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In Vitro Reduction of Aflatoxin B1 by Strains of Lactic Acid Bacteria Isolated from Moroccan Sourdough Bread

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ABSTRACT

The ability of some selected strains of lactic acid bacteria isolated from traditional sourdough ferments to remove aflatoxin B1 (AFB1) was studied in the present investigation. Isolates were grown for 48 h in MRS broth containing a known concentration of AFB1 at 30°C. The AFB1 in the medium was determined for each strain with HPLC and calculated with the initial and final concentration of AFB1 after fermentation. Results showed that *Lactobacillus* strains could remove more AFB1 than *Pediococcus* and *Leuconostoc* strains and the reduction of the initial amount of AFB1 ranged from 1.80 to 44.89% AFB1 for all strains studied. Five strains of *Lactobacillus rhamnosus*, one strain of a *L. lactis* and one strain of *L. casei* reduced AFB1 by more than 20%. *L. rhamnosus* strain Lb50 reduced AFB1 by 45%. These findings suggest that sourdough strains of lactic acid bacteria can be exploited as an approach of detoxification of aflatoxins from foods.

Key Words: Aflatoxin B1; Lactic acid; Sourdough bread; Fermentation

INTRODUCTION

Aflatoxins are the most studied mycotoxins produced by three common toxigenic *Aspergillus flavus, A. parasiticus* and *A. nomius* in foods and feeds (Kurtzman *et al.*, 1987). Aflatoxin B1 (AFB1) has been found to be a potent hepatocarcinogen and is classified by the International Agency of Research on Cancer as Group 1 carcinogen (IARC, 1993). The occurrence of aflatoxins contamination is global with severe problems especially prevalent in developing countries (Henry *et al.*, 1999). Aflatoxins are also of industrial importance due to the economic losses resulting from elimination of contaminated crops, impaired growth and feed efficiency of animals fed contaminated feeds. Consequently there is a demand for novel strategies to prevent both the formation of aflatoxins in foods and feeds and the impact of existing aflatoxins contamination (Haskard *et al.*, 2001).

The protective effect of microorganisms against food mutagens such as aflatoxins has been studied. Lillehoj *et al.* (1967) reported the ability of *Flavobacterium aurantiacum* NRRL B-184 to irreversibly remove AFB1 from solutions. Smiley and Draughon (2000) demonstrated that the crude protein extracts from *F. aurantiacum* degrades AFB1.

Studies undertaken in the last two decades suggested that lactic acid bacteria and fermented dairy products possess anticarcinogenic activity (Goldin & Gorbach, 1984). Recently, strains of lactic acid bacteria were also reported to remove AFB1 from liquid media. The removal of aflatoxins was strain and dose dependant and did not affect the viability of lactic acid bacteria. Removal of AFB1 by *Lactobacillus rhamnosus GG* and *L. rhamnosus Lc-705* was a rapid process with approximately 80% AFB1 removed without further incubation (El-Nezami *et al.*, 1998a). Physicochemical treatments of both strains by acid and temperature to kill bacteria enhanced the ability of the two strains to remove AFB1 suggesting that binding may explain the interaction between the AFB1 and the lactic strains studied (El-Nezami *et al.*, 1998b). In addition, specific dairy strains of lactic acid bacteria were also reported to remove Aflatoxin M1 from reconstituted milk (Pierides *et al.*, 2000).

In Morocco, cereals are the major food of the population especially in rural area. The worldwide distribution of contamination of cereals and other crops by mycotoxin-producing moulds has been amply documented in the literature. Toxic metabolites were found to occur naturally on foods and feeds from cereals. Davegowda *et al.* (1998) reported that as much as 25% of the world's cereals are contaminated with known mycotoxins.

The production of bread in rural areas in Morocco is still home made by traditional methods. In previous studies, Microbiological composition of Moroccan traditional sourdough ferments was studied (Boraam *et al.*, 1992; Faid *et al.*, 1994). Lactic acid bacteria are responsible for the production of flavour compounds and acidity and showed an interaction with yeasts during the process of fermentation and bread making (Faid *et al.*, 1993). In a recent survey, we have demonstrated the ability of some traditional ferment used in Moroccan sourdough fermentation to reduce

amounts of aflatoxin B1 and aflatoxin G1 in contaminated flour and we have suggested that the decrease of aflatoxins may be due to lactic acid bacteria, which constitute normal flora of Moroccan traditional bread (Zinedine *et al.*, 2004).

The purpose of the research described herein was to study the ability of some strains of lactic acid bacteria isolated from some Moroccan traditional sourdough ferments to reduce amounts of mycotoxins in a contaminated medium. AFB1 was selected among mycotoxins because of its wide occurrence in raw products and its high toxicity to human and animals.

MATERIALS AND METHODS

Micro-organisms. Lactic acid bacteria strains used in this work were isolated from traditional sourdough ferments collected in various rural areas in Morocco and the collection of the Department of Food Microbiology and Biotechnology. (I.A.V. Hassan II, Rabat). Lactic acid bacteria studied are: *L. brevis* strain Lb1; *L. casei* strain Lc12; *L. lactis* strains (Lb5 & Lb8); *L. plantarum* strains (Lb7 & Lb9); *L. rhamnosus* strains (Lb44, Lb21, Lb31, Lb103 & Lb50)*; Leuconostoc mesenteroides* strain Ln13; and *Pediococcus acidilactici* strain P55.

Standard aflatoxins preparation and medium contamination. Solid AFB1 (Promochem, France), was suspended in benzene/ acetonitrile (93:7v/v) to obtain a concentration of approximately 10 µg/mL. The concentration of the stock solution was checked by recording an ultraviolet /visible spectrum (Specord 200) of an AFB1 sample and calculating its actual value from the Lambert-Beer equation (A=ε.c.l).

To prepare an aqueous solution, benzene /acetonitrile were evaporated by heating in water bath (80°C for 10 min) and AFB1 was suspended again in methanol. MRS broth (De Man, Rogosa & Sharpe, Germany) was contaminated with the solution of AFB1 in methanol to make a final concentration of 5 µg/mL.

Culture conditions. 10 mL of the contaminated MRS (pH 6.5) were dispensed in tubes and inoculated with the strains and incubated for 48 h at 30°C. MRS contaminated with AFB1 and none inoculated, serving as a control assay, was analysed in the same conditions.

AFB1 determination by HPLC. Determination of AFB1 content in MRS after fermentation was carried by the method described by Gizzarelli *et al.* (1993). The extract was evaporated to dryness with an evaporator system and dissolved with 100 µL of chloroform.

The HPLC system consisted of a pump solvent delivery system, a programmable fluorescence detector and an ODS Hypersil (100 x 4 mm) column C18. The sample injection volume was set to 10 µL. The mobile phase (filtered & degassed) consisted of acetonitrile (15%), water (70%) and methanol (15%). The flow rate was 1mL/min. The detection wavelengths for excitation and emission were set at 360 and 420 nm, respectively. The retention time of AFB1 was approximately 6.3 min. The percentage of AFB1 removed from MRS broth was calculated by the following formula:

$$\%AFB1 = 100 \times [1 - (\frac{AFB1\ peak\ area\ of\ sample}{AFB1\ peak\ area\ of\ control})]$$

Effect of pH and temperature. The effect of temperature on the reduction of AFB1 by lactic acid bacteria was studied by incubation of each strain studied at 15, 25 and 37°C. Determination of AFB1 was done after 48 h of incubation. For the effect of pH, each strain of lactic acid bacteria studied was inoculated in triplicate in MRS broth contaminated with AFB1; the pH of the medium was adjusted to 5.5, 4.5, and 3.0 with a solution of lactic acid. AFB1 was quantified after incubation for 48 h at 30°C.

Statistical analysis. All assays in this study were carried out in triplicate. Statistical analysis of the data was carried out using Student's *t*-test (Microsoft® Windows XP, Excel version 5.1.2600) to identify significant differences between bacterial strains and different conditions. The results are considered to be statistically different at $p < 0.05$.

RESULTS AND DISCUSSION

Several strategies for the elimination or inactivation of mycotoxins have been reported in the literature (Galvano *et al.,* 2001). Nevertheless, only few of them have been accepted for practical use (ammonia treatment), and none is entirely effective. Some specialists are of the opinion that the best approach for decontamination of mycotoxins should be degradation by selected microorganisms (Bata & Lasztity, 1999).

The aflatoxin removing assay of sourdough strains of lactic acid bacteria are presented in Table I. The ability of lactic bacteria strains to reduce AFB1 after fermentation ranged from 1.8% to approximately 45%.

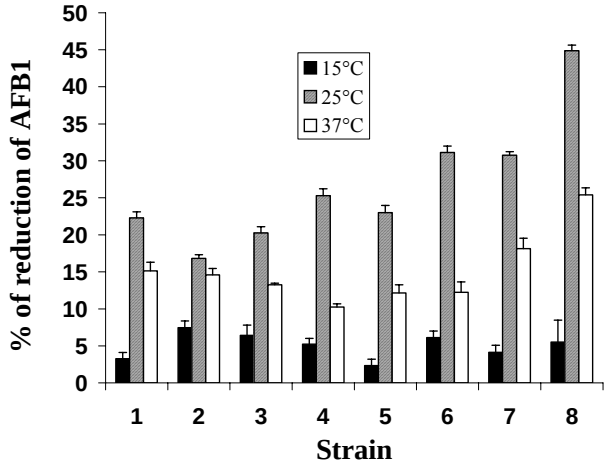
L. rhamnosus strains removed AFB1 from 23.01 to 44.89%. *L. lactis* strains Lb5 and Lb8 removed 16.81 and

Table I. AFB1 reduction *in vitro* by lactic acid bacteria (pH 6.5 at 30°C)

Strain	% of reduction ± SD*
Lactobacillus brevis Lb 1	4.46 ± 1.0^{c}
Lactobacillus casei Lc12	$22.28 \pm 1.3^{a,b}$
Lactobacillus lactis Lb5	16.81 ± 0.2^{d}
Lactobacillus lactis Lb8	$20.26 \pm 0.4^{a,b}$
Lactobacillus plantarum Lb7	2.14 ± 0.2^{c}
Lactobacillus plantarum Lb9	5.21 ± 0.7^{c}
Lactobacillus rhamnosus Lb 44	$25.27 \pm 1.5^{a,b}$
Lactobacillus rhamnosus Lb 21	$23.01 \pm 1.3^{a,b}$
Lactobacillus rhamnosus Lb 31	31.12 ± 0.9^{f}
Lactobacillus rhamnosus Lb 103	30.77 ± 0.4^{f}
Lactobacillus rhamnosus Lb 50	44.89 ± 2.1^{e}
Leuconostoc mesenteroides Ln 13	2.15 ± 0.5^{c}
Pediococcus acidilactici P5	1.80 ± 0.1^{c}

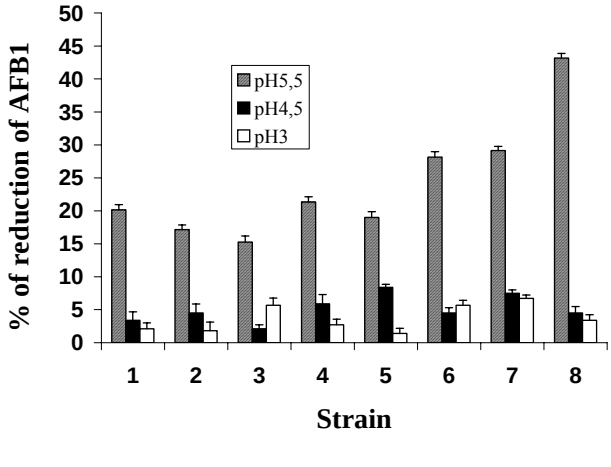
* Each Value is a mean of triplicate analyses ± SD (standard deviation). Means shearing the same letter dot not differ significantly at $p < 0.05$.

Fig. 1. Effect of temperature on the reduction of AFB1 by lactic acid bacteria. Error bars represent standard deviations (SD)



1: *L. casei* Lc12; **2**:*L. lactis* Lb5*;* **3:** *L. lactis* Lb8*;* **4***: L. rhamnosus* Lb21*;* **5***: L. rhamnosus* Lb31*;* **6***: L. rhamnosus* Lb21*;* **7***: L. rhamnosus* Lb103*;* **8***: L. rhamnosus* Lb50.

Fig. 2. Effect of pH on the reduction of AFB1 by lactic acid bacteria. Error bars represent standard deviations (SD)



1: *L. casei* Lc12; **2**:*L. lactis* Lb5*;* **3:** *L. lactis* Lb8*;* **4***: L. rhamnosus* Lb21*;* **5***: L. rhamnosus* Lb31*;* **6***: L. rhamnosus* Lb21*;* **7***: L. rhamnosus* Lb103*;* **8***: L. rhamnosus* Lb50.

20.26% AFB1, respectively. *L. casei* strain Lc12 removed about 22.28% AFB1; *L. brevis* strain Lb1 removed about 4.46% AFB1. *L. plantarum* strains Lb7 and Lb9 removed respectively 2.14 and 5.21% AFB1. *Leuconostoc mesenteroides* strain Ln13 removed about 2.15% AFB1 and *Pediococcus acidilactici* strain P55 removed 1.80% AFB1. *L. rhamnosus* strain Lb50 remove more AFB1 than other sourdough strains used in this study ($P < 0.05$). This is in agreement with other research carried out on the interactions between lactic acid bacteria and aflatoxins using *L. rhamnosus* strains GG and Lc705 which removed approximately 80% AFB1 (El- Nezami *et al.*, 1998) and *L rhamnosus* $Lc^{1/3}$ which removed about 54.6% AFB1 (Peltonen *et al*., 2001).

Sourdough strains of *L. casei* Lc12, *L. lactis* Lb5 and *L. lactis* Lb8 remove AFB1 in moderate amounts (16.81 to 20.26%). However, other strains used in this work such as *L. plantarum* strains, *P. acidilactici* P55, *L. mesenteroides* Ln33 and *L. brevis* Lb1 remove weak amounts of AFB1 (1.8 to 5.21%). Haskard *et al.* (2001) reported that viable cells of *L. lactis subsp lactis* and *L. casei Shirota* (YIT 901) remove 59 and 21.8% AFB1, respectively.

The effect of pH and temperature (Fig. 1 & 2) on the reduction of AFB1 showed that more AFB1 removal occurred at pH 5.5 (than pH 3 or pH 4.5) and at a temperature of 25°C (than 15 or 37°C) for all strains of lactic acid bacteria studied. These results suggest that a relationship between reduction of AFB1, pH of the medium and temperature of incubation typical of an enzymatic reaction could exist. Smiley and Draughon (2000) demonstrated that the degradation of AFB1 by *F. aurantiacum* is enzymatic. Other investigations reported the transformation of AFB1 by lactic acid bacteria into the non toxic aflatoxins B_{2a} in acidogenous yogurt (Megalla & Hafez, 1982) and showed also that the fermentation of yogurt and acidified milk contaminated with AFB1 reduced the amount of the toxin (Rasic *et al.*, 1991).

Even though the mechanism of AFB1 removal by Lactic acid bacteria is still unknown, it has been suggested that aflatoxins molecules are bound to the bacterial cell wall components of bacteria. Haskard *et al.* (2001) suggested that AFB1 is bound to the bacteria by weak noncovalent interactions, such as associating with hydrophobic pockets on the bacterial surface. Peltonen *et al.* (2001) showed the ability of both strains of lactic acid bacteria and strains of Bifidobacteria to remove the AFB1 from contaminated solution, the binding process was reversible and AFB1 was released by repeated aqueous washes.

More recently, other mycotoxins have been reported to be removed by specific strains of lactic acid bacteria. El-Nezami *et al.* (2002) demonstrated that strains of *L. rhamnosus GG* and *L. rhamnosus Lc-705* have the ability to remove zearalenone and its derivative α-zearalenol (55%) with a rapid reaction instantly after mixing with the bacteria. Turbic *et al.* (2002) showed that AFB1 (77- 99%) and ochratoxin A (36 -76%) were removed by *L. rhamnosus* strains in high and moderate amounts. In addition, only minimal amounts of other aromatic dietary substances such as caffeine, vitamin B12 and folic acid were also removed (9-28%).

CONCLUSION

Our studies demonstrate the ability of some strains of lactic acid bacteria (especially *L. rhamnosus, L. lactis and L. casei* strains) isolated from Moroccan sourdough ferments to reduce the initial concentration of AFB1 in MRS broth. *L. rhamnosus* strains significantly reduced more AFB1 as compared to other *Lactobacillus* strains used in this work ($P < 0.05$). The future trends are to include beneficial microorganisms in a process of dietary detoxification of contaminated foods to constitute an approach for reduction of the availability of aflatoxins in the human diet and animal feed.

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