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Biocontrol potential of *Lactobacillus kefiri* probiotic strain against *Aspergillus flavus* and aflatoxin production in almonds

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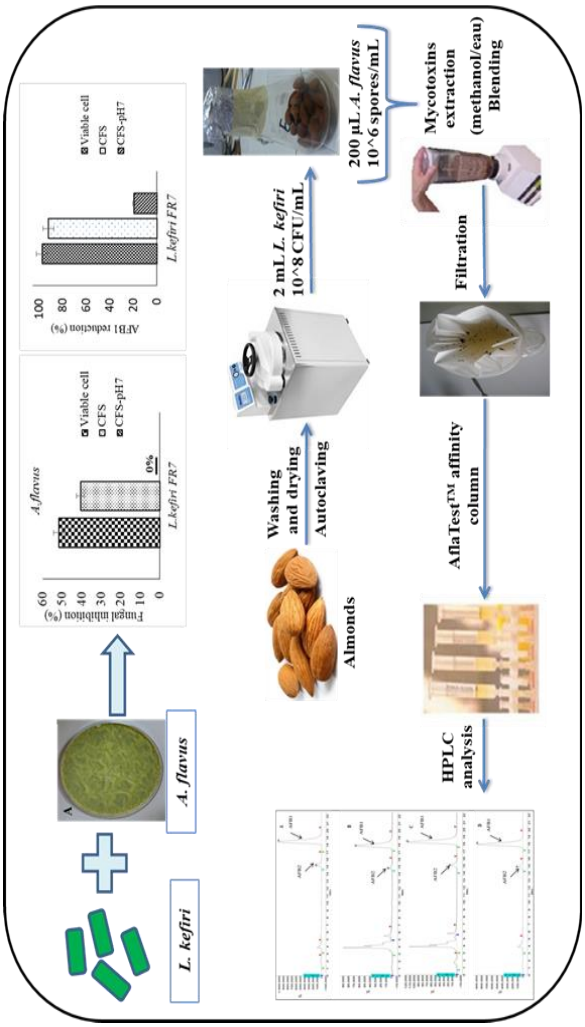
Graphical Abstract



Abstract.

Aspergillus flavus is a major producer of aflatoxin in almonds, causing fungal decay and posing a significant threat to human health. *Lactobacillus kefiri* MH107106 was used to inhibit the growth of *A. flavus* 15UA005, an aflatoxin producing strain previously isolated from curcumin. The inhibition effect *in vitro* was tested in Malt Extract Agar (MEA), while *in vivo* effect was examined in almonds. Results showed that *L. kefiri* strain inhibited 51.67% of *Aspergillus flavus* growth in agar medium. The cell-free supernatants (CFS) from *L. kefiri* reduced 40.56 % of fungal growth, while, no inhibition was observed for *A. flavus* treated with the cell-free supernatants at pH7 (CFS-pH7). Additionally, the strain *L. kefiri* inhibited the production of AFB1 and AFB2 by 97.22% and 95.27%, respectively. In the other hand, the inoculation of *L. kefiri* in almonds artificially contaminated with *A. flavus* decrease 85.27% of AFB1 and 83.94% of AFB2 content after 7 days of incubation. This study reveals that *L. kefiri* could be a promising natural agent for an effective bio-control, non-toxic bio-preservative, and an eco-friendly alternative to synthetic additives against *A. flavus* in almonds.

Keywords: *L. kefiri*, *A. flavus*, almonds, antifungal activity, mycotoxins

1. Introduction

Almonds (*Prunus dulcis),* are widely consumed in Tunisia due to their desirable sensory and nutritional qualities. They are a significant source of proteins, vitamins and unsaturated fatty acids. However, they can be contaminated by various mycotoxigenic fungi belong to genera *Aspergillus, Eurotium, Penicillium* and *Rhizopus,* regardless of the stage of production (Rodrigues, Venâncio, & Lima, 2012). These nuts are susceptible to aflatoxin contamination, endangering food security and global markets for Tunisian almonds.

Aflatoxins (AFs) are mycotoxins produced by certain species of *Aspergillus,* particularly *Aspergillus flavus, Aspergillus parasiticus* and *Aspergillus nomius* (Taheur, Mansour, Kouidhi, & Chaieb, 2019). Aflatoxin B1 (AFB1) is carcinogenic and genotoxic *in vitro* and *in vivo*, and it has been classified in the group 1 by the International Agency for Research on Cancer (IARC, 2016). AFB1 has toxic, carcinogenic, mutagenic and teratogenic effects in laboratory animals (Paterson & Lima, 2010).

Biological control of mycotoxins through different microorganisms such as bacteria, atoxigenic *Aspergillus flavus* strains, and yeasts are considered efficient and environmentally friendly approaches in reducing the risk of food mycotoxins content (Moradi et al., 2020).

Lactic acid bacteria (LAB) have received much attention for food and feed biopreservation (Le Lay et al., 2016). Indeed, LAB produced numerous active antimicrobial compounds mainly lactic, acetic benzoic and propionic acids, phenyllactic acid, hydrogen peroxide, bacteriocins, reuterine, fungicins, phenolic compounds and hydroxyl fatty acids, which are involved in their antagonistic effects (Cortés-Zavaleta, López-Malo, Hernández-Mendoza, & García, 2014; Ruggirello et al., 2019). Several studies reported that LAB may inhibit fungi growth and mycotoxin production (Le Lay et al., 2016; Taheur et al., 2019). LAB can also biodegrade or adsorb mycotoxins (L. Abrunhosa et al., 2014; Taheur et al., 2017).

There is scarce information on competitive abilities of *L. kefiri* and *A. flavus* and aflatoxin production in almonds. Therefore, the present study was undertaken to assess the potential ability of *L. kefiri* strain to reduce the population density of *A. flavus* and aflatoxin production in culture medium and almonds under *in vitro* experiments.

2. Material and methods

2.1. Microorganisms and culture conditions

L. kefiri was cultured in Man, Rogosa and Sharpe (MRS) (Oxoid) broth at 30°C for 72 h under anaerobic conditions before experiments. *A. flavus* 15UA005 was selected as AFB1 and AFB2 producer. Fungal strain was grown in Malt Extract Agar (MEA) (Himedia) for 7 days at 25° C. Spore suspension of fungal strain was prepared in sterile peptone water (0.1% w/v) supplemented with Tween 80 (Fisher Scientific) (0.001%) and counted with Malassez cell. Suspension concentration of the inocula were adjusted to 10^6 spores/mL.

2.2. Inhibition of mycelium growth and mycotoxin production

2.2.1. Preparation of cell-free supernatants (CFS)

L. kefiri was grown in MRS broth for 48 h at 30 °C. Then, cells were removed by centrifugation (7200×g, 10 min), and the obtained cell-free supernatants (CFS) were filter sterilized through a 0.22-µm pore-size filter and stored at -20 °C for further use.

2.2.2. Determination of antifungal activity

Antifungal activity of bacteria viable cell (VC), CFS and neutralized CFS, (CFS-pH7) of *L. kefiri* was evaluated as described by (Cortés-Zavaleta et al., 2014) with minor modifications. VC, CFS or CFS-pH7 (10%, v/v) was mixed with MEA at 45 °C, poured into Petri dishes (20 mL per plate) and kept to stand overnight at room temperature for media solidification. Then, 10 µL of mould spores suspension (10^6 spores/mL) of *A. flavus* were spotted onto the center of the surface of the agar layer in each plate. Control MEA plates were mixed with sterile MRS broth in the same proportions and inoculated. Experiments were repeated in duplicate. All the plates were incubated aerobically at 25 °C. Diameters of the mould growth were measured daily during 7 days and the percentage of mould growth inhibition was calculated according the formula:

$$I= [(A_c – A_T)/ A_c]*100$$

Where, (I) was the percentage of growth inhibition, (A_T) and (A_C) the diameter of mycelial growth in both treated and control plates.

2.2.3. Mycotoxins extraction from agar medium

After incubation for 7 days, agar containing fungi treated with VC, CFS or CFS-pH7 was cut and transferred to a 50 mL falcon tube. A volume of 30 mL of extraction solution acetonitrile/methanol/acetic acid (HPLC grade, Fisher Scientific) (78/20/2, v/v/v) was added. Then tubes were strongly mixed and kept to stand overnight at room temperature in the dark. All samples were filtered to clean 2 mL vials using a syringe filter (0.2 µm, Nylon) and conserved at -20 °C until HPLC analysis.

2.3. Antifungal activity of *L. kefiri* against *A. flavus* in almonds

2.3.1. Experimental design

L. kefiri was tested against *A. flavus* on almonds. *L. kefiri* strain was cultivated anaerobically in MRS broth for 48 h at 30°C. Almonds that achieve commercial level of maturity were washed twice with tap water, then surface-disinfected with 0.1% sodium hypochlorite for 1 min, cleaned with tap water and air dried. Twenty grams of almonds were placed in 250 mL flasks and autoclaved. Two milliliters of bacteria cell suspension (10^8 CFU/mL) were added in each flask. Substrates were mixed using a sterile spreader to ensure the same bacterial cells distribution. Controls were prepared by inoculating substrate with 2 mL of sterile MRS broth without *L. kefiri*. After incubation at room temperature for three hours, 200 µL of *A. flavus* spores (10^6 spores/mL) were inoculated into each flask and mixed to ensure even samples homogenization. The flasks were incubated at 25°C for seven days. Fungal growth was evaluated every day visually. Experiments were repeated in duplicate.

2.3.2. Mycotoxins extraction

After 7 days of incubation, AFB1 extraction was achieved as follow: twenty gram of almond were placed in blender jar with 4g of NaCl. Then, 100 mL of methanol/water (60:40, v/v) solution were added and mixture was blended at high speed for 1 minute. The obtained extract was filtered through filter paper and filtrate was collected in a clean vessel. A volume of 20 mL filtered extract was diluted with 20 mL of purified water. Dilute extract was filtered through glass microfibre filter. A volume of 10 mL of filtered diluted extract was passed through AflaTest^{TM} affinity column at a rate of about 1-2drops/second until air comes through column. Elution of AFB1 was performed by adding methanol through the column and collecting all of the sample eluate (1.0 mL) in a glass cuvette. Then, 1 mL of purified water was added to eluate. AFB1 quantification was performed using high performance liquid chromatography (HPLC).

2.4. Mycotoxin analysis

Mycotoxins were analyzed on a high performance liquid chromatography (HPLC) that consists of a column C18 reversed-phase YMC-Pack ODS-AQ (250 × 4.6 mm I.D., 5 µm) connected to a pre-column with the same stationary phase. The HPLC system was equipped with of a Varian Prostar 210 pump, a Varian Prostar 410 autosampler, a Jasco FP-920 fluorescence detector. The oven temperature was maintained at 30 °C using a column a Jones Chromatography 7971 column heater. The instrument and the chromatographic data were managed by a Varian 850-MIB data system interface and a Galaxie chromatography data system, respectively.

AFB1 determination was done as described by (Soares, Rodrigues, Freitas-Silva, Abrunhosa, & Venâncio, 2010). A mixture of deionized water/acetonitrile/methanol (3:1:1, v/v/v) was used as the mobile phase at a 1.0 mL/min flow rate. The detection wavelength was set at ex: 365 nm; em: 435 nm.

2.5. Statistical analysis

Experiments were prepared in duplicate (n= 2). The data were subjected to statistical analyses using the Statistica 6.1 software. One-way ANOVA with one factor comparisons by Tukey's test was carried out on data. Significant difference was considered at a P value < 0.05.

3. Results and discussion

3.1. Inhibition of mycelium growth and mycotoxin production

In vitro evaluation of *L. kefiri* antifungal activity by "dual culture" against *A. flavus* was performed. As presented in Figure 1, fungal growth diameter decrease significantly (P<0.05) during incubation at 25 °C for 7 days, when treated with bacteria viable cell (VC) and bacteria supernatant. However, no significant difference between fungal diameter of control (inoculation only with *A. flavus*) and fungal treated by bacteria neutralized supernatant (CFS-pH7).

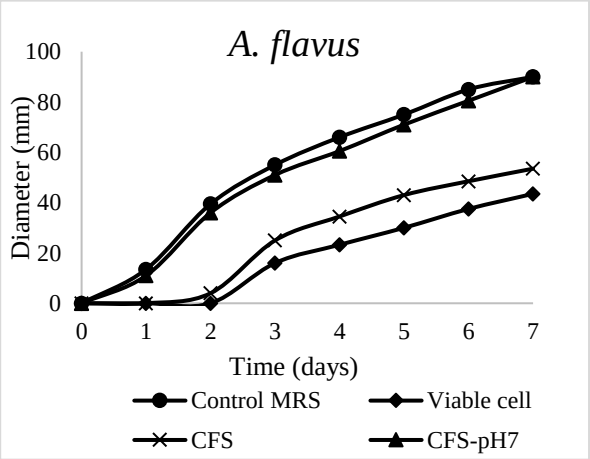


Fig.1. Graphical illustration of *A. flavus* diameter co-cultured with *L. kefiri* viable cell (VC), supernatant (CFS) and neutralized supernatant (CFS-pH7), in comparison to control samples (Plates inoculated only with *A. flavus*) during incubation at 25 °C for 7 days.

Fungal growth inhibition percentages are illustrated in Figure 2. Indeed, the tested probiotic strain exhibited inhibition effect of 51.67% for *A. flavus*. Our results revealed also that CFS obtained from *L. kefiri* reduced 40.56% of fungal growth. However, CFS-pH7 did not inhibited fungal growth (0%). Our results were in agreement with those reported by (Gerbaldo, Barberis, Pascual, Dalcero, & Barberis, 2012; Sadeghi, Ebrahimi, Raeisi, & Nematollahi, 2019).

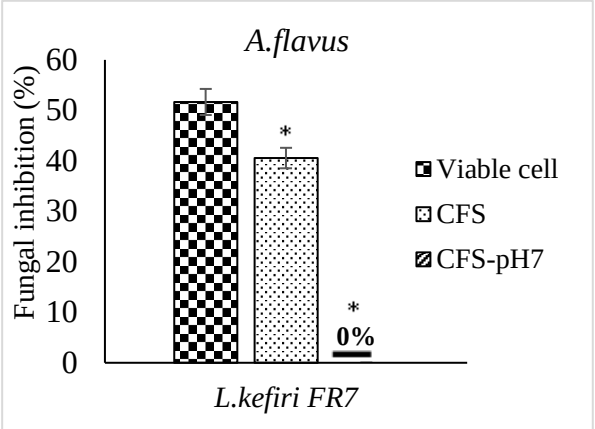


Figure 2. Percentages of growth inhibition of *A. flavus* by *L. kefiri* viable cell (VC), supernatant (CFS) and neutralized supernatant (CFS-pH7) after incubation for 7 days at 25 °C. The bars represent the mean ± SE. Asterisks indicate significant differences between CFS, CFS-pH7 and VC (ANOVA, $p < 0.05$).

The biopreservative activity of the *L. kefiri* against *A. flavus* was associated to the type and the amount of the produced inhibitory compounds and the low pH. In fact, low pH facilitated the crossing of the un-dissociated form of the antifungal metabolites through the cell membrane and cause the loss of viability. Moreover, the metabolites produced by LAB such as acetic and propionic acids, lactic acid, monohydroxy octadecenoic acid, caproic and hexanoic acids, formic acid, butyric acid, phenyllactic acid, hydroxy fatty acids, phenolic compounds, cyclic dipeptides, reuterin and fungicins contributed in their antifungal inhibitory (Dalié, Deschamps, & Richard-Forget, 2010).

The main mode of actions reported for the aforementioned inhibitory compounds include intracellular acidification, inhibition of glycolysis, either the increase or alters fungal membrane permeability, the inhibition of electron transport chain, uncoupling of phosphorylation, reduction of colonization, inhibition of phenylalanine dehydrogenase and inhibition of fungal spore synthesis (Hassan, Zhou, & Bullerman, 2016).

On the other hand, statistical analysis showed that VC, CFS and CFS-pH7 affected significantly fungal mycotoxins production ($P<0.05$). In addition, the VC was more effective in AFB1 and AFB2 reduction than CFS and CFS-pH7. VC of *L. kefiri* reduced 97.22% and 95.27 % of AFB1 and AFB2, respectively (Figure 3 and 4). Moreover, the fungi reduction growth by CFS-pH7 from *L. kefiri* FR7 reached 19.67% and 16.11% for AFB1 and AFB2, respectively. The pH affected the LAB antifungal potential. Previous study reported that CFS from *L. plantarum* UM55 inhibited AFB1 production in *A. flavus* MUM 17.14 by 91% and it lost its activity to 13% after pH neutralization (Guimarães, Santiago, Teixeira, Venâncio, & Abrunhosa, 2018). Furthermore, the reduction of aflatoxin amount is related to low mycelia formation and consequently to low secondary metabolites (mycotoxins) synthesis in the presence of LAB (Gerbaldo et al., 2012).

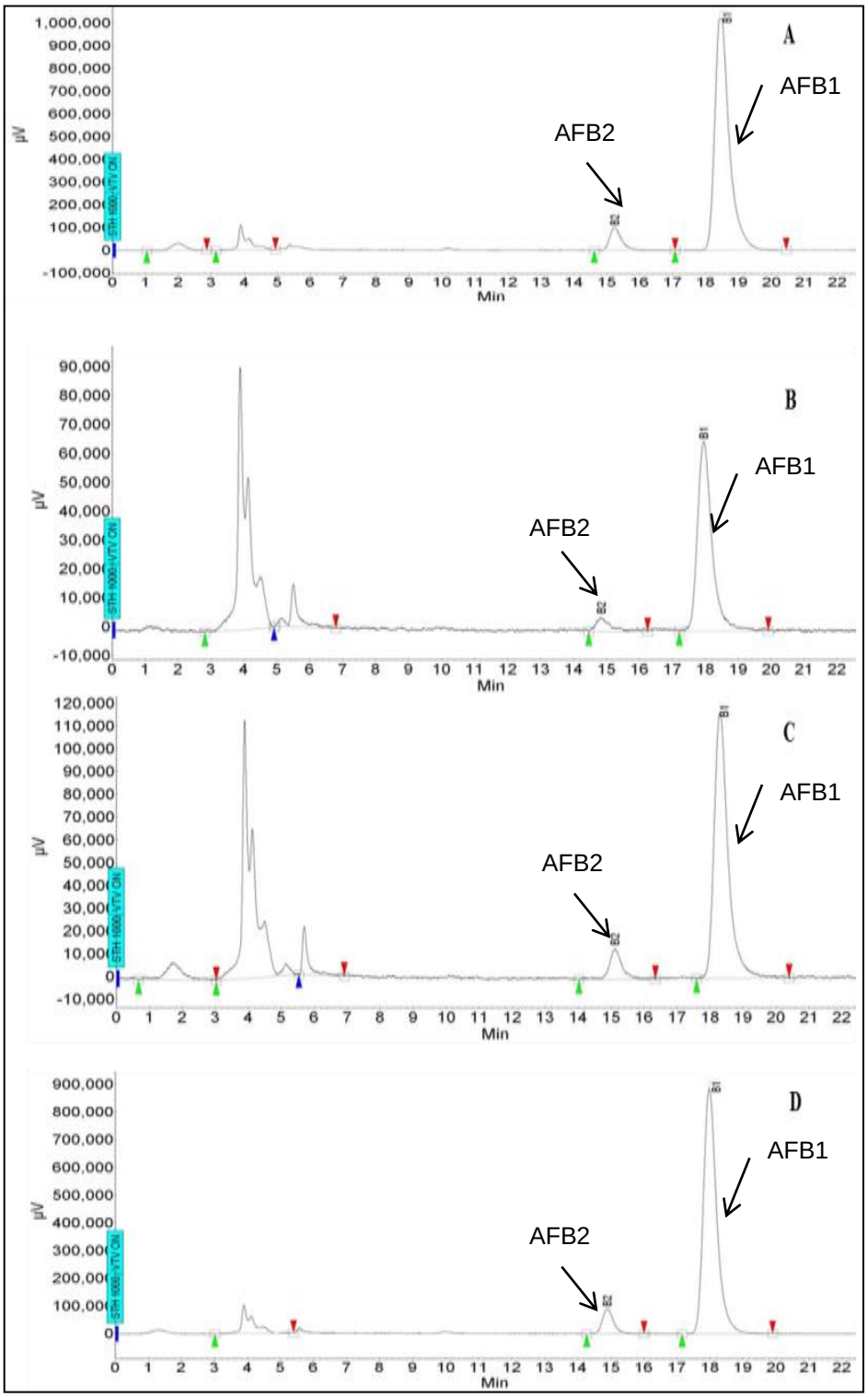


Figure 3. HPLC chromatograms indicating the extent of AFB1 production by A) untreated *A. flavus* (control), B) *A. flavus* treated with *L. kefiri*, C) *A. flavus* treated with *L. kefiri* cell-free supernatant, and D) *A. flavus* treated with neutralized *L. kefiri* cell-free supernatant after incubation for 7 days at 25 °C.

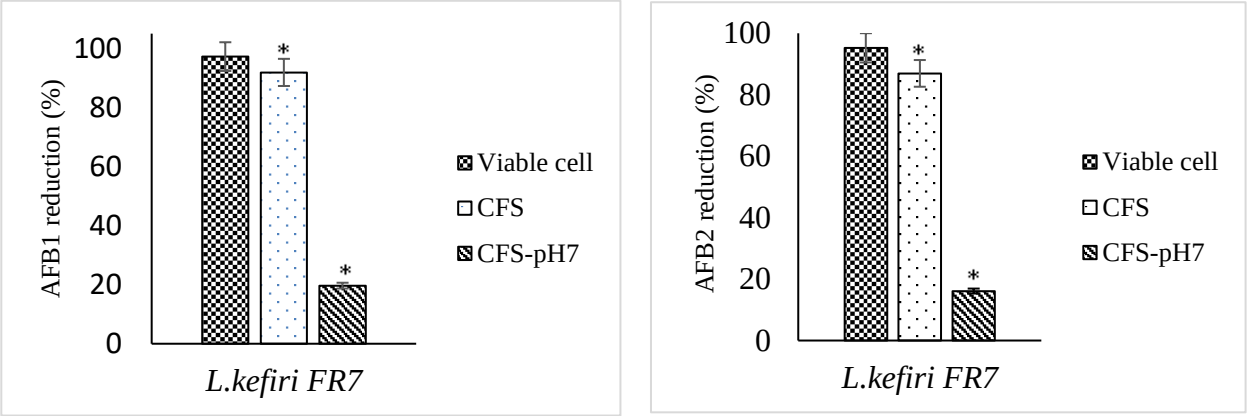


Figure 4. Percentages of AFB1 and AFB2 reduction by *L. kefiri* viable cell (VC), supernatant (CFS) and neutralized supernatant (CFS-pH7) after incubation at 25 °C for 7 days. The bars represent the mean ± SE. Asterisks indicate significant differences between CFS, CFS-pH7 and VC (ANOVA, p <0.05).

3.2. Antifungal activity of *L. kefiri* against *A. flavus* in almonds

In order to assess the potential of the selected LAB to inhibit growth and mycotoxin production, we tested *L. kefiri* FR7 against *A. flavus* on almonds. Results showed that in the control experiment (almond inoculated with fungi only), the visual growth of *A. flavus* started in the third day of incubation. The control exhibited moderate mould growth from day 4 and was completely covered in green for almond on day 5. However, in almonds treated with *L. kefiri* FR7, the visual fungal growth of *A. flavus* was observed at the fifth day of incubation.

Our results revealed, after 7 days of incubation, that in control essay, *A. flavus* produce 82.85 µg/kg of AFB1 and 6.85 µg/ kg of AFB2 in almonds. However, the inoculation of almonds with *L. kefiri* FR7 contributes to the decrease of production of AFB1 and AFB2 by 85.27% and 83.94%, respectively (Table 1). Earlier studies reported the antagonistic effect of LAB in food (Luís Abrunhosa et al., 2014; Melo Pereira et al., 2016).

Table 1
Reduction of aflatoxin production by *A. flavus* in almonds after treatment with *L. kefiri*

	Control	***L. kefiri* + *A. flavus* (in almonds)**
AFB1 (µg/Kg)	82.85*± 3.6	12.2 ± 1.3 (85.27 %)[a]
AFB2 (µg/Kg)	6.85 ± 1.8	1.1 ± 0.2 (83.94 %)

*Data are expressed as mean ± standard deviation of three replicates.
[a]Pourcentage of mycotoxin reduction.

4. Conclusions

The use of *L. kefiri* can be considered as an effective approach for biocontrol of toxin-producing fungi (*A. flavus*), especially in edible food such as almonds with high nutritional and economic values. The present assays have shown the high potential ability of *L. kefiri* strain against *A. flavus* growth and aflatoxin production in culture medium and in almonds. The commercial applications of this strain under field conditions require further studies.

Acknowledgments

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