Efficacy of Potentially Probiotic Fruit-Derived *Lactobacillus fermentum*, *L. paracasei* and *L. plantarum* to Remove Aflatoxin M$_1$ In Vitro

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Abstract: This study evaluated the efficacy of potentially probiotic fruit-derived *Lactobacillus* isolates, namely, *L. paracasei* 108, *L. plantarum* 49, and *L. fermentum* 111, to remove aflatoxin M$_1$ (AFM$_1$) from a phosphate buffer solution (PBS; spiked with 0.15 µg/mL AFM$_1$). The efficacy of examined isolates (approximately 10$^9$ cfu/mL) as viable and non-viable cells (heat-killed; 100 °C, 1 h) to remove AFM$_1$ was measured after 1 and 24 h at 37 °C. The recovery of AFM$_1$ bound to bacterial cells after washing with PBS was also evaluated. Levels of AFM$_1$ in PBS were measured with high-performance liquid chromatography. Viable and non-viable cells of all examined isolates were capable of removing AFM$_1$ in PBS with removal percentage values in the range of 73.9–80.0% and 72.9–78.7%, respectively. Viable and non-viable cells of all examined *Lactobacillus* isolates had similar abilities to remove AFM$_1$. Only *L. paracasei* 108 showed higher values of AFM$_1$ removal after 24 h for both viable and non-viable cells. Percentage values of recovered AFM$_1$ from viable and non-viable cells after washing were in the range of 13.4–60.6% and 10.9–47.9%, respectively. *L. plantarum* 49 showed the highest AFM$_1$ retention capacity after washing. *L. paracasei* 108, *L. plantarum* 49, and *L. fermentum* 111 could have potential application to reduce AFM$_1$ to safe levels in foods and feeds. The cell viability of examined isolates was not a pre-requisite for their capacity to remove and retain AFM$_1$.

Keywords: aflatoxin M$_1$; detoxification; *Lactobacillus*; probiotics; binding

Key Contribution: Viable and non-viable cells of all examined *Lactobacillus* isolates removed AFM$_1$; viable and heat-killed cells had a similar AFM$_1$ removal capability; AFM$_1$ retention efficacy of test isolates increased when contact time increased.

1. Introduction

Aflatoxins are fungal secondary metabolites toxic to humans and animals, causing carcinogenic, mutagenic, teratogenic, and immunosuppressive effects [1]. Aflatoxins are produced by toxigenic *Aspergillus flavus*, *A. parasiticus*, and *A. nomius* isolates growing in a variety of food and feed commodities [2]. These metabolites are very stable to autoclaving, pasteurization, and other food processing procedures [3].

Aflatoxin M$_1$ (AFM$_1$) is a 4-hydroxy derivative of aflatoxin B$_1$ (AFB$_1$), which, although approximately ten-fold less toxigenic than aflatoxin B$_1$, exerts cytotoxic, genotoxic, and carcinogenic effects in a variety of species [2], being classified as belonging to group 1 (i.e., carcinogenic to humans) by the International Agency for Cancer Research [4]. AFM$_1$ is...
formed in the liver and excreted through the milk of lactating animals that have consumed feed contaminated with AFB$_1$. Approximately 0.3–6.2% of AFB$_1$ ingested by livestock is converted to AFM$_1$ in milk [5]. In Brazil and the USA, the maximum allowable limit of AFM$_1$ in raw milk is 0.5 µg/L [6,7]. The European Union has set a maximum limit of AFM$_1$ of 0.05 µg/L for raw milk, heat-treated milk, and milk used in dairy products formulation [8].

Control of aflatoxin in food and feed can be primarily achieved by a prevention of mold contamination and growth with the adoption of improved agricultural practices and control of storage conditions, as well as by the detoxification of contaminated products through chemical (e.g., ammonia, hydrogen peroxide, alkalis, and acids) or physical methods (e.g., heat, radiations, ultraviolet, and microwave) [9]. Some methods used for aflatoxins decontamination, although they have been shown to be effective to a certain extent, may have some drawbacks, such as negative impacts on nutritional and sensory characteristics of foods, production of potentially toxic by-products, or non-suitability for use in solid foods [2,9].

Use of lactic acid bacteria (LAB) has been considered a safe and environmentally friendly biological method for the detoxification of aflatoxins in foods and feeds [10,11]. Studies have found a variable capability among probiotic Lactobacillus species or isolates to bind aflatoxins [12–14]. These studies have mostly used commercial Lactobacillus cultures or isolates from dairy origin. Although a number of Lactobacillus isolates recovered from fruit, vegetables, or their processing by-products have shown good performance in in vitro tests for the selection of probiotics [15–17], none of these isolates have been examined for their capacity to remove aflatoxins. The use of select probiotic Lactobacillus isolates has been considered a promising biological tool for removing aflatoxins from foods through adsorption when compared to chemical and physical treatments. Furthermore, although still the fastest method for retaining high detoxification efficacy [18,19], many chemical agents are nonedible materials and need to be eliminated after aflatoxin decontamination [20,21], while Lactobacillus species have been usually considered safe for use in foods [16,17].

Considering the available evidence, it was expected that fruit-derived L. fermentum, L. paracasei, and L. plantarum isolates with aptitudes to be used as probiotics would be able to remove AFM$_1$ in a prospective view for application in food and feed detoxification. To test this hypothesis, this study evaluated the efficacy of these isolates as viable and heat-killed (non-viable) cells, in the removal of AFM$_1$ in PBS, with removal percentage values in the range of 73.0 ± 1.2–80.0 ± 1.7% and 72.9 ± 1.1–78.7 ± 1.2%, respectively. Viable and heat-killed cells of the three examined isolates had similar values ($p > 0.05$) after 24 h for both viable and heat-killed cells compared to 1 h. Higher values of AFM$_1$ removal ($p \leq 0.05$) after 1 h were found for L. plantarum 49 and L. fermentum 111, but the three examined isolates had similar values of AFM$_1$ removal ($p > 0.05$) after 24 h.

2. Results and Discussion

Chromatograms for the quantification of AFM$_1$ in positive control, negative control, as well as in samples with viable cells of L. paracasei 108, L. plantarum 49, and L. fermentum 111 are shown in Figure 1. Chromatograms for the quantification of AFM$_1$ in assays evaluating the recovery of AFM$_1$ from cells after 1 h of incubation are shown in Figure 2.

Results of the capability of viable and heat-killed (non-viable) cells of L. paracasei 108, L. plantarum 49, and L. fermentum 111 for removing AFM$_1$ in PBS are presented in Table 1. Viable and heat-killed cells of all examined Lactobacillus isolates were able to remove AFM$_1$ in PBS, with removal percentage values in the range of 73.0 ± 1.2–80.0 ± 1.7% and 72.9 ± 1.1–78.7 ± 1.2%, respectively. Viable and heat-killed cells of the three examined isolates had similar values ($p > 0.05$) of AFM$_1$ removal. Only L. paracasei 108 had higher values ($p \leq 0.05$) of AFM$_1$ removal after 24 h for both viable and heat-killed cells compared to 1 h. Higher values of AFM$_1$ removal ($p \leq 0.05$) after 1 h were found for L. plantarum 49 and L. fermentum 111, but the three examined isolates had similar values of AFM$_1$ removal ($p > 0.05$) after 24 h.
Figure 1. Chromatograms of aflatoxin M₁ (AFM₁) quantification in positive and negative control. (I) Positive control: phosphate buffer solution (PBS) with AFM₁. Rₜ = Retention time of AFM₁ in phosphate buffer solution; chromatographic peak area corresponding to AFM₁; (II) Negative control after 1 h of incubation: PBS + L. paracasei 108; (III) Negative control after 1 h of incubation: PBS + L. plantarum 49; (IV) Negative control after 1 h of incubation: PBS + L. fermentum 111.
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Figure 2. Chromatograms of aflatoxin M1 (AFM1) quantification in PBS. (I) Chromatogram of assays after 1 h of incubation: PBS + AFM1 + L. paracasei 108; (II) Chromatogram of assays after 1 h of incubation: PBS + AFM1 + L. plantarum 49; (III) Chromatogram of assays after 1 h of incubation: PBS + AFM1 + L. fermentum 111; (IV) AFM1 recovery chromatogram of L. paracasei 108 and AFM1 complex after 1 h of incubation; (V) AFM1 recovery chromatogram of L. plantarum 49 and AFM1 complex after 1 h of incubation; (VI) AFM1 recovery chromatogram of L. fermentum 111 and AFM1 complex after 1 h of incubation. (A) Retention time (min) of aflatoxin M1 in phosphate buffer solution; (B) chromatographic peak area corresponding to aflatoxin M1.

Table 1. Percentage (average values ± standard deviation) of aflatoxin M1 (AFM1) removal in phosphate buffer solution by L. paracasei 108, L. plantarum 49, and L. fermentum 111.

| Isolates          | 1 h-Incubation | 24 h-Incubation |
|-------------------|----------------|-----------------|
|                   | Viable Cells   | Heat-Killed Cells | Viable Cells | Heat-Killed Cells |
| L. paracasei 108  | 73.0 ± 1.2 b,B | 72.9 ± 1.1 b,B   | 78.9 ± 0.5 a,A | 78.7 ± 1.2 a,A   |
| L. plantarum 49   | 78.1 ± 1.6 a,A | 75.8 ± 1.0 a,A,B | 77.0 ± 2.7 a,A | 76.6 ± 1.5 a,A   |
| L. fermentum 111  | 78.6 ± 2.1 a,A | 78.4 ± 0.65 a,A  | 80.0 ± 1.7 a,A | 78.3 ± 2.5 a,A   |

Different small letters in the same row (a,b) denote a significant difference (p ≤ 0.05) among values, based on Tukey’s test; different capital letters in the same column (A,B) denote a significant difference among values (p ≤ 0.05), based on Tukey’s test.

Previous studies have also verified that the capacity of LAB—either as viable or non-viable cells, of binding aflatoxins (e.g., aflatoxin B1, ochratoxin, trichothecene, and AFM1) in PBS, laboratory media, or dairy matrices (e.g., milk and yoghurt)—varies in an isolate-dependent manner [2,11,22,23]. Aflatoxins bind to the surface components of LAB.
cells and variations in aflatoxin’s binding capacities among LAB species or isolates could be associated with differences in the bacterial cell wall and cell envelope structures [7]. Early investigations have found lower capacity of AFM$_1$ removal by viable and/or heat-killed cells of different LAB (e.g., *L. plantarum*, *L. acidophilus*, *L. reuteri*, *L. johnsonii*, *L. rhamnosus*, *L. bulgaricus*, and *Streptococcus thermophilus*) [2,22,23], including probiotic *L. casei* [10], compared to *L. paracasei* 108, *L. plantarum* 49, and *L. fermentum* 111. The efficacy of AFM$_1$ removal from PBS as high (>60%) as those found for *Lactobacillus* isolates examined in this study was reported to *L. plantarum* MON03 and *L. rhamnosus* GAF01 after 6 or 24 h of incubation [24].

Results of the AFM$_1$ retention capacity of the viable and heat-killed cells of *L. paracasei* 108, *L. plantarum* 49, and *L. fermentum* 111 after washing with PBS are presented in Table 2. Percentage values of recovered AFM$_1$ from viable and heat-killed cells were in the range of 13.4 ± 1.5–60.6 ± 1.6% and 10.9 ± 1.2%–47.9 ± 1.5%, respectively. The highest values of recovered AFM$_1$ after 1 and 24 h were found for *L. fermentum* 111 and *L. paracasei* 108, respectively, for both viable and heat-killed cells. Only for *L. fermentum* 111 did the values of recovered AFM$_1$ decrease after 24 h for viable and heat-killed cells; for *L. paracasei* 108 and *L. plantarum* 49, these values varied with the viability/non-viability of cells and incubation time period. Overall, *L. plantarum* 49 had the higher AFM$_1$ retention capacity after washing. Variations in aflatoxin release have been linked to the differences in binding sites in different LAB isolates, or even in these binding sites being very similar. They could have minimal differences depending on each isolate [13,25,26].

### Table 2. Percentage (average values ± standard deviation) of recovered aflatoxin M$_1$ (AFM$_1$) in solution after washing with phosphate buffer solution.

| Isolates         | 1 h-Incubation | 24 h-Incubation |
|------------------|----------------|-----------------|
|                  | Viable Cells   | Heat-Killed Cells | Viable Cells | Heat-Killed Cells |
| *L. paracasei* 108 | 34.6 ± 1.1 b,B | 28.5 ± 1.7 d,C | 31.7 ± 1.2 c,A | 40.3 ± 1.6 a,A |
| *L. plantarum* 49 | 13.4 ± 1.5 c,C | 43.8 ± 1.5 a,B | 18.8 ± 1.0 b,B | 10.9 ± 1.2 d,C |
| *L. fermentum* 111 | 60.6 ± 1.6 a,A | 47.9 ± 1.5 b,A | 14.1 ± 1.4 c,C | 14.9 ± 1.6 c,B |

Different small letters in the same row (a–c) denote a significant difference (p ≤ 0.05) among values, based on Tukey’s test; different capital letters in the same column (A,B) denote a significant difference among values (p ≤ 0.05), based on Tukey’s test.

For all examined isolates, the values of recovered AFM$_1$ decreased after 24 h of incubation, indicating that AFM$_1$ retention capacity increased when the length of the contact time increased. There was no clear association between the capability of removing AFM$_1$, initially, and of retaining AFM$_1$ after washing among examined isolates. Interestingly, a study with different *Lactobacillus* species found lower AFM$_1$ removal values than those found in this study, although the recovery of AFM$_1$ from bacterial cells was lower in the former [11].

Heat treatment positively affected the capability of retaining AFM$_1$ in *L. paracasei* 108 after 1 h of incubation, as well as of *L. plantarum* 49 and *L. fermentum* 111 after 24 h of incubation. Heating could increase the interaction capacity of bacterial cells/aflatoxin complexes by causing an increased exposure of the cell wall components, primarily polysaccharides and peptidoglycans, which act as binding sites to aflatoxin [14]. However, the destruction of specific components of the bacterial cell wall by heating, causing the denaturation of proteins and increased cell surface hydrophobicity, has been cited to result in a decreased capability of LAB cells of binding AFM$_1$ [7]. An increased capability of removing aflatoxin B1 was also found in *L. rhamnosus* after heating [27].

The recovery of the AFM$_1$ bound to the cells of examined *Lactobacillus* isolates after washing indicates that the binding was not strong and could not involve a non-covalent weak bond, but probably a physical association of AFM$_1$ with hydrophobic sites in the
bacterial cell wall [13,20,25]. The lower AFM₁ recovery values found for the examined isolates could be linked to the interaction of AFM₁ molecules retained in the bacterial cell wall with other AFM₁ molecules retained in adjacent cells, forming a type of cross-linked matrix that avoids aflatoxin release during washing [10]. Probably, the efficacy of this type of cross-linked matrix decreased over time for L. paracasei 108 and L. plantarum 49. Although some authors have reported that a part of non-recovered AFM₁ might be degraded or biotransformed by a Lactobacillus metabolism [2,7], most of the available literature has indicated that aflatoxins are not removed by the metabolism of LAB, but because of a physical bound to the molecular components of bacterial cells, primarily peptidoglycans from the cell wall [19,21,25].

In agreement with available literature, the results of this study showed that the cell viability of the examined isolates is not a prerequisite for the removal and retaining of AFM₁ [13,28]. Cell concentration as high as 10⁸–10⁹ CFU/mL of viable or non-viable LAB is typically needed to reach a level of aflatoxin removal of ≥ 50% [22,28].

3. Conclusions

Results showed that potentially probiotic L. fermentum 111, L. paracasei 108, and L. plantarum 49 isolated from fruit processing by-products are capable of binding AFM₁ in vitro when assayed as either viable or non-viable cells. The recovery of AFM₁ from bacterial cell complexes varied with the examined isolate and contact time. Non-viable cells had a higher capability for retaining AFM₁ after 1 or 24 h of incubation. These results indicate that Lactobacillus isolates recovered from fruit with performance compatible to use as probiotics could have a satisfactory aflatoxin binding capacity, which could be exploited as a biological tool for the detoxification of foods and feeds, particularly, for the removal and restoration of AFM₁ to safe levels. Further studies are needed to investigate the mechanisms involved in removal of AFM₁ by these isolates and possible factors affecting the stability of formed complexes, including when exposed to conditions mimicking the human gastrointestinal tract.

4. Materials and Methods

4.1. Chemicals, Bacterial Isolates, and Inoculum Preparation

The AFM₁ standard was obtained from Sigma Aldrich (St. Louis, MO, USA). High-performance liquid chromatography (HPLC) grade solvents were obtained from Merck (Darmstadt, Germany).

The isolates Lactobacillus plantarum 49, L. fermentum 111, and L. paracasei 108 were examined separately for the removal of AFM₁. These isolates were recovered from fruit processing by-products, identified with a partial 16S rRNA gene sequence analysis and characterized as potential candidates for use as probiotics [17]. Stocks were stored at −20 °C in de Man, Rogosa, and Sharpe (MRS) broth (HiMedia, Mumbai, India) with glycerol (20 mL/100 mL; Sigma-Aldrich, St. Louis, MO, USA). Working cultures were maintained aerobically on MRS agar (HiMedia, Mumbai, India) at 4 °C and transferred to a new media monthly. Prior to use in assays, each isolate was cultivated anaerobically (Anaerobic System Anaerogen, Oxoid, Hampshire, UK) in MRS broth at 37 °C for 20–24 h (to reach the stationary growth phase), harvested by centrifugation (4500 × g, 15 min, 4 °C), washed twice, and resuspended in phosphate buffer solution (PBS; 50 mM K₂HPO₄/KH₂PO₄; pH 6.9) to obtain cell suspensions with an optical density reading at 660 nm (OD₆₆₀) of 0.5. This suspension had viable counts of approximately 1.1 × 10⁹ CFU/mL for each isolate when plated in MRS agar.

4.2. Evaluation of AFM₁ Removal and Recovery of AFM₁ from Bacterial Cells

The capability of examined Lactobacillus isolates to remove AFM₁ in PBS was assessed with viable and non-viable bacterial cell suspensions. To obtain non-viable bacterial cells, Lactobacillus cell suspensions were inactivated by boiling at 100 °C for 1 h. No visible colonies were found when heat-treated cell suspensions (named heat-killed cells) were
plated onto MRS agar and followed by anaerobic incubation (using Anaerobic System Anaerogen, Oxoid, Hampshire, UK) for 48 h. For testing the AFM$_1$ removal capability, 1 mL of test isolate suspension (pure culture of viable and heat-killed cells) was mixed with 1.5 mL of PBS, previously spiked with 0.15 µg/mL AFM$_1$, and incubated aerobically at 37 °C [28]. After 1 and 24 h of incubation, the mixture was centrifuged (1500 × g, 15 min, 4 °C) and the AFM$_1$ content in the supernatant was determined by HPLC, as detailed in Section 4.3.

Cell pellets collected from each monitored incubation period (contact time) were evaluated for the recovery of AFM$_1$ from cell complexes. Obtained pellets were washed with 1.5 mL of fresh PBS, the cells were re-pelleted (1500 × g, 15 min, 4 °C), and supernatant was collected for the quantification of released AFM$_1$ [18]. For each isolate, a positive control consisting of free cells suspended in PBS with 0.15 µg/mL AFM$_1$, and a negative control, consisting of bacterial cells (viable or heat-killed), suspended in PBS were used.

4.3. Quantification of AFM$_1$

The quantification of AFM$_1$ in supernatants was done with high-performance liquid chromatography (HPLC) using a Shimadzu (Prominense, Tokyo, Japan) HPLC system, equipped with an auto sampler SIL 20A HT (Prominense, Shimadzu, Tokyo, Japan), fluorescence detector RF-20A (Prominense, Shimadzu, Tokyo, Japan), an LC-20AT pump (Prominense, Shimadzu, Tokyo, Japan), oven CTO-20A (Prominense, Shimadzu, Toquio, Japan), a CBM-20A controller (Prominense, Shimadzu, Tokyo, Japan), a CLC-ODS (M) reverse phase column (4.6 × 150 mm; Shim-Pack, Prominense, Shimadzu, Tokyo, Japan) and pre-column G-ODS-4 (1.0 × 4.0 mm; Shim-Pack, Prominense, Shimadzu, Tokyo, Japan).

Chromatographic conditions were the same as those described in a previous study [7]. Excitation and emission wavelengths were 366 and 428 nm, and the injection volume was 20 µL. The mobile phase was water:methanol:acetonitrile (6:2:2) and the flow rate was 1 mL/min. The calibration curve was constructed using six concentrations of AFM$_1$ standard diluted in acetonitrile (20–60 ng/mL), performed in triplicate. From this analysis, the equation $y = 2E+07x + 873,267$ ($r^2 > 0.99$) was obtained. The limit of detection (LOD) and limit of quantification (LOQ) were estimated based on Resolution n° 899 of the Brazilian Agency for Health Surveillance [29]. The LOD and LOQ of AFM$_1$ were 0.20 and 0.67 ng/mL, respectively.

The percentage of AFM$_1$ removed by each isolate was determined with the Equation (1) [22,27,30]:

$$100 \times \left[1 - \frac{\text{peak area of chromatographic peak of sample}}{\text{area of positive control chromatographic peak}}\right].$$

4.4. Statistical Analysis

Assays were done in triplicate in three independent experiments (repetitions). A Kolmogorov–Smirnov normality test was run to assess whether obtained results had normal distribution. Results (average data ± standard deviation) were submitted to a one-way analysis of variance (ANOVA), followed by Tukey’s test, considering a $p$ value of ≤ 0.05 for significance. Statistical analyses were done with IBM SPSS Statistics 20 (Armonk, NY, USA).

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