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# Removing aflatoxin M1 from milk with native lactic acid bacteria, centrifugation, and filtration

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In order to minimise human exposure to aflatoxin $M_1$ ($AFM_1$) the levels of this highly carcinogenic mycotoxin in milk, heat-treated milk, and other dairy products have been limited to <0.05 μg $kg^{-1}$. However, its removal from dairy products presents a challenge for dairy producers, as commercial additives change organoleptic properties, and filtration alone yields poor results. The aim of this study was to find a strain of lactic acid bacteria (LAB) from milk or dairy products that most effectively binds $AFM_1$ and to see whether heat treatment of the selected LAB affects the binding efficiency. We also wanted to investigate whether centrifugation can improve filtering of the obtained $AFM_1$-LAB complexes from milk. To do that, we isolated and identified 10 native LAB species/strains, incubated their viable or heat-treated cells ($10^8$ CFU $mL^{-1}$) in milk spiked with 0.5 μg $L^{-1}$ of $AFM_1$ at 4 °C for 0, 2, 4, and 24 h, and quantified the amount of unbound $AFM_1$ with HPLC. $AFM_1$ binding efficiency ranged from 21 to 92 % for viable cells and from 26 to 94 % for the heat-treated ones. Since both viable and heat-treated *Lactobacillus plantarum* KM showed the best results, we used it for the next step in $AFM_1$ removal from milk. Heat treatment in combination with filtration and centrifugation yielded removal as high as 96 %.

KEY WORDS: *HPLC, lactic acid bacteria, L. plantarum KM, secondary fungal metabolites, viable and heat-treated cells*

Although grain and cereal-based products are the main source of mycotoxins for humans, mycotoxins can enter human body through meat, eggs, milk, and dairy products of animals fed with fodder contaminated with mycotoxins or moulds that synthesise them (*carryover* effect) (1-5). Aflatoxin $M_1$ ($AFM_1$), a highly carcinogenic mycotoxin, is a product of aflatoxin $B_1$ ($AFB_1$) metabolism in the liver (6). It is excreted in the tissues, biological fluids, and milk of lactating animals, including humans (7, 8). Its content in milk correlates with the $AFB_1$ level in feed (9). Once it enters milk, $AFM_1$ cannot be removed by heat treatment such as pasteurisation or sterilisation (10). Instead, it is carried over to dairy products, including milk powder, cheese, yogurt, and butter (11).

For this reason, the EU countries have limited $AFM_1$ levels in raw milk, heat-treated milk, and milk for the production of dairy products to <0.05 μg $kg^{-1}$ (12, 13). Milk with $AFM_1$ levels above this limit should not be used for human consumption or dairy production nor should it be mixed with uncontaminated milk to bring $AFM_1$ level down to acceptable.

This limit, of course, creates serious problems for dairies who cannot control $AFM_1$ levels, as their products become unmarketable. Physical and chemical methods for removing mycotoxins from milk are limited because of safety issues, quality losses in dairy produce, poor efficiency, and high cost (14). One of the ways to remove mycotoxins from milk is to use additives/mycotoxin deactivation products and intestinal adsorbents such as aluminium silicates, clay, or zeolite. Detoxification additives are efficient in bringing contamination levels below the European limit for $AFM_1$, while their residues in milk present no concern for human health (3). Intestinal adsorbents, in turn, are used with animal feed to reduce $AFB_1$ absorption in animal body and prevent further toxin metabolism, distribution, and carryover to milk. However, as these adsorbents alter the nutritional properties of food (3, 15-17), alternative solutions have been proposed, such as the use of microorganisms (18), lactic acid bacteria (LAB) in particular. LAB showed success, albeit varying, in binding $AFM_1$ in phosphate buffer and milk solutions (11, 19-24).

Problems arise when commercial starter cultures that bind $AFM_1$ compete with native microflora and change traditional organoleptic properties of the fermented product. Another problem is the removal of LAB-$AFM_1$ complex, since the data about mycotoxin complex removal are still scarce.

The aim of our study was to determine the binding parameters of $AFM_1$ in milk by native LAB (viable and

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heat-treated cells) that would not affect the organoleptic properties of a product and also determine the LAB-$AFM_1$ complex removal efficiency with the membrane filtration method.

## MATERIALS AND METHODS

*Isolation and identification of LAB*

To obtain as many native LAB strains as possible for further analysis, we isolated and identified them from fresh cow milk, cottage cheese, and cream of randomly selected dairy producers. Milk and dairy samples (N=5 of each) were prepared for microbiological testing according to the standard procedure (25) by serially diluting 10 mL samples of milk and cream or 10 g of cottage cheese (n=5) homogenised in 90 mL of sterile buffered solution, until we obtained 0.1 mL samples that were inoculated on MRS agar (Biolife, Milano, Italy) and incubated at 30 °C for 48 h.

Lactic acid bacteria were isolated from the milk, cream, and cheese samples following the ISO 15214:1998 procedure (26). We randomly took 150 LAB colonies cultivated on MRS agar and identified them based on colony and cell morphology and Gram staining. Identification was confirmed with the API 50 CHL biochemical test kit (BioMérieux, Marcy l'Etoile, France) (27, 28) following the manufacturer's instructions. The isolated LAB strains were stored in MRS broth (Biolife) containing 30 % glycerol (in volume) at -70 °C until further analysis.

*Standard $AFM_1$*

The working solution of $AFM_1$ was prepared by diluting the standard 0.5 μg $mL^{-1}$ solution in acetonitrile (LGC Promochem, Leeds, UK) to 0.05 μg $mL^{-1}$ and storing it at 4 °C until further analysis.

*Determination of $AFM_1$ to LAB binding in milk*

To determine $AFM_1$ binding to LAB in milk, the LAB were grown in MRS broth at 30 °C for 48 h. For each bacterial strain the cells were harvested by centrifugation (3000×*g* for 10 min) under aseptic conditions at room temperature, washed three times with 5 mL of sterile deionised water, resuspended in sterile deionised water, and divided in two groups. The first group contained viable cells and the second cells heated in a water bath at 100 °C for 60 min (heat-treated cells). Heat-treated cells have already been reported to bind $AFM_1$ (11, 19, 20, 24).

Before the experiment began, we verified that the sterile milk samples were not contaminated with $AFM_1$. Two hundred millilitres of milk were then inoculated with a suspension of either viable or heat-treated LAB cells ($10^8$ CFU $mL^{-1}$) and 0.5 μg $AFM_1$ per litre of milk. The samples were then incubated at 4 °C to see if the LAB strains could reduce $AFM_1$ levels in milk at refrigeration temperature. $AFM_1$ binding was determined with high-performance liquid chromatography (HPLC, see detailed description below) at 10–15 min after the sample was homogenised (0 h) and then at 2, 4, and 24 h of incubation by analysing the amount of unbound $AFM_1$ in the supernatant obtained by centrifuging the samples at 3000×*g* for 20 min.

*Determining $AFM_1$ residues after filtration*

To find the most efficient filtration method for the removal of $AFM_1$ from milk – alone or bound to viable or heat-treated *L. plantarum* KM cells, which showed the best binding results in the previous test (see below) – we tested two methods: the first involved centrifuging and filtering 50 mL milk samples and the second only filtering without centrifugation. The centrifugation parameters were 6000×*g* for 30 min followed by vacuum filtration on 0.45 μm pore size cellulose-nitrate filters (Sartorius Stedim Biotech GmbH, Goettingen, Germany) at a flow rate of 0.5 mL $min^{-1}$. Ten to 15 min after centrifugation and/or filtering, 15 mL filtrates were collected and $AFM_1$ was quantified using HPLC.

*HPLC analysis of residual $AFM_1$*

Before HPLC analysis, the samples (25 mL) were purified on immunoaffinity columns (VICAM, Milford, MA, USA) conditioned with 10 mL phosphate buffer saline (PBS). Sample flow rate did not exceed 3 mL $min^{-1}$. The imunoaffinity columns were then washed with 10 mL of distilled water and dried under vacuum. $AFM_1$ was eluted with 4 mL of acetonitrile. The eluate was collected in a tube and evaporated to dryness in a stream of nitrogen.

The concentration of residual $AFM_1$ in the samples was determined on an Agilent 1100 HPLC System (Agilent, Santa Clara, CA, USA) equipped with a CSI-6150 online vacuum column degasser (Cambridge Scientific Instruments, Witchford, Ely, England), an Agilent isoquantine pump (model G 1310 A9), and a fluorescent detector (Agilent 1100, FLD G1321A) with wavelength excitation and emission of 365 nm and 455 nm, respectively. Separation was carried out on a Synergi Polar column (150x3.0 mm, 4 μm) (Agilent). For the mobile phase we used water/acetonitrile (60/40, v/v) at a flow rate of 0.5 mL $min^{-1}$ (29).

*HPLC method validation*

For validation we used a standard of $AFM_1$ in acetonitrile ($AFM_1$ solution), milk free of $AFM_1$ ($AFM_1$-free milk), and milk spiked with $AFM_1$ ($AFM_1$-spiked milk). Linearity was tested by injecting seven concentrations of the working standard. Each concentration was injected three times, and the regression line and correlation coefficient determined.

The linear equation was:

$y=8.788x-1.193$

where y stands for area and x for concentration.

The correlation coefficient was 0.999. The selectivity of the method was determined by comparing the chromatograms of the standard $AFM_1$ solution, $AFM_1$-spiked milk, and $AFM_1$-free milk. The limit of detection (LOD) of $AFM_1$ in milk was 0.005 μg $kg^{-1}$, and the limit of quantitation (LOQ) 0.010 μg $kg^{-1}$. The recoveries of spiked $AFM_1$ in milk at different levels show that this method is reliable for the determining $AFM_1$ in milk (Table 1).

*Statistical analysis*

All experiments were carried out in triplicate. For statistical analysis we used STATISTICA v. 7.1 for Windows 10.0 (StatSoft, Inc, Tulsa, OK, USA). We compared mean percentages of bound $AFM_1$ between viable and heat-treated cells for each strain using the two-way analysis of variance (ANOVA) and set the level of significance at $p<0.05$.

**Table 1** *HPLC validation of $AFM_1$ recoveries*

| Analyte | Spiking (μg $kg^{-1}$) | Recovery range (%) | RSD (%) |
|---|---|---|---|
| $AFM_1$ | 0.01 | 92–96.5 | 2.2 |
| | 0.05 | 91.5–95.4 | |
| | 0.10 | 91.9–94.3 | |

## RESULTS AND DISCUSSION

*$AFM_1$ binding by LAB*

Among the 150 isolated colonies the biochemical API tests identified five LAB species of two genera (*Lactobacillus* 83.3 % and *Lactococcus* 16.7 %) with 99–99.9 % accuracy (Table 2). The predominant species in cow milk was *L. plantarum* (51.9 %), while *Lactobacillus helveticus* and *Lactobacillus paracasei* dominated in the cottage cheese and cream (51.1 % and 45.3 %, respectively).

The results were expressed as the percentage of $AFM_1$ bound in the LAB+$AFM_1$ complex to see which LAB species/strain, viable or heat-treated, was the most effective in $AFM_1$ binding. Every measurement included positive control (only $AFM_1$ in milk).

Table 3 shows $AFM_1$ binding efficiency of the identified native LAB species. The most efficient was *L. plantarum* KM at 4 h of incubation, heat-treated or not. Heat treatment, however, significantly improved its efficiency (from 92.7 % to 94.5 %, $p<0.05$).

These findings are consistent with the ones reported by Kabak and Var (11), who also observed a rise in $AFM_1$ binding between viable (7.9–25.9 %) and heat-treated cells (12.9–27.3 %) of *Lactobacillus* and *Bifidobacterium* after 4 h of incubation. Pierides et al. (24) also found significantly improved $AFM_1$ removal by *Lactobacillus* strains when they were heat-treated.

All these findings, including ours, suggest that LAB do not need to be alive to bind $AFM_1$. Although the mechanism of action of these microorganisms on aflatoxin has not yet been clarified, some authors suggest that $AFM_1$ molecules physically attach to bacterial cell components, mainly polysaccharides and peptidoglycans, instead of creating covalent bonds with them or being metabolised by them (30, 31). Furthermore, heat treatment seems to increase the attachment surface for $AFM_1$ by making available not only the LAB cell wall but also the plasma components, as the cell wall collapses (32).

Our findings have also shown that $AFM_1$ binding is reversible; all the isolated LAB released some of the bound $AFM_1$ as incubation continued. In fact, viable *L. plantarum* KM cells released back a great percentage of $AFM_1$ after 24 h, which underscores the advantages of heat treatment.

*$AFM_1$ removal by filtration*

Figure 1 shows the removal efficiency of $AFM_1$ from milk by filtration with or without centrifugation. Its removal from $AFM_1$-spiked milk without LAB by filtration alone was 81.2±0.2 % and in combination with centrifugation it reached 83.3±0.2 %. In contrast, the removal of the viable LAB-$AFM_1$ complex (obtained with the treatment with viable cells) in combination with centrifugation and filtration was less effective: 78.9±0.3 %. However, removal with combined centrifugation and filtration significantly increased to 95.7±0.2 % for the heat-treated LAB-$AFM_1$ complex. Of course, this increase has to do with the higher

**Table 2** *Native LAB species in cow milk, cottage cheese, and cream*

| Sample | N | Identified LAB | N isolates/N samples |
|---|---|---|---|
| Fresh cow milk | 5 | *Lactobacillus plantarum* | 27/5 |
| | | *Lactobacillus rhamnosus* | 18/5 |
| | | *Lactococcus lactis* | 7/3 |
| Cow cheese | 5 | *Lactobacillus plantarum* | 5/5 |
| | | *Lactococcus lactis* | 7/5 |
| | | *Lactobacillus helveticus* | 23/5 |
| | | *Lactobacillus rhamnosus* | 10/2 |
| Cream | 5 | *Lactobacillus plantarum* | 18/5 |
| | | *Lactobacillus paracasei* | 24/5 |
| | | *Lactococcus lactis* | 11/2 |

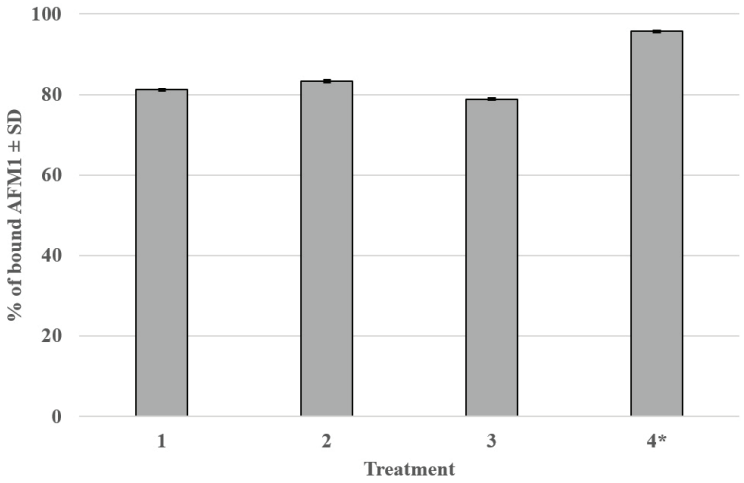


**Figure 1** *Comparison of $AFM_1$ removal efficiency between methods. 1 – $AFM_1$ removal from spiked milk (0.5 μg $L^{-1}$ of $AFM_1$) with filtration alone; 2 – $AFM_1$ removal from spiked milk (0.5 μg $L^{-1}$ of $AFM_1$) with filtration and centrifugation; 3 – removal of the $AFM_1$ + viable L.* plantarum *KM cell complex with filtration and centrifugation; 4 – removal of the $AFM_1$ + heat-treated L.* plantarum *KM cell complex with filtration and centrifugation. *statistically significant difference from treatments 1, 2, and 3*

$AFM_1$ binding ability of the heat-treated *L. plantarum* KM strain.

We believe that these findings greatly contribute to the current knowledge about $AFM_1$ removal from milk. Assaf et al. (19) have only recently studied the retention of $AFM_1$ on filters and proposed that it is owed to $AFM_1$ adsorption on the filter membrane. In combination with LAB treatment (*L. rhamnosus* GG) and centrifugation, the retention on filter paper was even greater, which the authors explained with greater contact surface between the LAB and $AFM_1$. Additionally, centrifugation improved the retention, because $AFM_1$ was bound to milk components such as fat globules or casein separated from milk by centrifugation and subsequent deposition. $AFM_1$ preference for milk proteins had been demonstrated earlier by Brackett and Marth (33).

However, centrifugation in combination with viable *L. plantarum* KM cells yielded lower $AFM_1$ removal from milk. We believe that this is because centrifugation breaks up electrostatic bonds between viable cells and $AFM_1$.

## CONCLUSION

Our study has singled out the most efficient $AFM_1$ removal procedure using the native LAB in order to avoid organoleptic changes in milk. It consists of refrigeration at 4 °C with heat-treated *L. plantarum* KM and then centrifugation and filtering, which in our experiment ensured over 95 % removal of $AFM_1$ from contaminated milk.

In practical terms, we believe that this procedure can find wide application among dairy producers, as it does not require special skills or equipment, provided that the native *L. plantarum* KM strain is available.

Our future studies shall focus on better understanding the interaction between bacteria and $AFM_1$. To do that, it is necessary to examine multiple LAB strains and other cultures used in the food industry.

*Conflicts of interest*

None to declare.

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**Table 3** *$AFM_1$ binding efficiency by lactic acid bacteria strains (viable and heat-treated) and incubation time*

| Treatment | % of bound $AFM_1$±SD | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| -- | 0 h | | 2 h | | 4 h | | 24 h | |
| Strain | Viable | Heat-treated | Viable | Heat-treated | Viable | Heat-treated | Viable | Heat-treated |
| *L. plantarum* SM1 | 80.6±0.1 | 80.2±0.1 | 24.9±0.1 | 76.1±0.2* | 79.6±0.2 | 79.2±0.2 | 35.9±0.2 | 26.1±0.1 |
| *L. plantarum* SMB | 91.0±0.1 | 84.6±0.2* | 73.1±0.2 | 75.7±0.1 | 82.45±0.2 | 46.94±0.1* | 21.4±0.2 | 64.1±0.2* |
| *L. plantarum* MM | 80.6±0.2 | 78.8±0.1 | 58.2±0.1 | 55.9±0.2 | 72.24±0.2 | 84.3±0.2* | 47.6±0.3 | 48.2±0.2 |
| *L. plantarum* KM | 62.45±0.2 | 83.3±0.2* | 75.5±0.2 | 62.7±0.1* | **92.7±0.1** | **94.5±0.3** | 26.1±0.1 | 85.7±0.1* |
| *L. paracasei* KM | 43.1±0.1 | 87.14±0.1* | 38.2±0.3 | 71.84±0.2* | 57.4±0.1 | 86.5±0.2* | 68.8±0.2 | 77.14±0.3 |
| *L. rhamnosus* KM | 41.0±0.1 | 83.3±0.2* | 32.04±0.2 | 63.3±0.2* | 84.7±0.1 | 77.6±0.1 | 43.1±0.2 | 84.1±0.1* |
| *L. plantarum* SMA | 29.6±0.2 | 51.44±0.3* | 44.14±0.1 | 43.2±0.2 | 34.6±0.2 | 37.6±0.2 | 35.7±0.3 | 37.04±0.2 |
| *L. plantarum* SS1 | 42.9±0.3 | 71.2±0.3* | 47.0±0.2 | 55.1±0.3 | 86.6±0.2 | 63.2±0.1* | 36.5±0.1 | 46.64±0.2* |
| *L. helveticus* S9 | 56.6±0.2 | 33.9±0.1* | 52.9±0.2 | 69.4±0.2* | 40.5±0.2 | 47.8±0.3 | 51.44±0.1 | 42.4±0.1 |
| *L. lactis* 5MS1 | 77.9±0.2 | 71.9±0.2 | 33.8±0.1 | 34.2±0.1 | 38.4±0.3 | 44.3±0.2 | 38.6±0.1 | 37.04±0.2 |

** statistically significant difference in comparison to viable cells ($p<0.05$)*


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**Uklanjanje aflatoksina $M_1$ iz mlijeka s autohtonim bakterijama mliječne kiseline centrifugiranjem i filtriranjem**

Kako bi se smanjila izloženost ljudi aflatoksinu $M_1$ ($AFM_1$), razina toga visokokancerogenoga mikotoksina u mlijeku, termički obrađenome mlijeku i drugim mliječnim prerađevinama ograničena je na <0,05 μg $kg^{-1}$. Međutim, njegovo uklanjanje iz mliječnih prerađevina pravi je izazov za proizvođače mlijeka, jer komercijalni aditivi mijenjaju njegova organoleptička svojstva, a sama filtracija daje slabe rezultate. Cilj ovoga rada bio je pronaći soj bakterije mliječne kiseline (BMK) iz mlijeka ili mliječnih prerađevina koji najučinkovitije veže $AFM_1$ te vidjeti utječe li termičko tretiranje izabrane BMK na učinkovitost vezanja. Također, željeli smo istražiti može li centrifugiranje poboljšati filtriranje dobivenog $AFM_1$-BMK kompleksa iz mlijeka. Kako bismo to učinili, izolirali smo i identificirali 10 autohtonih vrsta/sojeva BMK, inkubirali žive ili termički tretirane stanice ($10^8$ CFU $mL^{-1}$) u mlijeku s 0,5 μg $L^{-1}$ $AFM_1$ na 4 °C tijekom 0, 2, 4 i 24 sata te kvantificirali nevezani $AFM_1$ koristeći se HPLC-om. Učinkovitost vezanja $AFM_1$ živim stanicama kretala se od 21 do 92 % te od 26 do 94 % termički tretiranim stanicama. Budući da su i žive i termički tretirane stanice *Lactobacillus plantarum* KM pokazale najbolje rezultate, navedeni se soj koristio u sljedećem koraku uklanjanja $AFM_1$ iz mlijeka. Tretman toplinom u kombinaciji s filtracijom i centrifugiranjem rezultirao je uklanjanjem do 96 % $AFM_1$ iz mlijeka.

KLJUČNE RIJEČI: *HPLC;* L. plantarum *KM; sekundarni metaboliti plijesni; termički tretirane stanice; žive stanice*