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Effect of Storage Time and Concentration of Aflatoxin M_1 on Toxin Binding Capacity of *L. acidophilus* in Fermented milk Product

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ABSTRACT

Aflatoxins are potent carcinogenic and immunosuppressive agents. Acute exposure to high level of aflatoxins leads to aflatoxicosis, which cause rapid death due to liver failure. Immune modulating effects of probiotic bacteria have good prospects to detoxification of natural foods. This study was aimed to investigate the ability of *Lactobacillus acidophilus* strain LA-5 in the presence and absence of yoghurt starter culture for removing Aflatoxin M_1 (AFM_1) in comparison with yoghurt starter cultures (10^8 CFU ml^{-1}). AFM_1 detoxification was evaluated for 21 days of yoghurt storage at 4°C at different concentrations of Aflatoxin (0.1, 0.5 and 0.75 µg L^{-1}). The amounts of unbound AFM_1 were determined using competitive Enzyme-Linked Immunosorbent Assay (ELISA). *L. acidophilus* combined with yoghurt starter culture and alone could significantly ($P\leq 0.05$) remove AFM_1 compared to control group. The results indicated that increasing initial AFM_1 concentration in the yoghurt samples and storage time affected the capacity of AFM_1 binding.

Keywords: Aflatoxin M_1, Biological detoxification, Enzyme-linked immunosorbent assay, Lactic acid bacteria, Yoghurt.

INTODUCTION

Mycotoxins are secondary metabolites produced by mycelia or spores of filamentous fungi (Gonçalez *et al.,* 2001). Aflatoxins are one of the most carcinogenic substances known until now (Nierman *et al.,* 2008). Various food resources may be contaminated by aflatoxins such as corn, peanuts, cotton seeds, rice, pistachio, almonds, chestnuts, pumpkin seeds, as well as other oily seeds and sorghum (Chu, 1991; Tajkarimi *et al.,* 2007). The changing rate of ingested Aflatoxin B_1 (AFB_1) to AFM_1 is highly variable, ranging from 0.3 to 6.2%. There is a linear relationship between the AFM_1 concentration in milk and of AFB_1 in contaminated feeds consumed by the livestock (Bakirci, 2001; Creppy, 2002; Mohamadi and Alizadeh, 2010). Chronic exposure to low levels of aflatoxins may threaten the public health followed by serious economic burdens (Oliveira and Germano, 1997). The International Agency for Research on Cancer (IARC, 2002) classifies AFM_1 as Group 1 that leads to human cancer; however, AFM_1 is about 10

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times less carcinogenic than AFB_1. Since AFM_1 is detected frequently in milk and other dairy products that are commonly consumed (especially in countries that advanced ranching industry does not exist), a wide variety of methods were in order to control and decrease AFM_1 contamination of foods and feeds. Elimination of aflatoxin with chemical and physical methods have some disadvantages which limit their use. For example, insufficiency of toxin elimination, high costs and losing the nutritional value of the product (Line and Brackett, 1995; El-Nezami *et al.,* 1998). Biodegradation of aflatoxins by microorganisms offers an attractive alternative for the control, reduction or elimination of aflatoxins to maintain their quality and safety (Alberts *et al.,* 2009). Among all types of available microorganisms that may be utilized to remove aflatoxins from a contaminated medium, Lactic Acid Bacteria (LAB) would be a suitable choice for reducing the bioavailability of aflatoxins because of their unique characteristics. They are Generally Recognized As Safe (GRAS) by USFDA, also some of them confer beneficial effects on health which are called probiotics (El-Nezami *et al.,* 2002; Fuchs *et al.,* 2008). This study was carried out to investigate the ability of yoghurt starter culture and *Lactobacillus acidophilus* LA-5 for removing AFM_1 from contaminated probiotic yoghurt.

MATERIALS AND METHODS

Culture Preparation

The Direct Vat Set (DVS) lyophilized pouches of yoghurt starter culture (YoFlex) which contain *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, also, *L. acidophilus* LA-5® as a probiotic strain were originally obtained from Chr. Hansen's Lab (Denmark). The lyophilized cultures were maintained at -20°C until use. Both bacterial cultures (1 g per 100 ml) were inoculated directly into MRS broth (Merck™, Germany) and incubated at 37°C for 24 hours. After centrifuging (3,400×g at 4°C for 10 minutes), the bacterial pellets were collected and washed three times with 20 ml Phosphate Buffer Saline (PBS) (pH= 7.4). To achieve cell count of 10^8 CFU ml^{-1}, the Optical Density ($OD_{600\ nm}$) of bacterial suspension in PBS was adjusted to a McFarland standard (No. 1) by a spectrophotometer (Martin and Palomino, 2009). After centrifugation, 20 ml of PBS was drained gently from each bacterial suspension and 30 ml of contaminated milk was replaced.

Growth of Starter Culture and Probiotic Bacteria

The bacterial cell count was determined using traditional plate counting at MRS agar (Merck™, Germany) for the starter culture alone (Y), a combination of starter culture and *L. acidophilus* LA-5 (AY), also *L. acidophilus* alone (A). To assess the viability of *L. acidophilus* in the presence of starter culture (AY), according to Ashraf and Shah (2011), Ox-bile (0.15% v/v) (Fluka, Sigma-Aldrich™, Germany) was added into the MRS medium. All samples were incubated at 37°C for 72 hours, aerobically.

Milk Contamination with AFM_1 and Yoghurt Manufacturing

Aflatoxin M_1 (from *Aspergillus flavus*, 10 µg) was purchased from Sigma-Aldrich™ (Germany). AFM_1 stock solution (10 µg ml^{-1}) was prepared by dissolving the entire powder in 1 ml of high-performance liquid chromatography grade acetonitrile (Sigma™ Chemical Co. Ltd., USA). The concentration was verified by HPLC method according to the Institute of Standards and Industrial Research of Iran (ISIRI-7133, 2011). Working solution (100 µg L^{-1}) was prepared accurately by pure acetonitrile solution as a diluent and samples were stored at -20°C until use. Reconstituted milk was prepared by diluting skimmed milk powder (Code-15363; Merck™, Germany) in distilled water. Before adding AFM_1 to reconstituted milk, a portion of that was set apart as negative control. For yoghurt preparation, reconstituted milk was heated at 90-95°C for 5 minutes then cooled to 42°C. The pasteurized milk was contaminated with AFM_1 working solution at three different levels (0.1, 0.5 and 0.75 µg L^{-1}). The bacterial pellets were then inoculated into the contaminated milk and incubated at 42-45°C for 4 hours in order for the yoghurt to be set by the starter cultures (control group), also, 6-7 hours for yoghurt made by *L. acidophilus* La-5 alone. The prepared yoghurts were stored at 4°C for 21 days and samples were taken at 7 days intervals to determine unbound AFM_1.

Measurement of pH

The pH value of yoghurt samples were measured throughout the experiment by pH meter (Jenway™, UK) during a 21 day period (at 1, 7, 14 and 21 days).

Analysis of AFM_1 in Samples by Competitive ELISA

The yoghurt samples were centrifuged (3,400×g at 4°C for 5 minutes) at the end of each storage period (at days 1, 7, 14 and 21) and unbound AFM_1 content of the supernatants were determined by ELISA method. ELISA procedure was performed according to instructions provided by EuroProxima. One-hundred microliters of standard solutions and prepared samples were added into separate microtiter wells (pre-coated with anti-aflatoxin M_1) and incubated at room temperature (25°C) for 60 minutes in a dark environment. Next, the liquid was poured out and the wells were washed three times with washing buffer (300 µl) by microplate strip washer (ELx50; Bio-Tek Instruments, USA). Then, 100 µl of the diluted enzyme conjugate was added to the wells, mixed gently by shaking the plate manually and incubated at room temperature for 30 minutes. Again, the wells were washed three times with washing buffer. After that, 100 µl of substrate/chromogen was added, mixed gently by hand and incubated in a dark place at room temperature for 30 minutes. Finally, 100 µl of the stop reagent was mixed by the wells contents and the absorbance was measured at λ_{max}= 450 nm using ELISA plate reader (ELX808; Bio-Tek Instruments, USA). According to 5121AFM guidelines, the limit of detection (LOD) for the milk is < 0.006 ng ml^{-1} and < 10 pg ml^{-1} for cheese.

Quantification of Residual AFM_1 in Supernatant Samples by High-Performance Liquid Chromatography (HPLC)

In this study, the number of each toxin concentration of all the samples tested by ELISA kit, were randomly selected to confirm by High Performance Liquid Chromatography method according to ISIRI (2011). The linearity was evaluated by linear regression analysis using the least squares method and expressed as correlation coefficient (R^2).

The method is based on the immunoaffinity clean-up of the milk samples followed by the determination of the AFM_1 content by HPLC as follows:

At first, the fat of yoghurt samples were separated by centrifugation. Then immunoaffinity column that contains monoclonal antibodies to aflatoxin M_1 bound to a solid support, was applied for purification of defatted samples. The maximum volume of the affinity column shouldn't be less than 10 ng AFM_1 and the recovery rate shouldn't be lower than 70%. 50 µl of the reconstituted samples were injected in the HPLC using Waters 474 fluorescence detector at 360 and 440 nm for excitation and emission, respectively.

The chromatography was carried out with Water HPLC system with Waters Alliance 2695 HPLC pump. The column and guard column used were 4.6×200 mm reverse phase ODS-5 μm C18 column (Phenomenex, USA) and Onyx™ Monolithic C18 with 10×4.6 mm LC guard cartridge, respectively. The mobile phase was composed of methanol and water (40:60 v/v). The flow rate of the injected sample was 2.0 ml per minute to achieve the optimum resolution of aflatoxin. Based on aflatoxin standards injected into the device, the retention time of aflatoxin M_1 in samples was 3.69 minutes. The Limit Of Detection (LOD) is defined as the lowest amount reproducibly detected with at least 3:1 (signal to noise ratio) and in this method LOD was 0.01 ng ml^{-1} and the limit of quantitation was 0.03 ng ml^{-1}.

Statistical Analysis

All experiments were performed quadruplicate and the presented data are their means. Statistical analysis was carried out with IBM SPSS Statistics™ 20 software. Significant differences between the means were estimated by ANOVA and Duncan's tests at $P \leq 0.05$. All graphs were generated using Microsoft Excel™ software.

RESULTS AND DISCUSSION

Survival of Yoghurt Bacteria and *L. Acidophilus* La-5

Enumeration of *L. acidophilus* was done during 21 days by 7-day intervals during the refrigerated storage by standard plate counting on MRS-bile medium, when a combination of starter culture and probiotic strain were cultivated, and on MRS agar when probiotic strain grew alone. The number of lactic acid bacteria and yoghurt starter culture were showed in Figure 1. The initial viable cell counts of starter cultures and *L. acidophilus* La-5 both were 3×10^8 CFU g^{-1} immediately after yoghurt manufacturing before keeping in refrigerator. Survival of *L. acidophilus* La-5 in yoghurt in the absence of starter cultures remained stable throughout the storage period until day 14. From day 14 to 21, just 1 log cycle of cell count reduction was observed ($P \leq 0.05$). Survival of La-5 in the

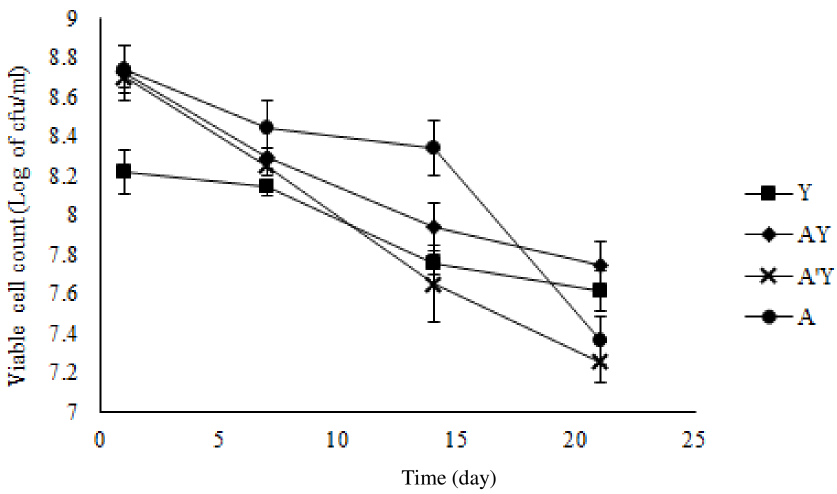

Figure 1. Enumeration of yoghurt starter culture and *L. acidophilus* La-5 survived, during 21 days of storage period at 7-day intervals. Data shown are representatives of quadruplicate. Starter culture alone (Y); combination of starter culture and *L. acidophilus* La-5 (AY); *L. acidophilus* La-5 separated from AY samples (A´Y), and *L. acidophilus* La-5 alone (A).

presence of starter culture showed 1.5 log cycle of cell count reduction throughout the 21 days of storage.

Acid and osmotic stress, as consequences of lactic acid production and application of food additives, are the most predominant stress factors during yoghurt manufacture and cold storage (Settachaimongkon *et al.,* 2015).

Many studies have reported low pH or the accumulation of organic acids, especially lactic acid which is mainly produced by *L. delbrueckii* ssp. *bulgaricus*, as one of the most influential factors which reduces the viability of probiotic cells in the products, (Kailasapathy and Chin, 2000; Lourens-Hattingh and Viljeon, 2001). Some scientists explained that acid can passively diffuse through the bacterial cell membrane and rapidly dissociate into protons inside the cytoplasm, then charge derivatives to which the cell membrane is impermeable causing an internal acidification that disorders the activity of acid sensitive enzymes, DNA and damages proteins. Thus, it is essential to monitor these qualifications during the storage time in order to favor bacterial growth (Bovo *et al.,* 2014). In this study, the pH of all three different yoghurt samples were declined to 4.5 after 21 days storage (unpublished data). It is reported that the least tolerable pH is 2.5 for *L. acidophilus* (Zhao *et al.,* 2012). The survival of *L. acidophilus* in acidic environments has been studied, and this species proved to be highly resistant to acid (Shah, 2000). Lorca and de Valdez (2001a; 2001b) expressed survival of *L. acidophilus* may be affected by physiological adaptation known as Acid Tolerance Response (ATR). Fundamental mechanisms of acid tolerance utilized by gram-positive bacteria include proton pumps, proteins involved in repair or degradation of damaged cell components, activity of arginine deaminase that cause increase of alkalinity of cytoplasm, urease and glutamine decarboxylase, and conversions in the composition of the cell envelope (Cotter *et al.,* 2001; Cotter and Hill, 2003; De Angelis and Gobbetti, 2004; Ruiz *et al.,* 2011). In the presence of organic acids, the F_1F_0-ATPase plays an important role in maintaining the intracellular pH (pHi). According to Tamime *et al.* (2005) and Demers-Mathieu *et al.* (2015), *L. delbrueckii* ssp. *bulgaricus* is known for the ability of post-acidification process and high production of hydrogen peroxide (H_2O_2) that have an impact on the growth of probiotic strains. As a result of lacking catalase, *L. acidophilus* is subjected to oxidative stress and this may damage the proteins and DNA of the cells and eventually kill them.

Changes of AFM_1 during Yoghurt Storage

The aflatoxin-binding capacity of different strains tested at 4°C during 21 days of storage are displayed in Figure 2. Yoghurt starter culture and *L. acidophilus* tested in this study were able to bind AFM_1. The significant difference ($P \leq 0.05$) between AFM_1 binding ability of *L. acidophilus* LA-5 and starter culture in yoghurt was demonstrated.

Preliminary investigations have expressed that yoghurt starter culture and probiotic bacteria could be used to remove AFM_1 from food and feed. Sarimehmetoğlu and Küplülü (2004) reported that *S. thermophiles* ST-36 (29.42–36.16%) has a great potential to bind a high percentage of AFM_1 in comparison with *L. delbrueckii* ssp. *bulgaricus* CH-2 (18.7–27.56%) in PBS and milk, respectively ($P < 0.01$). Elgerbi *et al.* (2006) assessed the ability of strains of *Lactobacillus* spp., *Lactococcus* spp. and *Bifidobacterium* spp. to bind the AFM_1 in buffered aqueous solution. They found that the percentage of AFM_1 bound by these strains ranged from 4.5-73.1% after 96 hours. El-Khoury *et al.* (2011) found that the yogurt bacteria, *L. bulgaricus*, *Str. thermophilus* and a combination of these two bacterium reduced AFM_1 content of milk to 58.5, 37.7 and 46.7% respectively, after incubation at 37°C for 6 hours. Bovo *et al.* (2012) evaluated the ability of some

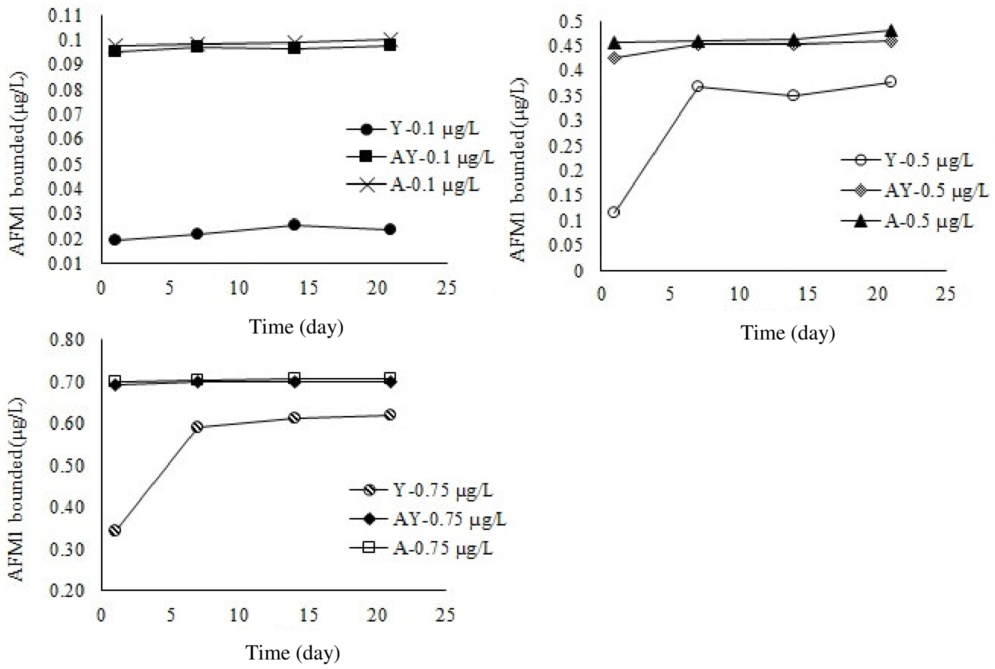

Figure 2. AFM_1 removal at three different levels of toxin in yoghurt during 21 days of storage in refrigerator with starter culture and *L. acidophilus*. Data shown are representatives of quadruplicate experiments. Means with the same letter for each types of yoghurt do not differ significantly at $P \leq 0.05$. Yoghurt made with starter culture (Y); probiotic yoghurt made with a combination of *L. acidophilus* LA-5 and starter culture (AY), and Fermented milk made with *L. acidophilus* La-5 alone (A).

probiotic strains to remove AFM_1 in skimmed milk and reported that the tested strains bound AFM_1 within a range from 13.51 to 37.75% for 15 minutes at 37°C. Serrano-Niño *et al.* (2013) assessed the ability of some spices of probiotic bacteria and reported that the bioaccessibility of AFM_1 reduced in range of 22.72 to 45.17% in the presence of the tested strains.

Decrease of AFM_1 levels in yoghurt might be assigned to some factors such as low pH, formation of organic acids or other fermentation by-products (Govaris *et al.,* 2002). Reduction of pH during the fermentation alters the structure of caseins in milk proteins. These changes lead to the formation of a network like yoghurt gel which hold the aflatoxin inside the precipitate (Montazeri *et al.*, 2014).

The results of this research showed a significant reduction in unbound AFM_1 content through the storage time. Analysis of the data in Figure 2 indicated that the binding of AFM_1 is a strain specific characteristic. At the first day of storage, *L. acidophilus* La-5 removed over 90% of the AFM_1 from the yoghurt samples. Then, until the end of the storage time a significant reduction ($P \leq 0.01$) in the amount of unbound aflatoxin was observed.

These results were in good agreement with previous reports (Elgerbi *et al.,* 2006; Biernasiak *et al.,* 2006). Motawee and El-Ghany (2011) evaluated the ability of eight dairy strain of lactic acid bacteria to remove aflatoxin M_1 and B_1 in yoghurt and noted that for all examined starters, the percentage of aflatoxins AFM_1 and AFB_1 reduction in yoghurt after 5 hours was considerably less than that at the end of storage period. Contrary to our observations, other authors found no reduction of AFM_1 in yoghurt during the cooled storage period Blanco *et al.,* 1993; Iha *et al.,* 2013). Factors such as toxin concentration, temperature of storage, time

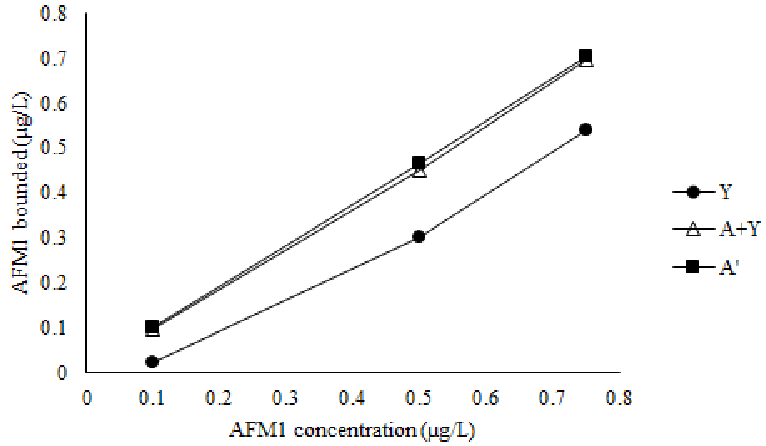

Figure 3. Effect of AFM_1 levels on toxin-binding capacity of yoghurt starter culture and *L. acidophilus* La-5. (Y) Yoghurt made with starter culture; (AY) Probiotic yoghurt made with a combination of *L. acidophilus* LA-5 and starter culture, and (A) Fermented milk made with *L. acidophilus* La-5 alone.

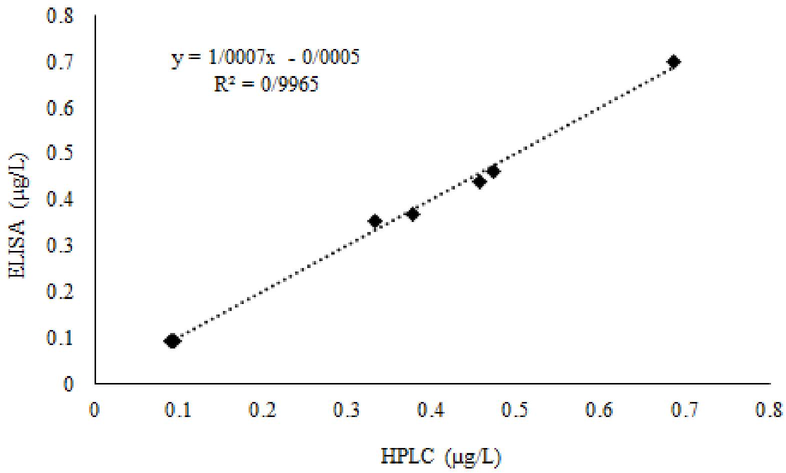

Figure 4. The correlation between HPLC and ELISA data for AFM_1 determination. Axes *x* and *y* show level of AFM_1 bounded which have been determined independently by ELISA and HPLC method, respectively.

elapsed before sample analysis, the difference in type of starter cultures used to make dairy product, variability in milk composition and milk contamination method may cause differences in results (Ismail *et al.,* 1989; Mohammadi *et al.,* 2009). The reduction of AFM_1 in yoghurt during storage period might be due to the oxidation of glucose that produces gluconolactone and hydrogen peroxide which will be distributed in yoghurt. H_2O_2 can form single reactive oxygen which may react with the double bond in the terminal of dihydrofuran moiety of the aflatoxin molecule (Elsanhoty *et al.,* 2014). Some authors showed that non-viable cells could also remove higher amounts of aflatoxin from different media (Pierides *et al.,* 2000; Shahin, 2007; Kabak and Var, 2008; Bovo *et al.,* 2014). Therefore, increasing non-viable cell contents during the yoghurt storage as a result of pH value reduction or increase of bacterial second metabolite in a fatal overdose case, may help to remove more aflatoxin from media.

Analysis of our data indicated that *L. acidophilus* La-5 removed over 90% of the

AFM_1. This observation confirmed that La-5 could bind the AFM_1 quickly which was in agreement with El-Nezami *et al.* (1998a) for *L. rhamnosus* strains GG and LC705. At the time of adding LAB, both strains removed 80% of the AFB_1 approximately. Some authors suggested that the significant differences existing among aflatoxin binding ability of lactic acid bacteria depends on distinctive cell-wall structure (El-Nezami *et al.,* 1998b; Pierides *et al.,* 2000; Peltonen *et al.,* 2001; Lahtinen *et al.,* 2004; Zinedine *et al.,* 2005; Hernandez-Mendoza *et al.,* 2009). Strength of mycotoxin–LAB is a fast physicochemical interaction between the toxin and the functional groups of the cell surface (Bovo *et al.,* 2014; Zoghi *et al.,* 2014), and it is based on physical adsorption, ion exchange, and complexation, regardless of the bacterial metabolism. It was proposed that carbohydrate components of the bacteria cell walls particularly peptidoglycans were probably the compounds which were in charge of binding aflatoxin to the bacterial surface. Hernandez-Mendoza *et al.* (2009) indicated that except the peptidoglycans, teichoic acids were also an important part of the cell wall which could bind aflatoxin. Binding to macromoleculs are functions of fibril network of teichoic acids and polysaccharides; and the stability and strength of binding of microorganism to toxins depends on strain, amino acid composition of peptidoglycan structure and environmental conditions (Zoghi *et al.,* 2014).

By increasing AFM_1 concentration, AFM_1 binding ability of yoghurt starter culture was increased (Figure 3). However, *L. acidophilus* La-5 in presence and also in the absence of yoghurt starter culture significantly showed further reduction in AFM_1 content at all concentrations tested compared to the control group ($P\leq 0.05$).

In this study, accuracy of the ELISA method for detecting AFM_1 in samples was verified by HPLC and the correlation between ELISA and HPLC methods were evaluated and are shown in Figure 4. The correlation coefficient (R^2) between these two methods was 0.9965. Therefore, the ELISA method can be used as a reliable and cheaper method to evaluate the level of aflatoxin in milk and even in animal husbandry.

In our research, the AFM_1 binding ability of yoghurt starter culture and *L. acidophilus* La-5 were increased, by increasing initial AFM_1 concentration. Our results were supported by some studies showing that the amount of bound AFM_1 by bacteria in milk and PBS is raised with the increase of AFM_1 concentration (Rašić *et al.,* 1991; Kabak and Var, 2008).

CONCLUSIONS

Since milk and dairy products are an important part of people's daily food basket of the world, aflatoxin contamination of these products and human body's inability to reduce or eliminate these toxins, can endanger the health of a large number of people in the world. This study was an attempt to show the capability of *L. acidophilus* La-5 as a biological and safe method to reduce aflatoxin in dairy products. Also the assessment of storage time and increasing initial toxin level in products showed significant increase in toxin binding to bacterial cell wall and eliminating it from products. Favorable survival of *Lactobacillus acidophilus* La-5 in the product during storage indicated that this bacterium as a probiotic strain can not only reduce the level of aflatoxin contamination but also incorporate to the production of a probiotic product at the same time.

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ارزیابی زمان انبارمانی و غلظت سم آفلاتوکسین M_1 بر ظرفیت اتصال به سم در باکتری *لاکتوباسیلوس اسیدوفیلوس* در محصول لبنی تخمیری

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چکیده

آفلاتوکسین‌ها عوامل بالقوه سرطان‌زا و سرکوب کننده سیستم ایمنی بدن هستند. مواجهه شدید با سطح بالایی از آفلاتوکسین‌ها منجر به بروز آفلاتوکسیکوزیز شده، که مرگ سریع به دنبال نارسایی کبد را در پی خواهد داشت. اثرات تعدیل سیستم ایمنی مرتبط با باکتری‌های پروبیوتیک چشم‌انداز خوبی برای سم‌زدایی از موادغذایی پیش روی ما قرار می‌دهد. هدف این مطالعه ارزیابی توانایی *لاکتوباسیلوس اسیدوفیلوس* سویه La-5 در حضور و عدم حضور آغازگرهای ماست جهت کاهش و یا حذف آفلاتوکسین M_1 در مقایسه با آغازگرهای ماست به عنوان تیمار شاهد بوده است ($۱۰^۸$ CFU/ml). سم‌زدایی AFM_1 در طول ۲۱ روز دوره انبارمانی ماست در دمای ۴ درجه سانتی‌گراد در غلظت‌های متفاوت آفلاتوکسین (۰/۷۵، ۰/۵، ۰/۱ µg/L) ارزیابی شد. مقدار آفلاتوکسین اتصال نیافته به سطح باکتری با استفاده از روش الایزای رقابتی اندازه‌گیری شد. با توجه به نتایج به دست آمده، *لاکتوباسیلوس اسیدوفیلوس* در ترکیب با آغازگرهای ماست و همچنین به تنهایی توانست به حد معنی‌داری (P<۰/۰۵) در مقایسه با تیمار شاهد آفلاتوکسین را حذف کند. نتایج حاکی از آن بود که افزایش در مقدار اولیه غلظت آفلاتوکسین و مدت زمان انبارمانی بر ظرفیت اتصال به آفلاتوکسین موثر است.