International Food Research Journal 29(1): 78 - 85 (February 2022)

Journal homepage: *http://www.ifrj.upm.edu.my*

Aflatoxin M_1 reduction by microorganisms isolated from kefir grains

[1,3]Adriansyah, P. N. A., [1,2]*Rahayu, W. P., [1,2]Kusumaningrum, H. D. and [3]Kawamura, O.

[1]Department of Food Science, Faculty of Agricultural Engineering and Technology, IPB University,,
Jl. Raya Dramaga, Babakan, Kec. Dramaga, Bogor, Jawa Barat 16680, Indonesia
[2]SEAFAST Centre, IPB University, Jl. Raya Dramaga, Babakan, Kec. Dramaga, Bogor,
Jawa Barat 16680, Indonesia
[3]Department of Food Hygiene, Faculty of Agriculture, Kagawa University, 2393 Ikenobe, Miki, Kita District,
Kagawa 761-0701, Japan

Article history

Received: 16 October 2020
Received in revised form:
12 March 2021
Accepted:
25 June 2021

Keywords

AFM_1 reduction,
LAB,
yeast,
kefir

Abstract

Aflatoxin M_1 (AFM_1) is a mycotoxin that often contaminates milk. Like other mycotoxins, it is thermostable and potentially carcinogenic. The present work was carried out to evaluate the ability of microorganisms isolated from Indonesian kefir grains to reduce AFM_1 in contaminated phosphate buffer saline (PBS). Fourteen isolates of lactic acid bacteria, both aerobic (LAE) and anaerobic (LAN), and nine isolates of yeast (YEA) were used. The significantly highest AFM_1 reduction percentage was shown by the isolate LAE7 (29.3 ± 0.6%) after 4 h incubation. DNA sequencing of LAE7 and YEA2 isolates showed that these isolates had homology (level of similarity) with species of *Lactobacillus kefiri* strain A/K and *Saccharomyces cerevisiae* NRRL Y-12632, respectively. The present work proved that isolates from Indonesian kefir grains could reduce AFM_1 and have the potential for practical use.

Introduction

Aflatoxins are secondary metabolites produced mainly by *Aspergillus flavus* and *A. parasiticus* that usually contaminate foods and feeds. These aflatoxigenic fungi are widespread in warm and humid climates especially in tropical countries (Viegas *et al.,* 2012; Patel *et al.,* 2015) including Indonesia. Contaminated maize and maize products by *A. flavus* have been reported in several cities in Indonesia (Kusumaningrum *et al.,* 2010). Aflatoxin B_1 (AFB_1) in contaminated products can be biotransformed into AFM_1 (4-hydroxylated metabolite of AFB_1) in the liver, and excreted in milk (Gurbay *et al.,* 2010). Although the toxicity of AFM_1 is only one-tenth of that of AFB_1, it is still considered a potential hazard since AFM_1 has similar chemical properties and activities as AFB_1 (Fallah, 2010; MdQuadri *et al.,* 2013). IARC has changed the classification of AFM_1 from group 2B to group 1 (carcinogenic to humans) (IARC, 2002; CAST, 2003). Exposure to AFB_1 in raw materials has been linked to the occurrence of liver cancer in Yogyakarta, Indonesia (Rahayu *et al.,* 2020). Like other mycotoxins, AFM_1 is thermostable, thus can resist thermal treatment/processing and remain in several milk products such as pasteurised, powdered, and infant milk (Galvano *et al.,* 2010).

The permissible limits of AFM_1 are 0.05 µg/L as prescribed by European Union (EU, 2006), and 0.5 µg/L as prescribed by Food and Drugs Administration of United States (FDA, 2005) and Indonesia regulation (BPOM, 2018). Research on the occurrence of AFM_1 in milk has been done previously by Widiastuti *et al.* (2006) who found that of 17 milk samples from Bogor, Indonesia, 12 were positive AFM_1 in the range of 0.001 - 0.343 µg/L. The result did not exceed the Indonesian regulatory limit, but exceeded the European Union regulatory limit. Measures to reduce mycotoxin to improve the quality of dairy products in Indonesia should be undertaken.

Mycotoxin decontamination/detoxification through physical, chemical, and biological methods has been investigated. AFM_1 reduction by lactic acid bacteria (LAB) and yeasts has also been reported. The decontamination/detoxification mechanism of aflatoxins by microorganisms has not been fully clarified yet, but it seems that aflatoxins bind to the polysaccharides and peptidoglycans of microbial cell wall. This can be achieved by hydrogen bond and Van der Waals interactions (Shetty and Jespersen,

---

*Corresponding author.
Email: wpr@apps.ipb.ac.id

DOI:
https://doi.org/10.47863/ifrj.29.1.09

2006; Yiannikouris *et al.,* 2006).

LAB and yeasts can be found in milk fermentation products such as kefir and yogurt. Kefir is a traditional fermented milk beverage with health-promoting properties, and produced by a mixture of microbial species naturally occurring in the kefir grains which originate from the Caucasus region (Kabak and Dobson, 2011). Kefir grains contain complex LAB that has symbiotic interactions with each other. Microorganisms that can be found in kefir grains are LAB such as *Lactobacillus kefiri, Lactobacillus kefiranofaciens, Lactobacillus acidophilus, Levilactobacillus brevis* (formerly *Lactobacillus brevis*; Zheng *et al.,* 2020), *Lacticaseibacillus casei* (formerly *Lactobacillus casei*; Zheng *et al.,* 2020), and *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*; Zheng *et al.,* 2020); yeasts such as *Kluyveromyces marxianus, Kluyveromyces lactis, Saccharomyces cerevisiae, Candida kefir*, and *Kazachstania unispora*; and acetic acid bacteria that cohabitate in a matrix composed of proteins and polysaccharides (Garofalo *et al.,* 2015).

Several studies regarding the reduction of aflatoxins by kefir grains have been reported. Ansari *et al.* (2015) reported that kefir grains could reduce 96.8% AFG_1 in pistachio nuts with 6 h contact time. Kefir grains also reduced 91.9% AFM_1 in milk with a concentration of 0.5 μg/L (Isakhani *et al.,* 2014). Microorganisms isolated from kefir grains can also bind AFB_1, zearalenone, and ochratoxin up to 82 - 100% in milk. The main strains that contributed to mycotoxin binding are *Lactobacillus kefiri, Kazachstania servazzii*, and *Acetobacter syzgii*, with *Lactobacillus kefiri* being the most active (Taheur *et al.,* 2017). Studies regarding the reduction of AFM_1 by microorganisms isolated from kefir grains are still limited. Therefore, the present work aimed to evaluate AFM_1-reducing ability of microorganisms isolated from kefir grains with different incubation times, and to identify the strains of LAB and yeast with the highest AFM_1-reducing ability.

Materials and methods

Isolation of microorganisms from kefir grains

Indonesian home industry kefir grains were used in the present work. The activated kefir grains (10 g) were suspended in NaCl solution (0.85% w/v), and homogenised with stomacher for 30 s. Sequential decimal dilutions were prepared in the same dilutant, and 0.1 mL were inoculated on specific solid growth media by spread-plate technique in triplicate. LAB were isolated on de Man, Rogosa, and Sharpe (MRS) agar (Difco™, Sparks, USA), and incubated at 30°C under aerobic and anaerobic conditions for 7 d. Anaerobic condition was achieved using an anaerobic chamber with a gas generator (AnaeroPack, Mitsubishi, Japan). Yeasts were isolated on yeast extract peptone dextrose (YPD) agar (Sigma-Aldrich, Darmstadt, Germany) at 25°C for 5 d. Isolates of LAB, both anaerobic (LAN) and aerobic (LAE), and yeasts (YEA) were isolated, streak-plate purified, and microscopically examined. LAB isolates were further subjected to biochemical tests such as Gram-staining, catalase test, and oxidase test (Taheur *et al.,* 2017).

Reduction of AFM_1 by microorganisms isolated from kefir grains

Isolates of LAB and yeasts on growth media were inoculated in MRS and YPD broth, respectively, then incubated at 30°C (LAB) and 25°C (yeast) until the cells reached approximately 1.0×10^8 CFU/mL. The incubated culture was then centrifuged at 7,500 rpm for 15 min. The separated cells were re-suspended with 1 mL Dulbecco's PBS, and this was heated at 90°C for 1 h to become non-viable cells. The cells were centrifuged again at the same condition as previously, followed by washing the cells with 1 mL sterile Milli Q twice. The cells were added with 1 mL PBS artificially contaminated with 10 ng/mL AFM_1 (FUJIFILM Wako Pure Chemical Corporation, Japan), followed by incubation at 4°C for 4 and 24 h. After incubation, the cells were centrifuged at 7,500 rpm for 15 min, and AFM_1 residue was immediately passed through the immunoaffinity column (IAC) (Soontornjanagit and Kawamura, 2015).

The IAC was conditioned by passing through 10 mL of PBS before it was used. IAC clean-up was done by adding 5 mL of PBS, followed by 5 mL of Milli Q. AFM_1 was eluted with 1 mL $CH_3CN:CH_3OH$ (1:1), and the elution process was done twice. The collected eluate was added with 2 mL of Milli Q, then the mixture was centrifuged at 12,000 rpm for 10 min.

The HPLC analysis was done with 100 μL of eluate in the HPLC analysis vials. The analysis was done by Shimadzu HPLC equipped with autosampler (Shimadzu, Japan) and fluorescence detector (Shimadzu RF-20A, Japan). The condition was: column, Shim-pack XR-ODS 100 × 3.0 mm (0.3 μm); temperature, 40°C; mobile phase, $H_2O:CH_3CN:CH_3OH$ (7:1.5:1.5); injection volume, 50 μL; fluorescence detector, excitation 360 nm and emission 430 nm; running time, 15 min; and flow rate, 0.4 mL/min (Abdelmotilib *et al.,* 2018). The calibration curve was constructed with several concentrations of AFM_1 standard diluted with acetonitrile. The reduced AFM_1 by the samples after 4 and 24 h incubations was calculated using Eq. 1:

$$\%\ \text{AFM}_1\ \text{reduced} = \frac{\text{AFM1 concentration 0 hour} - \text{AFM1 concentration with sample}}{\text{AFM1 concentration 0 hour} - \text{Negative control}} \times 100\% \qquad \text{(Eq. 1)}$$

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated based on the standard deviation of the response and slope. The LOD and LOQ of AFM_1 were 0.84 and 2.54 ng/mL, respectively. The mean recovery rate of AFM_1 was 89.6 ± 0.57%.

Identification of LAB and yeast strains from kefir grains

Selected LAB strains isolated from Indonesian kefir grains were prepared as DNA templates for polymerase chain reaction (PCR). The DNA template of isolated strains was identified using the molecular method by Sanger with an automated DNA sequencer (ABI3730, Applied Biosystems™, United States). The amplification of 16S rDNA from the bacterial strains by PCR was performed with the primers 27F: 5′-AGA GTT TGA TCC TGG CTC AG-3′, and 1492R: 5′-GGT TAC CTT GTT ACG ACT T-3′.

Selected yeast strains isolated from Indonesian kefir grains were prepared as DNA templates for PCR. The amplification of the D1/D2 domain of the 26S rDNA by PCR was performed with the primers NL1 5’-GCA TAT CAA TAA GCG GAG GAA AAG-3’, and NL4 5′-GGT CCG TGT TTC AAG ACG G-3′. The PCR products of 16S rDNA and D1/D2 26S rDNA were sequenced, then the obtained sequences were trimmed and assembled with the Bio-edit program. The assembled sequences were processed with BLAST to determine species with the closest molecular homology (Evvyernie *et al.,* 2000; Srinivasan *et al.,* 2015).

Statistical analysis

The test results were processed statistically using analysis of variance (ANOVA) with a significance level of 0.05; and if there was a significant factor, then the data were processed by Duncan's test. The statistical software used was SPSS Statistics 22.

Results

Reduction of AFM_1 by microorganisms isolated from kefir grains

Generally, the AFM_1-reducing ability of microorganisms isolated from Indonesian kefir grains ranged from 1.6% (by LAN) to 29.3% (by LAE). LAN isolates yielded the lowest AFM_1 reduction ability of 1.6 to 12.8% (Table 1). All LAN isolates after 4 h incubation, except for LAN5, showed significant results on AFM_1 reduction, with LAN2, LAN3, and LAN4 showed a non-significant difference. Meanwhile, afetr 24 h incubation, LAN3 and LAN5 had a significant result on AFM_1 reduction, although AFM_1 reduction percentage by LAN3 and LAN5 at 24 h did not have a significant difference. An increase in incubation time affected AFM_1 reduction on LAN isolates significantly, meaning that longer incubation of LAN yielded a significant result of AFM_1 reduction. The means of AFM_1 reduction by LAN isolates were very low, and generally showed no significant difference of AFM_1 reduction ability between isolates.

Table 1. AFM_1 reduction percentage by LAN isolates.

Sample	**AFM_1 reduction percentage (%)**	
	4 h	**24 h**
LAN2	3.6 ± 1.4^a	4.1 ± 1.7^b
LAN3	-4.6 ± 1.8^b	10.0 ± 2.6^a
LAN4	1.6 ± 0.3^a	-0.9 ± 0.4^c
LAN5	1.7 ± 1.5^a	12.8 ± 2.1^a
Average	0.9 ± 3.4^A	6.2 ± 5.6^B

Means in each column followed by different lowercase superscripts differ significantly. Means in each row followed by different uppercase superscripts differ significantly.

Based on Table 2, LAE isolates after 4 h incubation yielded a significant result, with the highest reduction percentage was given by isolate LAE7 (29.3 ± 0.6 %), although the result was not significantly different than LAE1, LAE9, and LAE10. Meanwhile, after 24 h incubation, isolates LAE1 showed a considerable difference on AFM_1 reduction than the other isolates. This result showed that 4 h incubation yielded a significant result on AFM_1 reduction by LAE isolates percentage than 24 h incubation. Generally, AFM_1 reduction by LAE isolates decreased after 24 h incubation. Isolate LAE7 with the highest reduction percentage and significant result after 4 h incubation was selected for further strain molecular identification.

Table 2. AFM$_1$ reduction percentage by LAE isolates.

Sample	AFM$_1$ reduction percentage (%)	
	4 h	24 h
LAE1	24.9 ± 2.8^{ab}	24.4 ± 3.0^{a}
LAE2	24.0 ± 1.3^{b}	16.0 ± 5.0^{b}
LAE3	23.0 ± 3.0^{b}	15.4 ± 0.9^{b}
LAE4	15.9 ± 0.4^{c}	9.1 ± 3.0^{c}
LAE5	21.0 ± 3.5^{b}	17.9 ± 4.5^{b}
LAE6	23.8 ± 1.7^{b}	15.2 ± 1.7^{b}
LAE7	29.3 ± 0.6^{a}	15.9 ± 3.2^{b}
LAE8	21.6 ± 0.5^{b}	16.1 ± 1.2^{b}
LAE9	25.1 ± 3.2^{ab}	18.4 ± 3.1^{b}
LAE10	24.6 ± 1.1^{ab}	15.6 ± 4.0^{b}
Average	23.3 ± 3.5^{A}	16.7 ± 4.4^{B}

Means in each column followed by different lowercase superscripts differ significantly. Means in each row followed by different uppercase superscripts differ significantly.

The results of AFM$_1$ reduction percentage by YEA isolates are shown in Table 3. Incubations for 4 and 24 h showed non-significant results on all isolates. Isolate YEA2 yielded the highest reduction percentage after 4 and 24 h incubations despite having a non-significant difference with other isolates. However, reduction percentage of AFM$_1$ by almost all YEA isolates after 24 h incubation significantly increased. AFM$_1$ reduction by all YEA isolates did not differ from each other after 4 and 24 h incubations. Isolate YEA2 with the highest reduction percentage was selected for further strain molecular identification.

Table 3. AFM$_1$ reduction percentage by YEA isolates.

Sample	AFM$_1$ reduction percentage (%)	
	4 h	24 h
YEA1	9.8 ± 3.9^{a}	15.0 ± 3.8^{a}
YEA2	16.0 ± 4.9^{a}	20.6 ± 0.8^{a}
YEA3	14.0 ± 1.9^{a}	17.2 ± 2.5^{a}
YEA4	12.6 ± 4.6^{a}	17.2 ± 2.5^{a}
YEA5	15.0 ± 4.7^{a}	15.7 ± 3.3^{a}
YEA7	13.6 ± 1.7^{a}	17.2 ± 1.3^{a}
YEA8	12.4 ± 2.3^{a}	12.2 ± 0.3^{a}
YEA9	8.5 ± 4.1^{a}	13.0 ± 4.6^{a}
YEA10	8.2 ± 1.2^{a}	13.9 ± 1.8^{a}
Average	12.1 ± 3.9^{B}	15.7 ± 3.3^{A}

Means in each column followed by different lowercase superscripts differ significantly. Means in each row followed by different uppercase superscripts differ significantly.

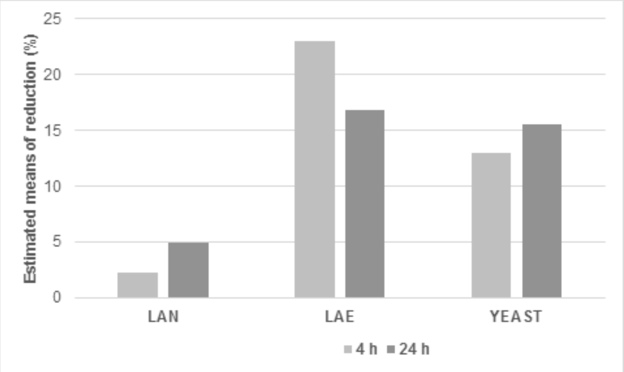

Figure 1. Estimated marginal means of AFM$_1$ reduction.

Based on Figure 1, it can be seen that there were interactions between the types of microorganisms and the incubation time factor. It is also clear that the mean reduction in LAE decreased after 24 h, while the average AFM$_1$ reduction of LAN and yeast increased after 24 h. LAE isolates yielded a higher mean of AFM$_1$ reduction percentage than yeast and LAN isolates. It can be seen that LAE showed different behaviour from LAN and yeast. It can be concluded that LAE is a bacterium that has the most influence on AFM$_1$ reduction among all microorganisms isolated from kefir grains.

Identification of LAB and yeast strains from kefir grains

The isolates with the highest AFM$_1$ reduction percentage were LAE7 (29.3 ± 0.6%) after 4 h incubation, followed by YEA2 (20.6 ± 0.8%) after 24 h incubation. Isolates LAE7 and YEA2 were identified by molecular method (PCR), and the result is shown in Table 4. DNA analysis using BLAST revealed that LAE7 had homology (level of

Table 4. Identification of isolate LAE7 and YEA2 from kefir grains.

Description	Sample code	
	LAE7	**YEA2**
Identified strain	*Lactobacillus kefiri* strain A/K	*Saccharomyces cerevisiae* NRRL Y-12632
Homology (%)	99.79	99.49
Max score (bits)	2606	1077
Total score	2606	1077
Query coverage (%)	100	98
E-value	0.0	0.0
Max Identities	1418/142 (99%)	590/593 (99%)
Accession number	NR_042230.1	NG_042623.1

similarity) of 99.79% with *Lactobacillus kefiri* strain A/K, while YEA2 had homology of 99.49% with *Saccharomyces cerevisiae* NRRL Y-12632.

Discussion

Non-viable cells were used in the present work since past studies have reported that they could reduce AFM_1 with a higher percentage in a short contact time (Bovo *et al.,* 2013). LAE isolates yielded the highest reduction ability among all isolates in the range of 9.1 to 29.3% (Table 3). LAE yielded higher AFM_1 reduction than LAN. This could be that aerobic LAB had higher cell yield than anaerobic LAB, as observed by Smetankova *et al.* (2012) who observed that *L. plantarum* had higher cell yield in aerobic condition than in anaerobic condition. The AFM_1 reduction ability varied among the isolates assessed in the present work. Despite similar genetic structure, ability of LAB can vary as observed by Pierides *et al.* (2000) who also found that *Lacticaseibacillus rhamnosus* (formerly *Lactobacillus rhamnosus* GG, Zheng *et al.,* 2020) strain had less reduction ability than *L. rhamnosus* strain GG. This could be due to the difference in biological activities of the strains.

In the present work, LAB isolates yielded higher AFM_1 reduction percentage than yeast isolates. Contrarily, another study found that yeast isolates reduced AFM_1 more than LAB (Abdelmotilib *et al.,* 2018). They observed that non-viable *L. plantarum* and *L. acidophilus* reduced 32.92 and 58.98% of AFM_1 in PBS after 72 h incubation, while *S. cerevisiae* reduced 64.52% of AFM_1 in the same condition. Mix isolates of LAB and yeast showed a maximum reduction of 100% after 60 min incubation. Another study suggested that yeast incubated longer than 24 h had high AFM_1 reduction percentage. Abdelmotilib *et al.* (2018) found that AFM_1 decreased gradually from 0 to 72 h by non-viable *S. cerevisiae* in PBS.

A higher concentration of yeast at 1.0×10^9 CFU/mL could also contribute to a higher percentage of AFM_1 reduction (Corassin *et al.,* 2013; Abdelmotilib *et al.,* 2018). Higher reduction percentage was also observed in incubation on different media with short incubation time. Corassin *et al.* (2013) found that LAB could reduce 11.5% of AFM_1 while *S. cerevisiae* could reduce 90.3% of AFM_1 in UHT skim milk after 30 min incubation.

In the present work, an increase in incubation time affected the reduction of AFM_1 by LAN and yeast isolates significantly. This finding agree with Elgerbi *et al.* (2006) who observed a significant difference in reduction ability of tested LAB strains after 24 and 96 h incubations in the range of 0 to 14.6% and 4.5 to 73.1%, respectively. Contrary to the previous study, Bovo *et al.* (2013) observed that AFM_1 reduction ability of all tested strains; *L. plantarum, Enterococcus avium, Pediococcus pentosaceus, Bifidobacterium lactis*, and *Lactobacillus gasseri* after 15 min and 24 h incubations had no significant difference. Meanwhile, in the present work, LAE isolates yielded a significant result on AFM_1 reduction after 4 h incubation. Attachment of AFM_1 to microbial cell walls is a rapid procedure, and the optimum attachment occurs within the first minutes of exposure (El-Nezami *et al.,* 1998; Bovo *et al.,* 2013).

The mean of AFM_1 reduction by LAE decreased after 24 h incubation, while the mean AFM_1 reduction by LAN and yeast increased after

24 h incubation. There is a possible symbiosis relation between AFM_1 reduction by LAE and yeast. The released AFM_1 by LAE cell wall after 24 h incubation can be absorbed by yeast, as shown by the increase in AFM_1 reduction percentage by YEA isolates. This showed the potential of the microbial isolates from kefir grains to reduce AFM_1 in milk due to kefir grains having complex microbial diversity.

The decrease in AFM_1 reduction percentage by LAE isolates after 24 h incubation was also observed by Elsanhoty *et al.* (2014) where non-viable *L. acidophilus, L. rhamnosus, L. plantarum,* and *L. bulgaricus* decreased gradually from 4 to 24 h incubation in PBS. Kuharic *et al.* (2018) also observed a decrease in AFM_1 reduction by *L. plantarum* isolates in milk. The AFM_1 reduction percentage of non-viable *L. plantarum* isolates incubated for 4 and 24 h were 79.2 and 26.1%, respectively.

A decrease in AFM_1 reduction after 24 h incubation might be due to the release of AFM_1 from the AFM_1-microorganism complex. Previous study found that aflatoxin could be removed from the AFM_1-microorganism complex by washing. Released AFM_1 by bacteria range from 40.57 to 87.37% (Bovo *et al.,* 2013). The amount of AFM_1 released by microorganisms is dependent on their species and strain. Bovo *et al.* (2013) found that viable *L. rhamnosus* released AFM_1 within 15 min after contact. Meanwhile, Kabak and Var (2008) found that AFM_1 released from bacterial cells ranged from 5.62 to 8.54%. The evidence that the LAB-AFM_1 complex could release aflatoxins after washing suggests that the binding is a weak bond *i.e.,* non-covalent binding between AFM_1 and the hydrophobic part of the bacterial cell wall (Haskard *et al.,* 2000). Therefore, the study on AFM_1 release from AFM_1-microorganism complex isolated from kefir grains must be conducted in the future to confirm the efficiency of the isolates; this was not done in the present work.

Aflatoxin release from the LAB-AFM_1 complex can also be explained by different binding sites or similar binding sites with slight differences between different strains. The lower amount of aflatoxin released from the complex can be explained by the interaction between aflatoxin molecules retained in the bacterial cell, thus forming a cross-linked matrix with aflatoxin molecules in the nearby bacterial cell, which in turn prevents aflatoxins from being released (Hernandez-Mendoza *et al.,* 2009).

The mechanism of aflatoxin reduction has not been clarified yet. Some researchers suggested that AFM_1 attaches to polysaccharides and peptidoglycans, parts of bacterial cell wall, instead of creating covalent bonds or getting metabolised by the bacteria (Lahtinen *et al.,* 2004; Shetty and Jespersen, 2006). Heat treatment on bacterial cell walls will cause denaturation, which will increase the hydrophobic nature of the cell surface or form products of the Maillard reaction. The disruption will allow aflatoxins to bind to bacterial cell wall and plasma membrane components which are inaccessible when the cell wall is not disrupted (Haskard *et al.,* 2001). The absence of AFM_1 metabolite peaks in HPLC chromatograms reported by Pierides *et al.* (2000) also further explains the possible AFM_1 reduction mechanism, which implies the involvement of physical interaction with microbial cell wall instead of a metabolic degradation reaction. Pierides *et al.* (2000) also stated that there was no metabolic degradation of AFB_1 because the toxin bound to the *Bacillus* can be extracted. It was also assumed that AFB_1 might be attached to the proteins in the *Bacillus megaterium* cell walls.

Studies on AFM_1 reduction ability of *L. kefiri* are yet to be done. *L. kefiri* has been shown to reduce other mycotoxins in previous study. Taheur *et al.* (2017) found that *L. kefiri* could reduce 80% AFB_1, 81% ochratoxin A, and 100% zearalenone when cultivated on milk. *S. cerevisiae* has been used for reducing aflatoxins in previous studies of Abdelmotilib *et al.* (2018) where *S. cerevisiae* could reduce 64.52% AFM_1 in PBS. *S. cerevisiae* also had a higher reduction ability on UHT milk medium with a 90.3% reduction (Corassin *et al.,* 2013). These data suggest that *L. kefiri* and *S. cerevisiae* isolated from Indonesian kefir grains have the potential to reduce mycotoxins in milk for further applications.

Conclusion

The highest AFM_1 reduction percentage among the tested microorganisms was shown by isolate LAE7 (29.3 ± 0.6%) in 4 h incubation time with significant result. In general, longer incubation of 24 h gave a significant result on LAN and YEA isolates, while longer incubation did not give LAE isolates significant results. The present work suggested that LAE showed different behaviours from LAN and yeast. This was indicated by the higher AFM_1 reduction mean value than the other two types of microorganism. It can thus be concluded that LAE had the most influence on AFM_1 reduction among all microorganisms isolated from kefir grains.

The DNA sequencing of LAE7 and YEA2 isolates using BLAST revealed that these isolates had homology (level of similarity) with *L. kefiri* strain A/K and *S. cerevisiae* NRRL Y-12632, respectively. The present work proved that isolates from kefir grains could reduce AFM_1 and have the potential for practical use.

References

Abdelmotilib, N. M., Hamad, G. M., Elderea, H. B., Salem, E. G. and El-Sohaimy, S. A. 2018. Aflatoxin M_1 reduction in milk by a novel combination of probiotic bacterial and yeast strains. European Journal of Nutrition and Food Safety 8(2): 83-99.

Ansari, F., Khodaiyan, F., Rezaei, K. and Rahmani, A. 2015. Modelling of aflatoxin G_1 reduction by kefir grain using response surface methodology. Journal of Environmental Health Science and Engineering 13(1): 1-7.

Badan Pengawas Obat dan Makanan (BPOM). 2018. Peraturan Badan Pengawas Obat dan Makanan Nomor 8 Tahun 2018 Tentang batas maksimum cemaran kimia dalam pangan olahan (Lampiran I). Retrieved from website: http://jdih.pom.go.id

Bovo, F., Corassin, C., Rosim, R. and de Oliveira, C. F. 2013. Efficiency of lactic acid bacteria strains for decontamination of aflatoxin M_1 in phosphate buffer saline solution and in skimmed milk. Food and Bioprocess Technology 6: 2230-2234.

Corassin, C. H., Bovo, F., Rosim, R. E. and Oliveira, C. A. 2013. Efficiency of *Saccharomyces cerevisiae* and lactic acid bacteria strains to bind aflatoxin M_1 in UHT skim milk. Food Control 31(1): 80-83.

Council for Agricultural Science and Technology (CAST). 2003. Mycotoxins: risks in plant, animal, and human systems. United States: CAST.

Elgerbi, A. M., Aidoo, K. E., Candlish, A. A. G. and Williams, A. G. 2006. Effects of lactic acid bacteria and bifidobacteria on levels of aflatoxin M_1 in milk and phosphate buffer. Milk Science International 61(2): 197-199.

El-Nezami, H., Kankaanpaa, P., Salminen, S. and Ahokas, J. 1998. Ability of dairy strains of lactic acid bacteria to bind a common food carcinogen, aflatoxin B_1. Food and Chemical Toxicology 36(4): 321-326.

Elsanhoty, R. M., Salam, S. A., Ramadan, M. F. and Badr, F. H. 2014. Detoxification of aflatoxin M_1 in yoghurt using probiotics and lactic acid bacteria. Food Control 43: 129-134.

European Union (EU). 2006. European Commission - commission regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of European Union L364: 5-24.

Evvyernie, D., Yamazaki, S., Morimoto, K., Karita, S., Kimura, T., Sakka, K. and Ohmiya, K. 2000. Identification and characterization of *Clostridium paraputrificum* M-21, a chitinolytic, mesophylic, and hydrogen-producing bacterium. Journal of Bioscience and Bioengineering 89(6): 596-601.

Fallah, A. A. 2010. Assessment of aflatoxin M_1 contamination in pasteurized and UHT milk marketed in central part of Iran. Food and Chemical Toxicology 48(3): 988-991.

Food and Drug Administration (FDA). 2005. Compliance program guidance manual. United States: FDA.

Galvano, F., Galofaro, V., Ritieni, A., Bognanno, M., De Angelis, A. and Galvano, G. 2010. Survey of the occurrence of aflatoxin M_1 in dairy products marketed in Italy: second year of observation. Food Additive and Contaminants 18: 644-666.

Garofalo, C., Osimani, A., Milanović, V., Aquilanti, L., De Filippis, F., Stellato, G., ... and Clementi, F. 2015. Bacteria and yeast microbiota in milk kefir grains from different Italian regions. Food Microbiology 49(1): 123-133.

Gurbay, A., Sabuncuoglu, S. A., Girgin, G., Sahin, G., Yigit, S., Yurdakok, M. and Tekinalp, G. 2010. Exposure of newborns to aflatoxin M_1 and B_1 from mothers breast milk in Ankara, Turkey. Food and Chemical Toxicology 48: 314-319.

Haskard, C. A., El-Nezami, H. S., Kankaanpää, P. E., Salminen, S. and Ahokas, J. T. 2001. Surface binding of aflatoxin B_1 by lactic acid bacteria. Applied and Environmental Microbiology 67(7): 3086-3091.

Haskard, C., Binnion, C. and Ahokas, J. 2000. Factors affecting the sequestration of aflatoxin by *Lactobacillus rhamnosus* strain GG. Chemico-Biological Interactions 128(1): 39-49.

Hernandez-Mendoza, A., Garcia, H. S. and Steele, J. L. 2009. Screening of *Lactobacillus casei* strains for their ability to bind aflatoxin B_1. Food and Chemical Toxicology 47(6): 1064-1068.

International Agency for Research on Cancer (IARC). 2002. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. France: IARC.

Isakhani, S., Marhamatizade, M. H. and Ebrahimi,

T. M. 2014. The assessment of reducing aflatoxin M_1 in kefir by *Saccharomyces kefir* and *Lactobacillus casei* TD4 by ELISA method. Trends in Life Science 3(4): 268-274.

Kabak, B. and Dobson, A. D. W. 2011. An introduction to the traditional fermented foods and beverages of Turkey. Critical Reviews in Food Science and Nutrition 51(3): 248-260.

Kabak, B. and Var, I. 2008. Factors affecting the removal of aflatoxin M_1 from food model by *Lactobacillus* and *Bifidobacterium* strains. Journal of Environmental Science and Health 43(7): 617-624.

Kuharic, Z., Jakopovic, Z., Canak, I., Frece, J., Bosnir, J., Pavlek, Z., ... and Markov, K. 2018. Removing aflatoxin M_1 from milk with native lactic acid bacteria, centrifugation, and filtration. Arhiv za Higijenu Rada i Toksikologiju 69(4): 334-339.

Kusumaningrum, H. D., Suliantari, Toha, A. D., Putra, S. H. and Utami, A. S. 2010. Contamination of *Aspergillus flavus* and aflatoxin at distribution chain of maize based food product and influencing factors. Journal of Food Technology and Industry 21(2): 171-176.

Lahtinen, S. J., Haskard, C. A., Ouwehand, A. C., Salminen, S. J. and Ahokas, J. T. 2004. Binding of aflatoxin B_1 to cell wall components of *Lactobacillus rhamnosus* strain GG. Food Additives and Contaminants 21(2): 158-164.

MdQuadri, S. H., Niranjan, M. S., Chaluvaraju, K. C., Shantaram, U. and Enamul, H. S. 2013. An overview on chemistry, toxicity, analysis and control of aflatoxins. International Journal of Chemistry and Life Science 2: 1071-1078.

Patel, S. V., Bosamia T. C., Bhalani H. N., Singh P. and Kumar, A. 2015. Aflatoxins: causes and effects. Agrobios Newsletter 13 (9): 140-142.

Pierides, M., El-Nezami, H., Peltonem, K., Salminen, S. and Ahokas, J. 2000. Ability of dairy strains of lactic acid bacteria to bind aflatoxin M_1 in a food model. Journal of Food Protection 63(5): 645-650.

Rahayu, W. P., Herawati, D., Broto, W., Indrotristanto, N., Ambarwati, S. and Adhi, W. 2020. Risk estimation of hepatocellular carcinoma due to exposure to aflatoxins in maize from Yogyakarta, Indonesia. Journal of Food Quality and Hazards Control 7: 45-50.

Shetty, P. H. and Jespersen, L. 2006. *Saccharomyces cerevisiae* and lactic acid bacteria as potential mycotoxin decontaminating agents. Trends in Food Science and Technology 17(2): 48-55.

Smetankova, J., Hladikova, Z., Valach, F., Zimanova, M., Kohajdova, Z., Greif, G. and Greifova, M. 2012. Influence of aerobic and anaerobic conditions on the growth and metabolism of selected strains of *Lactobacillus plantarum*. Acta Chimica Slovaca 5(2): 204-210.

Soontornjanagit, M. and Kawamura, O. 2015. Occurrence of aflatoxin M_1 in commercial powdered milk in Bangkok, Thailand. JSM Mycotoxin 65(2): 75-79.

Srinivasan, R., Karaoz, U., Volegova, M., Mackichan, J., Kato-Maeda, M., Miller, S. and Lynch, S. V. 2015. Use of 16S rRNA gene for identification of a broad range of clinically relevant bacterial pathogens. PloS One 10(2): article ID e0117617.

Taheur, F. B, Fedhila, K., Chaieb, K., Kouidhi, B., Bakhrouf, A. and Abrunhosa, L. 2017. Adsorption of aflatoxin B_1, zearalenone and ochratoxin A by microorganisms isolated from kefir grains. International Journal of Food Microbiology 251: 1-7.

Viegas, S., Veiga, L., Malta-Vacas, J., Sabino, R., Figueredo, P., Almedia, A., … and Carolino, E. 2012. Occupational exposure to aflatoxin (AFB) in poultry production. Journal of Toxicology and Environmental Health 75: 1330-1340.

Widiastuti, R., Maryam, R., Bahri, S. and Firmansyah, R. 2006. Aflatoxin residues (AFM_1) in fresh dairy milk in Pangalengan and Bogor district, west Java. Seminar Nasional Teknologi Peternakan dan Veteriner 23 (3): 239-243.

Yiannikouris, A., André, G., Poughon, L., Francois, J., Dussap, C. G., Jeminet, G., ... and Jouany, J. P. 2006. Chemical and conformational study of the interactions involved in mycotoxin complexation with beta-d-glucans. Biomacromolecules 7(4): 1147-1155.

Zheng, J., Wittouck, S., Salvetti, E., Franz, Charles M. A. P., Harris, H. M. B., Mattarelli, P., … and Lebeer, S. 2020. A taxonomic note on the genus *Lactobacillus*: description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. International Journal of Systematic and Evolutionary Microbiology 70: 2782-2858.