



# Use of scanning electron microscopy and high-performance liquid chromatography to assess the ability of microorganisms to bind aflatoxin M<sub>1</sub> in Minas Frescal cheese

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## Abstract

The aim of this study was to evaluate the capacity of two strains of lactic acid bacteria (LAB), *Lactobacillus rhamnosus* and *Lactococcus lactis*, and a yeast strain, *Saccharomyces cerevisiae*, inactivated by heat (121 °C, 10 min), from binding to aflatoxin M<sub>1</sub> (AFM<sub>1</sub>), as well as the interaction between these microorganisms, aflatoxin M<sub>1</sub> and the Minas Frescal cheese matrix after 2 and 30 days of storage. The ability of LABs and *S. cerevisiae* to bind AFM<sub>1</sub> to Minas Frescal cheese was evaluated by high performance liquid chromatography (HPLC) composed of a fluorescence detector. The interaction between these microorganisms and AFM<sub>1</sub> was evaluated using a scanning electron microscope composed of a backscattered electron detector with a voltage of 15 kV and magnifications of 1000 ×, 5000 × and 8000 ×. The use of microorganisms as a biological method is efficient in reducing AFM<sub>1</sub> in Minas Frescal cheese and does not affect the microbiological parameters. AFM<sub>1</sub> reduction varied according to the microorganism used in the treatments. *S. cerevisiae* showed greater capacity to bind AFM<sub>1</sub> over time, compared to LABs. Scanning electron microscopy was especially useful, confirming that lactic acid bacteria and *S. cerevisiae* were able to bind AFM<sub>1</sub> particles in Minas Frescal cheese.

**Keywords:** *S. cerevisiae*; lactic acid bacteria; mycotoxins; dairy; aflatoxins.

**Practical Application:** One of the main objectives of the food industry is to avoid contamination by mycotoxins in processes and products. Methods that can verify the effectiveness of decontamination of aflatoxin M<sub>1</sub> by microbial action are extremely important.

## 1 Introduction

Aflatoxins are secondary metabolites produced by toxigenic fungal species belonging to the *Aspergillus* genus, mainly *A. flavus*, *A. parasiticus* e *A. nomius*. Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is toxic, carcinogenic, teratogenic and/or mutagenic easily found as a contaminant in animal diet. Feeding dairy cows with fodder or any contaminated ingredient of the animal diet with AFB<sub>1</sub> results in the metabolic conversion of AFB<sub>1</sub> to aflatoxin M<sub>1</sub> (AFM<sub>1</sub>), which is excreted in milk, in addition, it will be presented in the final product due to heat treatment resistance (Öztürk Yılmaz & Altinci, 2019; Hajmohammadi et al., 2020; Min et al., 2020; Park et al., 2020).

Milk and dairy products are frequently consumed by a portion of the population considered vulnerable, children and the elderly (Park et al., 2019; Ahmadi, 2020). Minas Frescal cheese is a typical Brazilian product, it is one of the most highly consumed lactic products, showing wide national market acceptance. This is a fresh soft white cheese, slightly salted, with a slight lactic acid taste. It is produced by the enzymatic coagulation of pasteurized milk with rennet or other appropriate coagulating enzymes (Prezzi et al., 2020).

The negative impact of aflatoxins on health and economy has led to investigations of strategies to prevent their formation in food, as well as to eliminate, inactivate or reduce the availability of these toxins in contaminated products (Gonçalves et al., 2015a;

Bodobodak et al., 2018; Cagri-Mehmetoglu, 2018; Assaf et al., 2019; Martey et al., 2020).

In this context, biological methods of reducing mycotoxins arose, which come from the action of microorganisms, such as bacteria, yeast and others. Biological availability reduction methods have been extensively studied as they are efficient, cost-effective, and in many cases, already used in food production such as lactic acid bacteria and *S. cerevisiae* (Assaf et al., 2019; Barukčić et al., 2018; Cagri-Mehmetoglu, 2018; Campagnollo et al., 2020; Corassin et al., 2013; Gonçalves et al., 2015b, 2020; Ma et al., 2017).

Scanning electron microscopy is a versatile and useful technique, with a wide variety of commercial, industrial and research applications. It involves a finely collimated electron beam that scans the surface of the sample being analyzed. The beam is focused on a small probe that scans the surface of the sample. The interactions of the beam with the material result in the emission of electrons and photons when electrons penetrate the surface, and emitted particles are collected with the appropriate detector to provide surface information. The end product of the electron beam collision with the sample surface is an image (Lee et al., 2019).

In the present study, scanning electron microscopy was used to characterize the surface of biological additives and aflatoxin

Received 07 Sept., 2020

Accepted 15 Sept., 2020

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M<sub>1</sub> separately and together, depending on the treatment, in order to observe surface changes when aflatoxin is adsorbed. Additionally, the microstructure of cheese samples was evaluated.

## 2 Materials and methods

### 2.1 Material

Whole milk, probiotic culture composed of *Lactobacillus rhamnosus* (LRB, SACCO, Cadorago, Italy) and *Lactococcus lactis* (MWO 040, SACCO, Cadorago, Italy), a *Saccharomyces cerevisiae* strain (Fermentis K-97, SafAle, Belgium), liquid rennet (chymosin and bovine pepsin, Ha-La, Christian-Hansen®, Brazil) and Aflatoxin M<sub>1</sub> standard (Sigma Aldrich®), were used in this study.

### 2.2 Manufacture of Minas Frescal cheese

The Minas Frescal cheese were manufactured according to Fernandes et al. (2012). The cheese was produced at the Food Microbiology and Mycotoxicology Laboratory of the Faculty of Animal Science and Food Engineering at the University of São Paulo, Pirassununga campus, from milk produced on the same campus.

The cheese samples (16) were produced in duplicate for each of the following treatments: T1) negative control - cheese only; T2) positive control, cheese with addition of AFM<sub>1</sub> (0.5 µg/kg); T3) cheese with the addition of *L. rhamnosus* and *L. lactis* (10<sup>10</sup> cells/g); T4) cheese with the addition of *L. rhamnosus*, *L. lactis* and AFM<sub>1</sub>; T5) cheese with the addition of *S. cerevisiae* (concentration 10<sup>10</sup> cells/g); T6) cheese with the addition of *S. cerevisiae* and AFM<sub>1</sub>; T7) cheese with the addition of *L. rhamnosus*, *L. lactis* and *S. cerevisiae*; T8) cheese with the addition of *L. rhamnosus*, *L. lactis*, *S. cerevisiae* and AFM<sub>1</sub>. In all treatments, the lactic acid bacteria, *S. cerevisiae* and AFM<sub>1</sub> were in the same concentration.

The LAB and *S. cerevisiae* ability to bind AFM<sub>1</sub>, as well as the interaction between these microorganisms and aflatoxin M<sub>1</sub> in the Minas Frescal cheese were evaluated on days 2 and 30 after manufacture.

### 2.3 Sample preparation and determination of aflatoxin M<sub>1</sub>

AFM<sub>1</sub> determination in cheese samples was performed as described by Jager et al. (2013). Two grams of NaCl, 22 mL of methanol and 13 mL of ultrapure water were added to a tube containing 8 g of the sample. The mixture was centrifuged at 2,078 x g for 15 min., and the obtained supernatant was filtered. 20 mL of the filtrate was collected in another flask, and 40 mL of ultrapure water was added. Purification was performed by passing the total volume (60 mL) through a manifold-coupled immunoaffinity column (Aflatest WB, Vicam®) at 2-3 drops/sec flow. After this procedure, the column was washed by passing 20 mL of ultrapure water. Subsequently, the toxin was eluted by passing 1 mL of HPLC grade methanol. Then, the eluent was evaporated to dryness under a stream of nitrogen at 40 °C and resuspended with 1 mL solution of methanol and ultrapure water (50:50 v/v).

Final extracts of cheese 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). A Kinetex C<sub>18</sub> column (Phenomenex, Torrance, CA, USA) 4.6 × 150 mm, 2.6 µm particle size and an in-line filter of 0.5 µm were used. 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. The equipment used in this study included vortex mixer (Fanem, São Paulo, Brazil); centrifuge (SOLAB SL-700, Piracicaba, Brazil); column manifold (Supelco, Bellefonte, Pennsylvania, USA) and vacuum pump (Model 131, type 2V, Primatec, Itu, Brazil).

Calibration curves to detect AFM<sub>1</sub> from cheese samples were prepared using standard solution of AFM<sub>1</sub> (Sigma®), diluted in acetonitrile at concentrations of 0.625, 1.25, 5, 10, 20 µg/L. Integrated peak areas were linearly correlated with the concentrations. The limits of detection (LOD) and quantification (LOQ) were calculated for each method of analysis based on signal:noise ratio of 3:1 and 10:1, respectively. Linearity was evaluated by verifying the coefficient of determination (r<sup>2</sup>) and visual inspection of residual plots of analytical curves. The performance of methods used for determination of AFM<sub>1</sub> in cheese samples in the laboratory has been presented elsewhere (Jager et al., 2013), describing limits of detection (LOD) and quantification (LOQ) values of 0.017 and 0.055 µg/kg, in cheese samples respectively.

### 2.4 Scanning electron microscopy assay

The cheese samples were prepared for scanning electron microscopy according to proposed by Lobato-Calleros et al. (2002) and Fritzen-Freire et al. (2010) with adaptations. Cylindrical samples of 0.5 cm diameter by 0.5 cm height were fixed in 2% buffer glutaraldehyde (0.1 M phosphate buffer, pH 7.2) for 6 h. The samples were dehydrated in increasing concentrations of aqueous ethanol solutions (50%, 60%, 70%, 80%, 90% and 100%, 15 min for 3 times in each, totalizing 45 min) and placed in acetone for 1 h. After that, each sample was fractured perpendicular to its long axis and mounted on stubs with fractured face upwards. A scanning electron microscope (Hitachi, TM 3000, Japan) with a high sensibility semiconductor backscattered electron detector was used at 15 kV to observe each sample at a magnification of 1000×, 5000× and 8000×.

#### Image analysis

The analysis of the scanning electron microscopy images was performed without the use of a specific software, besides the SEM - Hitachi 3000 device itself, which was used only for image capture. The images were evaluated one by one, being selected according to the following criteria: (1) image sharpness and (2) magnification used, the magnification that presented the sharpest images in all treatments was sought. After choosing the best magnification, which was 5000×, the clearest images and the best images (day 2 and day 30) were screened for each treatment through a new screening.

### 2.5 Microbiological assay

Determination of *Salmonella* sp., *Listeria monocytogenes*, thermotolerant coliforms and coagulase positive staphylococci in Minas Frescal cheese were performed on days 2 and 30 after

manufacture following the procedures described by American Public Health Association (2004).

### 3 Results and discussion

Table 1 shows the results of the microbiological analyzes performed on Minas Frescal cheese after 2 and 30 days of storage. Thermotolerant coliforms counts in the samples is in conformity with Brazilian legislation, and all confirmatory tests for *Salmonella* sp. and *Listeria monocytogenes* were negative, as were the coagulase tests to verify the positive coagulase staphylococci count (Brasil, 2001). Therefore, it can be said that

the use of BAL and *S. cerevisiae* as a biological method to reduce AFM<sub>1</sub> in Minas Frescal cheese had no negative effect on the shelf-life of this cheese, which is generally 21 days. The shelf-life of Minas Frescal cheese is also related to the raw material used, the production process and the initial quantity of microorganism present (Prezzi et al., 2020).

The concentration of AFM<sub>1</sub> in cheese from treatments which AFM<sub>1</sub> was not included was below the detection limit of the analytical method (Table 2), while the concentration of AFM<sub>1</sub> in treatments that included AFM<sub>1</sub> ranged from 0.29 µg of AFM<sub>1</sub>/kg until undetected.

**Table 1.** Microbiological analysis of Minas Frescal cheese produced with or without the addition of inactivated cells of lactic acid bacteria and yeast and aflatoxin M<sub>1</sub> after 2 and 30 days of storage.

Treatment	Probiotic culture <sup>a</sup> (cells/kg)	Yeast <sup>b</sup> (cells/kg)	AFM <sub>1</sub> in curd (µg/kg)	Microbiological parameters during storage <sup>c</sup>							
				Thermotolerant coliforms (NMP/g)		<i>Salmonella</i> sp.		<i>Listeria monocytogenes</i>		Coagulase positive staphylococci (CFU/g)	
				Day 2	Day 30	Day 2	Day 30	Day 2	Day 30	Day 2	Day 30
T1	0	0	0	26	273	ND <sup>1</sup>	ND <sup>1</sup>	ND <sup>1</sup>	ND <sup>1</sup>	<10 <sup>2</sup>	<10 <sup>2</sup>
T3	2 x 10 <sup>10</sup>	0	0	28	250	ND <sup>1</sup>	ND <sup>1</sup>	ND <sup>1</sup>	ND <sup>1</sup>	<10 <sup>2</sup>	<10 <sup>2</sup>
T5	0	10 <sup>10</sup>	0	26	99	ND <sup>1</sup>	ND <sup>1</sup>	ND <sup>1</sup>	ND <sup>1</sup>	<10 <sup>2</sup>	<10 <sup>2</sup>
T7	2 x 10 <sup>10</sup>	10 <sup>10</sup>	0	33	270	ND <sup>1</sup>	ND <sup>1</sup>	ND <sup>1</sup>	ND <sup>1</sup>	<10 <sup>2</sup>	<10 <sup>2</sup>
T2	0	0	0.5	1	270	ND <sup>1</sup>	ND <sup>1</sup>	ND <sup>1</sup>	ND <sup>1</sup>	<10 <sup>2</sup>	<10 <sup>2</sup>
T4	2 x 10 <sup>10</sup>	0	0.5	16	19	ND <sup>1</sup>	ND <sup>1</sup>	ND <sup>1</sup>	ND <sup>1</sup>	<10 <sup>2</sup>	<10 <sup>2</sup>
T6	0	10 <sup>10</sup>	0.5	42	89	ND <sup>1</sup>	ND <sup>1</sup>	ND <sup>1</sup>	ND <sup>1</sup>	<10 <sup>2</sup>	<10 <sup>2</sup>
T8	2 x 10 <sup>10</sup>	10 <sup>10</sup>	0.5	0	160	ND <sup>1</sup>	ND <sup>1</sup>	ND <sup>1</sup>	ND <sup>1</sup>	<10 <sup>2</sup>	<10 <sup>2</sup>

<sup>a</sup>Commercially available lyophilized product (SACCO Brazil) containing *L. rhamnosus* at 1.0 x 10<sup>10</sup> cells/g + *L. lactis* at 1.0 x 10<sup>10</sup> cells/g; <sup>b</sup>Commercially available brewer's biological dry yeast (Fermentis K-97, SafAle, Bruggeman, Belgium) containing 1.0 x 10<sup>10</sup> yeast cells/g; <sup>c</sup>Values expressed as mean of samples analyzed in triplicate; <sup>1</sup>ND - Not detected. NMP - most

**Table 2.** Concentrations and percentage reductions of aflatoxin M<sub>1</sub> in Minas Frescal cheeses manufactured with or without the addition of AFM<sub>1</sub> and heat-killed cells of lactic acid bacteria and yeast during 30 days of storage.

Treatment	Probiotic culture <sup>c</sup> (cells/kg)	Yeast <sup>f</sup> (cells/kg)	AFM <sub>1</sub> in curd (µg/kg)	Aflatoxin M <sub>1</sub> in cheese during storage			
				Day 2		Day 30	
				Concentration (µg/kg) <sup>g</sup>	% Reduction <sup>h</sup>	Concentration (µg/kg) <sup>g</sup>	% Reduction <sup>h</sup>
T1	0	0	0	<LOD	-	<LOD	-
T3	2x10 <sup>10</sup>	0	0	<LOD	-	<LOD	-
T5	0	10 <sup>10</sup>	0	<LOD	-	<LOD	-
T7	2x10 <sup>10</sup>	10 <sup>10</sup>	0	<LOD	-	<LOD	-
T2	0	0	0.5	0.29 ± 0.01	42 <sup>aA</sup>	0.23 ± 0.03	54 <sup>aB</sup>
T4	2x10 <sup>10</sup>	0	0.5	0.09 ± 0.02	82 <sup>bA</sup>	0.03 ± 0.01	94 <sup>bB</sup>
T6	0	10 <sup>10</sup>	0.5	0.13 ± 0.02	74 <sup>cA</sup>	<LOD	100 <sup>cC</sup>
T8	2x10 <sup>10</sup>	10 <sup>10</sup>	0.5	0.13 ± 0.01	74 <sup>cA</sup>	<LOD	100 <sup>cB</sup>

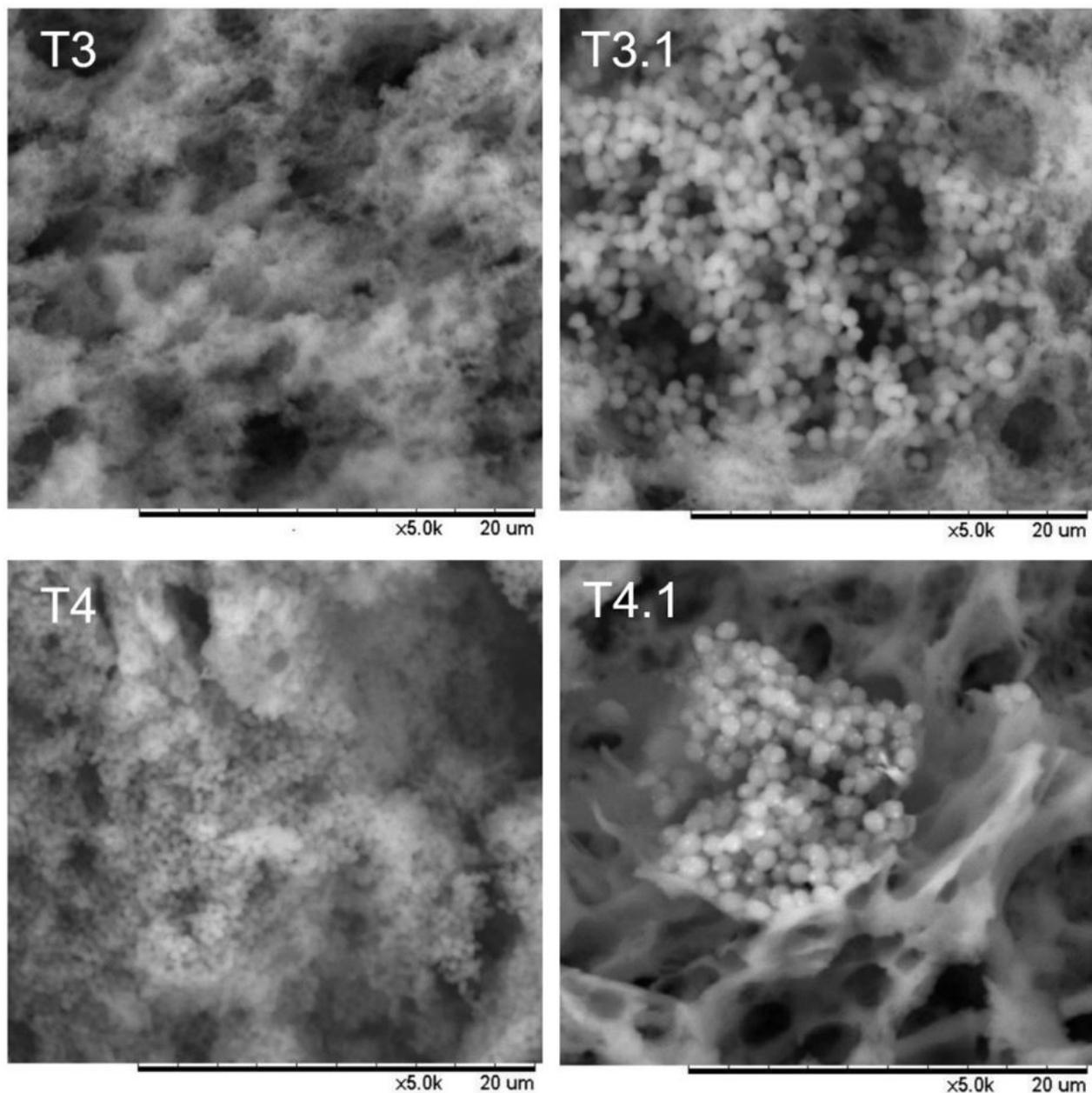
<sup>a-d</sup>In the same column, means followed by different letters differ significantly ( $P < 0.05$ ); <sup>A-C</sup>In the same line, means followed by different letters differ significantly ( $P < 0.05$ ); <sup>c</sup>Commercially available lyophilized product (SACCO Brazil) containing *L. rhamnosus* at 1.0 x 10<sup>10</sup> cells/g + *L. lactis* at 1.0 x 10<sup>10</sup> cells/g; <sup>f</sup>Commercially available brewer's biological dry yeast (Fermentis K-97, SafAle, Bruggeman, Belgium) containing 1.0 x 10<sup>10</sup> yeast cells/g; <sup>g</sup>Values expressed as mean ± standard deviation of samples analyzed in triplicate; <sup>h</sup>Cumulative percentages of reductions related to the concentration of aflatoxin M<sub>1</sub> added in cheese curd during processing. LOD: Limit of detection (0.017 µg/kg).

In the present study, the scanning electron microscopy assay was useful to support the results obtained through the analysis by HPLC, showing that there is a connection or interaction between the surfaces of LAB, yeast and aflatoxin  $M_1$ , separately and in associated treatment. In addition, it was possible to observe few changes in (1) LAB and *S. cerevisiae* when aflatoxin was attached and (2) in the microstructure of Minas Frescal cheese with aflatoxin and LAB and/or *S. cerevisiae* added versus cheese containing only these microorganisms.

Figures 1 to 3 show the micrographs of the different treatments applied to Minas Frescal cheese evaluated in this work, after days 2 and 30 of storage. Differences were observed in the microstructure of the cheese and in the morphology of the microorganisms used to reduce AFM<sub>1</sub>.

The cheese microstructure is a spatial arrangement of casein micelles that join in groups and chains to form a viscoelastic protein network through which moisture, fat globules, minerals and bacteria are dispersed (Aldalur et al., 2019).

Differences were observed in the microstructure of the cheese and in the structure of the lactic acid bacteria (Figure 1). Comparing the images of treatments 3 and 4 on days 2 and 30 (T3 and T3.1 and T4 and T4.1, respectively) it is possible to observe that the cheese matrix it has a more compact structure on day 2 of both treatments, and it is not possible to even visualize the lactic acid bacteria. After 30 days of production, the cheese network is less compacted, and it is possible to observe lactic acid bacteria in treatment 3 (T3.1) and in treatment 4 (T4.1), it is possible to observe AFM<sub>1</sub> (white dots) linked to lactic acid bacteria.



**Figure 1.** Scanning electron microscopy images of treatments 3 and 4 on days 2 and 30 after manufactured.

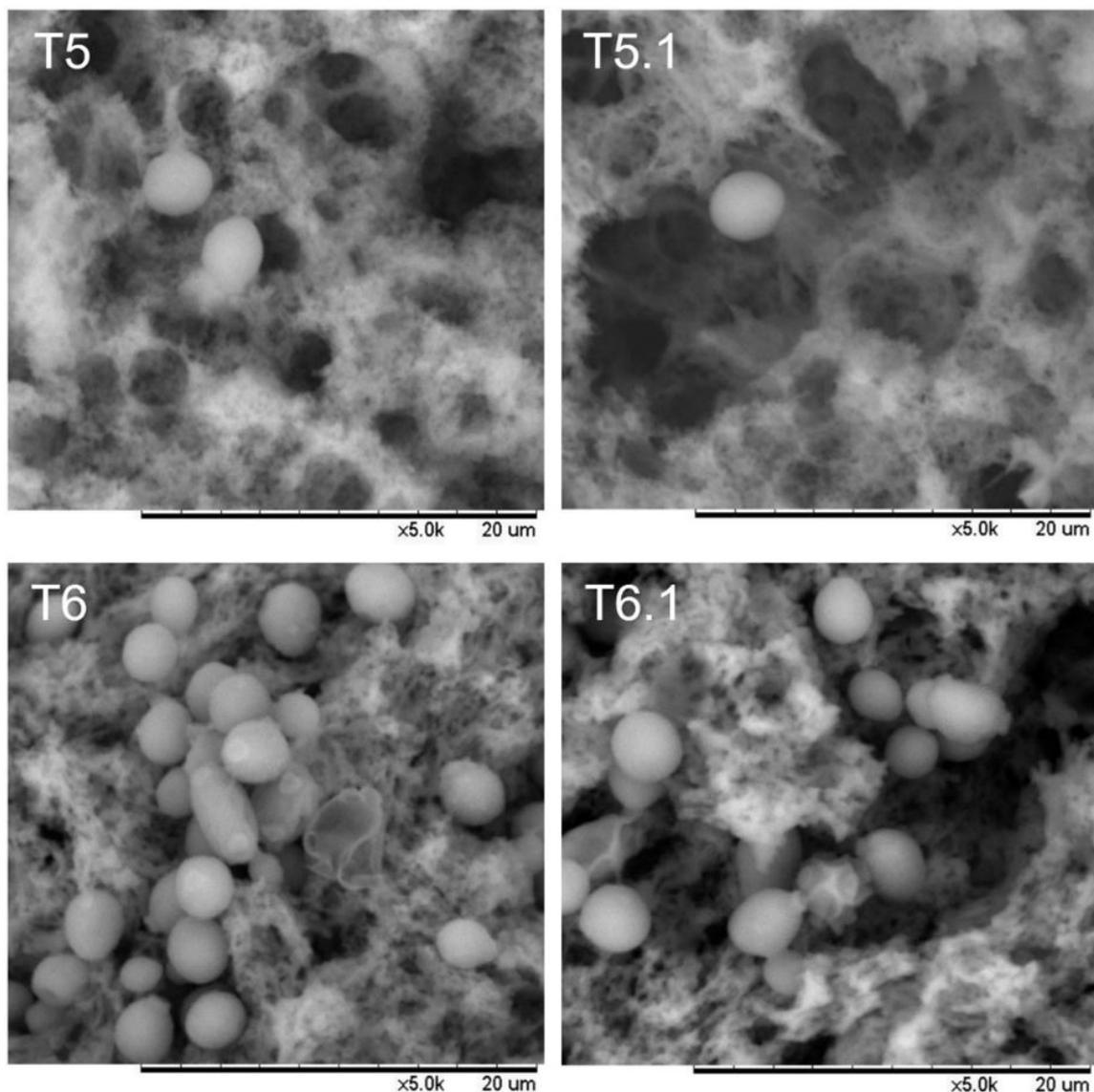
Fritzen-Freire et al. (2010) and Madadlou et al. (2007) reported that during cheese storage, structural changes may occur due to moisture loss and biochemical changes, as well as due to changes in cheese properties. In addition, another factor that can contribute to the formation or changes in the microstructure of the cheese is the use of lactic acid bacteria, due to their ability to improve the body and texture of the cheese (Centeno et al., 2002; Merrill et al., 1996). Also, Abdelmotilib et al. (2018) observed that strains of *Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Bifidobacterium bifidum* when in contact with AFM<sub>1</sub> had spots on the cell wall after adsorption of the toxin.

The effects of the microorganisms used to reduce AFM<sub>1</sub> can be observed in the LAB treatment (T4), supported by the SEM images. Analyzes by HPLC showed that LAB has a high capacity to reduce AFM<sub>1</sub>. On day 2, a reduction of 82% was

observed (Table 2), followed by an increase in the percentage of reduction on day 30, reaching 94%.

In Figure 2 its possible comparing the images of treatments 5 and 6 (T5, T5.1, T6 and T6.1 on days 2 and 30, respectively). Differences were observed in the cheese matrix between days 2 and 30, both between T5, T5.1 and between T6 and T6.1, in both cases there was an increase in the pores of the network. Both in T6 and T6.1 it is possible to observe AFM<sub>1</sub> binding to the wall of *S. cerevisiae*, which, when the toxin is bound, changes its shape, becoming elongated or oval.

In agreement with our study, Hamad et al. (2017) reported that SEM analysis showed that the microorganism's surface is able to adsorb the toxin particles that appear as a small dot on the microorganism's cell walls. Abdelmotilib et al. (2018) reported in their study that non-viable strains of the yeasts



**Figure 2.** Scanning electron microscopy images of treatments 5 and 6 on days 2 and 30 after manufactured.

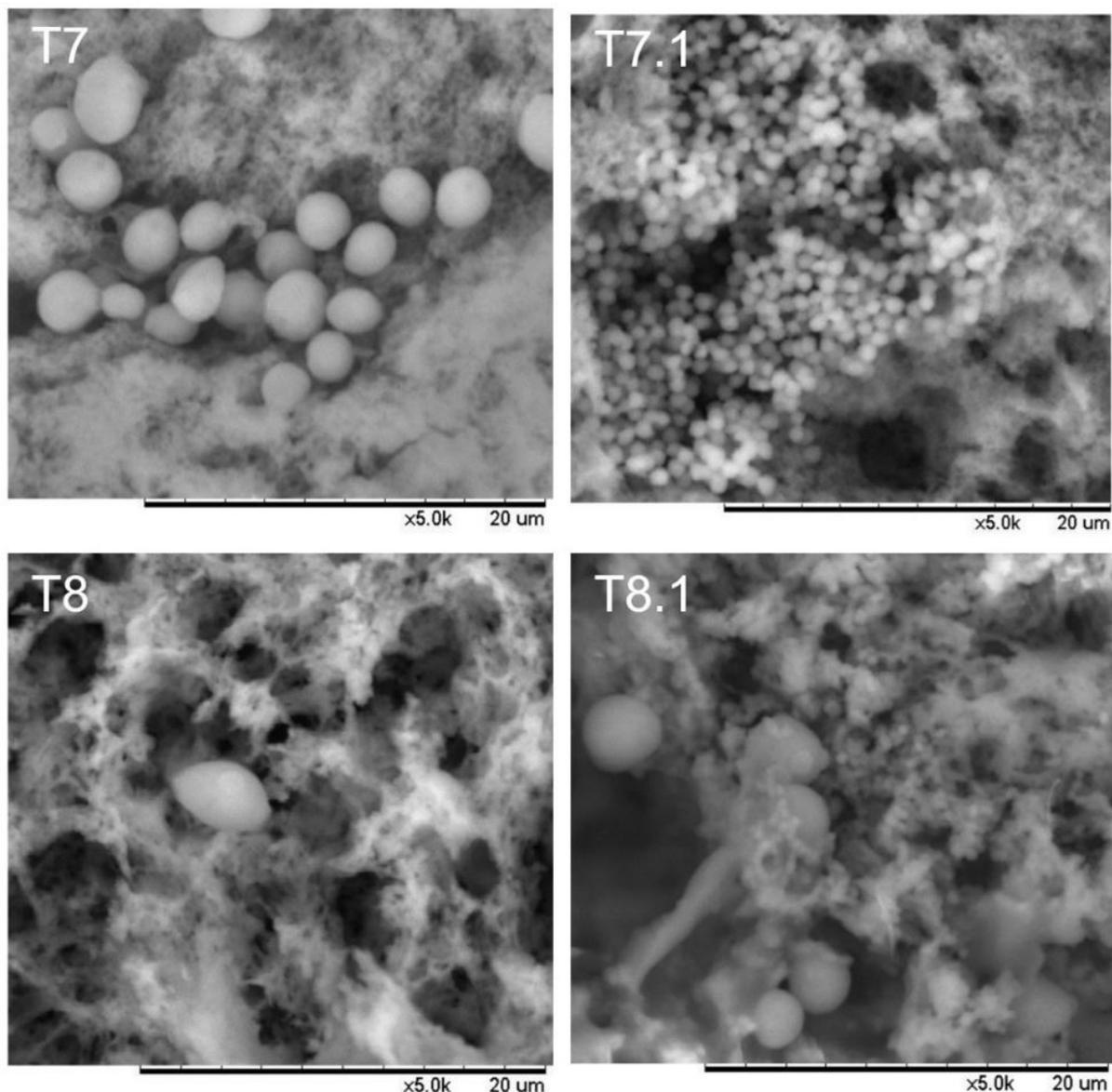
*Kluyveromyces lactis* and *S. cerevisiae* showed changes in the cell wall after adsorption of AFM<sub>1</sub>.

Supporting SEM images, HPLC analyzes showed *S. cerevisiae*, as a microorganism choice to reduce AFM<sub>1</sub>, once it presented a lower percentage of reduction than LAB initially, 74%, however, after 30 days it was more efficient, reaching a percentage reduction of AFM<sub>1</sub> of 100%.

Treatments 7 and 8 (T7, T7.1, T8 and T8.1 on days 2 and 30, respectively) are in Figure 3. Differences in the cheese matrix between treatments without AFM<sub>1</sub> (T7 and T7.1) and with AFM<sub>1</sub> (T8 and T8.1) and in the same treatment were observed between days, day 2 (T7 and T8) and day 30 (T7.1 and T8.1). When comparing T7 and T7.1 to T8 and T8.1, it is possible to observe that the first presents a cheese matrix with a more compact structure than the other. According to Fox et al. (2017) a less compacted microstructure may be the

result of demineralization (loss of calcium and phosphate in the casein micelle) that occurs due to the decrease in pH. A fact that may have occurred with Minas Frescal cheese over the course of thirty days, there may have been a decrease in pH due to the increase in lactic acid. Fritzen-Freire et al. (2010) reported that after 28 days, the Minas Frescal cheese with a higher lactic acid content showed a less compacted structure and less deformations.

Regarding AFM<sub>1</sub> binding to the yeast, when comparing T7 and T7.1 with T8 and T8.1 in Figure 3, it is possible to observe the stains on the cell wall of *S. cerevisiae* in T8 and T8.1. These spots indicate that AFM<sub>1</sub> is adsorbed to the yeast cell wall. In agreement with our study, Abdelmotilib et al. (2018) and Hamad et al. (2017) reported that the surface of the microorganism is able to adsorb AFM<sub>1</sub> particles that appear as small dots or spots on the cell wall of microorganisms. Supporting SEM images, the result



**Figure 3.** Scanning electron microscopy images of treatments 7 and 8 on days 2 and 30 after manufactured.

obtained through HPLC showed that on day 2, the treatment that received LAB and *S. cerevisiae* combined showed the same percentages of reduction as the treatment that received only *S. cerevisiae*. On day 2, a reduction of AFM<sub>1</sub> of 74% was observed and on day 30, 100%.

#### 4 Conclusions

The use of microorganisms as a biological method to reduce AFM<sub>1</sub> is efficient in Minas Frescal cheese and does not affect the microbiological parameters of the product. The reduction in AFM<sub>1</sub> varied according to the microorganism used in the treatments. *Saccharomyces cerevisiae* showed greater capacity to bind AFM<sub>1</sub> over time, compared to LABs. Scanning electron microscopy was especially useful, confirming that both lactic acid bacteria and *S. cerevisiae* were able to bind AFM<sub>1</sub> particles in Minas Frescal cheese. In addition, it allowed visualizing the changes that occurred in the cheese protein matrix over time and according to the different treatments.

#### Acknowledgements

The authors would like to thank: São Paulo Research of Foundation, FAPESP [Grants #2017/20081-6 and #2017/19683-1], Brazilian National Council for Scientific and Technological Development, CNPq [Grant #302195/2018-1], and this study was financed in part by the Coordenação de Aperfeiçoamento Pessoal de Nível Superior - Brasil (CAPES) – Finance Code 001.

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