Jager et al. (2013). Final extracts from yogurt samples were injected (20 µL) into a Shimadzu 10VP liquid chromatograph (Kyoto, Japan), equipped with a 10 AXL fluorescence detector (excitation at 360 nm and emission above 440 nm). The chromatographic run was achieved using a Kinetex C₁₈ column (Phenomenex, Torrance, CA, USA) 4.6 × 150 mm, 2.6 µm particle size, and the isocratic mobile phase consisted of methanol/water/acetonitrile (61.4:28.1:10.5, v/v/v) with a flow rate of 0.50 mL/min.

Five-point calibration curves containing AFLM₁ at levels from 0.1 to 1.0 µg/L were prepared using AFLM₁ standard prepared in acetonitrile. Integrated peak areas were linearly correlated with the concentrations. Identification of AFLM₁ was achieved by comparing the retention time of AFLM₁ peaks in the samples with the standards in the calibration curves. The limits of detection (LOD) and limits of quantification (LOQ) were calculated at a signal-to-noise ratio of 3 and 10, respectively, being 0.017 and 0.055 µg/kg, respectively. The analytical method was previously validated with contaminated yogurt samples at levels of 0.2 and 0.5 µg/kg (n = 3, for each concentration), which resulted in AFLM₁ recovery rates in yogurt samples ranging from 72 to 93% (Jager et al., 2013).

Analysis of the pH of spiked yogurts

The pH was determined in yogurt samples artificially spiked with AFLM₁ and/or yeast cell biomass as described by AOAC (2019).

Statistical analysis

The General Linear Model of SAS (2004) was approached as the statistical analysis of AFLM₁ binding assays, while a level of P < 0.05 was considered as significant.

Results and Discussion

The occurrence of aflatoxin M₁ in yogurt collected in dairy plants

AFLM₁ was detected in seven samples (9.8 %) of yogurt manufactured in dairy plants at São Paulo state, with a range of 0.017 to 0.130 µg/kg (Table 1). While no regulation for the levels of AFLM₁ in yogurt was established in Brazil, none of the analyzed samples presented levels higher than the Brazilian limit for milk (0.50 µg/L) (ANVISA, 2011). As AFLM₁ is frequent in dairy foods produced worldwide, many countries proposed some regulatory limits for AFLM₁ in milk and dairy products, with limits varying from 0.05 to 0.5 µg/kg (Iha et al., 2011). Studies have described the occurrence of AFLM₁ in yogurt worldwide, although the frequency is high; in most studies, the reported levels of AFLM₁ were considered low (Muaz et al., 2021; Souza et al., 2020).

The number of the contaminated samples (n = 7) and the mean level of AFLM₁ (0.051 ± 0.13 µg/kg) reported in the present study were similar to those reported by Cano-Sancho et al. (2010), who evaluated the occurrence of AFLM₁ in 72 samples of yogurt marketed in Spain and detected a low incidence of AFLM₁, 2.8% (n = 2), and low levels of AFLM₁, ranging from 0.04 to 0.052 µg/kg. However, in Iran, Fallah (2010) and Nilchian and Rahumi (2012) reported a higher incidence of AFLM₁ in yogurt, about 66.1% (n = 45) and 35% (n = 14), respectively. However, both studies reported ranges for AFLM₁, 0.015 to 0.119 µg/kg, and 0.011 to 0.116 µg/kg, respectively. Analogous to Iran, in Pakistan, Iqbal et al. (2013) reported a higher incidence of AFLM₁, 33.3% (n = 32), than in the present study and low levels of AFLM₁ (0.019 to 0.053 µg/kg) in the evaluated yogurt samples. In Turkey, as well as in Pakistan, Ertas et al. (2011) and Kocasari et al. (2012) reported a high incidence of AFLM₁ in the samples, 56% (n = 28) and 44.4% (n = 20), and low levels of AFLM₁, 0.002 µg/kg at 0.078 and 0.05 to 0.36 µg/kg, respectively. In Qatar, Hassan et al. (2018), despite reporting a high incidence of 76% (n = 16), the levels of AFLM₁ detected in the yogurt samples were less than 0.05 µg/L.

Several reports indicate that the occurrence of AFLM₁ in milk and dairy products strongly depended on several factors, including lactation stage, feed quality, season/climate, animal breed, and milk production performance beside the used technique for AFLM₁ assessment (Hassan et al., 2018; Iqbal et al., 2017;...

<table>
<thead>
<tr>
<th>Range of AFLM₁ level (µg/kg)</th>
<th>Number of samples</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; LOD</td>
<td>65</td>
<td>90.2</td>
</tr>
<tr>
<td>LOD-0.05</td>
<td>4</td>
<td>5.6</td>
</tr>
<tr>
<td>0.05-0.25</td>
<td>3</td>
<td>4.2</td>
</tr>
<tr>
<td>0.25-0.50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.50-1.00</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>72</td>
<td>100</td>
</tr>
</tbody>
</table>

*LOD: Limit of detection (0.017 µg/kg).*

Occurrence of AFM₁ in yogurts and yeast decontamination

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The pH of the yogurt, stored at 4°C, was not affected (P > 0.05) by using *S. cerevisiae* in any of the evaluated treatments, during the entire period (from days 0 to 30) of the study (Table 2).

As expected, AFM₁ concentrations in nonspiked yogurts were below the LOD of the analytical method (0.017 µg/kg). The mean levels of AFM₁ in spiked yogurts ranged from 0.27 ± 0.03 to 0.50 ± 0.01 µg AFM₁/kg during 30 days of storage (Table 3). In our study, in the treatment without *S. cerevisiae*, a percentage reduction of 10% in AFM₁ after 30 days was noted, which can be associated with the natural function of lactic acid bacteria in raw and pasteurized milk used in the processing of yogurt (Franciosi et al., 2009). Another explanation for the observed reduction in AFM₁ can be correlated with the low pH value. Corroborating with our study, Govaris et al. (2002) reported the stability of AFM₁ in yogurt

### Table 2. pH values of yogurts prepared with or without the addition of heat-killed cells of yeast and aflatoxin M₁ during 30 days of storage.

<table>
<thead>
<tr>
<th>Yeast*(cells/kg)</th>
<th>AFM₁ (µg/kg)</th>
<th>pH</th>
<th>Day 0</th>
<th>Day 10</th>
<th>Day 20</th>
<th>Day 30</th>
<th>Meanb</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td></td>
<td>4.01</td>
<td>4.14</td>
<td>4.03</td>
<td>4.05</td>
<td>4.06 ± 0.06</td>
</tr>
<tr>
<td>10⁵</td>
<td>0</td>
<td></td>
<td>4.35</td>
<td>4.34</td>
<td>4.28</td>
<td>4.3</td>
<td>4.32 ± 0.03</td>
</tr>
<tr>
<td>0</td>
<td>0.5</td>
<td></td>
<td>4.03</td>
<td>4.01</td>
<td>3.95</td>
<td>3.99</td>
<td>4.00 ± 0.03</td>
</tr>
<tr>
<td>10⁵</td>
<td>0.5</td>
<td></td>
<td>4.92</td>
<td>4.34</td>
<td>4.19</td>
<td>4.24</td>
<td>4.42 ± 0.34</td>
</tr>
</tbody>
</table>

*Commercially available brewer’s biological dry yeast (Fermentis K-97, SafAle, Bruggeman, Belgium) containing 1.0 × 10¹⁰ yeast cells/g.

*Values were expressed as mean ± standard deviation of samples analyzed in triplicate.

No significant differences were found between means in rows or columns (P > 0.05).

### Table 3. Mean aflatoxin M₁ (AFM₁) levels and percentage reductions (R) in spiked yogurts prepared with or without heat-killed cells of yeast during 30 days of storage.

<table>
<thead>
<tr>
<th>Yeast*(cells/kg)</th>
<th>AFM₁ (µg/kg)</th>
<th>Aflatoxin M₁ in yogurt during storage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean level (µg/kg)</td>
<td>% (R)c</td>
</tr>
<tr>
<td>0</td>
<td>&lt;LODd</td>
<td>–</td>
</tr>
<tr>
<td>10⁵</td>
<td>&lt;LOD</td>
<td>–</td>
</tr>
<tr>
<td>0</td>
<td>0.50 ± 0.01</td>
<td>0.0</td>
</tr>
<tr>
<td>10⁵</td>
<td>0.46 ± 0.01</td>
<td>8.0</td>
</tr>
</tbody>
</table>

*Commercially available brewer’s biological dry yeast (Fermentis K-97, SafAle, Bruggeman, Belgium) containing 1.0 × 10¹⁰ yeast cells/g.

*Values are expressed as mean ± SD of samples analyzed in triplicate.

*Cumulative reduction percentages of AFM₁ in relation to the initial concentration of AFM₁ in spiked yogurts.

*LOD: Limit of detection (0.017 µg/kg).
artificially contaminated with concentrations of 0.05 and 0.1 µg/L, during storage for 4 weeks, at 4°C, at two pH levels (4.0 and 4.6). Their findings demonstrated that at a pH of 4.6, no significant change in AFM₁ levels was observed. However, AFM₁ showed a significant decrease after the third and fourth weeks of storage. The authors quoted that the reduction of AFM₁ could be a function of the low pH.

The effect of *S. cerevisiae* in reducing AFM₁ was highlighted by the findings of our study. There was an 8% reduction in AFM₁ in yogurt on day 0, followed by an increase in reduction on day 10 (24%), continuing the reduction on day 20 (36%), and at day 30, the percentage of AFM₁ decreased, reaching a reduction of 46%. There is only one previous study that assessed the effect of *S. cerevisiae* on the removal of aflatoxin M₁ in yogurts. In a similar study to ours, Karazhiyan *et al.* (2016) reported AFM₁ reduction percentages much higher than ours, when they evaluated the ability of *S. cerevisiae* (viable, treated with acid, heat, and ultrasound) to bind to AFM₁ in yogurt over time (days 1, 7, 14, and 21 after manufacture). Among the treated yeasts, the one with the highest binding capacity to AFM₁ was treated with acid (76.46%). Yeasts treated with heat (76.39%) and ultrasound (74.20%) also showed high percentages of reduction. An important advantage of using *S. cerevisiae* as the AFM₁ binder in yogurts is the overall acceptance of this yeast without restrictions in the food industry, considering its classification as a GRAS organism (Van der Hoek *et al.*, 2019). Besides, the low costs of adding *S. cerevisiae* biomass in yogurts provide a viable alternative to the dairy industry to reduce the AFM₁ contamination in the product during the storage period. In this regard, further investigations are recommended to evaluate the involved mechanisms in the process of mycotoxin reduction by *S. cerevisiae*. In addition, the associated factors with the stability of the sequestration of toxins, such as the concentration of yeasts, acidity, and type of initial culture, should be considered (Karazhiyan *et al.*, 2016).

**Conclusion**

The limited survey performed in the present study indicates that milk received for the manufacture of yogurt in the dairy plants evaluated have low incidence (9.8%) and levels (mean: 0.071 ± 0.08 µg/kg) of AFM₁. The addition of *S. cerevisiae* biomass in yogurts containing 0.5 µg/kg of AFM₁ reduced its concentration to 0.27 µg/kg after 30 days of storage, thus providing a 46% decrease of AFM₁ in the period. Results of this trial indicate that the incorporation of *S. cerevisiae* could efficiently decrease the AFM₁ levels in yogurt. Further studies are required to examine the involved mechanisms in the process of aflatoxin reduction by *S. cerevisiae*. In addition, the associated factors with the stability of the sequestration of toxins, such as the concentration of yeasts, acidity, and type of initial culture, should be considered.

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