Alexandria Journal of Veterinary Sciences
www.alexjvs.com

AJVS. Vol. 59(1): 60-67. Oct. 2018
DOI: 10.5455/ajvs.299013

A New Trial in Egypt to Detoxify AFM1 in UHT Milk by Lactobacilli and their Bacteriocins

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Key words:

AFM1, UHT skim milk, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, bacteriocin.

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ABSTRACT

Aflatoxins are potent carcinogenic, mutagenic and immune suppressive agents. Acute exposures to aflatoxins cause rapid death due to liver failure. Several strains of probiotics, frequently used in food fermentation and preservation, have been reported to bind different types of toxins in liquid media. The purpose of this study was to evaluate the ability of heat killed *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* and their bacteriocins to detoxify aflatoxin M1 (AFM1) in UHT (ultra-heat- treated) skim milk spiked with 5, 10, 20 ng/ml AFM1.Both strains were heat killed (100 ^{o}C /1hr) before binding in spiked skim milk, the unbound AFM1 was measured by HPLC. Heat killed *Lb. acidophilus* had a significantly higher capability to bind AFM1 in UHT skim milk with mean percentage of (0 %, 27.3±2.9 % and 39.07 ±10.33%) in comparsion with *Lb. rhamnosus* (0%, 12.3±2.6% and 14.3± 3.48 %), respectively. On other hand, bacteriocins of both strains showed a significantly AFM1 reduction ranged from 33 to 77%. These findings shed light on the ability of heat killed *Lb. acidophilus*, *Lb. rhamnosus* and their bacteriocins to detoxify AFM1 in UHT skim milk.

1. INTRODUCTION

Milk is the primary source of human nutrition because of the fact that it contains appreciable amounts of macro and micro nutrients especially for infants and eldery (Dobrzanski *et al.*, 2005; Gonzalez-Montana *et al.*, 2012). Aflatoxins residues from foods of animal origin have the potential for introducing into the humane diet through milk (Galvano *et al.*, 2001). Aflatoxins are secondary metabolites produced by different kinds of fungi, namely *Aspergillus flavus* and *Aspergillus parasiticus* with intense mutagenic, carcinogenic, teratogenic, hepatotoxic and immunosuppressive properties that can cause adverse effects on animal and human health (Harper, 2003; Lewis *et al.*, 2005; Iha *et al.*, 2013).

Aflatoxin M1 (AFM1) is a hepatocarcinogen 4-hydroxy derivative of Aflatoxin B1 (AFB1), formed in liver and excreted into the milk of both human and lactating animals that have been fed with AFB1 contaminated diet (Gurbay *et al.*, 2010). The conversion rate of AFB1 into AFM1 ranges between 0.5 and 6% (Var and Kabak, 2009). Although AFM1 is about 10 times less toxigenic than AFB1, its carcinogenic effects has been demonstrated in several species, in addition to, it`s relative stability in raw and processed milk products and cannot be destroyed by heat treatments or pasteurization (Murphy *et al.*, 2006; Fallah, 2010; Iha *et al.*, 2013).

The International Agency for Research on Cancer has classified AFM1 as belonging to Group 1, a human carcinogen (IARC, 2002). Depending on the high incidence of AFM1 in milk is a serious public health problem; several countries have legislation for aflatoxins (Van Egmond and Jonker, 2004). Brazilian and USA regulations establishes the

maximum allowable limit of AFM1 in fluid milk is 0.5 µg/l (Agência Nacional de Vigilância Sanitária, 2011), although the European Union and Egyptian ministry of health considers 0.05 µg/kg as maximum level for AFM1 in raw milk, heat treated milk, and milk for the manufacture of dairy products (European Commission, 2006 and EOSQ, 2010).

Ideally, the best way to prevent aflatoxin contamination in the food chain is the adoption of improved agricultural practices and control of storage conditions of products. However, practical difficulties to effectively prevent contamination, along with the stability of aflatoxins under normal food processing conditions, have led to investigation of decontamination methods for food products that could be safe, effective, environmentally friendly and presenting a cost benefit (Elgerbi *et al.*, 2006 ;Wu *et al.*, 2009). In the recent years lactic acid bacteria (LAB) due to their generally regarded as safe (GRAS) status and use as probiotics, are of particular interest for reducing the bioavailability of AFM1 (Corassin *et al.*, 2013).

Among biopreservatives, bacteriocins that caught the attention of food researchers and industries to be used as natural food bio preservatives (Cleveland *et al.*, 2002). Bacteriocins are defined as ribosomally synthesized peptides or small proteins that have bactericidal or bacteriostatic action (Lemos *et al.*, 2008). Bacteriocins are non-toxic to human, don't alter the nutritional properties, effective at low concentration and active under storage conditions (Cotter *et al.*, 2005).

Bacteriocins could detoxify AFB1 very effectively. These proteins, which are colorless, odorless, and work at neutral pH levels, can be used in the food industry as an alternative to chemical preservatives for removing AFs (Sezer *et al.*, 2013).

Insight of these facts the present study aimed to detoxify of AFM1 by using heat killed *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* and their bacteriocins in UHT skim milk.

2. MATERIAL AND METHODS

2.1. Activation of *Lactobacillus rhamnosus* :

Lactobacillus rhamnosus *ATCC 7469* was obtained from Microbiology Department, Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt. The strain was activated in sterile 9 ml De Man, Rogosa and Sharpe, Biolife, Italy (MRS broth) at 37° C for 24 hr in anaerobic jar under CO_2 and N_2 atmosphere (Gas Pak System). Three subcultures were performed to activate the strain till obtaining the concentration of 10^{12}cfu/ml with some modification according to Magnuson and Schnürer (2001).

2.2. Activation of *Lactobacillus acidophilus* strain:

Lactobacillus acidophilus *DSMZ 20079* was obtained from Cairo MIRCEN (Microbiological Resource Center), Faculty of Agriculture, Ain Shams University, Cairo, Egypt. The strain was activated in sterile 9 ml MRS broth at 37 ° C for 24 hr, three subcultures were performed to activate the strain till obtaining the concentration of 10^{12} cfu /ml in order to meet the required level for active probiotic and then kept in refrigerator until use, within 24 hr (Ogunbanow et al., 2003).

2.3. Extraction of crude bacteriocins of *lactobacillus rhamnosus* and *Lactobacillus acidophilus:*

Ten ml of activated culture of *lactobacillus rhamnosus* and *Lactobacillus acidophilus* with a concentration of 10^{12}cfu/ml was inoculated into one liter of MRS broth under aseptic conditions and incubated at 37°C/16 hr as mentioned by Abdl El-Fattah (1999) and Chumchalova *et al.* (2004).

The crude bacteriocin (free cell extract) was obtained after heating cultured broth in water bath at 100°C for 5 min to get rid of H_2O_2 and cells were harvested by two successive centrifugations at 10,000 rpm for 20 min at 4°C. The supernatants were collected and neutralized at pH 7 by NaOH (1N) to exclude the effects of organic acids (lactic acid and acetic acid). Then the extract was sterilized by using 0.45 µm pore size Seitz filter with single sheet to eliminate the possible presence of viable bacterial cells to obtain *lactobacillus rhamnosus* cell free crude bacteriocin (Savadogo, *et al.*, 2004 and Simova *et al*., 2009).

2.4 Determination of protein concentration in crude bacteriocin:

Protein concentration in crude bacteriocins of *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* was determined by Kjeldahl`s method according to AOAC (2000).

2.5. Aflatoxin M1 binding assay in UHT milk:

2.5.1. Aflatoxin M1 raw material:

Solid AFM1 from *Aspergillus flavus* (Sigma Chemical Company, St. Louis, Mo.) was suspended in methanol in order to make 10 µg /ml stock solution. Various concentrations equivalent to (5 ng, 10 ng, 20 ng) AFM1/ml UHT milk were prepared for the assay (Pierides *et al.*, 2000).

2.5.2. Inoculation of heat killed *Lb. acidophilus* and *Lb rhamnosus* into skim milk UHT with different concentration of AFM1:

Activated strains of *Lactobacillus acidophilus* and *lactobacillus rhamnosus* at concentration 10^{10} cfu/ml were tested. Both strains

were heat-killed by boiling at 100 °C for 1 hr before the binding assays, to avoid any possible milk fermentation during the contact time (Corassin et al., 2013).

The AFM1 binding assay was performed using commercial UHT skim milk samples spiked with (5, 10 ,20) ng /ml AFM1 (Kabak and Var, 2008).

The AFM1 binding assays were performed in triplicates as evaluated by Pierides et al. (2000) convenient volumes of culture broths containing 10^{10} cells of each strain were transferred to Eppendorf tubes and centrifuged at 1800 g for 15 min.

The supernatant was discarded and the bacterial pellets were washed twice with sterile ultrapure water (Milli-Q, Millipore, Bedford, MA, USA). After that, the pellets were resuspended in 1.0 ml of UHT skim milk containing AFM1, vortexed for 3 min and incubated at 37°C for 24 hr. Following the contact times, the tubes were centrifuged again at 1800 g for 15min, the supernatant (milk layer) removed for analysis of AFM1.

(Bovo et al., 2013).

Positive control: only spiked skim milk containing 5, 10, 20 ng /ml AFM1.

Negative control: (only *Lactobacillus rhamnosus* and *Lactobacillus acidophilus*) and non-spiked UHT skim milk controls.

2.5.3. Inoculation of bacteriocins of *Lb. acidophilus* and *Lb rhamnosus* into skim milk UHT with different concentration of AFM1:

The assay was done by using 9000 ppm from the crude *Lb. rhamnosus* bacteriocin and 7000 ppm from the crude *Lb. acidophilus* bacteriocin and incubation in UHT milk spiked with 5, 10, 20 ng/ml AFM1 at 37^{o}C for 24 hr (Stancic et al., 2009).

2.5. 4. Analysis of AFM1 by using HPLC:

Analysis should be proceded by extraction and purification of the supernatant from the binding assays for AFM1 as mentioned by Fernandes et al. (2012), with some modifications proposed by the manufacturer of the immuno affinity columns. Identification and quantification of the AFM1 residues was achieved by injecting 20 ul of sample extracts in a high performance liquid chromatography (HPLC).

. Calibration curve of AFM1 was prepared using standard solutions of AFM1 (Sigma, St Louis, MO, USA) at concentrations of 3.125, 6.25, 12.5, 25 and 50 ng m/l. The detection limit of the analytical method was 0.01 ng/ml**.** HPLC analysis was carried out using an Aligent 1260 series. The separation was carried out using C18 column (4.6mm x 250mm i.d., 5 μm). The mobile phase consists of water: isopropanol: acetonitrile (80:12:8) at a flow rate 1ml/min. The fluorescence detector was monitored at 365 nm and excitation at 435 nm emission. The injection volume was 10 μl for each of the sample solutions. The column temperature was maintained at 35 ^{o}C.

2.6. Statistical analysis:

Statistical analysis of the data was done using the analysis of variance (ANOVA) in SPSS 16.0. Statistical comparisons were made by using one-way analysis of variance. The results were considered significantly different with $P < 0.05$ as described by Clarke and Kempson (1997).

2. RESULTS AND DISCUSSION

Bacteriocins have gained worldwide attention in recent years as a natural food preservative (Pal *et al.,* 2010). The results obtained showed that *Lactobacillus rhamnosus* bacteriocins had higher protein concentrations which were 0.9% than *Lactobacillus acidophilus* bacteriocins (0.7%) (Data not shown). Those results agreed to some extent with Al-Malky et al. (2017) and Srinivasan *et al.* (2013).

Kanatani et al. (1995) demonstrated that *Lb. acidophilus* culture supernatant contained lower protein concentrations which was two times higher compared to that of *Lb. plantarum* .These results are not surprising since it was well known that culture medium and incubation conditions greatly affected bacteriocins production in the genus of Lactobacilli (Todorov and Dicks, 2005).

3.1. Antimycotoxin activity (Detoxification activity) of heat killed *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* in UHT milk against AFM1.

Lactic acid bacteria act as an interesting alternative to physical and chemical methods of AFM1 control, these microbes can bind AFM1 and can reduce the bioavailability of AFM1 in the range of 5-50% (Khoury et al., 2011; Kabak and Ozbey, 2012; Bovo et al., 2013; Corassin et al., 2013). To avoid possible fermentation effects and for achieving higher binding rates heat killed microbial cells are preferred before binding assay (Elsanhoty et al., 2014).

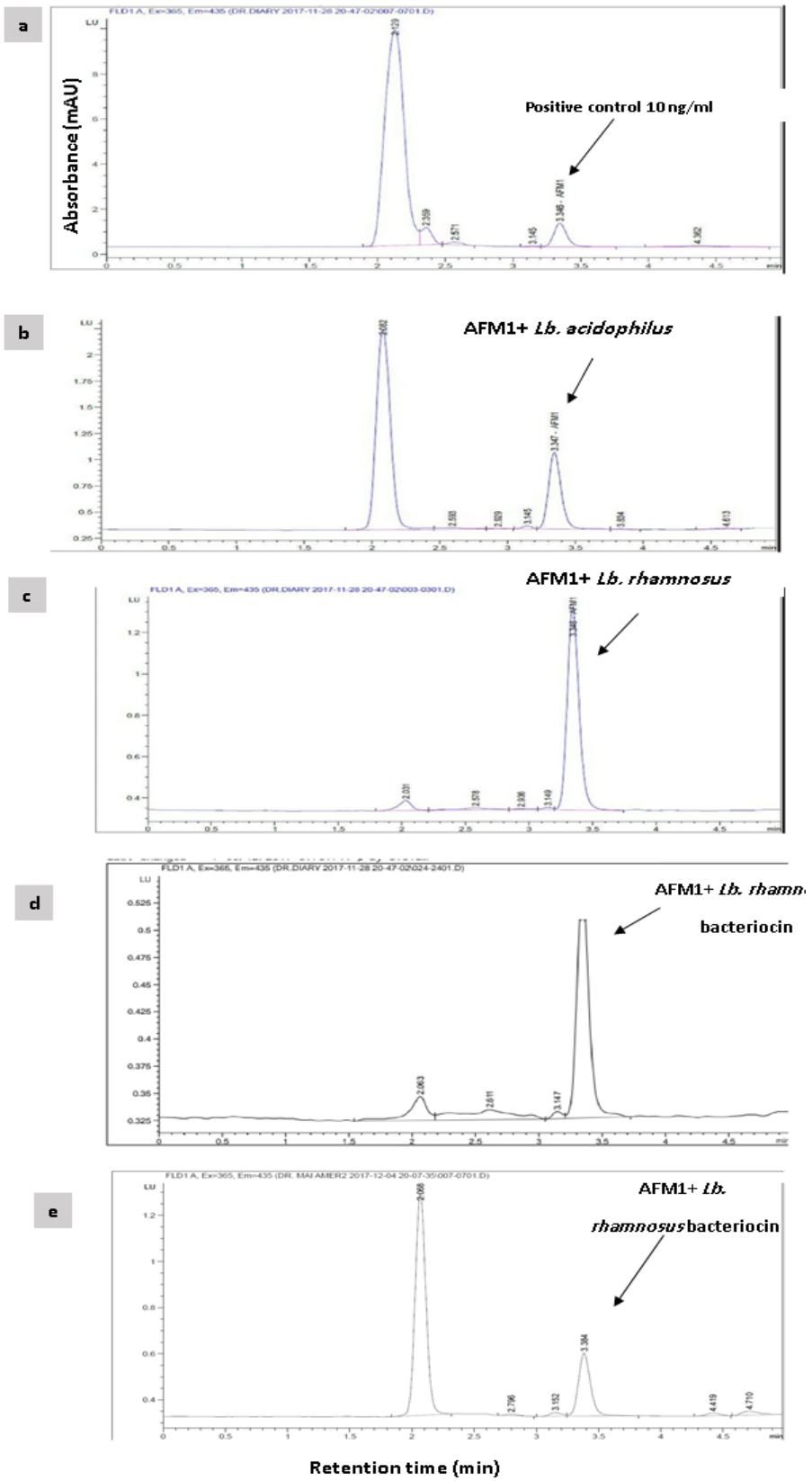

Figure (1): High performance liquid chromatography (HPLC) chromatograms of (a) Un treated (positive control 10 ng/ml) (b) UHT milk supplemented with AFM1 at 10 ng/ml after 24hr contact with *Lb. acidophilus* (c) UHT milk supplemented with AFM1 at 10 ng/ml after 24hr contact with *Lb. rhamnosus.* (d) UHT milk supplemented with AFM1 at 10 ng/ml after 24hr contact with *Lb. rhamnosus bacteriocin.*(e) UHT milk supplemented with AFM1 at 10 ng/ml after 24hr contact with *Lb. acidophilus* bacteriocin.

The percentages of AFM1 bound in UHT skim milk by *Lb. rhamnosus* and *Lb. acidophilus* (10^{10} cfu/ml) at different AFM1 concentrations were presented in Table (1). *Lactobacillus rhamnosus* showed mean percentages

of AFM1 bound of (0%, 12.3±2.6% and 14.3± 3.48 %) at (5, 10 and 20) ng/ml, respectively after contact time 24 hr. However, *Lb. acidophilus* had higher capability to bind AFM1 in UHT milk (0 %, 27.3±2.9 % and 39.07 ±10.33%) at (5, 10 and 20) ng/ml AFM1, respectively, after the same contact time Figure (1).

The percentages of AFM1 bound by the *Lb. rhamnosus* obtained in the present study are somewhat in agreement with those reported by Pierides *et al.* (2000). They observed that heat-killed cells of *Lb. rhamnosus* bound to18.8% of AFM1 in reconstituted skim milk powder and to 26.0% of toxin in reconstituted whole milk powder. Kabak and Var (2008) observed that binding percentages of AFM1 by non-viable Lactobacillus cells was 12.85%. Also, Bovo et al. (2013) found that binding percentage of AFM1 in UHT skimmed milk were 19.70 and 24.46 % at 4 °C and 37 °C, respectively.

Corassin et al. (2013) used a pool of three different heat killed lactic acid bacteria cells at the concentration of 10^{10} cells and found the binding percentages were in the range of 11- 12%.

The percentages of AFM1 bound by the *Lb. acidophilus* obtained in the present study are somewhat in agreement with those reported by Kabak and Var (2008) who found that heat-killed cells of *Lb. acidophilus* NCC 36 bound 24.13 and 25.09 % of AFM1 in reconstituted skim milk powder at concentration of 10 and 20 ng/ ml.

Elsanhoty *et al.* (2014) observed that heat treated *Lb. acidophilus* ATCC 20552 removed 43.9 % of AFM1 present in Phosphate buffer saline and 39.9 % of AFM1 in contaminated MRS broth.

The different binding abilities of lactic acid bacteria as being suggested in the current study were due to different cell-wall structure; in addition Pierides *et al.* (2000) reported that *Lb. rhamnosus* strain 1/3 has a less binding ability than *Lb. rhamnosus* strain GG in spite of the same genetic structure, and they presumed that this was caused by different biological activities of the strain.

Aflatoxin binding appeared to be a physical phenomenon with non-viable cells (Karazhiyan et al., 2016) through a physical union with the bacterial cell wall components, mainly to polysaccharides and peptidoglycans, (Lahtinen et al., 2004; Shetty and Jespersen, 2006).

It is apparent that bacterial viability is not a prerequisite for removal of AFM1 by LAB (Corassin *et al.*, 2013). Heat treated bacterial cells had higher binding abilities than viable ones (Ismail et al., 2017). The heat treatment greatly affected both polysaccharides and peptidoglycans of bacterial cell wall leading to denaturation of proteins and increasing the hydrophobic nature of its surface so it is considered that such disturbances still allow aflatoxin to bind to bacterial cell wall, and also to components of plasmatic membrane which were not available when cell wall was intact (Haskard et al., 2001).

Aflatoxin M1 binding ability of heat killed *Lb. rhamnosus* and *Lb. acidophilus* was increased, by increasing initial AFM1 concentration. This was explained by Lee et al. (2003) who reported that mycotoxin binding was dependent on its solution concentration and was always linear at low level of aflatoxin B1 and showed the transition to a plateau with higher toxin concentrations so the amount of toxin removed increased with increasing AFB1 concentration, but the percentage removed decreased with increasing toxin concentration (5 µg/ ml) because the saturation started.

These results are similar to those of Karazhiyan et al. (2016). The relative amounts of aflatoxin removed by viable as well as heat and acid-treated LAB bacteria depend on initial concentrations of toxin (El-Nezami et al., 1998; Elsanhoty et al., 2014; Peltonen et al., 2001)

These results disagree with Kabak and Var (2008) who found that the toxin concentration had no effect on the removal of AFM1 levels by both viable and heat-treated bacteria in reconstituted milk.

3.2. Antimycotoxin activity (Detoxification activity) of bacteriocins of both *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* in UHT milk against AFM1.

The result in Table (1) showed the ability of *Lactobacillus rhamnosus* bacteriocin and *Lactobacillus acidophilus* bacteriocin to reduce AFM1 concentration in UHT milk but this reduction was increasing by increase AFM1 concentrations.

Lactobacillus rhamnosus bacteriocin showed mean percentage of AFM1 reduction of (33.77±11.63, 77.3±6.67 and 46.93± 5. 99%) at (5, 10 and 20) ng/ml AFM1, respectively after contact time 24 hr. as compared to *Lactobacillus rhamnosus* bacteriocin, *Lactobacillus acidophilus* bacteriocin showed lower mean reduction percentage of AFM1 reduction of (33.20 ±8.26, 72.6 ±6.96 and 45.77±0.15 %) at (5, 10 and 20) ng/ml AFM1, respectively after the same contact time Figure (1).

Table (1): Percentages of Aflatoxin M1 bound by heat killed *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* and their bacteriocins in UHT skim milk.

Groups	% Binding of AFM1 (mean ± SE)		
	5 ng	10 ng	20 ng
Heat killed Lactobacillus rhamnosus	0	12.3 ± 2.6 [b]	14.3 ± 3.48 [b]
Heat killed Lactobacillus acidophilus	0	27.3 ± 2.9 [b]	39.07 ± 10.33 [a]
Lactobacillus rhamnosus bacteriocin	33.77 ± 11.63 [a]	77.3 ± 6.67 [a]	46.93 ± 5.99 [a]
Lactobacillus acidophilus bacteriocin	33.20 ± 8.26 [a]	72.6 ± 6.96 [a]	45.77 ± 0.15 [a]

[ab] Means of different groups within the same column having different superscripts are significantly different ($p \leq 0.05$). Results shown were SEM (Standard error of mean).

These results were in agreement with Sezer *et al.* (2013) who reported that *Lb. plantarum* bacteriocin could reduce 43.58 ± 1.30% of AFB1, *Lb. lactis bacteriocin* could reduce 16.18 ± 1.11% of AFB1 in phosphate buffer saline, while bacteriocin of *Lb. plantarum* and *Lb. lactis* co culture reduced 90% of AFB1, which may be due to bacteriocins which have strong toxin-binding ability with Aflatoxins.

3. CONCLUSION and RECOMMENDATION

Finally, it was concluded that *Lactobacillus rhamnosus* and their bacteriocin have ability to detoxify AFM1 in UHT skim milk effectively more than *Lactobacillus acidophilus* bacterial strains and their bacteriocin .So they can be used in the dairy industry as an alternative to chemical preservatives for removing AFM1. The application of this phenomenon in the removal of mycotoxins from contaminated food and feed is urgently needed to improve the safety of food and feed.

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