International Journal of
Food Science & Technology
International Journal of Food Science and Technology 2012, **47**, 1647–1653

Original article

Evaluation of aflatoxin decontaminating by two strains of *Saccharomyces cerevisiae* and *Lactobacillus rhamnosus* strain *GG* in pistachio nuts

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(Received 18 October 2011; Accepted in revised form 25 February 2012)

Summary In this study, the surface binding ability of *Saccharomayces cerevisiae* and *Lactobacillus rhamnosus* GG (LBGG) to aflatoxin in pistachio nuts was compared. Results showed that *Saccharomayces cerevisiae* and *Lactobacillus rhamnosus* strains had aflatoxin binding ability of 40% and 35% with initial concentration of 10 ppb and 70% and 60% with initial concentration of 20 ppb aflatoxin, respectively. Acid treatment increased this ability for yeast and bacterium to 60% and 85% in first concentration and 73% and 90% for second concentration of aflatoxin, respectively. Also, heat treatment could raise surface binding of yeast to 55% and 75% for two concentrations. In addition, heat condition for *Lactobacillus* improved binding to 85% and 90% for two concentrations of aflatoxin. Experiments showed that microorganism's immobilisation on contaminated pistachio had no effect on qualitative characteristics of pistachio such as colour, texture and peroxide value.

Keywords Aflatoxin, *Lactobacillus rhamnosus*, pistachio nut, *Saccharomayces cerevisiae*, surface binding.

Introduction

Mycotoxins are secondary metabolites produced by filamentous fungi cause a toxic response (mycotoxicosis) when ate by animals and humans. The most generally known mycotoxins are the aflatoxins that are naturally produced principally by two types of mould: *Aspergillus flavus* and *Aspergillus parasiticus* (Dakovic *et al*., 2008; Fernandez-Ibanez *et al*., 2009; Reddy *et al*., 2009).

Aspergillus flavus is a frequent constituent of the microflora in air and soil throughout the world. It is common in stored wheat, corn, and cotton seed, rice, barely, bran, flour, peanuts, tree nuts (pistachio, walnut and hazelnut), sorghum and other commodities (Park *et al*., 2000). In a study, Reddy *et al*. (2011) reported accumulation of AFB_1 by *A. flavus* strains on cereal grains such as barely, maize, rice, wheat and sorghum ranging from 785.6 to 15645.2 μg kg^{-1}. Different types of aflatoxins, such as B_1, B_2, G_1 and G_2, have already been identified. Aflatoxin B_1 (AFB_1), that is potent carcinogenic, mutagenic, hepatotoxic and immunosuppressive metabolite, has been distinguished by IARC and their presence in pistachio nuts are heavily monitored and regulated to ensure a safe food supply (Dorner *et al*., 2003; Bircan *et al*., 2008). There are many reports in the presence of aflatoxins in foods and feeds, which lead to serious problems such as economic loss in addition to their carcinogenic potential (Mabrouk & EL-Shayeb, 1992).

Aflatoxin contamination of pistachio can occur in the field (preharvest) when severe late-season drought stress occurs and during storage (postharvest) when abnormal climatic conditions of moisture and temperature exist (Dorner *et al*., 2003). Developing of new strategies to control the aflatoxin problem is obtained by understanding the effects of these factors on aflatoxin production (Dakovic *et al*., 2008; Fernandez-Ibanez *et al*., 2009; Reddy *et al*., 2009). The EU Official Journal has established action levels for AFB_1 incidence in all feed materials between 5 and 20 ppb (CE, 2003).

The pistachio nut is as one of the main commercial product of Iran. Pistachio nuts are a rich source of essential fatty acids, which are essential for the human diet. Developing strategies to control aflatoxin and to reduce the level of aflatoxin from agricultural commodities are reasonably considerable for about 15 years. Other decontamination approaches include food and feed processing, such as chemical, physical and biological methods.

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doi:10.1111/j.1365-2621.2012.03015.x

Any degradation process must be technically and economically feasible. However, no universally applicable, effective and practical methods are currently available. Therefore, it is important to find a large-scale, practical and cost-effective method for detoxifying aflatoxin contaminated feedstuffs. A wide array of organisms, including bacteria, yeasts and fungi, have been tested for the biological control of aflatoxin contamination. Evidently, the best decontaminant should be detoxification by biodegradation, giving a possibility for the removal of mycotoxins under mild condition. Several reports have been published about AFB_1 reduction by some bacteria. In a research by Farzaneh *et al.* (2012), *Bacillus subtilis* UTBSP1 could considerably remediate AFB_1 from nutrient broth culture and pistachio nut by 85.66% and 95%, respectively. The best conditions for enzymatic degradation of AFB_1 were at 35–40 °C for 24 h.

Among the different decontaminating microorganisms, we selected *Saccharomyces cerevisiae* and *Lactobacillus rhamnosus* GG (LBGG) representing unique groups, which are widely used in food industry (Shetty & Jespersen, 2006) as an effective microorganism for the removal of aflatoxin in pistachio. In this research, we used the applied procedure of cell immobilisation of microorganisms in sodium alginate which is a polymer that was used as a coating for the pistachio samples. The objective of this study is the comparison of biological decontamination of aflatoxin through surface adsorption of *S. cerevisiae* and *L. rhamnosus* GG (*LBGG*) immobilised in calcium alginate.

Materials and methods

Chemicals and microorganisms

Aflatoxin solution was obtained from Sigma Co. (St Louis, MO, USA) and methanol, acetone, chloroform, n-hexane, phosphate-buffered saline (PBS), acetonitrile, potassium bromide and all culture media used in this research were supplied by Merck Co (Stutgart, Germany). All chemicals were high-performance liquid chromatography (HPLC) or analytical grade. Ultrapure water (Milli-Q system Millipore, France) was used for analytical purposes. Calcium chloride was purchased from Scharlau Co. (Germany) and sodium alginate from BDH Co. (Dubai, UAE). *S. cerevisiae* (ATCC 9763) was obtained from a Persian-type culture collection in Iran. *L. rhamnosus* GG (ATCC 53103) was provided from Probiotical Company in Italy.

Sample preparation

Akbari is the main pistachio cultivar grown in Iran; therefore, this cultivar was selected for this study. Freshly harvested pistachios were purchased from Kerman, Iran. Pistachios were then subjected to de-hulling, washing and sorting (to keep apart nuts from nonsplit ones). Finally, samples were kept at −20 °C before experiments.

Culture preparation

Yeast and bacterium strains grown in yeast mould broth (YM Broth) and MRS broth media for 48 h at 25 °C and 37 °C were used as inoculums, respectively. Erlenmeyer flasks containing 100 mL of selected broth medium were inoculated with the desired culture at 10^2 cells mL^{-1} and incubated at 25 °C and 37 °C until the cells reached an OD_{600} of $1.8–2 \times 10^{10}$ cells mL^{-1}. Cells harvested at the required OD were centrifuged at 5000 ***g*** for 10 min, washed twice with PBS (pH = 6.0), turning of cells 5000 ***g*** for 10 min after every washing. Heat-treated (autoclaved in 10 mL PBS at 120 °C for 20 min) and acid-treated (incubated at 25 °C and 37 °C for yeast or LBGG in 10 mL 2 M HCl for 90 min with mild shaking) samples were washed twice with 4 mL PBS and centrifuged at 5000 ***g*** for 10 min prior to use (Nezami *et al.*, 1998; Shetty *et al.*, 2006). Each test was conducted in triplicate. All experimental work with microorganisms was done in a microbiological hood. Protective clothes and gloves were worn during the experiments. Aflatoxin analysis has been carried out immediately after each trial.

Cell immobilisation

In this study, a sodium alginate solution (3%) was prepared by dissolving sodium alginate in 100 mL boiling water and autoclaved at 121 °C for 15 min. Cells were harvested in every approach of treatment, resuspended in 2 mL of saline and added to sterilise alginate solution. To immobilise the cells in alginate, pistachio was dipped in a 0.2 M $CaCl_2$ solution immediately and incubated for 1.5, 3, 8 or 12 h at 25 °C and 37 °C for yeast and LGG, respectively (Beshay, 2003; Peinado *et al.*, 2006).

Extraction and clean-up procedures for HPLC analysis

Samples were analysed using a HPLC method (AOAC, 1995). A ground pistachio sample (50 g) was blended with 2.5 g of NaCl and 200 mL of methanol in water solution (80%) for 5 min. Then, the mixture was filtered through Whatman filter paper. After filtration, the extract was diluted with water and filtered through a glass microfibre filter. Aflatest IACs were used to clean up the samples. 15 mL of PBS were first passed during the IAC. Then, 70 mL of the filtrate were passed through the IAC at a flow rate of ca.1 drop s^{-1}. The column was washed with 15 mL water and dried under vacuum condition. Finally, AF was eluted with

methanol using the following procedure. First, 0.5 mL of methanol was applied on the column which passed through by gravity. After 1 min, the second part of 1 mL of methanol was applied and collected. The eluate was diluted with water and analysed by HPLC (AOAC, 1995).

Bound aflatoxin measurement

Percentage of binding was determined from the amount of unbound AFB_1 remaining on the pistachio after incubation when compared to control (without microorganisms' cells) before coating with alginate: % Bound = 100 [1 – counts in the sample after coating/counts in control].

Colour measurement

Pistachio nut colour (before and after coating) was determined using a colourimeter (Minolta CR 300 series; Minolta Camera Co., Ltd., Osaka, Japan) that measures lightness (L) and chromaticity parameters a [a (+) = red, a (–) = green] and b [b (+) = yellow, b (–) = blue]. The total colour difference (ΔE) was calculated using the following equation, where L*, a*, and b* refer to the colour reading of the pistachio sample without coating. Uncoated pistachio was used as a reference and a ΔE donates colour change from the reference material. All measurements were performed in triplicate.

$$\Delta E = \sqrt{(L^* - L)^2 + (a^* - a)^2 + (b^* - b)^2} \qquad (1)$$

where L^*, a^* and b^* are the colour parameter values of the reference and L, a and b are the colour parameter values of the sample.

Texture measurement

Pistachio texture changes were measured by compressing samples with a Testometric apparatus (M350-10CT; Rochdale, UK). The greatest force (N) required to compact the sample was recorded as the firmness of the pistachio sample. Pretest speed, test speed and post-test speed were all set at 10 mm min^{-1}. Triplicates of each treatment were evaluated.

Peroxide value measurement

The peroxide value of the pistachio nuts samples were measured by using the iodometric titration method recommended by AOAC (1995). The peroxide value was calculated as meq of peroxide kg^{-1} of oil. All chemicals used in this experiment were purchased from the Merck (Darmstadt, Germany). All measurements were carried out in triplicate.

$$\text{Peroxide value} = \frac{(S - B) \times Nthiosulfa\ t \times 1000}{\text{Weight of sample(g).}} \qquad (2)$$

where S = mL of $Na_2S_2O_3$ for sample, B = mL of $Na_2S_2O_3$ for blank and N = the concentration of the $Na_2S_2O_3$ solution.

Statistical analysis

Experiments were conducted in triplicate and data sets were subjected to the analysis of variance (ANOVA) and the Duncan's multiple range tests using Minitab software (Minitab 16; Minitab Inc., Minneapolis, MN, USA). In all cases, a value of $P < 0.05$ was considered significant. The data were expressed as mean ± standard deviation (SD).

Results

One of the most proper methods for cell immobilisation is its entrapment in calcium alginate because this procedure is quite simple and economical (Kourkoutas *et al*., 2004). This procedure in food science is an interesting and rapidly expanding study area because of its technical and economic advantages compared with a free cell system. However, for applications in the food industry, immobilisation supports should satisfy additional fundamentals (Kourkoutas *et al*., 2006).

Immobilisation comprises methods that involve the trapping of the biocatalyst into a network formed by one or several polymers such as carrageenan, alginate or synthetic resins (Peinado *et al*., 2006). Alginic acid is a copolysaccharide extracted from brown algae consisting of D-mannuronic and L-guluronic acid monomers. Sodium alginate is a water soluble salt of alginic acid, a naturally occurring nontoxic polysaccharide (Cha *et al*., 2002).

This natural polymer was selected as a support for immobilising yeast and bacterium on pistachio samples (Sallam *et al*., 2005). It shows to be a valuable way for entrapping of microorganisms to bind to AFB_1. *S. cerevisiae*, the strain used in this study, is recognised as bakery yeast and its benefits have been proven in several ways (Madrigal-Santilla′n *et al*., 2006). According to previous studies, it is known that the cell wall of *S. cerevisiae* consists of network of β-1, three backbones with β-1, six glucan side chains, which in turn are attached to highly glycosylated mannoproteins that form the external layer. The glucans and proteins provide numerous easily accessible binding sites with different binding mechanisms, such as hydrogen binds, ionic bonds or hydrophobic interactions. Approximately 2×10^9 CFU mL^{-1} was required for significant AFB_1 elimination. This observation agrees with the findings of Line & Brackett (1995), indicating that viable cell populations of 1×10^9 CFU mL^{-1} or greater were

necessary for significant removal of AFB_1. *L. rhamnosus* GG is currently used in various dairy products, introduced as a probiotic bacterium and has beneficial effects on healthy. According to previous studies, carbohydrate and protein components of the bacterium surface are important for AFB_1 binding like as yeast. That is, peptidoglycans in cell wall are important factors for AFB_1 removal and heat treatment does not reduce this binding (Gratz *et al*., 2007). Results indicate that the binding stage is a quick process that reaches to maximum point after 3 h. Figures 1 and 2 indicates aflatoxin binding at three treatments including viable cells, heat treated and acid treated by means of S. *cerevisiae* ATTC 9763 was immobilised on pistachio with 10 and 20 ppb AFB_1. These figures demonstrate that heating, even at 120 °C for 20 min, could increase yeast binding ability to 55% and 75% with primary AFB_1 concentrations of 10 and 20 ppb, respectively. Also, the cell treatment under acid condition could increase the binding ability of yeast to 60% and 73% for two investigated concentrations of aflatoxin, respectively.

Results for LBGG are shown in Figs 3 and 4, which is similar to yeast. Binding percentage of viable cells from 35% and 60% increased to 85% and 90% for heat treatment, using two different concentrations of aflatoxin. As well, binding ability of bacterium in acidic condition reached to 85% and 90% for 10 and 20 ppb of aflatoxin. Yeast binding ability in the exponential phase was highest comparable with LBGG binding (Table 1).

Effect of immobilisation on pistachio qualitative characteristics

Colour changes

Colour measurements provide an objective index of food quality. Table 2 shows the *L*, *a*, and *b* Hunter Lab values and total colour difference (ΔE) of pistachio nuts with viable yeast. In addition, these parameters for pistachio nuts with viable LBGG (without acid or heat treatment) were 54.88 ± 1.45, 12.13 ± 1.5 and 14.77 ± 1.5 (Table 3).

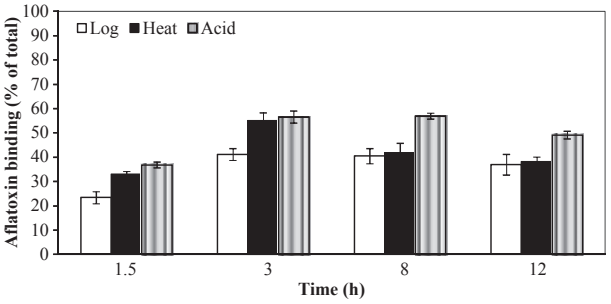

Figure 1 Aflatoxin binding at three treatments: Exponential phase, Acidic and Heat condition, *Saccharomayces cerevisiae* ATTC 9763 was grown at 25 °C were immobilised on pistachio with 10 ppb AFB_1.

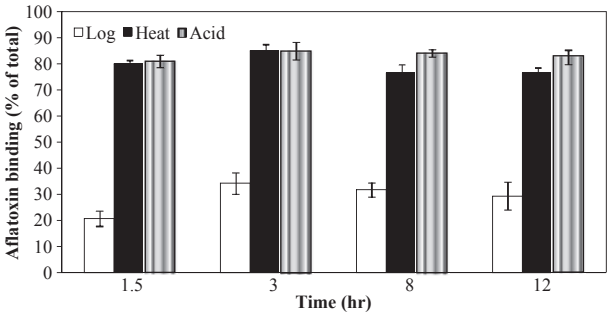

Figure 3 Aflatoxin binding at three treatments: Exponential phase, Acidic and Heat condition, *LBGG* was grown at 37 °C were immobilised on pistachio with 10 ppb AFB_1.

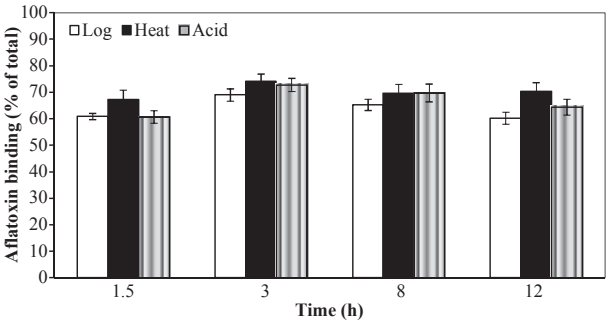

Figure 2 Aflatoxin binding at three treatments: Exponential phase, Acidic and Heat condition, *Saccharomayces cerevisiae* ATTC 9763 was grown at 25 °C were immobilised on pistachio with 20 ppb AFB_1.

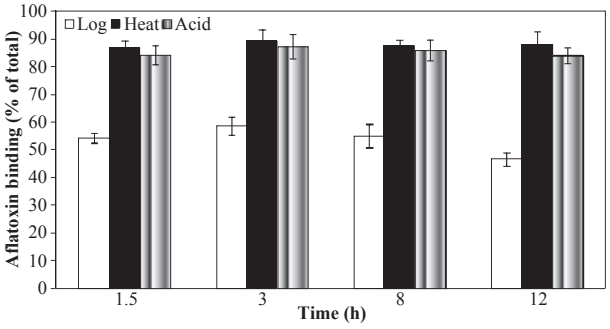

Figure 4 Aflatoxin binding at three treatments: Exponential phase, Acidic and Heat condition, *LBGG* was grown at 37 °C were immobilised on pistachio with 20 ppb AFB_1.

Table 1 The comparison of aflatoxin binding ability (%) and the amount of unbound AFB1 after coating with yeast and bacterium strains in the nuts contaminated with 10 and 20 ppb AFB_1

Contamination level (ppb)											
10 ppb AFB1						20 ppb AFB1					
Saccharomyces cerevisiae			*Lactobacillus rhamnosus*			*Saccharomyces cerevisiae*			*Lactobacillus rhamnosus*		
Viable	Heat	Acid	Viable	Heat	Acid	Viable	Heat	Acid	Viable	Heat	Acid
6 ppb (40%)[c]	4.5 ppb (55%)[b]	4 ppb (60%)[b]	6.5 ppb (35%)[c]	1.5 ppb (85%)[a]	1.5 ppb (85%)[a]	6 ppb (70%)[b]	5 ppb (75%)[b]	5.4 ppb (73%)[b]	8 ppb (60%)[c]	2 ppb (90%)[a]	2 ppb (90%)[a]

*values with same letter have no significant difference.

Table 2 Changes in colour values of coated pistachio samples treated with *Saccharomyces cerevisiae* for 12 h

Time (hr)*,†	L	a	b	ΔE
0	41.81 ± 1.25	13.38 ± 1.21	14.98 ± 0.28	0.28 ± 0.07
1.5	41.57 ± 2.26	13.09 ± 0.23	14.95 ± 0.65	0.25 ± 0.12
3	41.82 ± 0.85	13.22 ± 0.98	14.50 ± 1.22	0.40 ± 0.08
8	41.46 ± 0.67	13.48 ± 1.09	15.77 ± 1.03	1.02 ± 0.19
12	41.15 ± 1.05	13.69 ± 1.12	14.42 ± 0.47	0.99 ± 0.11

*Values are mean ± SD of three replications.
†No significant different ($P < 0.05$) was observed in all of data.

Table 3 Changes in colour values of coated pistachio samples treated with *Lactobacillus rhamnosus* for 12 h

Time (hr)*,†	L	a	b	ΔE
0	54.88 ± 1.45	12.13 ± 1.50	14.77 ± 1.50	13.10 ± 0.78
1.5	54.78 ± 2.45	12.14 ± 1.15	14.60 ± 1.15	13.00 ± 1.25
3	54.50 ± 1.65	12.50 ± 1.22	14.30 ± 2.22	12.71 ± 1.42
8	54.80 ± 2.85	12.65 ± 1.75	14.80 ± 1.35	12.99 ± 1.39
12	55.90 ± 2.15	12.70 ± 2.5	14.70 ± 1.05	14.09 ± 1.74

*Values are mean ± SD of three replications.
†No significant different ($P < 0.05$) was observed in all of data.

However, no significant ($P > 0.05$) difference in colour parameter values for these viable microorganisms were detected.

Texture analyses

Texture is a mechanical behaviour of foods measured by sensory or physical means. Hardness and fracture force are widely used in nuts as textural attributes. In this study, the immobilisation of cells (yeast or bacterium) did not affect coated pistachio nuts and did not cause any changes in texture (Table 4).

Peroxide value

The peroxide value is an index for the quality of pistachios describing their fat content and freshness. Peroxides can also react with proteins and vitamins causing losses in nutritional value and functionality properties of nuts. The results of peroxide values did not show any considerable changes after immobilisation of yeast or bacterium cells on pistachio nut samples (Table 4).

Table 4 Peroxide value and required force for punch experiment of coated pistachio nuts by sodium alginate

Before coating		After coating	
Saccharomyces cerevisiae	*Lactobacillus rhamnosus*	*Saccharomyces cerevisiae*	*Lactobacillus rhamnosus*
Peroxide value (meq peroxide kg^{-1} oil)			
0.226 ± 0.005	0.235 ± 0.003	0.212 ± 0.007	0.228 ± 0.001
Texture (Punch force, *N*)			
22.68 ± 0.27	23.50 ± 0.09	21.28 ± 0.34	22.80 ± 0.12

Discussion

Appearance characteristics particularly colour is an important factor in a product's acceptability to consumers. Hunter colourimetry showed no significant changes in colour after coating of pistachio for AFB_1 removal. This can probably be attributed to the transparency of alginate and also its very thin thickness.

Texture is one of the most important attributes used by consumers to assess food quality. According to the analysis of variance (ANOVA) of texture, differences in coated and uncoated pistachio nuts were not significantly different.

As hydroperoxides are the major products of lipid oxidation, their content, that is peroxide value, is often used as an indicator for the initial stages of oxidation (Maskan & Karatas, 1999). Peroxide value of all pistachio nut samples was not significantly different. This may be due to the fact that alginate is permeable to air and O_2.

Our results demonstrate that the qualitative attributes of pistachio nuts contaminated with different amounts of aflatoxin did not significantly change under microbial

cell immobilisation. However, to the best of our knowledge, reports on these characteristics are relatively limited.

With increasing knowledge about AFB_1 as a potent source of health hazards to human, a great deal of effort has been made to eliminate it completely or reduce its content in nuts to significantly lower levels. Physical methods for aflatoxin degradation have less harmful effects than chemical ones on nutritional value. Yazdanpanah *et al*. (2005) reported that roasting as an effective method to reduce aflatoxin content in nuts and decreased it about 17–63% in pistachio nut samples. However, reduction of aflatoxin by this physical method is time- and temperature-dependent and may have some effects on nutritional contents and changes of physical appearance of pistachio nuts. However, this study shows that microbiological reduction in aflatoxin varies from 30% to 90% depending on used strains without any significant changes in qualitative attributes such as colour or peroxide value of pistachio nuts.

Bueno *et al*. (2007) proposed an absorption model for aflatoxin binding. This model estimated two parameters including the number of binding sites per microorganism (M) and the reaction equilibrium constant (K_{eq}), both of which are variables for estimating the adsorption effectiveness of particular microorganism. According to this model, binding sites for this yeast is 100–1000 times more than LGG, and maybe, this finding is the main reason for observed differences between yeast and LGG in viable condition, and though, in this field, other studies are still necessary. LBGG cells indicated higher AFB_1 binding percentage than yeast cells in acid and heat conditions, and this may be due to that the cell wall of bacterium is weak and easily break under heat or acid treatment compared with yeast cells. Heating may raise the permeability of the external layer of the cell wall because of the suspension of mannans from the cell surface, leading to an increase in the availability of hidden binding sites. It is possible that the acidic conditions could affect polysaccharides by releasing monomers, which are further fragmented into aldehydes after the breaking down of glycosidic linkages. Moreover, it was shown that an increase in AFB_1 concentration enhanced the binding ability of cells. Binding ability of acid- and heat-treated yeast or LBGG cells are better than viable cells and acts in a similar manner. Furthermore, acid treatment is better than heat treatment or acts similar to it. The better binding ability of the physically and chemically modified cells against the viable cells indicates the physical nature of binding rather than a metabolic process. Furthermore, it was shown that the studied yeast strain were more efficient than other yeast strains in indigenous fermented foods, as studied by Shetty & Jespersen (2006). Gratz *et al*. (2006) showed that HCl acid in stomach, intestinal enzymes and intestinal mucosal did not have any effect on aflatoxin binding by LGG. However, further studies are needed to identify the mechanism of binding, to detect the cell surface binding structures and to study the stability of complex under physicochemical conditions (*in vitro*) comparable with *in vivo* condition. The nature of the cell wall components engaged in mycotoxin binding is still unclear.

Conclusion

The effect of decontaminant microorganisms on the carcinogenic and mutagenic aflatoxin B_1 reduction, colour, peroxide value and textural properties of pistachio nuts was investigated. The results showed that *S. cerevisiae* and *L. rhamnosus* GG bind considerable amounts of the aflatoxin. Aflatoxin binding appears to be a fast process with nonviable and physically altered cells binding significantly higher levels of aflatoxin than their viable counterpart. It was found that *L. rhamnosus*, viable or nonviable, is effective aflatoxin binder especially where high aflatoxin level is a potential health risk. The results of study indicated that coating with aflatoxin binders do not have any significant influence on the colour, texture and peroxide value on pistachio nuts, which are important in nuts acceptance. The method investigated and developed in this study is expected to be useful for future researches in this area.

Acknowledgments

We would like to express our thanks to the 'Iran Industrial Researches and Standard Institution' and to 'Research Council of the University of Tehran' for their financial assistance for this study. Also, we want to thank Dr. Gioergio Cerena from Probiotical Company in Italy for providing of *Lactobacillus rhamnosus* GG (LBGG).

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