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Aflatoxin B_1 adsorption/desorption dynamics in the presence of *Lactobacillus rhamnosus* RC007 in a gastrointestinal tract simulated model

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Running head: **AFB_1 adsorption/desorption under simulated model of gastrointestinal tract**

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Abstract

Aims: 1) to determine the aflatoxin B_1 (AFB_1) adsorption and desorption dynamics in the presence of *L. rhamnosus* RC007 under simulated transit of AFB_1 at each gastrointestinal tract (GIT - saliva, stomach and intestine) stage consecutively and then, separately, 2) to study the ability of *L. rhamnosus* RC007 to biotransform AFB_1 as a strategy that complements the adsorption process.

Methods and results: the AFB_1 adsorption and desorption assay simulating the GIT passage of AFB_1 (93.89 ng/g) in presence of *L. rhamnosus* RC007 (10^8 CFU/mL) was conducted. Moreover, lactic acid production was determined. Results demonstrated that predominant environmental conditions in salivary solution (SS) induced a low AFB_1 adsorption, while the transit through the gastric solution (GS) and intestinal solution (IS) allowed high percentages of adsorption and did not generate significant AFB_1 desorption.

Conclusions: The AFB_1 adsorption and desorption dynamics in the presence of *L. rhamnosus* RC007 was favoured by gastric and intestinal environment. **Significance and impact of study:** the knowledge of the adsorption dynamics of AFB_1 with a microorganism of interest will allow predicting its behavior at each stage of the GIT.

Key words: adsorption/desorption dynamics, aflatoxin B_1, degradation, gastrointestinal tract, *Lactobacillus rhamnosus*.

Introduction

Mycotoxins are toxic secondary metabolites produced by some species of fungi that exert deleterious effects in animals and humans. Not all fungi are capable of producing mycotoxins and those who produce them, do it under specific environmental conditions (CAST 2003). Major agricultural important mycotoxins are produced mainly by three genera of fungi: *Aspergillus*, *Fusarium* and *Penicillium* and although there are other genera, only the mycotoxins produced by these three genera have been studied in depth (Magan and Aldred

2007). Mycotoxin contamination affects overall livestock and cereal sectors as well as in the human health (Pinotti *et al.* 2016). It reduces the efficiency of growth, decreases food conversion and reproduction rates, harms the resistance to infectious diseases, reduces the vaccination effectiveness, and induces pathological damage to the liver and other organs. Each toxin has features and specific effects, and the occurrence of two or more mycotoxins, as normally found in nature, may cause more adverse effects than the presence of a single one (CAST 2003).

Pre and post-harvest strategies to reduce mycotoxins contamination in food and raw materials have been proposed; however, once mycotoxins have reached to contaminate an ingredient or a food, it is extremely difficult to achieve the total elimination. FAO (2006) defines probiotics as "viable microorganisms which, when administered in adequate amounts, confer benefits on the host health". Among great potential probiotic microorganisms are included some species of Lactobacilli that have been widely used in the food additives production being recognized as safe (GRAS). They also have a high multiplication rate, are able to grow in a wide variety of carbon sources and do not produce toxic compounds. The union of probiotics to mycotoxins reduces their availability and, consequently, their absorption in the gastrointestinal tract (GIT). It has been demonstrated in vitro the adsorption capacity of major mycotoxins such as aflatoxin B_1 (AFB_1) exerted by probiotic *Saccharomyces cerevisiae* (Armando *et al.* 2011, 2012) and probiotic *Pediococcus acidolactici and P. pentosaceus* (Martínez *et al.* 2017). The use of these microorganisms at commercial level requires a design of the culture medium, one of the most important tasks within the biological technology, evaluating the use of economic substrates for the biomass production at large-scale, allowing also the conservation of the biological properties previously demonstrated in the laboratory.

Previous studies works allowed selecting lactic acid bacteria (LAB) from maize silage, such as probiotic *Lactobacillus rhamnosus* RC007 capable of inhibiting the growth of *Fusarium graminearum* and *Aspergillus carbonarius* (Dogi *et al.* 2013). This strain did not possess genes for veterinary importance antibiotics and showed the interaction with the

intestinal microbiota by modulating the immune system of the host, increasing its resistance against pathogens, or by stimulating the immune system of mice and pigs through the release of anti-inflammatory cytokines (Dogi *et al.* 2016, Garcia *et al*. 2018).

Studies have demonstrated the ability of LAB to adsorb mycotoxins; however, none of them have determined in detail the AFB_1 adsorption and desorption dynamics and how it is occurring at the different stages of the simulated GIT (Lahtinen *et al.*, 2004; Niderkorn *et al.*, 2006). The present work aims to 1) determine the AFB_1 adsorption and desorption dynamics simulating the passage of AFB_1 at each stage of the gastrointestinal tract (GIT - saliva, stomach and gut) consecutively, in the presence of *L. rhamnosus* RC007 and then, considering the AFB_1 adsorption and desorption behaviour at each of the simulated stages, independently, 2) to study the ability of *L. rhamnosus* RC007 to biotransform AFB_1 as a strategy that complements the adsorption process.

Materials and methods

Microorganism, growth medium and cultural conditions

Lactobacillus rhamnosus RC007 isolated from maize silage was obtained from the collection centre at the National University of Rio Cuarto, Argentina. Stock cultures were maintained at -80°C in15% (v v^{-1}) glycerol. The LAB strain was identified from both the fermentation pattern (API 50 CHL test) and the 16S rRNA gene sequence (Dogi *et al.* 2013). *Lactobacillus rhamnosus* RC007 was grown at 37°C for 24 h without agitation in Man, Rogosa and Sharpe (MRS) broth (Britania, Buenos Aires, Argentina).

Effect of simulated gastrointestinal conditions on aflatoxin B_1 binding

To conduct the AFB_1 binding assay, inoculations were made from the working cultures into each artificial solution simulating the GIT. After being incubated, cells were collected by centrifugation (5000 g 10 min) and washed twice with phosphate-buffered saline (PBS, pH 7). The LAB pellet (1 x 10^8 cells ml^{-1}) concentration was determined by viable counts.

Aflatoxin B_1 solutions

From a solution of 7500 ng ml^{-1}, new AFB_1 solutions containing 93.89 ng ml^{-1} were prepared using artificial salivary solution (SS), simulated gastric juice (GS) or artificial intestinal fluid (IS), separately. Positive (AFB_1 suspended in the adequate simulated condition) and negative (non - AFB_1 inoculated simulated condition) controls were included in all the experiments. The assays were conducted in triplicate.

Simulated gastrointestinal tract solutions composition

Simulated GIT solutions were prepared as follows:

SS: lisozime 2 mg ml^{-1} (Sigma 47700 U/mg) in saline solution pH 6.5;

GS: NaCl 125 mmol, KCl 7 mmol, $NaHCO_3$ 45 mmol, pepsin 3 g l^{-1} adjusted to pH 3 with HCl; IS: trypsin 1 mg ml^{-1} (Fluka 11531 U/mg), chymiotrypsin 1 mg ml^{-1} (Fluka 80 U/mg), oxgall bile salts 0.3 % (w v^{-1}) adjusted to pH 8 with NaOH 5 mmol l^{-1}.

Aflatoxin B_1 adsorption and desorption assay simulating the gastrointestinal tract passage

Simulated GIT passage was performed according to Armando *et al.* (2011) with some modifications. The assays were conducted by two different ways:

1- A complete simulated GIT passage (the SS, GS and IS consecutively) was simulated in order to know the dynamics of the adsorption process, LAB cells were resuspended in SS (1 ml) containing AFB_1 (93.89 ng ml^{-1}) and incubated at 37°C for 5 min under agitation in an orbital shaker (100 rpm) to simulate the mastication process. After centrifugation, the same cells were added to GS (1 ml) and incubated at 37°C for 45 min under agitation in an orbital shaker (50 rpm) simulating the peristalsis process. After centrifugation, the same cells were added to IS (1 ml) and incubated at 37°C for 30 min under agitation in an orbital shaker (100 rpm). After that, LAB cells were centrifuged at 5000 g for 15 min, and the supernatant containing unbound mycotoxin was collected and stored at -20°C until HPLC analysis.

2- Each GIT stage (SS, GS and IS) was simulated separately. So, each stage had AFB_1 (93.89 ng ml^{-1}) in presence of *L. rhamnosus* RC007 (10^8 CFU ml^{-1}) and was conducted as follows:

- LAB cells ($1x10^8$ cells ml^{-1}) were resuspended in SS containing AFB_1 (93.89 ng ml^{-1}) and incubated at 37°C for 5 min under agitation in an orbital shaker (100 rpm) to simulate the mastication process. After centrifugation, the supernatant containing unbound mycotoxin was collected and stored at -20°C.

- LAB cells ($1x10^8$ cells ml^{-1}) were resuspended in GS containing AFB_1 (93.89 ng ml^{-1}) and incubated at 37°C for 45 min under agitation in an orbital shaker (50 rpm) simulating the peristalsis process. After centrifugation, the supernatant containing unbound mycotoxin was collected and stored at -20°C.

- LAB cells ($1x10^8$ cells ml^{-1}) were resuspended in IS containing AFB_1 (93.89 ng ml^{-1}) and incubated at 37°C for 30 min under agitation in an orbital shaker (100 rpm). After that, cells were centrifuged at 5000 g for 15 min, and the supernatant containing unbound mycotoxin was collected and stored at -20°C. All supernatants from each GIT stage (salivary, gastric and intestinal conditions) were analysed by High-Performance Liquid Chromatography (HPLC).

Aflatoxin B_1 degradation assay by cell-free extracts

The toxin degradation technique was performed according to the methodology proposed by Teniola *et al.* (2005) with modifications. The experiment was run in 15 ml tubes; a cell-free extract from a 24 h culture of *L. rhamnosus* RC007 was placed and contaminated with a solution of AFB_1 (93.89 ng ml^{-1}). The mixture was incubated in dark at 37°C without stirring for 12, 24 and 48 h. Extraction with chloroform (1:1, v v^{-1}) of the remaining AFB_1 was then performed for further quantification by HPLC. All experiments were carried out in triplicate.

Detection and quantification of aflatoxin B_1

Aflatoxin B_1 was quantified by HPLC (Waters e2695; Waters, Milford, MA, USA) with fluorescence detection (Waters 2475 multi λ fluorescence detector) according to Trucksess *et al.* (1994) with some modifications. Chromatographic separations were performed on a reversed-phase C18 column (150 × 4.6 mm i.d., 5 µm particle size; Phenomenex, Luna, Torrance, CA, USA). The fluorescence of AFB_1 derivatives was recorded at excitation and emission wavelengths of 360 and 460 nm, respectively. The mobile phase (water:acetonitrile:methanol, 4:1:1) was pumped at 1.5 ml min^{-1}. The injection volume was 100 ul and the retention time was around 4 min. The detection limit of the technique was 1 ng g^{-1}.

Aflatoxin B_1 recovery assays

Aflatoxin B_1 recovery assays were carried out in triplicate in the different solutions simulating the GIT. The percentage (%) recovery was tabulated by taking the difference of the amount of AFB_1 spiked into each different solutions and the amount of AFB_1 recovered from the assay divided by the amount of AFB_1 spiked into each different solutions multiplied by 100%.

Determination of total viable Lactobacillus rhamnosus counts

Aliquots (100 µl) of *L. rhamnosus* cells prior to GI passage and after salivary, gastric and intestinal conditions were taken. Total viable counts of L. rhamnosus were determined by a plate method using MRS agar after serial 10-fold dilutions. Plates were incubated at 37°C for 24 h.

Statistical analyses

Means were analysed by analysis of variance (ANOVA) ($p<0.0001$). The variables that showed significant differences were compared using the Least Significant Fisher LSD

difference test ($p \leq 0.05$). The analysis was performed using Info Stat for Windows 2012 Version 2.03 (SPSS Inc.).

Results

Effect of simulated gastrointestinal conditions on aflatoxin B_1 binding

The ability of *L. rhamnosus* RC007 to adsorb and desorb AFB_1 in the GIT solutions was determined, simulating temperature, pH, peristaltic movements, as well as the enzymes and specific salts of each stage of the tract (saliva, stomach and intestine).

Table 1 shows the levels (ng) and percentages (%) of AFB_1 adsorption and desorption when the GIT of AFB_1 (93.89 ng) in presence of *L. rhamnosus* RC007 was simulated. The results were expressed as levels (ng) of AFB_1 present in SS or present in the same LAB pellet that transited through GS and IS. It is important to note here that cell-free supernatant solutions containing the non-adsorbed-mycotoxin by the LAB pellet were removed and did not continue the subsequent steps of the assay according to the employed methodology. In this way, the levels (ng) of AFB_1 (Table 1 - first column) present in the SS and those present in the LAB pellet were reported and subsequently passed to the other stages of the GIT, showing that in each one of them there was also a desorption process. Both, levels (ng) and percentages (%) of desorption at each GIT stage were considered taking into account the residual levels of AFB_1 as well as relative percentages (percentage of AFB_1 adsorbed by *L. rhamnosus* RC007 in relation with the AFB_1 added to SS or present in the *L. rhamnosus* RC007 pellet in GS and IS) and adsorbed AFB_1 total percentages (percentage of AFB_1 adsorbed by *L. rhamnosus* RC007 in relation to the total AFB_1 added at the start of the experiment in the SS).

The relative AFB_1 adsorption percentage in the passage through simulated SS showed a low adsorption percentage, as did the passage through the GS; while IS allowed to retain a high percentage of AFB_1. If the total percentage of adsorbed AFB_1 is observed, the IS showed the

highest retention. These results are directly related to the relative and total percentages of desorption that were between 81.20% and 96.11% at the different GIT stages.

In a subsequent trial, the LAB pellet was confronted with AFB_1 for each of the solutions that simulated the GIT separately (Table 2). Again, it was observed that when mycotoxin was in contact with SS, the percentage of AFB_1 adsorption was low. However, this did not occur in the GS or IS since the mycotoxin adsorption rates were significantly higher ($p \leq 0.0001$), as can be observed in the levels of adsorbed mycotoxin (ng – Table 2). These results showed that in both, GS and IS, the adsorption rates were higher than 88%. If the mycotoxin desorption percentages are observed, they are directly related to the adsorption percentages previously shown.

Aflatoxin B_1 degradation assay

Table 3 shows the AFB_1 degradation percentages by *L. rhamnosus* RC007 cell-free extracts. The strain showed a range between 74.43% and 80.40% of AFB_1 degradation between 12 and 48 h exposure, showing significant differences between 12 and 24 h in relation with 48 h where the best degradation observed.

Aflatoxin B_1 recovery assays

The overall % recoveries for salivary, gastric and intestinal solutions were 105.71%, 97.55% and 88.57%, respectively

Effect of simulated gastrointestinal tract on Lactobacillus rhamnosus RC007 viability

After carrying out the viable cell count, after incubation it was observed that all the strains kept the number of viable cells constant after the simulation to the gastrointestinal passage ($10^{7\text{-}8}$ CFU ml^{-1}), indicating in this way that they resist the stomach and intestinal conditions.

Discussion

There is a growing interest in the research of strategies to prevent the AFs production in food and feed, as well as to eliminate, inactivate or reduce their bioavailability in contaminated products (Hernandez-Mendoza *et al*. 2009). The use of microorganisms for AFs control or elimination is a very attractive alternative and several studies have shown that both viable and non-viable LAB cells have a great ability to bind to AFs (Bovo *et al.* 2014).

In this work, the results showed the adsorption and desorption dynamics that exist when the AFB_1 transits through the GIT different stages in presence of *L. rhamnosus* RC007. When AFB_1 transit the predominant environmental GIT conditions consecutively, the SS generated a low adsorption of the mycotoxin, while the transit through the other stages (GS and IS) allowed high percentages of adsorption and did not generate significant desorption of AFB_1 from the LAB pellet. When AFB_1 transit through GIT, simulating each of the stages, separately, the results verified again the low adsorption in the SS, while both in GS and in IS an adsorption greater than 88% was observed. Most of the studies carried out in the world with LAB as mycotoxin reducing agents have studied *L. plantarum* strains (Valero *et al.* 2004, Broberg *et al.* 2007, Laitilla *et al.* 2012). Different authors have reported the binding model of AFB_1 molecules to the surface of the bacterial cell wall suggesting two processes in consideration, the binding (adsorption) and the release (desorption) of the aflatoxin molecule to and from the binding site in the surface of microorganisms (Lee *et al.* 2003, Bueno *et al.* 2007). Using this model, it has been shown that the different capacities of the strains to trap AFB_1 were directly related to the number of binding sites present by each microorganism in the cell wall. The cell wall of one of the most studied LAB, *L. rhamnosus*, has polysaccharides where it binds aflatoxins. These polysaccharides occur in three main forms: cell wall polysaccharide, peptidoglycan and teichoic or lipoteichoic acids. The environmental conditions of the different portions, given by pH, enzymes, etc. would be influencing the three-dimensional structure of the bacterial cell wall and therefore the binding sites thereof to the aflatoxins. Haskard *et al.* (2000) showed that hydrophobic interactions were expected in acid treated bacteria where the protein denaturation treatment may expose

more hydrophobic binding areas to mycotoxins. Moreover, they emphasized that salts were expected to influence the bacterial surface charge and the results show that electrostatic interactions would have some effects on binding. Although in the present work the cells were subjected to different times according to each stage of the GIT, Bueno *et al.* (2007) stated that only 30 s would be enough to achieve maximum mycotoxin adsorption. In future studies, it would be interesting to perform these tests taking into account the adsorption and desorption process at the time permanence of food at each stage of the GIT, considering each animal species in which this probiotic additive adsorbent of AFB_1 would be used.

The search for an effective, specific, feasible and environmentally sound decontamination technology is a great demand. The biological detoxification of mycotoxins, using microorganisms and/or enzymes to degrade mycotoxins to less toxic or non-toxic compounds may be a choice of such technology. In this work, the cell-free extract from *L. rhamnosus* RC007 was tested in interaction with AFB_1 and high percentages of degradation were obtained, demonstrating the potential of this bacterium for its use as an antimycotoxin feed additive that complements the adsorption and degradation abilities of AFB_1. González Pereyra *et al.* (2018) evaluated soil-borne *Bacillus* spp. strains on aflatoxigenic *A. parasiticus* growth and AFs production and the culture supernatant of the most effective strain was studied. They found that six *Bacillus* spp. strains were able to reduce *A. parasiticus* growth rate significantly (p<0.05). Among them, *Bacillus* spp. RC1A inhibited fungal growth almost completely and could also reduce AFB_1 concentration produced by *A. parasiticus* (p<0.0001). The ability of some strains of LAB isolated from different sources to eliminate OTA, AFLA and ZEA has allowed recognizing their potential biotechnological application to reduce the health hazards associated with these mycotoxins. Cell-free supernatant of LAB isolated from vegetable (wine, olives, fruits and silage) and animal (milk and dairy products, sausages) sources were evaluated for their ability to eliminate mycotoxins. Among them, 10 strains isolated from milk were able to eliminate AFB_1, belonging to *L. casei*, *L. paracasei*, *L. plantarum* and one to *Leuconostoc mesenteroides*. Two strains of *Enterococcus faecium* and one *of E. faecalis* from sausage eliminated ZEA. Concerning to strains of vegetal origin,

one *L. plantarum* isolated from elderberry fruit, *one L. buchnerii* and one *L. parafarraginis* both isolated from silage eliminated ZEA. Other two strains *of L. plantarum* from silage were able to degrade both ZEA and OTA, and one *L. buchnerii* showed activity over AFB_1 (Inés *et al.* 2015). Based on the present results, future studies should be conducted to characterize the cell-free supernatant and demonstrate the presence of the compound responsible for such degradation.

The AFB_1 adsorption and desorption dynamics in the presence of *L. rhamnosus* RC007 was strongly affected by the salivary environment. The knowledge of the adsorption dynamics of AFB_1 with a microorganism of interest will allow to predict its behavior at each stage of the GIT, and future encapsulation strategies based on the GIT influence on mycotoxin adsorption could be designed. As the greatest desorption was observed in saliva, a portion of *L. rhamnosus* RC007, produced in a molasses containing medium, could be encapsulated to resist the saliva environment and allow its release in the stomach, while another portion should be designed using other encapsulation strategy to resist saliva and stomach environment for its final release in the intestine to exert an AFB_1 adsorption/degradation process. Latter, some candidate degradation products could be analysed (e.g. using HR-MS). In this way, *L. rhamnosus* RC007 is promissory for its use as an antimycotoxin feed additive that complements the AFB_1 adsorption/degradation ability.

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Conflict of interest

The authors have no conflict of interest to declare.

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Table 1. Adsorption and desorption of aflatoxin B_1 (AFB_1) in the presence of *Lactobacillus rhamnosus* RC007 (10^8 CFU ml^{-1}) through the different stages of the gastrointestinal tract consecutively (GIT).

GIT *	Aflatoxin B_1 adsorption				Aflatoxin B_1 desorption		
	Levels (ng ml^{-1})		Percentage (%)		Residual levels (ng ml^{-1}) ¶	Relative **	Total ††
	in solution or in pellet †	adsorbed	relative ‡	total §			
SS	93.89 ± 5.12	17.74 ± 3.88	18.8		76.15 ± 2.34	81.2	
GS	17.74 ± 3.88	4.43 ± 2.03	24.97	4.71	13.31 ± 1.85	75.03	95.28
IS	4.43 ± 2.03	3.65 ± 0.48	82.39	3.89	0.78 ± 0.03	17.61	96.11

* GIT: gastrointestinal tract. SS: salivary solution, GS: gastric solution, IS: intestinal solution.

† Aflatoxin B_1 present in the salivary solution at the start of the experience or present in the pellet in the gastric and the intestinal solutions (GS – IS).

‡ Percentage of aflatoxin B_1 adsorbed by *L. rhamnosus* RC007 in relation with the AFB_1 added to the salivary solution (SS) or present in the *L. rhamnosus* RC007 pellet (GS and IS).

§ Percentage of aflatoxin B_1 adsorbed by *L. rhamnosus* RC007 in relation with the total AFB_1 added at the start of the experiment in the salivary solution (SS).

¶ Residual amount of AFB_1 that is not adsorbed by *L. rhamnosus* RC007 and is removed in the supernatant at each stage of the GIT. This AFB_1 does not enter the next tract again.

** Percentage of AFB_1 desorbed by *L. rhamnosus* RC007 in relation with the AFB_1 added to the salivary solution (SS) or present in the *L. rhamnosus* RC007 pellet (GS ans IS).

†† Percentage of AFB_1 desorbed by *L. rhamnosus* RC007 in relation with the total AFB_1 added at the start of the experiment in the salivary solution (SS).

Table 2. Adsorption and desorption of aflatoxin B_1 (AFB_1) in the presence of *Lactobacillus rhamnosus* RC007 (10^8 CFU ml^{-1}) simulating each stage of the gastrointestinal tract, independently.

GIT *	AFB_1 (ng ml^{-1}) †	AFB_1 adsorption		AFB_1 desorption (%)
		(ng ml^{-1})	percentage (%)	
SS	93.89 ± 5.12	17.74 ± 3.88[a]	18.8	81.2
GS	93.89 ± 5.12	83.49 ± 5.02[b]	88.92	11.08
IS	93.89 ± 5.12	91.07 ± 5.28[b]	96.99	3.01

***** GIT: gastrointestinal tract. SS: salivary solution, GS: gastric solution, IS: intestinal solution.
† Aflatoxin B_1 present in the salivary, gastric and intestinal solutions.
Different letters mean significant differences ($p<0.05$) between the values according to Fisher´s LSD test.

Table 3. Aflatoxin B_1 degradation percentages by *Lactobacillus rhamnosus* RC007 cell-free extracts at different time of exposure.

Time (h)	Degradation percentage (%) (mean ± SD)
12	74.43 ± 0.68 a
24	75.87 ± 2.33 a
48	80.40 ± 3.10 b

SD: standard deviation. Values corresponding to the same letter are not significantly different according to Fisher´s protected LSD test ($p<0.05$).