Acta Alimentaria 52 (2023) 4, 579–588
DOI: 10.1556/066.2023.00139

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Aflatoxin M1 binding by probiotic bacterial cells and cell fractions

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ORIGINAL RESEARCH PAPER

Received: June 6, 2023 • Accepted: October 3, 2023
Published online: November 9, 2023

ABSTRACT

Food industrial bacterial cells eliminate aflatoxin M1 (AFM1) at different ratios. The study aimed to investigate the effect of AFM1 on probiotic industrial bacteria (*Lactococcus lactis* ssp. *lactis* R703, *Bifidobacterium animalis* ssp. *lactis* BB12, and *L. paracasei* subsp. *paracasei* 431) and evaluating their AFM1 binding ability in naturally contaminated milk. The growth of the R703 strain was affected by AFM1 at 1.47 μg L^{-1} concentration. Peptidoglycan (PG) cell wall fractions of R703 and BB12 bound a significant amount of AFM1 from naturally contaminated milk under one-hour treatment, while *L. paracasei* 431 was not effective. PG was better absorbent for AFM1 than viable cells of BB12, while the difference was insignificant for the R703 strain. Increasing the time did not significantly change the mycotoxin binding of BB12, while for R703 PG the absorption seemed reversible. BB12 PG needs further analysis for biotechnological application in dairy products.

KEYWORDS

aflatoxin, peptidoglycan, lactic acid bacteria

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1. INTRODUCTION

Mycotoxins are low-molecular-weight organic compounds produced as secondary metabolites by filamentous fungi and cause severe diseases in animals and humans. Three genera of fungi (*Aspergillus*, *Fusarium*, and *Penicillium*) are the most important mycotoxin producers (Marin et al., 2013).

Aflatoxins (AF) are the most toxic mycotoxins produced mostly by aspergilli, which cause a significant economic loss in agriculture (Mitchell et al., 2016). The most dangerous aflatoxin form is aflatoxin B1 (AFB1), which is hepatotoxic and genotoxic. AFB1 has been considered a Group 1 carcinogen by the International Agency for Research on Cancer (IARC, 2012; Ostry et al., 2017). Aflatoxin M1 (AFM1), a hydroxylated metabolite of aflatoxin B1, is excreted into milk in mammals. About 0.3%–6.2% of AFB1 from the feed is presented as AFM1 in cow milk (European Food Safety Authority, 2004). Its excretion depends on several factors, such as animal genetics, seasonal changes, the milking processes, and other environmental conditions (Unusan, 2006). AFM1 is structurally like AFB1 but is associated with lower cytotoxicity, carcinogenicity, genotoxicity, mutagenicity, and teratogenicity (Awuchi et al., 2022). AFM1 in milk and dairy products is a real health safety issue as people of all ages regularly consume milk and dairy products in their diets (Benkerroum, 2016).

Among bacteria taken into consideration, lactic acid bacteria (LAB) and *Bacillus* spp. are two groups that are tested on food the most due to their benefits to the host. Their role has been reported not to be restricted to antimicrobial ability, specific strains can inhibit mycotoxin production or decrease mycotoxin concentrations (Nasrollahzadeh et al., 2022; Mateo et al., 2023). LAB have a significant and well-known role in food fermentation and are found in the human and animal intestines and on mucous membranes. These bacteria are Gram-positive with natural lactic acid fermentation. LAB are also the most popular probiotic microorganisms with beneficial health properties (Zoghi et al., 2014). *Lactococcus* and *Lactobacillus* among LAB could prevent or limit the growth of potential mycotoxin-producing fungi like penicillia, aspergilli, and fusaria (Dalié et al., 2010; Bangar et al., 2021), so it was proven that LAB could reduce fungal growth and aflatoxin production. Beside antimicrobial activity, LAB and bifidobacteria have several significant abilities such as antioxidant activity, and vitamin and exopolysaccharide production. In addition, binding, absorbing, and biotransforming metal ions (Zoghi et al., 2014; Martínez et al., 2020) from the media into their organic forms is one characteristic documented in LAB.

LAB and bifidobacteria are Generally Regarded as Safe (GRAS; FDA, USA) organisms or granted QPS (Qualified Presumption of Safety) status by EFSA in Europe (EFSA, 2018) and are good candidates for food-grade mycotoxin binders.

Considering the composition, physicochemical characteristics, and milk's aflatoxin binding capacity (on casein fraction; Indyk et al., 2021), the situation of toxin availability is highly complex. Second derivative ATR-FTIR spectroscopy results confirmed the occurrence of hydrophobic interactions between the AFM1 and milk proteins and suggested that the affinity towards casein can be attributed to its porous structure and AFM1 interaction with the C=O bond existing in the proteins (Harshitha et al., 2023). Unfortunately, in most studies focused on applying bacteria to remove aflatoxins, phosphate-buffered saline (PBS) solution amended with mycotoxin was used in the experiments (Peles et al., 2021) instead of milk.

The study aimed to investigate the effect of AFM1 on probiotic industrial bacteria (*Lactococcus lactis* ssp. *lactis* R703, *Bifidobacterium animalis* ssp. *lactis* BB12, and *L. paracasei* subsp. *paracasei* 431) and testing the AFM1 binding ability of the different cell fractions in naturally contaminated milk.

2. MATERIALS AND METHODS

2.1. Culture conditions

L. lactis ssp. *lactis* R703, *B. animalis* ssp. *lactis* BB12, and *L. paracasei* ssp. *paracasei* (*Lactobacillus casei*) 431 (Chr. Hansen A/S, Denmark) strains were cultured in de Mann–Rogosa–Sharp (MRS) Broth medium (Scharlab, Barcelona, Spain) and incubated for 24 h at 30 °C. The bacterial cultures were centrifuged (Biofuge, Pico, Heraeus) at 8,000 r.p.m. for 10 min (4 °C) under sterile conditions to remove the supernatant. The collected biomass was washed three times with PBS and distributed into Eppendorf tubes in 100 µL aliquots for the treatments. The cell concentrations were 10^9 CFU mL^{-1}. The washed, resuspended culture was diluted in PBS and the concentrations were checked with plate count method on MRS agar. The aliquots were stored at −18 °C.

2.2. Cell growth

Cell growth was measured with densitometry at 630 nm using a microtiter plate reader (Synergy HTX multi-mode reader, BioTec Hungary Ltd., Szigetszentmiklós, Hungary), where 10 µL of the 16 h cultures (10^9 CFU mL^{-1}) were loaded with 200 µL MRS Broth and 5 µL (0–1.47 µg L^{-1}) AFM1 toxin (Biopure, Romer Labs, Tulln, Austria) at the inoculation time. The measurement was continued up to 24 h at 30 °C. The cultures were shaken before every measurement time. Data ($n = 4$) gained for all measurement points were analysed statistically ($P < 0.05$).

2.3. Cell fractionation

The biomass was washed three times with 200 µL sterile phosphate-buffered saline (PBS), followed by centrifugation at 8,000 r.p.m for 10 min (4 °C) and the supernatant was removed. After the washing steps, different treatments were applied. 10 w/v % trichloroacetic acid (TCA) for the peptidoglycan fraction; H_2O for the cell debris; 2% w/v sodium dodecyl-sulphate (SDS) for the cell wall fraction; 0.1 M HCl for the teichoic acid and other glycophosphate fractions, all at 100 °C for 15 min (Niderkorn et al., 2009). Untreated viable cells were washed only with PBS. After the treatments, the supernatants were removed, and the pellets were washed 3 times with 200 µL PBS. The fractions were stored at −18 °C.

2.4. Quantification of AFM1 in the ELISA system

Aflatoxin M1 High Sensitivity ELISA (enzyme-linked immunosorbent assay) assays (Romer Labs, Tulln, Austria) were carried out using a direct competitive assay according to the manufacturer's instructions applying TS50 microplate washer (BioTec Hungary Ltd., Szigetszentmiklós, Hungary). The samples were measured at 450 nm using a microtiter plate reader (Synergy HTX multi-mode reader, BioTec Hungary Ltd., Szigetszentmiklós, Hungary). Measurements were done in four repetitions with CV <5%.

2.5. AFM1 binding of cell fractions

The naturally contaminated raw milk sample was centrifuged (4,000 *g*, 10 min, 4 °C) to reduce the fat content, and the AFM1 content was measured by the Aflatoxin M1 High Sensitivity ELISA (Romer Labs, Tulln, Austria). 450 µL of the non-fat milk was pipetted into Eppendorf tubes, and 50, 100, or 150 µL of the peptidoglycan fractions, cell debris, cell wall fraction, teichoic acid, and other glycophosphate fractions, and untreated viable cells were added to test the AFM1 binding ability. Slow horizontal mixing (100 r.p.m.) was carried out at 4 °C for 1 h or 2 h for proper homogenisation. After 10 min of centrifugation, the remaining AFM1 concentration was measured by ELISA from the supernatant. PBS mixed with milk was applied as the control sample. Binding assays were repeated three times with between-run CV < 15%, and within-run CV <5%.

2.6. Statistical analysis

Data analysis was done with Microsoft Excel Data Analysis ToolPac. Growth data analyses were processed in Gen5 3.05 software (BioTec) and Microsoft Excel Analysis ToolPac, where Pearson's *t*-probe (at $P \leq 0.05$) was performed for the significance analysis.

3. RESULTS AND DISCUSSION

3.1. Bacterial growth under aflatoxin M1 stress

Aflatoxin M1 (AFM1) was tried at different concentrations against the tested probiotic strains. No significant changes in the growth were detected under the AFM1 treatment of the viable cells in the MRS medium except for *L. lactis* ssp. *lactis* R703 culture, where a small but significant ($P < 0.05$) decrease in cell density was detected in the exponential growth phase of the cultures (Fig. 1) at high AFM1 concentration (1.47 µg L^{-1}).

3.2. AFM1 binding of viable biomasses and cell fractions

The AFM1 concentration of the defatted naturally contaminated milk was 30 ± 5 ng kg^{-1}. After treatment, the residual AFM1 content of the milk was the lowest with the peptidoglycan fraction of *L. lactis* ssp. *lactis* R703 biomass (58%). Following the peptidoglycan fraction, the residual AFM1 content of milk was high for each fraction, purified cell wall (75%), teichoic acid fraction (84%), and cell debris (91%) (Fig. 2).

Meanwhile, 67% of the initial concentration of the AFM1 remained in the milk with untreated live biomass. Interestingly, the increase in peptidoglycan cell fraction did not result in a decreased AFM1 concentration in the milk.

Binding of AFM1 by *B. animalis* ssp. *lactis* BB12 cell preparations are shown in Fig. 2. The treatment with TCA gaining the peptidoglycan fraction showed the highest AFM1 binding capacity. The residual AFM1 content of the milk was the lowest with the peptidoglycan fraction of *B. animalis* ssp. *lactis* BB12 biomass (60%). Following the peptidoglycan fraction, the AFM1 content of milk increased for each fraction, purified cell wall (78%), the teichoic acid fraction (70%), cell debris (75%), and viable cells (81%), but the difference in the binding capacity from the one of the viable cells was insignificant.

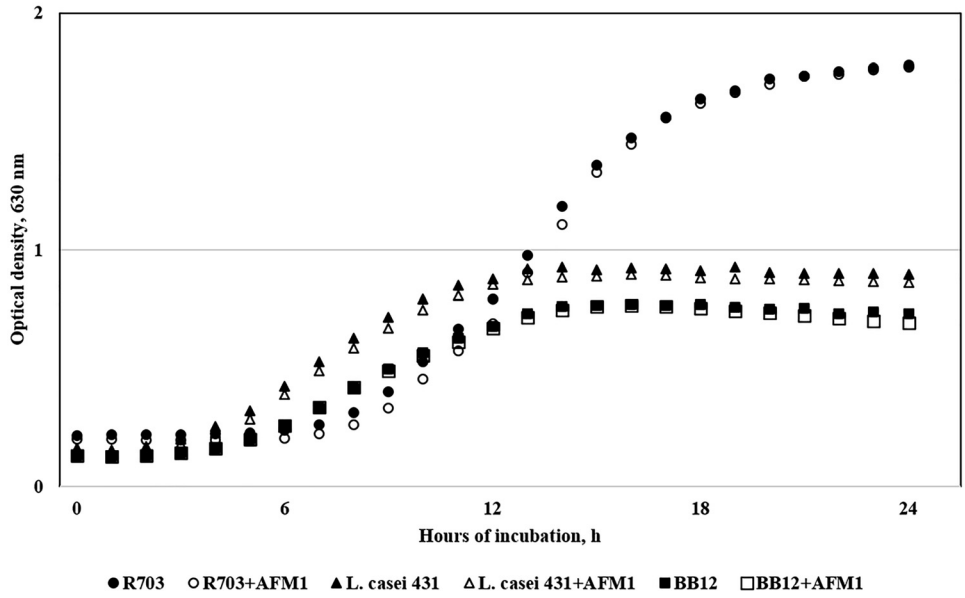

Fig. 1. Growth of *Lactococcus lactis* ssp. *lactis* R703, *Bifidobacterium animalis* ssp. *lactis* BB12, and *L. paracasei* subsp. *paracasei* (*L. casei*) 431 with and without aflatoxin M1 (1.47 μg L^{-1}) treatment (n = 4; CV < 5%)

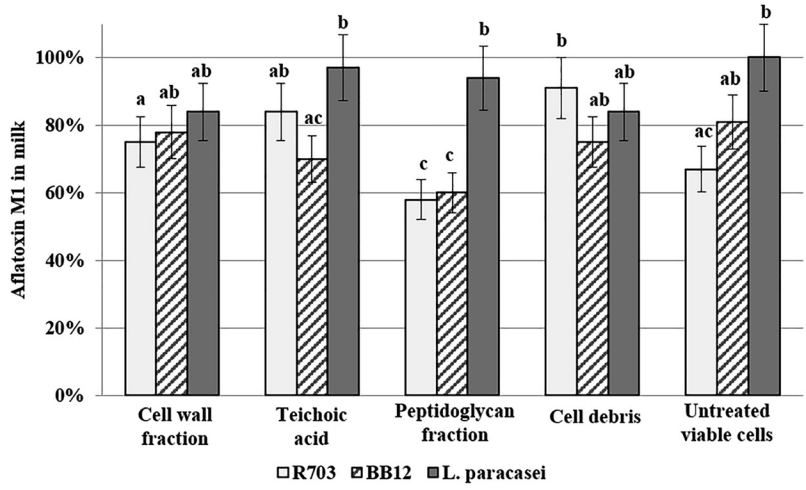

Fig. 2. AFM1 content of milk after treatment with *Lactococcus lactis* ssp. *lactis* R703, *Bifidobacterium animalis* ssp. *lactis* BB12, and *L. paracasei* subsp. *paracasei* (*L. casei*) 431 bacterial cell fractions (n = 3; CV < 10%). Different letters show significant differences (P < 0.05)

The AFM1 binding of *L. paracasei* subsp. *paracasei* lactic acid bacterium was weak; 84% of the initial concentration of the AFM1 remained in the milk with SDS and H_2O treated fractions (Fig. 2).

3.3. The time dependence of the AFM1 elimination

AFM1 binding ability of the R703 and BB12 biomass preparations was tested with increased incubation time (Fig. 3). After 2 h of incubation, the AFM1 binding of the two cell preparations was not increased as compared to one-hour incubations of the same biomass preparations (Figs 2 and 3). *L. lactis* ssp. *lactis* R703 and *B. animalis* ssp. *lactis* BB12 bacterial preparations's AFM1 binding was tested after 2 h of incubation. Residual AFM1 remaining in the system was 78% in the case of the R703 purified peptidoglycan and 68% with BB12 purified peptidoglycan. The untreated viable BB12 cells did not eliminate AFM1. We measured 79% AFM1 in the milk with the untreated R703 bacterium (Fig. 3).

Interestingly, for the BB12 strain, the mycotoxin binding capacity of the peptidoglycan fraction was better than that of the viable cells. While for the R703 strain, there were no significant differences between the binding capacity of the cells and their peptidoglycan fraction. Increasing the reaction time did not result in increased AFM1 binding by the cells and peptidoglycan fractions of both R703 and BB12 strains.

Using different amounts (50, 100, and 150 µL) of bacterial peptidoglycan fraction resulted in similar residual AFM1: 60%, 68%, and 62% in milk, without significant differences. Therefore, it can be concluded that the preparation applied in increasing amounts did not increase AFM1 binding.

The TCA treatment released peptidoglycan fraction proved to be the best cell preparation of R703 and BB12 strains with an increased binding capacity of AFM1. R703 and BB12 strains were treated with TCA and heat (100 °C), contributing to their significant affinity for AFM1.

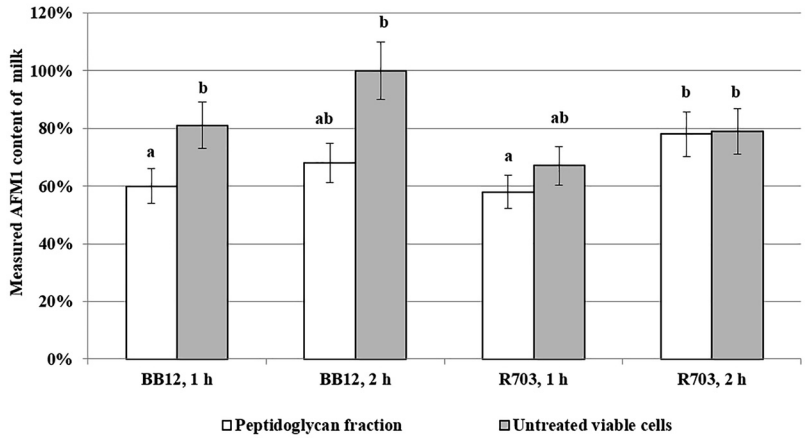

Fig. 3. AFM1 content of milk after treated with purified peptidoglycan and untreated viable *Bifidobacterium animalis* ssp. *lactis* BB12 and *Lactococcus lactis* ssp. *lactis* R703 cells for 1 and 2 h incubation ($n = 3$). Different letters show significant differences ($P < 0.05$)

These reduced the AFM1 content of the milk by around 40–50%, as was shown for several other LAB (Peles et al., 2021). In the case of *Lactobacillus rhamnosus* GG strain, Lahtinen et al. (2004) subjected the cells to various enzymatic and chemical treatments. They revealed that aflatoxin B1 also binds to the cell wall peptidoglycan or compounds strongly connected with the peptidoglycan fraction. Polysaccharides and peptidoglycans were confirmed to bind aflatoxins and were suggested to be responsible for the binding of aflatoxin B1 and M1 (Lahtinen et al., 2004; Shetty and Jespersen, 2006). The peptidoglycan structure influences the bacterial potency of mycotoxin binding (Niderkorn et al., 2009).

Heat treatment caused protein degradation, and acid treatment disrupted glycoside linkages of polysaccharides and destroyed amide bonds, thus changing the peptidoglycan structure, which allowed the bacterial cell to bind to more aflatoxin B1 (El-Nezami et al., 1998). El-Nezami et al. (1998) also reported the binding of heat and acid-treated bacteria to mycotoxin zearalenone. The sufficient binding of aflatoxins by bacteria depended on the LAB strain's inherent features, incubation time, temperature, pH, and the matrix itself (Ahlberg et al., 2015; Adibpour et al., 2016). However, increasing the incubation time of AFM1 with the peptidoglycan did not increase toxin binding by the R703 and BB12 strains. It has also been proved by Zhao et al. (2015) and Mahmood Fashandi et al. (2018) that the increase in the incubation time did not intensify the mycotoxin binding capacity of the LAB strains. Also, the AFM1 binding was partially reversible, and the toxin was released after repeated washes (Ismail et al., 2017; Assaf et al., 2018), which suggested a non-covalent interaction between the mycotoxin and the hydrophobic pockets on the bacterial surface (Haskard et al., 2001), and that interaction behaved in a concentration-dependent manner (Peltonen et al., 2001; Hernandez-Mendoza et al., 2009). Our experiment showed that increasing the applied bacterial biomass did not increase the aflatoxin M1 binding capacity of the LAB tested, which was controversial to the above statements. It could be explained by the reversibility of the binding.

The role of the peptidoglycan fraction of the bacterial cell wall in aflatoxin binding is undeniable. Since all Gram-positive bacteria usually have a thick peptidoglycan layer surrounding the cell membrane, and considering the relatively low amount of aflatoxin M1 bound to the peptidoglycan fractions, other factors can also modify the availability of this structure. Heat and TCA treatment seem to increase availability, and mycotoxin binding efficiency can be seen in the peptide moiety structure of the peptidoglycan as for fumonisin mycotoxins (Niderkorn et al., 2009).

4. CONCLUSIONS

Probiotic industrial strains are crucial in the dairy industry and can be further exploited. In the case of xenobiotics LAB's role in detoxification is well known. Peptidoglycan cell wall fractions of R703 and BB12 bound a significant amount of AFM1 from naturally contaminated milk under one-hour treatment while *L. paracasei* 431 was not effective. Moreover, peptidoglycan fraction was better absorbent for AFM1 than viable cells of BB12, while the difference was insignificant for the R703 strain. Increasing the reaction time did not significantly change the mycotoxin binding in BB12 while for R703 peptidoglycan the absorption seemed reversible. BB12 peptidoglycan needs further analysis for biotechnological application in dairy products.

Our investigation of the cell wall fractions of the bacterial cells revealed that purified BB12 peptidoglycan fraction is much more suitable and available for binding of AFM1 than the viable cells.

Further investigation of the cell surface forms and compounds of the bacterial cells is needed. It could result in the development of biotechnological applications, e.g., molecular sieves against AFM1 contamination or selecting probiotics with increased mycotoxin binding efficiency.

ACKNOWLEDGMENTS

Project no. TKP2021-NKTA-32 has been implemented with the support provided by the Ministry of Culture and Innovation of Hungary through the National Research, Development, and Innovation Fund, financed under the TKP2021-NKTA project.

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