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Aflatoxin B1 detoxification potential of viable and killed *Lactobacillus rhamnosus* by *in vitro* method#

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Abstract

Aflatoxin B1 (AFB1) is a potent mycotoxin produced by Aspergillus flavus and related fungi, posing severe health risks to humans and animals. This study evaluated the aflatoxin detoxification ability of viable and killed Lactobacillus rhamnosus using an in vitro binding assay. Five experimental groups were included: Treatment 1 contained viable L. rhamnosus with poultry layer feed adjusted to 30 ppb AFB1 by adding contaminated maize, while treatment 2 used killed L. rhamnosus under the same conditions. A positive control was maintained with 30 ppb AFB1 alone. Two negative controls were prepared using feed with naturally occurring AFB1 (concentration determined after preparation) and supplemented with either viable or killed L. rhamnosus. AFB1 levels were quantified at 3h and 6h post-treatment using ultra-high-performance liquid chromatography (UHPLC) following immunoaffinity column (IAC) cleanup. Statistical analysis was performed using RMANOVA and one-sample t-tests. The results demonstrated a significant reduction in AFB1 levels in the presence of both viable and killed L. rhamnosus. AFB1 reduction ranged from 24.4% to 28.2% in Treatment 1 and Treatment 2, while in the negative controls, the reduction was between 16.7% and 17.8%. These findings highlight the potential application of L. rhamnosus in aflatoxin detoxification strategies, particularly for improving feed safety in the poultry industry.

Keywords: *Aflatoxin B1, Lactobacillus rhamnosus, in vitro, UHPLC*

Aflatoxins are secondary metabolites predominantly produced by *Aspergillus* spp., particularly *A. flavus*, *A. parasiticus*, and *A. nomius*. They are among the most toxic mycotoxins found in food and feed. Aflatoxin B1 (AFB1) is the most potent and carcinogenic, causing hepatotoxicity, immunosuppression, and economic losses in the agriculture and livestock industries (Petrova *et al*., 2022).

A global survey by Gruber-Dorninger (2019) reported that AFB1 was detected in 82.2% of feed and food samples collected from South Asia, the highest detection rate among all regions surveyed over ten years. A study conducted in Kerala, India, found AFB1 concentrations ranging from 1 to 400 ppb in 709 feed and feed ingredient samples, with poultry, duck, and quail feeds frequently exceeding recommended safety limits. AFB1 accounted for 66–82% of total aflatoxins in these samples (Becha and Devi, 2013). These findings highlight both the widespread prevalence and regional severity

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of AFB1 contamination, underscoring the urgent need for effective detoxification strategies.

Various approaches have been employed to reduce aflatoxin levels, including physical, chemical, and biological methods. However, chemical and physical treatments often suffer from limitations such as low efficacy, high cost, complex procedures, and the potential to produce toxic residues. These methods may also negatively impact the nutritional and sensory qualities of the treated feed.

Biological detoxification, particularly using microorganisms such as bacteria, yeasts, and non-toxigenic *Aspergillus* strains, has emerged as a promising alternative (Alberts *et al*., 2006; Darsanaki *et al*., 2014). Among microbial agents, lactic acid bacteria (LAB) are promising agents due to their health-promoting properties and antifungal effects. *Lactobacillus rhamnosus*, a well-studied LAB strain, has demonstrated significant aflatoxin-binding capacity. However, the efficacy of detoxification varies with the strain, cell viability, and mechanism involved. While both viable and heat-killed cells have shown potential in binding AFB1, the role of metabolic activity remains unclear. This uncertainty raises an important question about whether detoxification is primarily enzymatic or due to passive adsorption by cell wall components.

Accordingly, this study aimed to evaluate and compare the *in vitro* AFB1 detoxification potential of viable and heat-killed *L. rhamnosus*. The findings are expected to provide mechanistic insights and support the development of safe, efficient, and biologically based interventions for aflatoxin mitigation in animal feed.

Materials and methods

Bacterial culture and preparation

The *Lactobacillus rhamnosus* culture was obtained from the Verghese Kurien Institute of Dairy and Food Technology (VKIDFT), Mannuthy. The bacteria were grown in De Man, Rogosa and Sharpe (MRS) broth at 37°C for 24 hours. The viable cell suspension was standardised to 10^9 CFU/mL using both spectrophotometry (measuring optical density at 600 nm) and traditional spread plating on MRS agar, followed by incubation at 37°C for 48 hours. The bacterial suspension was autoclaved at 121°C for 15 minutes in phosphate-buffered saline (PBS) to prepare the killed culture.

Fungal culture and maintenance

The fungal culture, *Aspergillus flavus* NRRL 6513 strain was obtained from the Central Avian Research Institute (CARI), Indian Veterinary Research Institute (IVRI), Izatnagar, India and maintained in Sabouraud dextrose agar (SDA).

Preparation of aflatoxin-contaminated maize

The preparation of contaminated maize was carried out following the method of Shotwell *et al*. (1966). Fifty grams of maize was placed in a 250 mL conical flask, and 25 mL of distilled water was added. The maize was then autoclaved at 121°C and 15 psi for 15 minutes. After cooling, a loopful of fresh *A. flavus* spores was introduced. The flask was incubated for 10 days with regular shaking to prevent substrate clumping. After the incubation period, the contaminated maize was autoclaved, dried and once dried, it was finely ground into a powder.

Feed preparation

The feed for the experiment was prepared according to the BIS standards for poultry layer phase 1 (IS: 1374, 2007) the composition of which is given in Table 1.

Table 1. Ingredient composition of experimental feed (g/kg)

Poultry layer phase 1	
Ingredients	Parts (g/kg)
Maize	522
DORB	150
Soybean meal	230
wheat bran	49
Calcite	25
Grit	5
DCP	10
Methionine	2
Lysine	2
Salt	5

Experimental groups

The experiment was conducted in five groups to evaluate the aflatoxin detoxification potential of *L. rhamnosus*. Treatment 1 included a viable culture of *L. rhamnosus* (10^9 CFU) added to poultry layer feed containing 30 ppb AFB1, while treatment 2 consisted of a killed culture of *L. rhamnosus* (10^9 CFU) with the same AFB1 concentration. The positive control group comprised poultry layer feed with 30 ppb AFB1 but without *L. rhamnosus*. Negative control 1 contained poultry layer feed without added AFB1, supplemented with a viable culture of *L. rhamnosus*, whereas negative control 2 included poultry layer feed without added AFB1 but with a killed culture of *L. rhamnosus*.

Aflatoxin B1 quantification using UHPLC

AFB1 levels in samples were analysed using ultra high-performance liquid chromatography (UHPLC) at the Central Instruments Laboratory, College of Veterinary and Animal Sciences, Mannuthy.

Aflatoxin B1 standard (Trilogy, 25µg/ml, TAS-M11DA1-10) and immunoaffinity columns (Aflarhone, wide, RBRP116/100) from R-Biopharm for the cleanup procedure were purchased from BVN Instruments (Madras) Private Limited, Chennai. HPLC-graded methanol (Himedia) and acetonitrile (Himedia) were used. Type I water was collected from the Milli-Q purification system.

The analysis was conducted using a Dionex UltiMate 3000 UHPLC-FLD system (Thermo Fisher Scientific, USA), which included an autosampler, a fluorescence detector (FLD), a UV-Vis detector, and a diode array detector (DAD). Chromatographic separation was achieved through a reverse-phase C18 column (5 µm, 4.6 × 250 mm, Agilent Technologies India Pvt. Ltd.) fitted with guard cartridges. The column temperature was maintained at 40°C, with an injection volume of 10 µL and a flow rate of 0.4 mL/min. The mobile phase was a mixture of 0.1% acetic acid in water (A), methanol (B), and acetonitrile (C) in a 30:35:35 ratio. The total run time was 12 minutes under isocratic conditions. Fluorescence detection was performed at an excitation wavelength of 365 nm and an emission wavelength of 456 nm (Kumar *et al*., 2020).

Statistical analysis

Statistical analysis was performed using SPSS version 24.0. Data were analysed using RMANOVA using the mixed model method to compare AFB1 reduction across treatments. A one-sample t-test was applied with test values given as the initial AFB1 levels.

Results and discussion

This study evaluated the ability of viable and heat-killed *L. rhamnosus* to reduce AFB1 in poultry layer feed. Both viable and killed *L. rhamnosus* significantly reduced AFB1 levels over time, with reductions confirmed by statistical analysis (Table 2). The initial AFB1 level of 30.79 ppb in treatment 1 and treatment 2 significantly declined after 3 and 6 hours of incubation, as did the levels in the negative control groups (initially 13.97 ppb); the data is graphically represented in Figure 1. These results indicate that metabolic activity is not required for AFB1 detoxification, as heat-killed *L. rhamnosus* exhibited a binding efficiency comparable to that of viable bacteria. A similar observation was seen in a study, in which AFB1-exposed lactic acid bacterial cells, when incubated in toxin-free buffer or organic solvent, were found to release the toxin in its original form (Bueno *et al*., 2007).

Table 2. Effect of treatment and time on AFB1 levels[1]

Treatment group	3h	6h	p value
Treatment 1	23.29^{Ab} ±0.377	22.14^{Bb} ±0.305	**0.025***
Treatment 2	22.11^{Ac} ±0.455	22.78^{Ab} ±0.493	0.174^{ns}
Positive control	29.85^{Aa} ±0.181	30.98^{Ba} ±0.412	**0.027***
Negative control 1	11.63^{Ad} ±0.451	11.48^{Ac} ±0.618	0.771^{ns}
Negative control 2	11.50^{Ad} ±0.415	11.59^{Ac} ±0.304	0.862^{ns}
P value	<0.001	<0.001	

[1]Mean values based on 6 replicates with SE; ns-non significant (p>0.05)

*Mean is significantly different from the test value (p<0.05)

AFB1 reduction percentages ranged from 24.4% to 28.2% in treatment groups and 16.7% to 17.8% in negative control groups, suggesting that higher toxin concentrations enhance bacterial binding efficiency. Statistical analysis using a one-sample t-test confirmed that these reductions were significant at both time points.

These findings are similar to previous studies that reported detoxification capabilities of lactic acid bacteria. Shah and Wu (1999) and El-Nezami *et al*. (1998) reported AFB1 reductions ranging from 20-80%, depending on the *Lactobacillus* strain used.

Importantly, the results of this study also support the concentration-dependent nature of AFB1 binding. The greater reduction observed in groups with higher initial AFB1 levels (30.79 ppb) is consistent with the findings of Bueno *et al*. (2007), who reported that AFB1 removal initially increased with rising toxin concentrations, up to a saturation point beyond which removal efficiency declined. A linear relationship between AFB1 concentration and adsorption rate in various bacterial strains was observed by Liew *et al*. (2018), suggesting that adsorption is due to the availability of binding sites on the bacterial cell surface. Additionally, Lee *et al*. (2003) proposed that at higher AFB1 concentrations, a cross-linked matrix may form among bound AFB1 molecules, resulting in more stable adsorption on the bacterial surface.

Binding efficiency varies among bacterial strains and incubation times. While El-Nezami *et al*. (2000) observed immediate AFB1 binding, Peltonen *et al*. (2001) found a time-dependent increase, followed by possible desorption. The present study found no significant difference between 3 h and 6 h incubations, except in treatment 1, indicating rapid binding within the initial hours.

AFB1 detoxification appears to occur primarily through adsorption to bacterial cell wall components rather than enzymatic degradation. Similar binding efficacy observed between viable and heat-killed *L. rhamnosus* in this study reinforces this hypothesis. Previous studies have identified key structural components of the bacterial cell wall such as carbohydrate moieties, peptidoglycan, and

teichoic acids as critical for AFB1 adsorption (Haskard *et al*., 2000; Lahtinen *et al*., 2004).

Lactobacilli, being Gram-positive bacteria, possess a thick peptidoglycan layer enriched with molecules such as teichoic acids, polysaccharides (e.g., β-D-glucan), and proteins. Liew *et al*. (2018) noted the presence of teichoic acids and β-D-glucan on the *Lactobacillus* cell wall, which may contribute to toxin binding. Although the precise mechanism of AFB1 detoxification by lactic acid bacteria (LAB) remains unclear, current evidence suggests that it is primarily a physical interaction mediated by reversible, non-covalent bonds rather than metabolic degradation (Liu *et al*., 2020). This interaction is due to adhesion by specific cell wall components such as polysaccharides and peptidoglycans (Darsanaki *et al*., 2014).

Hernandez-Mendoza *et al*. (2009) also highlighted teichoic acids as a key structural component in AFB1 adsorption.

It can be concluded that *L. rhamnosus* have effective AFB1-binding capability, highlighting its potential for aflatoxin mitigation in poultry feed. The adsorption is mediated by cell wall components, making both viable and heat-killed cells promising candidates for practical applications in feed safety.

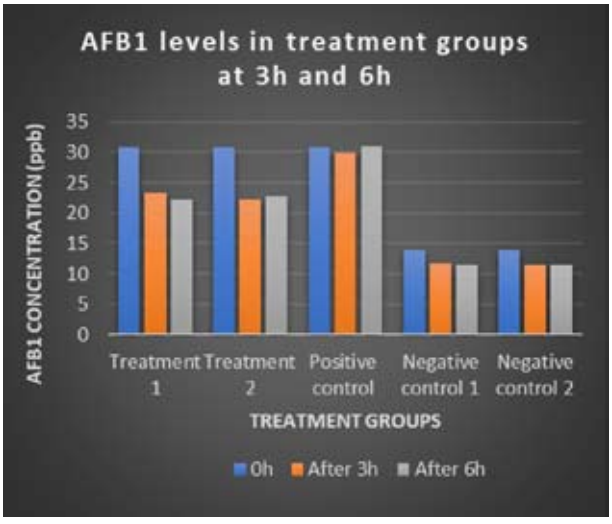

Fig. 1. Aflatoxin B1 levels in various treatment groups at 3h and 6h

Conclusion

This study demonstrated the AFB1 reduction potential of *Lactobacillus rhamnosus* in poultry layer feed. Both viable and heat-killed *L. rhamnosus* significantly reduced AFB1 concentrations, with no substantial difference in binding efficiency, confirming that metabolic activity is not required for detoxification. The extent of AFB1 reduction was influenced by initial toxin concentration and incubation time. These findings highlight the potential of *L. rhamnosus* as a biological tool for AFB1 mitigation in animal feed, offering a promising strategy to enhance feed safety. Further studies on bacterial cell wall components responsible for AFB1 binding will be useful in the food and feed industries.

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Conflict of interest

The authors declare that they have no conflict of interest.

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