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The Ability of *Lactobacillus rhamnosus* in Solution, Spray-Dried or Lyophilized to Bind Aflatoxin B_1

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Abstract

Aflatoxin B_1 (AFB_1) can cause carcinogenic, mutagenic, teratogenic and immunosuppressive effects in humans and animals. Several lactic acid bacteria species have the ability to bind AFB_1 *in vitro*, showing a potential application for reducing the bioavailability of AFB_1 in contaminated products. Thus, the aim of this study was to evaluate the capacity of *Lactobacillus rhamnosus*, non-viable and dried, in removing the AFB_1 from a contaminated medium. *L. rhamnosus* were cultured in MRS broth, sterilized (121 ºC, 15 min.) to inactivate their metabolism and then dried by spray-drying or freeze-drying (lyophilization). Binding assays using AFB_1 (1.0 µg/ml) and *L. rhamnosus* cells (1×10^{10} cells, in suspension or spray-dried or freeze-dried) were conducted at pH 3.0 and 6.0, room temperature and contact time of 60 min. Quantification of AFB_1 was achieved by high performance liquid chromatography. Scanning electron microscope was also performed in order to analyze the drying effect on the atomized and lyophilized *L. rhamnosus* cells. For pH 3.0 and 6.0, there were no significant differences between AFB_1 binding efficiency by *L. rhamnosus* cells in solution (45.9 ± 8.8% and 35.8 ± 7.7%, respectively) or freeze-dried (36.6 ± 7.1% and 27.2 ± 4.0%, respectively). However, the spray-dried cells lost completely the AFB_1 binding capacity during atomization, which damaged the structural and functional properties of the bacterial cell wall. In conclusion, *L. rhamnosus* retained its AFB_1 binding ability only when its cell wall remained intact as observed in the lyophilization procedure. Lyophilized *L. rhamnosus* cells therefore can be a practicable alternative for decontamination of food products susceptible to aflatoxin contamination.

Keywords: lactic acid bacteria, binding, aflatoxin B_1, freeze-drying, atomization

1. Introduction

Aflatoxins, produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*, are distinguished by their wide distribution in food and feed and pronounced toxic properties (Alberts, Engelbrecht, Steyn, Holzapfel, & van Zyl, 2006). Their carcinogenic, mutagenic, teratogenic and immunosuppressive effects are well documented in humans and animals, causing health and economic problems (Hernandez-Mendoza, Garcia, & Steele, 2009). Aflatoxin B_1 (AFB_1), the most prevalent and toxic of all aflatoxins, is classified by the International Agency for Research on Cancer [IARC] (2002) as belonging to Group 1, carcinogenic to humans.

In view of the high toxicity of aflatoxins, there is an increasing research interest on strategies to prevent their formation in food as well as to eliminate, inactivate or reduce their bioavailability in contaminated products (Hernandez-Mendoza et al., 2009). The use of microorganisms for control or elimination of aflatoxins is a very attractive alternative, and several studies have shown that both viable and non-viable cells of lactic acid bacteria (LAB) have a great capacity to bind aflatoxins (Bovo, Corassin, Rosim, & Oliveira, 2012; El-Nezami, Kankaanpää, Salminen, & Ahokas, 1998; Haskard, El-Nezami, Kankaanpää, Salminen, & Ahokas, 2001; Pierides, El-Nezami, Peltonem, Salminen, & Ahokas, 2000; Shetty & Jespersen, 2006). According to Lahtinen, Haskard, Ouwehand, Salminen, and Ahokas (2004), bacterial cell wall components, especially polysaccharides and peptidoglycans, are responsible for a physical union with aflatoxins instead of covalent bonding or degradation by microbial metabolism. However, mostly of the studies on aflatoxin binding used LAB cells in solution, which makes it difficult to access their applicability in food and feed products. A possible solution to this problem would be the drying of LAB cells and their application as food additives to reduce the exposure level to aflatoxins, without compromising the final product characteristics. In this context, the objective of the

present study was to determine the ability of LAB strain, *Lactobacillus rhamnosus*, nonviable and dried by spray-drying or lyophilization, in removing the AFB_1 from a contaminated medium.

2. Materials and Methods

2.1 L. rhamnosus and Culture Conditions

The LAB strain used in this study was *L. rhamnosus* HOWARU® LYO 40 DCU, kindly donated by Danisco Brazil Ltda. The lyophilized strain was reactivated in MRS broth (de Man, Rogosa, & Sharpe - Acumedia®, Lansing, MI, USA) at 37 ºC for 24 hours and grown under these conditions until achieving a high concentration of cells ($>10^9$ cells/ml). Estimation of bacterial concentration was determined by a turbidmetric method (Begot, Desnier, Daudin, Labadie, & Lebert, 1996). Bacterial concentration curve was constructed using absorbance measured at 600 nm (spectrophotometer Spectrumlab 22PC - Shanghai Lengguang Technology Co. Ltd, Shanghai, China) and the logarithm of bacterial concentration was obtained by dilution and pour plate counting (Wehr & Frank, 2004) after incubation in MRS agar (Acumedia®, Lansing, MI, USA) at 37 ºC for 48 hours under anaerobic conditions. From these data, it was generated an equation to calculate bacterial concentration in the medium, which has adapted perfectly to data as its coefficient of determination (R^2) was 0.999.

2.2 Drying of L. rhamnosus Broth by Spray-Drying and Lyophilization

Before spray-drying and lyophilization (freeze-drying) processes, *L. rhamnosus* cells in MRS broth were sterilized (121 ºC, 15 minutes) to inactivate their metabolism, since even under this condition, cells are capable of binding the AFB_1 in a contaminated medium. Spray-drying was performed using a MSD 1.0 Spray Dryer (LabMaq, Ribeirão Preto, SP, Brazil) with the following operating parameters: inlet and outlet air temperature in the drying chamber of 160 ºC and 105 ºC, respectively; inlet air velocity of 2.5 m/s; bacterial broth feeding volume of 10 ml/minute; and air flow of 30 l/min. Maltodextrin (MOR RER® 1910 - Corn Products Brazil, Mogi-Guaçú, SP, Brazil) was added in a rate of 15% to help the drying process since the broth containing the bacterial cells has an extremely low percent of solids. The determination of the process yield took into account the number of bacterial cells in the medium, the volume of medium prior to drying, losses during the process and the final weight after drying, including the added maltodextrin.

The freeze-drying process started with centrifugation (Excelsa II 206 BL - Fanem, São Paulo, Brazil) of bacterial broth containing the inactive *L. rhamnosus* cells at 1500 × g for 5 minutes. The supernatant was completely removed and the bacterial pellets were washed with ultra-pure water (Simplicity 185 – Millipore®, Billerica, MA, EUA). An additional centrifugation under the same conditions was made to remove the excess of water resulting in sediment containing the concentrated bacterial cells, which was frozen at -18 ºC for 24 hours. After, the sediment was placed into the lyophilizer (LC 1500 - Terroni Equipment Ltda., São Carlos, SP, Brazil) for 24 hours under vacuum (DV-142N vacuum pump - JB Platinum, Aurora, IL, USA) and gradual temperature increase (initial and final temperature of -5.0 ºC and 19.5 ºC respectively). The determination of process yield took into account the same parameters used for spray-drying process, except for the addition of maltodextrin.

2.3 AFB_1 Binding Assay With L. rhamnosus Cells

AFB_1 standard (Supelco™, Bellefonte, PA, USA) was dissolved in toluene and acetonitrile (9:1) and calibrated in spectrophotometer (Spectrumlab 22PC – Shanghai Lengguang Technology Co. Ltd, Shanghai, China) according to Scott (1990), in order to obtain a 10.0 µg AFB_1/ml stock solution. A 1.0 µg AFB_1/ml working solution was prepared in citrate-phosphate buffer solution (pH 3.0 and pH 6.0) using a combination of solutions of 0.1 M citric acid (Synth, Diadema, SP, Brazil) and 0.2 M di-basic sodium phosphate (Süd Chemie, Jacareí, SP, Brazil). The solvent was completely evaporated by direct air injection in a heating bath (TE-019 –Tecnal, Piracicaba, SP, Brazil) at 40 ºC.

The AFB_1 binding assays were conducted using *L. rhamnosus* cells inactivated by sterilization in suspension (before undergoing the drying process) and with the dryed cells (spray-dried or lyophilized). For the *L. rhamnosus* suspension assays, the method described by El-Nezami et al. (1998) was used with some adaptations. Aliquots of the culture broth containing 1×10^{10} cells were transferred to test tubes and centrifuged (CT-14 000 - Cientec, Piracicaba, SP, Brazil) at 1800 g for 5 min. The bacterial pellets were washed with sterile ultra-pure water, and re-suspended in 1.5 ml of buffer solutions (pH 3.0 and 6.0) containing AFB_1 and incubated under agitation of 180 rpm (TE-140 - Tecnal, Piracicaba, SP, Brazil) for 60 min. at room temperature.

The procedures using spray and freeze-dried *L. rhamnosus* cells were performed according to the method described by Ledoux and Rottinghaus (1999), designed for the efficiency evaluation of adsorbents to bind AFB_1 in a contaminated medium. For both pHs 3.0 and 6.0, an amount of dried mass containing 1×10^{10} cells was suspended in 5 ml of buffer solution with AFB_1 and also incubated under agitation of 180 rpm for 60 min. at

room temperature.

Following incubation in the assays with *L. rhamnosus* cells in suspension or dried, the solutions were centrifuged at 1800 g for 5 min., and the supernatant was analyzed for quantification of AFB_1 by high performance liquid chromatography (HPLC). Analyses were performed in triplicate and, for each sample, negative (*L. rhamnosus* cells suspended in buffer solution) and positive (AFB_1 in buffer solution) controls were incubated and analyzed.

2.4 Scanning Electron Microscopy

Samples were observed in a scanning electron microscope (SEM), for a better understanding of the effect of the drying process on the atomized and lyophilized *L. rhamnosus* cells, as well as to evaluate the cell surface structure. A small amount of each *L. rhamnosus* cell dried sample was deposited on a carbon conductive tape double coated 8 mm (Ted Pella, Inc., Redding, CA, USA) and the observation was made in a TM 3000 Tabletop Microscope (Hitachi High-Technologies Corporation, Tokyo, Japan) with an increase of 3000 times.

2.5 Quantification of AFB_1 by HPLC

AFB_1 quantification in the buffer solutions was achieved by injection into a HPLC system equipped with a fluorescence detector RF-10A XL and an autosampler SIL-10AF (Shimadzu®, Tokyo, Japan), and an ODS column 5 μm 4.6 × 150 mm (Phenomenex®, Torrance, USA). A flow rate of 1.0 ml/min was used with a mobile phase containing water, acetonitrile and methanol (60:20:20). Excitation and emission detection were set at a wavelength of 360 nm and 440 nm, respectively. The limit of detection (LOD) for AFB_1 was 0.01 ng/ml, and its retention time was 10.5 minutes with a retention window of ± 10%. The equation 1 was used for quantification of AFB_1, where A represents the percentage of AFB_1 bound by the sample, and B, C and D are the areas of chromatographic peaks of positive control, sample and negative control, respectively.

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

2.6 Statistical Analysis

The results were subjected to ANOVA, in accordance with procedures established in the General Linear Model of SAS Institute (1992) by using Fisher LSD test for significant differences between means at $P<0.05$.

3. Results

The results of AFB_1 binding capacity by *L. rhamnosus* are shown in Table 1. For both pHs, there were no significant differences ($P<0.05$) between the bacterial cells in MRS broth and freeze-dried cells. However, the spray-dried cells lost completely the AFB_1 binding capacity at pH 3.0 and decreased to 2.6% at pH 6.0, whereas lyophilized *L. rhamnosus* still preserved the ability to bind AFB_1 from the medium. There were no significant differences ($P<0.05$) between pH 3.0 and 6.0 for any of bacterial cells conditions (in solution, spray or freeze-dried).

Table 1. AFB_1 binding capacity of *Lactobacillus rhamnosus* cells[1]

Cell conditions	AFB_1 binding capacity (%)[2]	
	pH 3.0	pH 6.0
In suspension (MRS broth)	45.9 ± 8.8 [a]	35.8 ± 7.7 [a]
Spray-dried	0.0 ± 0.0 [b]	2.6 ± 1.5 [b]
Freeze-dried (lyophilized)	36.6 ± 7.1 [a]	27.2 ± 4.0 [a]

[1] Values expressed as mean ± standard deviation of samples analyzed in triplicate.

[2] Percentage of AFB_1 bound in a buffer solution containing 1.0 μg AFB_1/ml.

[a-b] In the same column, means followed by different letters differ significantly ($P<0.05$).

Figure 1 shows the images obtained by SEM for *L. rhamnosus* cells dried by spray-drying and lyphilization. In the samples of spray-dried cells viewed at a magnification of 3000× (Figure 1A), only the molecules of maltodextrin added in order to facilitate the drying process and some components of the MRS broth could be seen, but not the bacterial cells. The absence of intact cells in the spray-dried samples is a consequence of their destruction during the atomization process, probably because of the high temperature used. In the lyophilized

samples (Figure 1B), the *L. rhamnosus* cells remained intact, hence indicating that the lyophilization is milder, conservative process to preserve the bacterial cells and its ability to bind to aflatoxins.

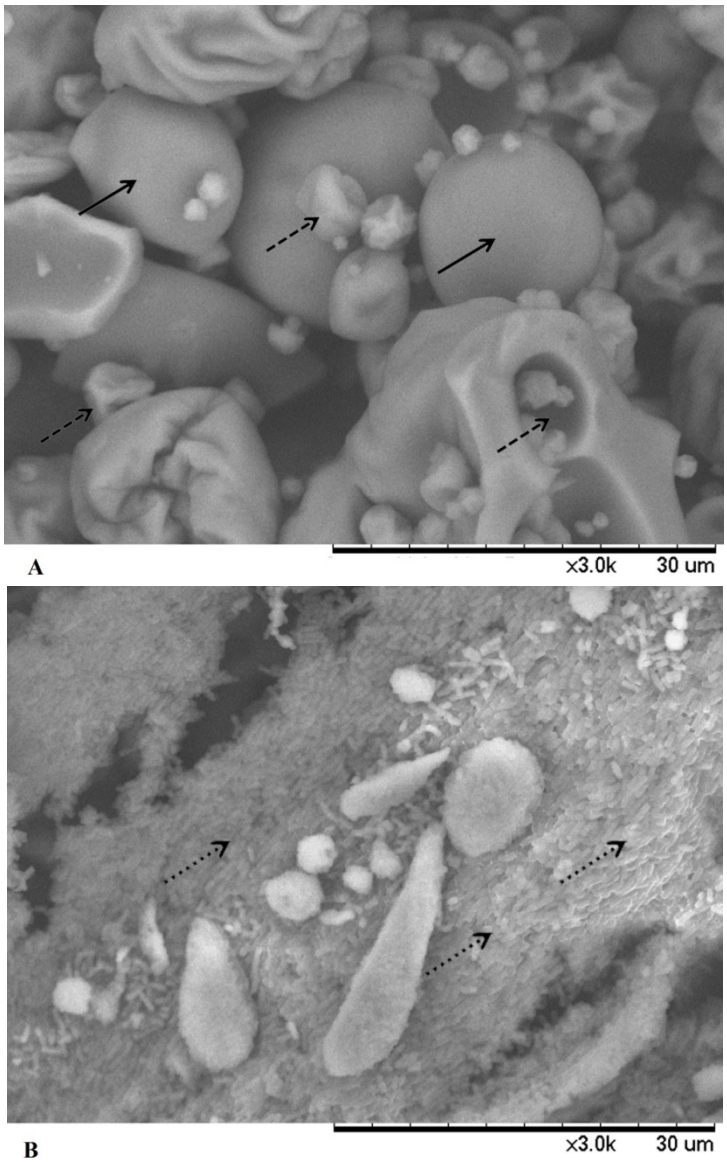

Figure 1. Scanning electron microscopy of *Lactobacillus rhamnosus* dried by spray-drying (A) and lyophilization (B), seen at a magnification of 3000×. In the spray-dried bacteria (A), it is not possible to see the bacterial cells, probably because of their destruction due to the high temperature applied during the atomization process; only the maltodextrin molecules (solid arrows) and residual components of MRS broth (dashed arrows) could be observed. In the lyophilized bacteria (B), in which maltodextrin was not added, it is possible to see the bacteria cells as clusters of rods (dotted arrows)

4. Discussion

The results expressed in Table 1 showed that autoclaved cells in MRS broth had the best AFB_1 binding capacity compared to the autoclaved and dried cells, although freeze-dried cells did not differ from the cells in solution. In the present work, non-viable bacterial cells were chose for aflatoxin decontamination because viable cells probably would ferment the medium and this is not desirable in some susceptible food products. Lahtinen et al. (2004) explained that, as non-viable cells can also bind the toxin, it is assumed that a physical union occurs with the cell wall components, particularly polysaccharides and peptidoglycans, instead of covalent bonding or degradation by microbial metabolism. Peltonen, El-Nezami, Haskard, Ahokas, & Salminen (2001) compared the

ability of viable and heat treated cells of Bifidobacteria to bind AFB_1 and noted that viable cells bound 4 to 56% of AFB_1 while non-viable cells bound 12 to 82%. Azab et al. (2005) evaluated the influence of inactivation treatments on the ability of 4 strains of *Lactobacillus* spp. to adsorb AFB_1 and observed that acid (58.6 to 87.0%) and heat (33.5 to 71.9%) treatments increased the binding capability when compared to the viable cells in buffer solution (16.3 to 56.6%), but alkaline (8.3 to 27.4%) and ethanolic (15.9 to 46.5%) treatments decreased the amount of adsorbed AFB_1.

It was also observed from the results of Table 1 that there were not significant differences between pH 3.0 and 6.0 for any of bacterial cells conditions. Haskard, Binnion, and Ahokas (2000) also did not observe changes in the amount of AFB_1 adsorbed by *L. rhamnosus* GG cells when the pH of the medium varied from 2.5 to 8.5. The stability of the LAB/aflatoxin complex in a wide range of pH is an important factor in the use of these microorganisms to remove aflatoxin from foods, once gastric release of the toxin would have negative health implications. Therefore, the complex formed has to resist environmental stress caused by the gastrointestinal tract, such as low pH and presence of bile (Oliveira, Bovo, Corassin, Jager, & Reddy, 2013).

One of the hypotheses for the total loss of AFB_1 binding capacity by spray-dried *L. rhamnosus* is the severe damage to the bacteria cell wall caused by high temperature during the atomization process, as the inlet and outlet temperatures were 160 ºC and 105 ºC, respectively. It is known that spray-drying significantly reduces cell viability, however in our case, at first it would not be a problem since bacterial cells were already non-viable prior to drying. According to Lievense and van't Riet (1994), it is generally assumed that there are four main primary sites for thermal damage that can cause injury or death. These sites are DNA, RNA (including ribosomes - rRNA), proteins (enzymes) and cell membrane. The authors explained that not a single factor, but probably a multiplicity of effects can cause cell injuries. Teixeira, Castro, Mohácsi-Farkas, & Kirby (1997) observed that during heating at temperatures greater than 65 ºC, ribosomes and/or proteins denaturation as well as cell wall damage may be responsible for irreversible thermal damage.

Corcoran, Ross, Fitzgerald, and Stanton (2004) concluded that cytoplasmic membrane is one of the most susceptible sites of cellular injury during spray-drying, and thermal stress is not solely the responsible for it, dehydration, oxygen exposure and osmotic stress are also a factors that exacerbate cellular damages. According to Ananta, Volkert, and Knorr (2005), the water in the cells contributes to cell stability and its removal can cause irreversible changes in the structural and functional integrity of the bacterial membrane. The same authors also observed that the higher the temperature during spray-drying, the greater the damage to the cell membrane. Additionally, Peighambardoust, Tafti, and Hesari (2011) stated that the cells are in contact with a large volume of air during atomization, which consequently causes lipid oxidation in the membrane. Hernandez-Mendoza et al. (2009) explained that the integrity of bacterial cell wall is important in the aflatoxin binding process by viable and nonviable cells. In their study with AFB_1, they verified that the bacterial cell wall and its purified fragments were able to bind AFB_1 from the medium, however when occurred the loss or destruction of bacteria cell wall (total or partial) in response to enzymatic treatments, a significant decrease in the binding capacity was observed.

Similar studies using the non-viable dried bacterial cells for AFB_1 binding were not found, as the vast majority of these works acquired the bacteria in a viable and lyophilized form, which was reactivated and then inactivated by thermal, acid, enzymatic or others treatments, maintaining the bacteria in solution without undergoing the drying process. Spray-drying was chosen because is a relatively inexpensive process and widely used in the food industry. In addition, the commercialization of dried bacteria would facilitate the transportation and storage conditions, and could be added directly in food products through two distinct ways. The first way, applied to liquid products, dried bacterial cells would be added for a period of time, the toxin binding would occur without damaging the product or cause fermentation and, then, the cells would be removed from the medium resulting in a total or partially decontaminated product. The second way would be applied for solid products, and dried bacterial cells would be used as a food additive and aflatoxin binding process would occur within the gastrointestinal tract.

Unlike the spray-dried bacteria, lyophilized (freeze-dried) cells retained the capacity for binding of AFB_1, as they did not differ significantly from bacterial cells in solution. To perform a lyophilization process, firstly it is necessary to freeze the samples, and then dry them by water sublimation. During freezing, extra-cellular ice crystals are formed and increase the extra-cellular osmolality, so as soon as ice forms outside of the cell in solution, the cell dehydration begins. As temperature drops, the intracellular and extra-cellular solution concentrations will increase until a eutectic point is reached (Meng, Stanton, Fitzgerald, Daly, & Ross, 2008). The ice crystals can be too large and eventually damage the cell structure, especially the bacterial cell wall (Morgan, Herman, White, & Vesey, 2006). Furthermore, according to Carvalho et al. (2004), the lyophilization (the drying phase itself) of bacterial cultures can have consequences as damage to the cell wall, cell membrane

and DNA, besides membrane lipid oxidation. Although freeze-dried bacteria did not differ significantly from bacterial cells in solution, all these factors can be considered as causes of a lower AFB_1 binding capacity by freeze-dried *L. rhamnosus*. El-Nezami et al. (1998) observed that pre-cultured cells of *L. rhamnosus* strains GG and LC705 removed significantly more AFB_1 than freeze-dried cells, although the most efficient removal of AFB_1 was achieved by heat killed bacteria. The authors speculated that the differences between pre-cultured and freeze-dried bacteria could be related to pre-culturing changes in the cell wall components, which could enhance the ability of the bacteria to remove more AFB_1.

Although freeze-drying can maintain the ability of *L. rhamnosus* in binding the AFB_1, this process is much more expensive than spray-drying. According to Peighambardoust et al. (2011), fixed costs of operating a spray-dryer are equivalent to 12% of the costs of operating a lyophilizer. However, the most significant negative factor is that freeze-drying had an extremely low yield, since to achieve approximately 1.0 g of lyophilized product it was required about 1000 ml of MRS broth containing bacterial cells, although the resulting cellular concentration was extremely high (8.2×10^{11} cells/g, dry weight).

The high concentration of freeze-dried *L. rhamnosus* cells could be better observed through the image produced by SEM (Figure 1B). In contrast, it was not possible to visualize the spray-dried bacterial cells (Figure 1A), probably due to their destruction during the drying process and the presence of maltodextrin. This emphasizes the importance of integrity of bacterial cell, because they can be inactivated (dead), but after the drying process they have to be intact in order to bind the aflatoxin. Thus, alternatives to spray-drying bacterial cells in an attempt to cause less damage to bacterial cell wall would be the use of lower temperatures during the drying process or the use of protective agents such as sorbitol, skim milk, whey, trehalose, glycerol, betaine, sucrose, glucose, lactose and polymers such as dextran and polyethyleneglycol (Hubálek, 2003). Logically, the performance assessment of the protective agent during spray-drying would be required, besides checking for possible interference in the aflatoxin binding process.

5. Conclusion

It was clearly shown by this study that *L. rhamnosus* retained its AFB_1 binding ability only when its cell wall was intact, as occurred with the bacteria in solution and freeze-dried bacteria. The high temperatures applied during the spray-drying damaged the structural and functional maintenance of bacterial cells causing the loss of their capacity to bind aflatoxins. Lyophilized *L. rhamnosus* cells therefore can be a practicable alternative for decontamination of food products susceptible to aflatoxin contamination.

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