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Evaluation of *Lactobacillus rhamnosus* viability effect on reduction of aflatoxin M1 in probiotic yogurt

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ABSTRACT

Aflatoxins are a group of toxin compounds in many foods and animal feeds and have potent hepatocarcinogens in man as acute toxicants[1]. Physical and chemical approaches for elimination of mycotoxins are limited due to problems which they cause in food safety. Microbial detoxification is one of the aflatoxin removal methods such as aflatoxin M_1. Many reports implicate that some lactic acid bacteria strains are able to adsorb aflatoxins by their cell walls. This research was carried out on evaluation of *Lactobacilus rhamnosus* ability for aflatoxin M_1 adsorbtion in probiotic yogurt. In this research, Lactobacilus rhamnosus with starter culture were inoculated to non aflatoxin reconstituted skim milk about 10^8 cfu/ml. After that, the yogurt samples were contaminated by aflatoxin M_1 in 0.1, 0.5 and 0.75 ppb concentrations. Aflatoxin M_1 residuals were determined in the supernatant of the samples by competitive ELISA in first, seventh, fourteenth and twenty-once days after yogurt processing. The results showed, however, both tested probiotic bacteria (*Lactobacillus rhamnosus* subspecies GG) and starter cultures (*Streptococcus thermophilus* and *Lactobacillus bulgaricusphilus*) are able to adsorb toxins, but the ability of probiotic bacteria was statistically higher than starter cultures in yogurt.

KEYWORDS

Aflatoxin M_1; *Streptococcus thermophilus*; *Lactobacillus bulgaricus*; *Lactobacillus rhamnosus* and probiotic yogurt.

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INTRODUCTION

Aflatoxins are a group of mycotoxins with too poison compounds which are found in many foods and animal feeds and are also produced by some species of aspergillus such as *Aspergillus flavus*, *Aspergillus paraciticus* and *Aspergillus nomius*.

Milk and dairy products can been considered as the indirect resources of the aflatoxins. When cattle consume aflatoxin B_1 in animal feed, the liver microsomes metabolize it to aflatoxin M_1 by the complex function of an oxidase enzyme that can be excreted into breast milk[2, 3]. Based on epidemiological studies, there are many evidences to point to the dangers of aflatoxin M_1 because of the carcinogenic potential in particular liver cancer in the people who have used these contaminted foods.

In most countries, it has been applied the tough rules to limit the presence of aflatoxin in food and commercial products, however, the presence of such toxins in food are inevitable. According to the codified law by the FDA, the maximum allowable amount of aflatoxin M_1 in milk is 0.5 ppb. That has reduced by the European countries, for example the maximum of the aflatoxin M_1 in milk is 0.05 ppb in Switzerland. This magnitude for flavored milk and all milk heated in Iran, is 0.1 ppb right now. The importance of milk and dairy products consumption as a nutrition and also the present risks from aflatoxin M_1 in these foods are created to require a suitable method to degrade toxin[1].

The consumers' healthcare will be threaded since milk and dairy products daily are consumed by the majority of people, especially for individual sensitive people such as children and the elderly and it is also possible that they have been contaminated by aflatoxin M_1, therefore, it is necessary to find ways to remove or degrade aflatoxin M_1 in these products.

To utilize of many physical and chemical methods for removal of mycotoxins in the contaminated food is limited due to problems related to food safety, the possibility of the nutritional quality reduction in these products, lower efficiency and higher costs.

These reasons occasion that the research extensively carry out to find a safe, efficient and desirable method instead of the physical and chemical approaches. For this reason, the usage of a biological method is one of the best and most suitable options. Among of all biological methods, the microbial approach is the most important way to reduce mycotoxins in the contaminated foods. Reports indicate that some strains of lactic acid bacteria are able to adsorb and remove Aflatoxins through their cell walls, effectively.

MATERIALS AND METHODS

Solid aflatoxin M_1 (Sigma, Germany) and sodium chloride, potassium chloride, sodium hydrogen phosphate, potassium dihydrogen phosphate, sulfuric acid, barium chloride, culture media and other chemicals were purchased from Merck, Germany. The lyophilized probiotic (1637PTCC) *Lactobacillus rhamnosus* GG strain was obtained from Iranian Research Organization for Science and Technology (IROST)- Persian Type Culture Collection (PTCC)-Microbial Collection was purchased. Aflatoxin M_1 kit which had been used in this study was manufactured by EuroProxima B.V., Netherland.

Preparation of aflatoxin M_1solutions

Solid aflatoxin M_1 was prepared at a concentration of 100 ppb. For preparation of contaminated samples, aflatoxin M_1 solutions in 0.05, 0.25 and 0.5 ppb were obtained[4].

Preparation of the starter cultures and probiotics

Direct Vat Starter culture powders (yogurt starter and *Lactobacillus rhamnosus* GG) was activated in MRS broth medium for 24 h at 37°C. In order to determine the growth curve for each, turbidity equivalent of 0.05 was obtained and until to reach to the stationary phase, the magnitudes of turbidity and colonies was measured, alternatively.

The growth curves of the starter cultures and probiotic bacteria were utilized for the yoghurt processing and to achieve to the logarithmic growth phase. After that, these cultures were washed during the thrice centrifugation in 3400g, at 4 ° C for 10 min by the phosphate buffer solution (PBS) and so the microbial pellets were isolated. Bacteria concentration was determined based on 0.5 McFarland standard in phosphate buffer solution at a wavelength 600 nm by a spectrophotometer. Then, 20 mL of the bacterial suspension (20 mL for starter cultures, 10 mL for each from starter cultures and probiotic bacteria in the combined culture) was centrifuged in 3400g, 4°C and 10 min for isolation pellets from buffer and inoculation in milk samples.

The bacteria growth rate and their numbers were evaluated by Neubauer Lam and standard plate count using MRS agar in addition the bacterial suspension turbidity by a spectrophotometer[6, 7, 8, 9].

Yogurt preparation

For yogurt preparation, reconstituted Skim milk 12% was primarily heated up to 90-95°C for 5 min, then it was cooled to 42°C and added aflatoxin M_1 in 3 concentration levels including 0.1, 0.5 and 0.75 ppb and finally inoculated with starter cultures which mentioned above to prepare them and incubated for at least 3 h at 42°C. It is notable that the coagulation of the produced yogurt samples required about 3.5 h by bacteria[4].

The viability determination of microorganisms in yogurt samples during storage

It was sampled in duplicate replication from yogurt samples for the viability determine of the starter cultures and probiotic bacteria at intervals of 7 days during the 21 days storage period. After dilution in the Ringer solution, it was cultivated by not only the general MRS agar medium but also probiotics differentiation media in 10^{-5} to 10^{-7} concentrations.

Isolation and differentiation of *Lactobacillus rhamnosus* was performed using MRS-vancomycin agar medium[5].

Measurement of Aflatoxin M_1 by ELISA procedure

After each sampling, yogurt samples were centrifugated at 4°C for 5 min and the supernatants were isolated and stored at -20°C for determination of the unbound and, finally, absorbed aflatoxin M_1 content by ELISA procedure[4].

The used method in the purchased kit was the direct competitive ELISA. ELISA procedure was performed in this manner that the duplicate replication of each samples, for example, 100 µl of the zero standard (A1 and A2 wells for control), 100 µl of the zero standard (B1 and B2 wells for the maximum signal), 100 µl of aflatoxin M_1 standard solutions (C1 and C2 to H1 and H2 wells for 6.25, 12.5, 25, 50, 100 and 200 ppt) were replaced into one of the wells in 96 well microplate. Then, this microtiter plate was capped, shook for a few second on microstirrer and mixture was incubated at room temperature in the dark place for 1 h.

After that, the solution was discarded from microplate wells and washed by washing buffer for three times. 100 µL of the conjugated solution was added into each well. This final solution was discarded and wells were washed by buffer solution for triple replication. 100 µL of substrate solution was placed in each well and incubated for 30 min at room temperature. Finally, 100 µL of stop solution was added to each well and immediately read absorbance at 450 nm. Zero standard had to be 100% (maximum adsorption) and the rest ODs were calculated based on following formula and the maximum percentage.

$$\% \text{ Maximum Adsorption} = \frac{OD_{\text{Standard or Sample}}}{OD_0} * 100$$

STATISTICAL ANALYSIS

The Results of this study were analyzed based on a completely randomized factorial design by SPSS Ver.16. The results are averages of duplicate incubations. The one-way variance analysis was done for determining the difference as binding amount of aflatoxin M_1. DUNCAN test was used to evaluate significant differences between starter cultures, *Lactobacillus rhamnosus* and storage times in the aflatoxin M_1 adsoption. Values of $P< 0.05$ were regarded as significant. Excel-2003software was utilized for drawing of curves.

RESULTS AND DISCUSSION

In order to achieve the actived microorganisms for yogurt processing, the incubated time had to be determined to reach the growth exponential phase.

For this reason, the turbidity and microorganism enumerations over time must be measured, the growth curve for the mixed starter cultures (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) and probiotic bacteria were illustrated, and accordingly, the required time for activation each were caculated. Then, First of all, the growth curve of *Lactobacillus rhamnosus* was drew by measuring the number of the viable cells to time result (Fig. 1).

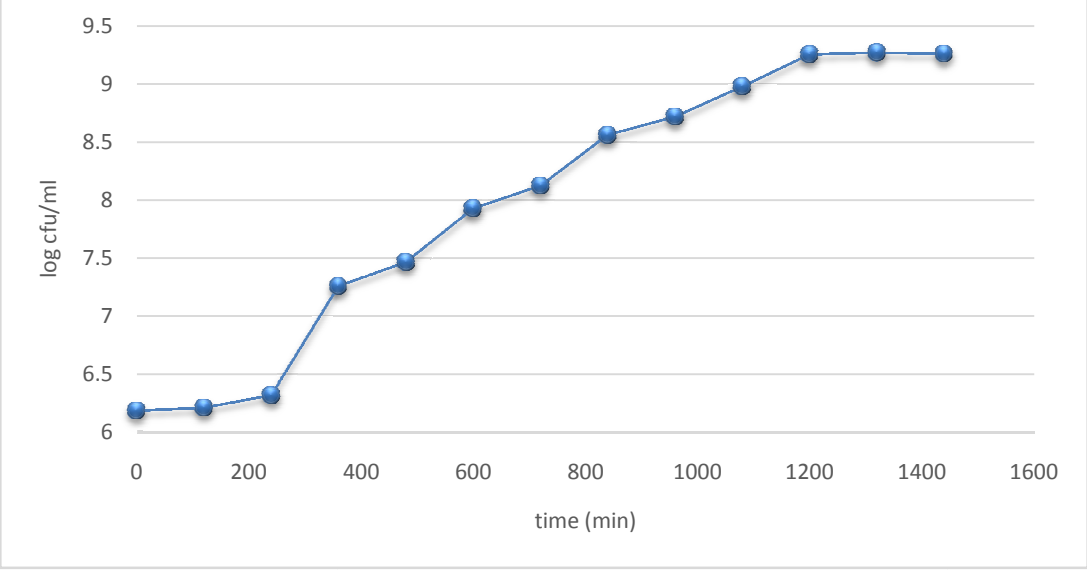

Figure 1 : The growth curve of *Lactobacillus rhamnosus* based on the cell count over time

Figure 2 was determined based on the generated turbidity of the bacterial growth curve in MRS Broth medium at 30°C during incubation. This curve the maximum amount of the viable bacteria cells showed how much *Lactobacillus rhamnosus* after incubation about 20 h and then they entered to their stationary phase.

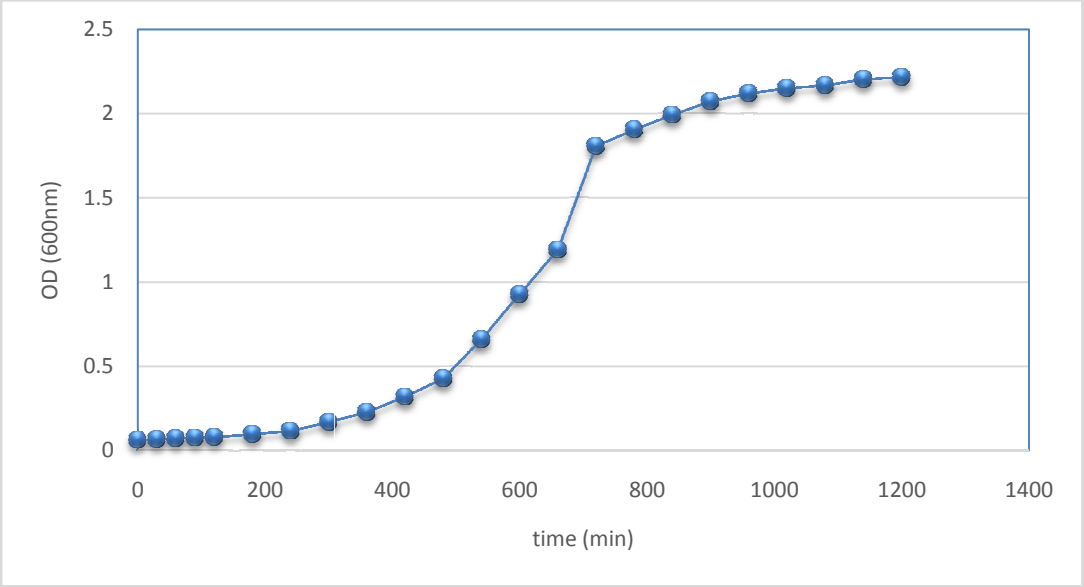

Figure 2 : The growth curve of *Lactobacillus rhamnosus* based on the cell density over time

Drug effects will indicate when the probiotic bacteria reach active and viabile to intestine. For this reason, it is necessary that the quantity of the viable bacteria must be at least 10^7 per gram of milk from the International Dairy Federation (IDF) point of view, while this amount for therapeutic effects has been recommended 10^8-10^9 cfu/ml by many different researches.

Therefore, about 10^8-10^9 cfu/ml of the starter cultures and probiotic bacteria were inoculated for regular and probiotic yogurt, then their bacteria viability were evaluated in presence the different aflatoxin M_1 concentrations and during shelf life (21 days). Assessment of aflatoxin M_1 effect on *Lactobacillus rhamnosus* viability indicated that the highest level of viability occurred in a yogurt which had the lowest aflatoxin M_1 concentrations and vice versa (Fig. 3).

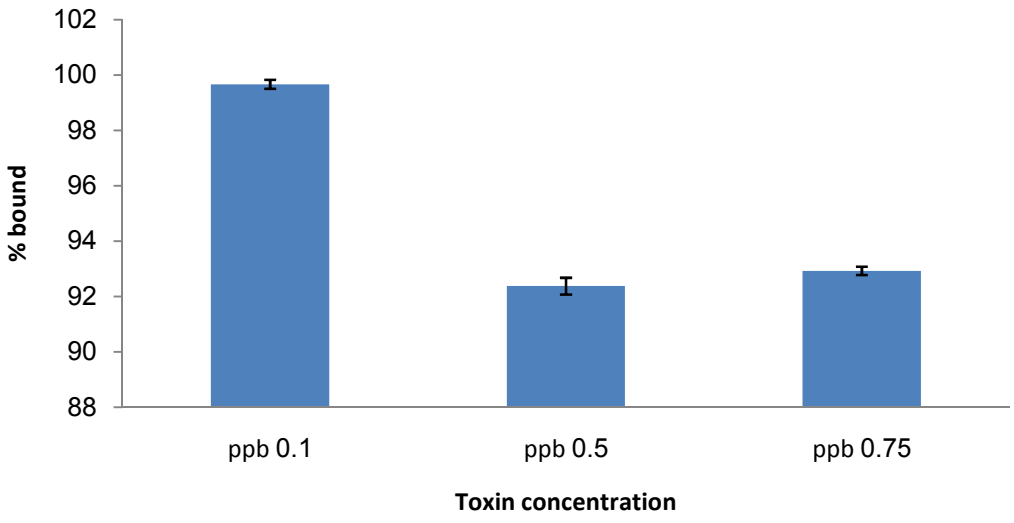

Figure 3 : Aflatoxin M_1 concentration effects on *Lactobacillus rhamnosus* viability in yogurt

It was observed more than 10^8 colonies per mL of sample in the first day after the probiotic with *Lactobacillus rhamnosus* yogurt processing. Then, its amount declined gradually over time, so the logarithm of the colonies number was about 7.85 after 21 days (Fig. 4). The most number of the probiotic bacteria viable cells in the contaminated yogurt samples with the three different concentrations were belonged to the firstt day of processing (Fig. 5).

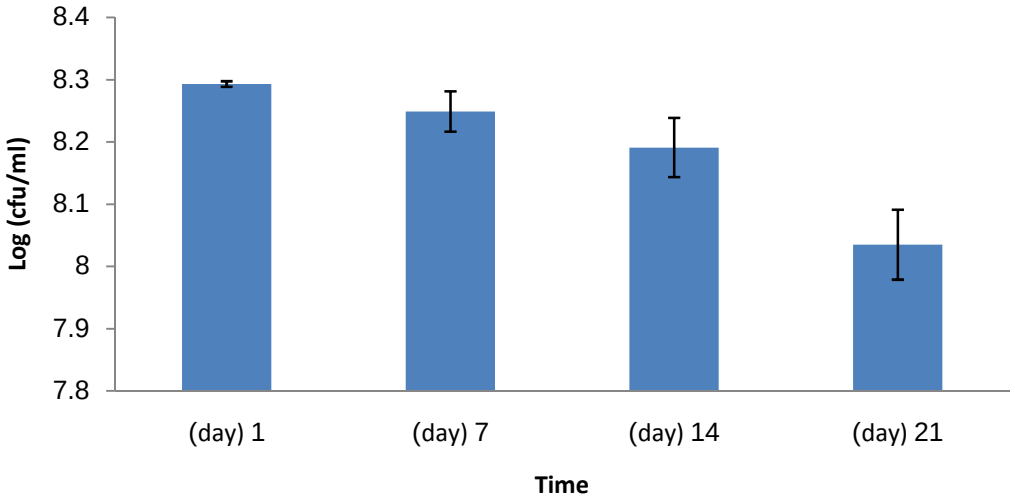

Figure 4 : Evaluation of *Lactobacillus rhamnosus* viability during probiotic yogurt shelf life

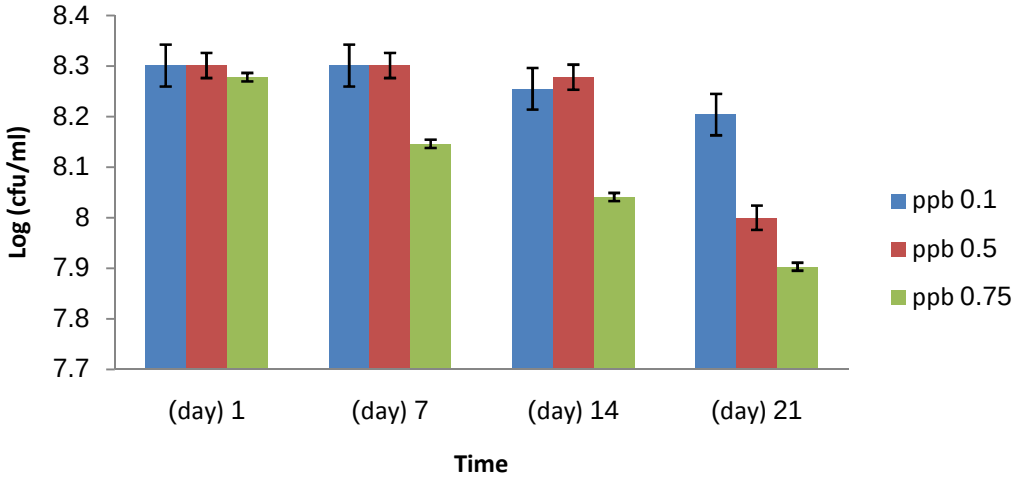

Figure 5 : Evaluation of contraction between toxin concentration and shelf life time on *Lactobacillus rhamnosus* viability in probiotic yogurt

CONCLUSION

This study had been aimed to evaluate the probiotic bacteria effect on aflatoxin M_1reduction in probiotic yogurt, to measure of the toxin adsorption percent by ELISA as well as to perform the measurement of probiotic bacteria and yogurt starter culture viability.

The found results of this study showed not only *Lactobacillus rhamnosus GG* but also the yogurt regular starter culture (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) are able to adsorb toxins and probiotic bacteria ability for the adsorption of the higher toxin amounts is significantly more than starter culture.

Also, the viability results during the shelf life time for yogurt samples illustrated that the microorganisms are properly able to survive until the end of the yogurt storage period (21 days) and are just about 1 logarithmic cycle lower than the first day.

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