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Short Communication

Detoxification of aflatoxin B_1 and patulin by *Enterococcus faecium* strains

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ABSTRACT

Aim of the present study was to investigate the detoxification of aflatoxin B_1 and patulin from aqueous solution by probiotic culture of *Enterococcus faecium* M74 and commercial culture of *E. faecium* EF031. The effect of the bacterial viability, incubation time and pH of the medium on the binding ability was tested. Also, binding stability was determined by washing the bacteria–mycotoxin complexes with phosphate buffer saline. Both M74 and EF031 strains have the ability to remove aflatoxin B_1 and patulin. While M74 removes 19.3 to 30.5% of aflatoxin B_1 and 15.8 to 41.6% of patulin, EF031 removes 23.4 to 37.5% of aflatoxin B_1 and 19.5 to 45.3% of patulin throughout a 48 h incubation period. The removal of aflatoxin B_1 and patulin was highest at pH 7.0 and 4.0, respectively. The stability of the aflatoxin B_1 and patulin complexes formed with the bacterial strains was found to be high. The viability of the bacteria did not have any significant effect on the detoxification of aflatoxin B_1 and patulin. Detoxification properties of *E. faecium* could represent new strategies for a possible application in the human diet and animal feed.

1. Introduction

Mycotoxins are food contaminants with harmful impact on human and animal health. Mycotoxin contamination is a severe problem in the world and is especially widespread in developing countries. Aflatoxin and patulin are within the most frequently found mycotoxins in human foods (El-Nezami et al., 1998; Fuchs et al., 2008).

Aflatoxins are a group of secondary metabolites of fungi that grow on a variety of food and feed commodities at any stage of production. They have detrimental effects on the health of humans and animals, including carcinogenic, mutagenic, teratogenic, and immunosuppressive effects (Eaton and Gallagher, 1994) and also cause economic losses in industry due to the contamination of food and feed (Haskard et al., 2001).

Patulin is a highly reactive unsaturated lactone produced by several species of *Penicillium*, *Aspergillus* and *Byssochlamys* (Moake et al., 2005). Patulin is found sometimes in higher concentrations as other mycotoxins in foods, especially in vegetables and fruits (in particular in apples and apple derived products) (Fuchs et al., 2008; Gökmen and Acar, 1999).

It has been stated that patulin has carcinogenic and teratogenic effects in animals (Dickens and Jones, 1961). It is of public health concern because of its potential carcinogenic properties (Park et al., 2001). It causes impairment of kidney functions, oxidative damage, and weakness of the immune system. It has also a negative impact on reproduction in males via interaction with hormone production (Fuchs et al., 2008; Selmanoglu and Kockaya, 2004).

Microorganisms, especially bacteria, have been studied for their potential to either degrade mycotoxins or reduce their bioavailability (El-Nezami et al., 1998; Fuchs et al., 2008; Haskard et al., 2001; Peltonen et al., 2001). Among bacteria, lactic acid bacteria (LAB) are the most important probiotic microorganisms typically associated with the human gastrointestinal tract. They are widely used in food industry because of their beneficial health effects in humans. One of the effects identified is the protection against toxins contained in foods such as heterocyclic aromatic amines, polycyclic aromatic hydrocarbons, amino acid pyrolysates and mycotoxins (Fuchs et al., 2008; Hosono and Hisamatsu, 1995; Knasmuller et al., 2001).

Several LAB strains of food or animal origin have been tested for their ability to bind aflatoxins and other mycotoxins to their surface (El-Nezami et al., 2002; Fuchs et al., 2008). Also, Peltonen et al. (2001) reported that different bacterium strains are able to bind aflatoxin B_1 (AFB_1) in significantly different amounts. Recently, Fuchs et al. (2008) showed that LAB strains can remove patulin and ochratoxin A from aqueous solution in different levels. Halttunen et al. (2008) stated that combining LAB strains together may be beneficial when several toxic compounds are removed together. However, in the same study, they suggest to use pure single strains to remove single compounds.

Enterococcus faecium is a member of LAB mostly found in nature, especially in foods and has various applications in the processing of some fermented dairy products (Giraffa, 2003). Nowadays, *E. faecium* is popular in cheese production as an adjunct culture. However, no investigations about the protective properties of *E. faecium* towards aflatoxin and patulin are available. Therefore, the objective of this study was to determine the removal of AFB_1 and patulin by two

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E. faecium strains (probiotic culture of *E. faecium* M74 and a commercial culture of *E. faecium* EF031). The effect of cell viability, incubation time, pH, and the stability of the bacteria–mycotoxin complexes were investigated.

2. Materials and methods

2.1. Chemicals and media

Brain Heart Infusion (BHI) Broth and Agar were obtained from Fluka (India), peptone water was purchased from Merck (Darmstadt, Germany). AFB_1 and patulin [4-Hydroxy-4H-furo[3,2-c]pyran-2(6H)-one] were obtained from Sigma-Aldrich (St. Louis, MO, USA). Phosphate buffered saline (PBS) was from Fluka (USA). All chemicals used in the experiments were obtained from Merck (Darmstadt, Germany), unless stated specifically.

2.2. Bacterial strains, culture conditions, and enumeration

Probiotic culture of *E. faecium* M74 and commercial culture of *E. faecium* EF031 were from Medipharm (Sweden) and Aroma-Prox (Cedex, France), respectively. These strains were selected based on either on available information regarding probiotic effects or on commercially available for food industry. *E. faecium* strains were cultivated in BHI Broth (24 h, 37 °C) for preparation of overnight cultures. Samples of 1 mL of cultivated bacterial suspensions were decimally diluted in sterile peptone water (0.1%, w/vol). The colony forming units in the overnight cultures were determined by plate counting on BHI Agar (Topcu and Bulat, 2010). In the present study, all incubations were carried out at 37 °C, and all centrifugations were at 5000 rpm for 15 min (5 °C) unless indicated otherwise.

2.3. Mycotoxin binding assay

Solid AFB_1 was suspended in benzene–acetonitrile (97/3, vol/vol) to obtain an AFB_1 concentration of 1000 mg/L. The benzene–acetonitrile was evaporated by nitrogen stream and AFB_1 was resuspended in methanol. The working solution of 5 mg/L AFB_1 was prepared in PBS at pH 7.0 by using a stock solution of AFB_1 in methanol (El-Nezami et al., 1998; Haskard et al., 2001). Solid patulin was suspended in ethyl acetate to obtain a patulin concentration of 40 mg/L (Gökmen and Acar, 1999). The ethyl acetate was evaporated by nitrogen stream and patulin was resuspended in PBS (pH 4.0) to obtain 1 mg/L of working solution. The patulin assay was studied at pH 4.0, because patulin has a high stability in the range of pH 3.5–5.5 (Drusch et al., 2007).

The cultures of each strain were divided in two aliquots. In one aliquot, a volume of the culture broth corresponding to approximately 1×10^{10} bacteria was transferred into tubes and centrifuged (viable cells). The second aliquot (approx. 1×10^{10} bacteria in tubes) was autoclaved at 121 °C for 20 min (nonviable cells) and centrifuged. Supernatants were removed and bacterial pellets (viable or nonviable cells) were washed with deionized water and centrifuged again (Peltonen et al., 2001; Topcu and Bulat, 2010). For each strain, either viable or nonviable bacterial pellets were resuspended in 2 mL of either AFB_1 (5 mg/L, pH 7.0) or patulin (1 mg/L, pH 4.0) working solutions in separate assays. Mycotoxin solutions without cells served as controls. The experiments were driven as following conditions.

To study the effect of incubation time on the reduction of mycotoxin concentration, samples were vortexed (Stuart, Staffordshire, UK) shortly (5 s) and incubated for 48 h on a Finemixer SH2000 orbital shaker (FINEPCR, Seoul, Korea) with soft agitation. Supernatant samples (500 µL) were collected by centrifugation after incubation for 1, 5, 24, and 48 h, and analyzed by HPLC (see below).

The effect of pH on the reduction of mycotoxin by bacterial strains was tested after incubation at 24 h. The pH of the PBS medium containing mycotoxins was adjusted to 3.0, 4.0, 5.0 and 7.0 with 1 M HCl.

The analyses of the effect of heat treatment on the ability of the bacteria to bind AFB_1 and patulin were performed according to the method as described above (pH and incubation time) (El-Nezami et al., 1998).

The stabilities of the bacteria-AFB_1 and bacteria–patulin complexes were evaluated by determining the amount of AFB_1 and patulin remaining bound following three washes. The binding assay was performed at pH 7.0 for AFB_1 or at pH 4.0 for patulin after incubation at 24 h. Bacterial pellets with bound AFB_1 or patulin were washed by suspending in 2 mL PBS (pH 7.0 for AFB_1, and pH 4.0 for patulin) containing no AFB_1 and patulin, and incubated for 10 min at 37 °C. The bacteria were pelleted, and a sample of the supernatant was collected for the quantification of AFB_1 or patulin released from bacteria. This washing procedure was repeated another two times (Haskard et al., 2001; Peltonen et al., 2001).

2.4. Quantification of mycotoxins by HPLC

Samples of the supernatant fluids were analyzed for AFB_1 and patulin residues using a reverse-phase HPLC method with no sample extraction step (El-Nezami et al., 1998; Peltonen et al., 2001). To quantify AFB_1, a ThermoFinnigan SpectraSystem HPLC system (Thermo Separation Products, CA, USA) equipped with a fluorescence detector (FL3000) was used. Separation was achieved by using a Phenomenex Jupiter C18 wide-pore analytical column (250×4.6 mm, 5 µm, Torrance, CA, US) at 30 °C with an injection volume of 20 µL. AFB_1 was eluted isocratically with mobile phase of deionized water/acetonitrile/methanol (60/30/10; vol/vol/vol) at a 0.8 mL/min flow rate. The detection wavelengths for excitation and emission were set at 360 and 440 nm, respectively.

For patulin quantification, the HPLC system as given above equipped with a photodiode-array detector (UV6000LP) was used. Patulin concentrations were determined isocratically using the method of Gökmen and Acar (1999) with some modifications. Briefly, 20 µ L of the sample was injected to a Phenomenex Jupiter C18 wide-pore analytical column (250×4.6 mm, 5 µm) at 40 °C and deionized water/acetonitrile (9/1; vol/vol) was used as the mobile phase at a 0.5 mL/min flow rate. The detection wavelength was set at 276 nm.

The percentage of mycotoxins bound to the bacteria was calculated using the equation:

$$\%\ Reduction = 100\ x\left(1 - \left[\frac{mycotoxin\ peak\ area\ of\ sample}{mycotoxin\ peak\ area\ of\ control}\right]\right)$$

2.5. Statistical analysis

All the analyses were carried out in triplicate and the values represented as the mean values. Data were analyzed by ANOVA using SPSS 13.0 statistical package (SPSS Inc., Chicago, III., U.S.A.). The results are considered to be statistically different at $P<0.05$. Tukey test was used for comparing treatment means.

3. Results

E. faecium M74 and EF031 bound AFB_1 and patulin with different efficiency ($P<0.05$). While M74 bound approx 19.3 to 30.5% of AFB_1 and 15.8 to 41.6% of patulin, EF031 bound approx 23.4 to 37.5% of AFB_1 and 19.5 to 45.3% of patulin. The small differences in binding ability between viable and nonviable cells were not statistically significant in all assays (Table 1).

The effects of incubation time on mycotoxin binding by viable and nonviable cells are given in Table 1. As a general trend, the extent of removal of AFB_1 and patulin increased with extended incubation time ($P<0.05$), independently on cell viability, for both strains. However, in contrast to AFB_1 removal, patulin reduction rate increased

Table 1
Effect of incubation time on AFB_1 (at pH 7.0) and patulin (at pH 4.0) detoxification by *E. faecium* strains.

Incubation time (h)		*E. faecium* M74		*E. faecium* EF031	
		Viable	Nonviable	Viable	Nonviable
Reduction of AFB_1 (%)	1	22.0 ± 2.49^a	19.3 ± 2.71^a	23.4 ± 1.60^a	27.2 ± 2.04^a
	5	27.5 ± 3.37^b	25.5 ± 3.58^b	30.6 ± 1.46^b	34.0 ± 1.97^b
	24	30.5 ± 1.87^b	27.1 ± 3.15^b	35.1 ± 2.01^b	33.5 ± 1.53^b
	48	29.0 ± 2.29^b	29.3 ± 3.04^b	33.7 ± 2.41^b	37.5 ± 2.09^b
Reduction of patulin (%)	1	15.8 ± 2.40^a	16.1 ± 0.16^a	21.1 ± 0.18^a	19.5 ± 0.09^a
	5	25.0 ± 0.70^b	23.5 ± 1.07^b	22.4 ± 0.81^a	19.7 ± 0.94^a
	24	39.9 ± 2.31^c	35.3 ± 1.24^c	35.8 ± 1.11^b	30.4 ± 1.67^b
	48	41.6 ± 1.70^c	38.6 ± 1.41^c	45.3 ± 0.55^c	36.4 ± 1.15^c

Each value is a mean ± (standard deviation) of triplicate assays.
Different letters in the column for each assay are significantly different ($P<0.05$).

continuously depending on incubation time for viable and nonviable cells of both strains.

The effect of pH on AFB_1 and patulin reduction by *E. faecium* strains was given at Table 2. Removal of AFB_1 by nonviable cells of M74, and viable and nonviable cells of EF031 was unaffected by pH in the range from 3.0 to 7.0. However, the effect of pH on AFB_1 removal by viable cells of M74 was statistically significant ($P<0.05$). The amount of removed AFB_1 by M74 was maximum (28.6%) at pH 7.0 and minimum (21.1%) at pH 5.0.

The maximum removal of patulin was occurred at pH 4.0 by both of the strains (Table 2). While the removal of patulin by viable and nonviable cells of M74 strain was 43.5 and 37.1%, viable and nonviable cells of EF031 detoxified 35.5 and 31.4% at pH 4.0, respectively. The detoxification of patulin by M74 and EF031 strains was decreased at higher pH values. The removal of patulin at pH 7.0 was the smallest (data not shown). But, depending of low stability of patulin at pH 7.0, the values obtained at that pH were not taken into consideration.

Binding stability of bacteria–mycotoxin complex was given at Fig. 1. Small amounts of bound mycotoxins were released by *E. faecium* strains at first wash ($P<0.05$). However, the complex was significantly more stable after first wash. Only 17 to 23% of bound AFB_1 and 19 to 25% of bound patulin were released from the complex after three washes. Binding stability between viable and nonviable cells was not statistically significant. The pH of washing solution (3.0, 4.0, 5.0, and 7.0), except pH 7.0 for patulin, did not affect the binding stability of bacteria–mycotoxin complex (data not shown).

4. Discussion

This study has been the first to demonstrate that specific strains of *Enterococcus* species have significant ability to bind both AFB_1 and patulin from aqueous solutions. Both of the *E. faecium* strains were able to remove AFB_1 and patulin, but in different amounts. EF031 was more efficient than M74. This may be a result of different cell wall structure. Similarly, Peltonen et al. (2001) noted that the differences in AFB_1 binding by the strains were probably due to different bacterial cell wall and cell envelope structures. It was reported that *Lactobacillus rhamnosus* strain GG and *L. rhamnosus* strain LC705 can significantly remove AFB_1 when compared with other strains of either Gram-positive or Gram-negative bacteria (El-Nezami et al., 1998).

Table 2
Effect of pH on AFB_1 and patulin detoxification by *E. faecium* strains at 24 h.

pH		*E. faecium* M74		*E. faecium* EF031	
		Viable	Nonviable	Viable	Nonviable
Reduction of AFB_1 (%)	3.0	26.7 ± 1.10^b	24.7 ± 2.21	32.4 ± 3.02	30.7 ± 1.50
	4.0	26.2 ± 2.37^{ab}	24.4 ± 1.67	33.4 ± 2.44	31.6 ± 1.73
	5.0	21.1 ± 2.19^a	23.8 ± 2.68	31.5 ± 2.19	30.4 ± 0.64
	7.0	28.6 ± 2.24^b	28.1 ± 0.80	31.1 ± 2.12	31.9 ± 2.04
Reduction of patulin (%)	3.0	38.6 ± 2.35^{ab}	33.7 ± 1.56^{ab}	28.7 ± 0.37^a	18.7 ± 1.04^a
	4.0	43.5 ± 2.11^b	37.1 ± 2.55^b	35.5 ± 1.45^b	31.4 ± 1.99^c
	5.0	32.9 ± 1.04^a	29.2 ± 0.88^a	35.7 ± 0.63^b	25.2 ± 0.86^b

Each value is a mean ± (standard deviation) of triplicate assays.
Different letters in the column for each assay are significantly different ($P<0.05$).

In our study, viable and nonviable cells of *E. faecium* strains responded in a similar manner and binding ability was statistically insignificant. Thus, it can be said that detoxification of AFB_1 by *E. faecium* is due to the binding of the mycotoxin to the bacterial cell wall components, a mechanism which has also been postulated by other studies (El-Nezami et al., 1998; Haskard et al., 2000; Haskard et al., 2001). Bacterial cell wall peptidoglycans and polysaccharides have been suggested to be responsible components for the mycotoxin binding by bacteria (Hosono et al., 1988). Oatley et al. (2000) noted that as heat treated bacteria were often more efficient to remove AFB_1 than viable cells, metabolic degradation cannot be the mechanism responsible for AFB_1 removal. Also, they said that the toxin was bound to the bacterial surface. However, there was no significant difference between the removal ability of viable and boiled cells of *L. lactis* ssp. *cremoris* ARH74 (Pierides et al., 2000). It can be concluded that the removal of mycotoxins from aqueous solution by viable or nonviable cells is strain dependent and each strain may response in a different manner. Unaltered detoxification capacity of nonviable bacteria is an important trait from a practical standpoint because the viability of bacteria decreases while passing through the stomach due to its low pH.

Conversely, a different mechanism could explain patulin detoxification because it was removed more efficiently than AFB_1 by viable *E. faecium* cells. Similarly, Fuchs et al. (2008) reported that viable bacteria bind patulin more efficiently than heat treated cells, which can indicate that metabolic conversion of toxins by release of specific enzymes may take place.

Peltonen et al. (2001) reported that the removal of AFB_1 was a rapid process and the AFB_1 binding by *Lactobacillus amylovorus* CSCC 5160 increased from 52.6 (0 h) to 73.2% (72 h). In another study, it was observed that at 0 h the percentage of removed AFB_1 was not significantly different from that at 72 h (El-Nezami et al., 1998). In the present study, at 1 h, M74 and EF031 strains removed AFB_1 which was approximately 65% of total removed AFB_1 throughout the whole incubation period of 48 h. It suggests that the detoxification of AFB_1 by M74 and EF031 strains is a rapid process. Also, the amount of removed AFB_1 by the viable cells of both strains increased with incubation time from 1 h to 24 h and then, it declined after 24 h. This shows that AFB_1 was not bound strongly by viable cells of M74 and EF031 strains, and that some AFB_1 could be released back into solution from bacteria-AFB_1 complex after 24 h. However, the detoxification of patulin by viable and nonviable cells of both strains increased with incubation time.

pH (range from 3.0 to 7.0) did not affect the detoxification of AFB_1 by the strains except viable cells of M74. Similarly, Haskard et al. (2001) noted that binding of AFB_1 to *L. rhamnosus* GG was not affected by pH in the range from 2.5 to 8.5, suggesting that a cation-exchange mechanism was not operating. In our study, the pH dependency of removal of patulin differs from that found with AFB_1. The pH profile of patulin detoxification suggests that there may be an effect of hydrogen bound interactions on removal of patulin by bacteria. In similar, Fuchs et al. (2008) found that optimal removal of patulin occurred at low pH values (pH 5.0).

The findings obtained from the washing of mycotoxin–bacteria complex suggest that the binding of mycotoxins (AFB_1, patulin) to *E. faecium* cell surface may be a reversible process. Also, the stability of the complexes formed between *E. faecium* strains and AFB_1 or patulin was strain specific. Similarly, it was reported that variable amounts of AFB_1 bound by bacteria were released back into the solution when washing the *Lactobacillus*-AFB_1 complexes (*L. rhamnosus* LC, *L. amylovorus* CSCC 5160 and CSCC 5197) (Peltonen et al., 2001). In

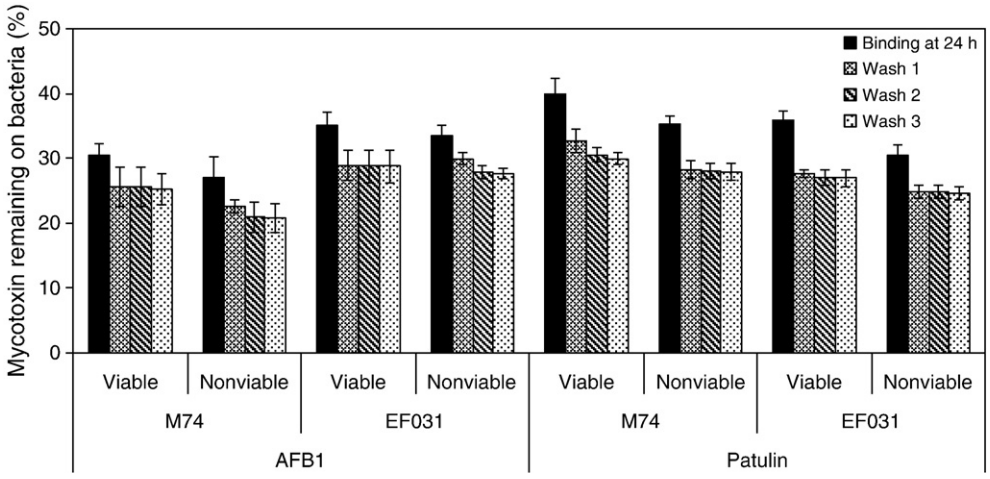

Fig. 1. Effect of washing bacterial pellets with phosphate saline buffer on releasing AFB_1 and patulin bound with viable and nonviable *E. faecium* strains.

addition, Haskard et al. (2001) noted that heat treatment significantly enhanced the stability of the complex formed with AFB_1 for *L. rhamnosus* GG and *L. rhamnosus* LC 705 strains. In contrast, there was no significant difference between viable and heat treated bacteria at binding stability of AFB_1 and patulin in our study.

In conclusion, the results of our investigation show that M74 and EF031 strains of *E. faecium* have the capacity to remove AFB_1 and patulin from aqueous solution. They can be used in the manufacturing of fermented dairy foods such as cheese; hence our results may be beneficial to the development of strategies for detoxification of contaminated foods and reduction of the bioavailability of aflatoxin and patulin in the human diet and animal feed. In addition, *E. faecium* has a potential to use in combination with other LAB strains. It needs to be studied in future experiments.

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