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Effect of different microbial concentrations on binding of aflatoxin M_1 and stability testing

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ABSTRACT

Aflatoxin M_1 (AFM_1) is a group 2b category carcinogenic compound of global concern due to its occurrence in milk. Various microbes have been employed for the binding of AFM_1 and to reduce its bioavailability in humans. In the current study three strains of lactic acid bacteria, a strain of *Saccharomyces cerevisiae* and a mixture of all four were used to evaluate their binding potentials for AFM_1. Milk samples were spiked with two different concentrations of AFM_1, 0.05 and 0.1 μg/l, and four concentrations of microbes, 10^7, 10^8, 10^9 and 10^{10} cfu/ml, were tested to evaluate their binding potentials. The concentration of AFM_1 and microbes were found to significantly affect the binding potentials of microbes. *Saccharomyces cerevisiae*, *Lactobacillus helveticus* and the mixture of microbes at the concentration of 10^{10} cfu/ml resulted in 100% binding of AFM_1. *Lactobacillus helveticus* was found to have a higher binding potential than any other lactic acid bacteria reported previously. The binding of AFM_1 with microbes was reversible as the washing test resulted in ~20–~70% release of AFM_1. These results indicate that the microbes can be effectively used for the reduction of AFM_1 levels up to safe limits in milk and milk products.

1. Introduction

Milk is the primary source of human nutrition because of the fact that it contains appreciable amounts of macro and micro nutrients. Milk and milk products in European countries contribute about 15% of daily food intake (Dobrzanski, Kolacz, Gorecka, Chojnacka, & Bartowiak, 2005; González-Montaña, Senís, Gutiérrez, & Prieto, 2012). FAO estimates that per capita consumption of milk was 50.70 kg in 2009 (FAO, 2014). Although milk is consumed irrespective of age group, however, the more frequent consumption is by infants who completely rely on it during the initial months of their lives and also by the elderly where it protects from the risks of osteoporosis and bone fractures (Ismail, Akhtar et al., 2015). These two age groups, i.e. infants and elderly, are also more prone to infections due to weak immunity systems. Therefore, the presence of any toxic element in milk is of extreme concern for researchers all around the globe.

Aflatoxins are the secondary metabolites of *Aspergillus* species which are reported in a number of food commodities including cereals, spices, cottonseeds and dried fruits (Kabak & Ozbey, 2012). Among 18 different types of aflatoxins, the most carcinogenic is aflatoxin B_1 (AFB_1) (Bognanno et al., 2006). AFB_1 is converted into aflatoxin M_1 (AFM_1) inside the liver of the animal and ultimately becomes a part of the animal milk. The conversion rate for AFB_1 into AFM_1 ranges between 0.5 and 6% (Abbas, Zablotowics, & Locke, 2004; Var & Kabak, 2009). AFM_1 is categorized as an animal carcinogen by the International Agency for Research on Cancer but as there is insufficient evidence in humans, it is classified as a group 2b carcinogenic compound, i.e., possible human carcinogen (IARC, 2002). The other health impacts of AFM_1 are teratogenic (Bbosa et al., 2013), cytotoxic (Neal, Eaton, Judah, & Verna, 1998) and genotoxic (Lafont, Siriwardana, & Lafont, 1989; Shibahara, Ogawa, Ryo, & Fujikawa, 1995). AFM_1 is reported in milk and milk

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products all around the globe, e.g., in Spain by Cano-Sancho, Marin, Ramos, Peris-Vicente, and Sanchis (2010), in Egypt by Ayoub, Sobeih, and Raslan (2011), in Turkey by Kabak and Ozbey (2012), in Saudi Arabia by Abdallah, Bazalou, and Al-Julaifi (2012), in China by Han et al. (2014) and in Brazil by Silva, Janeiro, Bando, and Machinski (2015).

The severe health impacts of AFM_1 have resulted in worldwide regulations for monitoring the level of AFM_1 in milk and milk products. The most accepted permissible limit for AFM_1 (0.05 μg/l) in milk is by Codex Alimentarius Commissions (2001). There has been much research into means to control the level of AFM_1 below the permissible limits. The most studied method in the recent years for the binding of AFM_1 is through microbes and especially the lactic acid bacteria. These microbes can bind AFM_1 and as a result can reduce the bioavailability of AFM_1. Various studies have reported the binding of AFM_1 with lactic acid bacteria in the range of 5–50% (Bovo, Corassin, Rosim, & de-Oliveira, 2013; Corassin, Bovo, Rosim, & Oliveira, 2013; Kabak & Ozbey, 2012; Khoury, Atoui, & Yaghi, 2011). To avoid possible fermentation effects and for achieving higher binding rates heat killed microbial cells are preferred. The binding of AFM_1 with microbes has proved to be reversible as the washing of microbial and AFM_1 complex or the bioaccessibility studies resulted in the release of AFM_1 (Corassin et al., 2013; Elsanhoty, Salam, Ramadan, & Badr, 2014; Serrano-Nino et al., 2013). Some more studies are therefore required to find out the potential of LAB to ensure 100% decontamination of these toxins to avoid any possible danger to human health from the consumption of milk. Moreover, Efficacy of buffer or water washing to break up the AFM1-microbe complex and bio accessibility via human digestive models are direly needed.

The purpose of the current study was to evaluate the effects of various concentrations of microbes and AFM_1 on the AFM_1 binding percentages and to find a best possible solution for AFM_1 decontamination in milk by using different microbes. Different concentrations (10^7,10^8, 10^9 and 10^{10} log) of *Saccharomyces cerevisiae* and three different strains of lactic acid bacteria were utilized for binding of AFM_1. Two different concentrations of AFM_1 (0.05 and 0.1 μg/l) were spiked in milk samples for binding studies. Moreover, the effects of PBS washing of AFM_1-microbial complexes were also studied.

2. Materials and methods

2.1. Microbial strains

Three strains of lactic acid bacteria including *Lactobacillus plantarum* NRRL B-4496 (LP), *Lactobacillus helveticus* ATCC 12046 (LH) & *Lactococcus lactis* JF 3102 (LL), a yeast strain of *Saccharomyces cerevisiae* HR 125a (SC) and a mixture of yeast and lactic acid bacteria (SC + LP + LH + LP in the ratio of 2:1:1:1) were used to bind AFM_1. Lactic acid bacteria were grown in MRS broth while SC (BDH) was grown in YM broth (BDH). The growth temperature for SC and LL was 32 °C while for LP and LL it was 37 °C. Microbial concentrations were estimated through turbidity metric method as adopted by Bovo et al. (2013). Shortly, the microbial growth curves were constructed by correlating the microbial growth concentrations calculated by pour plated method and the respective absorbance was obtained at 600 nm. Microbial cells were heat killed in water bath at 100 °C for 1 h to avoid possible fermentation problems in milk.

2.2. Quantification of aflatoxin M_1

AFM_1 quantity in spiked and non spiked skimmed milk samples was measured through ELISA method. The ELISA kits for AFM_1 were purchased from Helica Biosystem Inc. (Cat. No. 961AFLM01M-96) with a detection limit of 0.002 μg/l. Briefly, 200 μl samples of milk were added in the wells of ELISA kit and were incubated at room temperature. After 2 h, the liquid from the wells was poured out and the wells were washed three times with washing buffer and were tapped faced downward on an absorbent paper towel. Then, 100 μl of ready to use enzyme conjugate was added in each well and the plate was incubated for 15 min at ambient temperature. After the due time the plates were again washed three times wish washing buffer followed by moisture removal by using paper towels. Then, a 100 μl of substrate was added in each well and the plate was incubated for 15 min at room temperature in dark. At the end of incubation, 100 μl of stop solution was added in each well and the blue color of solution immediately turned into yellow. Absorbance was measured at 450 nm through an ELISA reader. The concentration of AFM_1 in standard and samples was measured by using the following formula.

$$\%\text{Absorbance} = \frac{\text{Mean absorbance value of sample or standard solutions}}{\text{Absorbance value of standard}}$$

2.3. Standard solution

The standard solution for AFM_1 was purchased from Sigma-Aldrich (Saint Louis, MO). The working solutions were prepared by following the method of Serrano-Nino et al. (2013). Briefly, the powdered AFM_1 was dissolved in a mixture of HPLC grade chloroform/methanol (1:1) supplied by Merck chemicals (Darmstadt, Germany) to a concentration of 4 μg/ml. The standard solution was further diluted with PBS (pH 7.2, 0.5 M). Chloroform/methanol was evaporated by heating in a water bath at 80 °C for 10 min. Lambert-Beer equation ($A = \varepsilon cl$) was used to calculate the final concentration of solution.

2.4. Preparation of spiked milk samples

From the above prepared AFM_1 solution (0.1 μg/ml) 25 μl were added in 975 μl of methanol to prepare a solution of 0.0025 μg/ml, then 20 and 40 μl from the earlier solution were added in 980 and 960 μl skim milk to prepare 0.05 and 0.1 μg/l solution of AFM_1, respectively.

2.5. Microbial binding of AFM_1

Heat killed microbial cells were centrifuged at 3000×*g* for 15 min, the microbial pellets were washed with double distilled water and re-suspended in PBS. In vitro binding ability of selected microbes was evaluated by introducing 1 ml of microbial suspension (10^7, 10^8, 10^9, 10^{10} log) in 1 ml of spiked milk (0.05 and 0.1 μg/l). The contact time between microbial cells and AFM_1 solutions was 1 h. After the due time the microbial-AFM_1 solution was centrifuged at 3000×*g* for 15 min, the microbial cells were removed and the remaining supernatant was analyzed for AFM_1. Microbe free spiked milk samples were run as positive controls and the AFM_1 free milk having microbial pellets of each strain in it were employed as negative controls.

2.6. Microbial-AFM_1 complex stability

The stability of microbial-AFM_1 complex was determined according to the method of Bovo et al. (2013). Briefly, the microbial pellets were washed three times by PBS. The pellets were vortexed in PBS for 20 s. The microbial cells were centrifuged again at