

Aflatoxin M₁ in Milk and Milk Products in Jordan and Methods for its Reduction

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ABSTRACT

Aims: The aim of this study was to assess AFM₁ levels in liquid and powdered milk and other dairy products in Jordan using ELISA technique and assess antiaflatoxin M₁ (AFM₁ reduction) effect of fermentation, high heat treatments and the addition of plant extracts during milk fermentation.

Place and Duration of Study: Department of Nutrition and Food Technology, The University of Jordan, Amman, Jordan during July 2006 and March 2007.

Methodology: A total of 22 samples of milk and dairy products were randomly collected from local markets and were grouped as follows: raw (n=3), pasteurized (n=4) and powdered milk (n=3), infant formula (n=3), yoghurt (n=3), labaneh (low moisture sour yoghurt) (n=3) and jameed (traditional dry yoghurt) (n=3). Dairy products were analyzed for their aflatoxin M₁ (AFM₁) content ppt. Anti-aflatoxin treatments of fermentation, high heat, garlic (*Allium sativum*), black cumin (*Nigella sativum*) and carrot (*Ducaus carota sativus*) water extracts were studied to determine their influence on AFM₁ content.

Results: The results show that AFM₁ was detected in 81.8% of tested samples with varying levels ranging from 25 ppt to zero no detection. The highest levels were found in 2 labaneh samples purchased from local brands (25.8 and 22 ppt of AFM₁). Levels of AFM₁ of 19, 15.5, 14, ppt were detected in a yoghurt, raw milk, and jameed samples respectively. No AFM₁ was detected in imported infant formula samples, pasteurized milk and one labaneh brand. Carrot water extract had the highest impact by decreasing AFM₁ by 72% of the total toxin followed heating (56%) and fermentation (40%).

Conclusion: These AFM₁ levels found in milk and dairy products in Jordan are considered low and within the European maximum limit of 50 ppt. An important finding for AFM₁ reduction was our finding that a natural product such as carrot extract can be effective in lowering AFM₁ in milk samples.

Keywords: Aflatoxin M₁, ELISA, , AFM₁ Reduction, Plant extracts, Milk products

1. INTRODUCTION

Aflatoxins are a group of mycotoxins, which came to our knowledge in the early 1960s of the 20th century. In the early months of 1960 at total of 100,000 turkeys poultries died in mysterious circumstances in the United Kingdom. Through investigations showed that the

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22 turkeys died from acute liver necrosis and bile duct hyperplasia after they consumed infected
23 groundnuts [1].

24 Aflatoxins (AFs) are produced primarily by some strains of *Aspergillus flavus* and *A.*
25 *parasiticus*, as well other related species. These strains produce four major classes of
26 aflatoxins: AFB1, AFB2, AFG1 and AFG2 in addition to major metabolites derivatives such
27 as aflatoxin M1 (AFM1) and aflatoxin Q1 (AFQ1) that are developed either by metabolism in
28 humans, animals, and microorganism or by environmental reactions [2]. AFB1 was found to
29 be the most common and the most hazardous of all mycotoxins found in human food and
30 animal feed. AFs were classified as group 1 carcinogens by IARC [3] AFB1 is categorized as
31 class 1 carcinogen (definitely carcinogenic) for human, whereas AFM1 is a group 2B
32 carcinogen (probably carcinogenic) for human [4]. AFM1 is a hydroxylated metabolite of
33 AFB1, first detected in the milk of lactating cows that had injected feeds contaminated with
34 AFB1 [5]. AFM1 is released with milk produced by cows, sheep, goats and other lactating
35 animals that have ingested feeds contaminated with AFB1. The relationship between the
36 amount of AFB1 ingested by animal and quantity of AFM1 recovered in milk is quite variable
37 and estimated to be between 1-3% [6]. It was also estimated that about 0.3-6.2% of AFB1 in
38 feed consumed by lactating animals is transformed to AFM1 in their milk [7]. The daily intake
39 of AFM1 is estimated to be 6.8ng/person for the European diet, 3.5ng/person for the Latin
40 American diet, 12ng/person for the Far Eastern diet, 0.7ng/person for Middle Eastern diet
41 and 0.1ng/person for the African diet [8, 9]. Several studies from Turkey [10], Iran [11, 12],
42 Morocco [13] and Jordan [14] demonstrated high levels of AFM1 in milk products that reach
43 levels above the accepted maximum levels determined by the European Union. All these
44 studies recommend development of approaches to address this public health problem.

45 Although the potency of AFM1 is one tenth that of AFB1, AFM1 still represents a potential
46 carcinogen for humans [2]. The international Agency for Research on Cancer (IARC)
47 categorized AFB1 as Class 1 carcinogen (definitely Carcinogenic for humans), whereas
48 AFM1 is a group 2B carcinogen (probably carcinogenic) for humans [13]. Therefore, The
49 contamination of dairy products with AFM1 has been recognized as a significant human
50 health hazard, particularly for babies, since milk is their basic food.

51 Quantitative and qualitative methods for detecting AFM1 in dairy products were established.
52 These methods include thin layer chromatography (TLC), high performance liquid
53 chromatography (HPLC), plus immunochemical tests such as radioimmunoassay (RIA) and
54 enzyme linked immunosorbent assays (ELISA).

55

56 The tolerance limit for AFM1 in milk varies from one country to another. The European
57 Community and Codex Alimentarius Commission put a maximum level of AFM1 in liquid milk
58 not to exceed 50ng/l whereas the USA, FDA stretched the safety levels to 500ng/Kg [8].

59 Studies on the reduction of AFM1 in dairy products by using natural products are very
60 limited. The aim of this study is to assess antiaflatoxin M1 (AFM₁ reduction) effect of
61 fermentation, high heat treatments and the addition of plant extracts (Garlic (*Allium sativum*),
62 black cumin (*Nigella sativa*) and carrots (*Daucus carita sativus*)) during milk fermentation.
63 We also assess AFM1 levels in liquid and powdered milk and other dairy products (yoghurt,
64 labaneh and jameed) marketed and consumed in Jordan using ELISA technique.

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2. MATERIAL AND METHODS

2.1 Sampling and sample preparation

A total of 22 samples ~~and/or brands~~ of raw (n=3), ~~and~~ pasteurized milk (n=4) and powdered milk (n=3), infant formula (n=3), yoghurt (n=3), labaneh (low moisture sour yoghurt) (n=3) and jameed (traditional dry yoghurt) (3) were collected randomly from local markets in Amman, Jordan during the period July 2006 and March 2007. Ten gm (10 gm) of each of powdered milk, infant formula and Jameed were suspended in 100 ml of deionized water and homogenized. One ml (1 ml) ml of each of liquid milk samples and reconstituted powdered milk, infant formula and Jameed were cooled to 10° C and centrifuged at 2500 rpm for 10 min. to separate and remove the fat layer. The upper, creamy layer was aspirated with a Pasteur pipette, and the supernatant (defatted skimmed milk) was used to evaluate AFM1 (100 µl per well). ~~Ten gm (10 gm) of each of powdered milk, infant formula and Jameed were suspended in 100 ml of deionized water and homogenized.~~ One ml of labaneh is homogenized with 2 ml of deionized water. One ml (1 ml) of each sample was prepared for AFM1 assay similar to milk samples.

2.2 Quantification of AFM₁ by ELISA

Samples were analyzed for AFM1 content according to ELISA manufacturer ~~procedure and AFM1 standards provided by the same source~~ (R-Biopharm AG, Darmstadt, Germany).

The ELISA assay is based on the affinities of the monoclonal or polyclonal antibodies against AFM1. ELISA is a fairly cheap, sensitive and quick method for the determination of AFM1 and is suitable for routine diagnostic application [15, 16]. The sensitivity of ELISA method for AFM1 detection has been ~~highlighted investigated~~ by many investigators and compared to other methods, such as HPLC and found to be a more sensitive and a reliable ~~method than~~ HPLC [16]. ~~The AFM1 ELISA kit has a quantitation range of 5-100ppt.~~

~~The~~ microtitre plate wells are pre-coated with mouse anti- AFM1. Before the first incubation step, AFM1 standards or samples are examined for the presence of AFM1 added to the pre-coated wells. The free AFM1 (in the standard solution or in the sample) is bound by the specific antibody binding sites. Other unbound material of samples are washed away in the washing step by buffer (10 Mm phosphate buffer containing 0.05% Tween 20) leaving only the AFM1 bound to the binding sites. After a washing step and before the second incubation step, enzyme labeled aflatoxin (enzyme conjugate) is added into the wells. The enzyme conjugate binds with free specific antibody binding sites. After the second incubation, the non-bound (enzyme labeled) reagents are removed in a washing step. The amount of bound enzyme conjugate is visualized by the addition of chromogen substrate (tetramethylbenzidine, TMB). The bound enzyme transforms the chromogen into a colored product. The substrate reaction is stopped by the addition of stop solution (sulphuric acid).

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111 The color intensity is measured photometrically at 450nm and is inversely proportional to
112 the AFM1 concentration in the sample up to 80. The AFM1-ELISA kit has a quantitation
113 range of 5-100ppt.

114

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116 Optical density readings measured by ELISA reader were converted to absorbency % which
117 is :

118
$$\text{Absorbance \%} = (\text{Absorbance of standard (or sample)} \div \text{Absorbance of zero standard}) \times 100$$

119

120 A standard curve was prepared by plotting % absorbency against 6 concentrations of the
121 standard solution of AFM1 (0, 5, 10, 20, 40 and 80 ppt). Results for each concentration were
122 recorded as the average of duplicates.

123 **2.3 Reduction of AFM1**

124 Testing for AFM1 reduction was carried out with high heat treatment, fermentation, and the
125 addition of water extracts of garlic, black cumin and carrots added to milk samples before
126 fermentation. High heat treatment, fermentation and the addition of water extracts of garlic,
127 black cumin and carrots added to milk samples before fermentation, were experimented
128 tested for AFM₁ reduction attempts of artificially contaminated samples with AFM₁. AFM₁
129 was artificially added at initial levels of 7.5 - 12.5 ng/ml milk or milk product and mixed
130 vigorously for 5 min. by vortex mixer. Five equal portions of milk samples, each sample was
131 containing 3 ml were used in each reduction AFM₁ experiment.

132 **2.3.1 Effect of heat treatment**

133 The 3 milk sample containing AFM1 is divided into 4 portions. They are H1 (0.5 ml) control
134 (no heat treatment), H2 (0.5 ml) pasteurization at 72°C for 15 sec., H3 (1.0 ml) boiling for 30
135 sec. and H4 (1.0 ml) sterilization for 15 min. Each sample is heated in a water bath tempered
136 at each given temperature, and the last sample is autoclaved. All samples were cooled
137 immediately after heat treatment before ELISA assay.

138 **2.3.2 Effect of fermentation**

139 Three ml (3 ml) of fresh milk with AFM1 was fermented to yoghurt then divided into 4
140 portions in test tubes (F1 & F2, 0.5 ml each; F3 & F4, 1.0 ml each) and incubated at 42°C
141 for 0 times as control, 3hrs, 6hrs, 24 hrs and 48 hrs, respectively. Samples were prepared as
142 in paragraph 2.1 for ELISA assay for possible anti-AFM₁ (detoxifying) effect of fermentation
143 process on AFM₁ reduction.

144 **2.3.3 Effect of plant extracts during milk fermentation**

145 Water extracts of garlic (*Alium sativum*) black cumin (*Nigella sativum*) and carrots (*Daucus*
146 *carota sativus*) were used for an attempt to detoxifying AFM1 by these extracts. Garlic
147 extracts were prepared according to the methods used by Al-Delaimy and Ali; Yin and
148 Change [17, 18]. Milk samples with AFM1 were prepared as described above and

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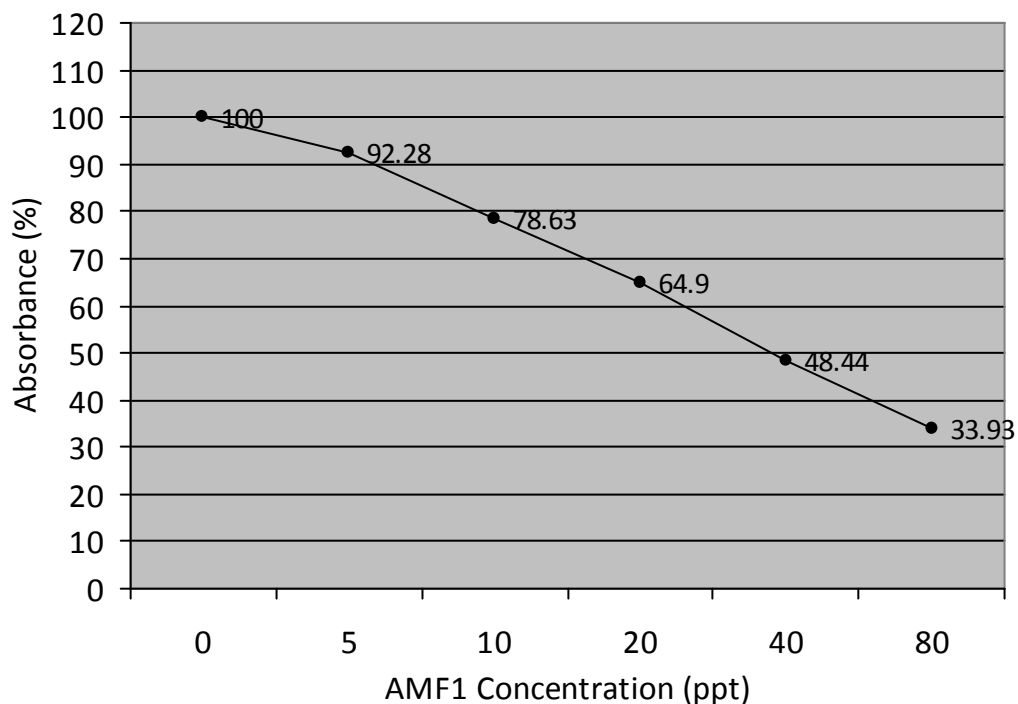
149 designated as G0, G1, G2, G3 and G4. Ten percentage (10 %) of garlic extracts was added
150 to each of 4 milk samples (except the control G0) before fermentation at 42° C and
151 incubated for 3, 6, 24 and 48 hrs, respectively. Samples were assayed for AFM₁ by ELISA
152 technique to observe the AFM₁ reduction effect of garlic extract.

153 Black cumin seeds extracts was prepared as was described by Garcia et al [19] and
154 experimented with similar procedure as was for garlic water extract. Was used for black
155 cumin water extract.

156 Fresh carrots roots produced in Jordan were washed thoroughly with tap water and three
157 times with sterilized water. The ends of the roots were removed then peeled and cut into
158 pieces by a sharp flame sterilized knife. Fifty grams of carrot pieces were blended with 100
159 ml distilled sterilized water for 3 min. Extract was filtered and 10% of the extract was used for
160 each of the 5 samples of fermented milk as was in garlic and black cumin water extract.

161 3. RESULTS AND DISCUSSION

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163 Standard curve (Fig. 1) of the absorbency % against known AFM₁ concentrations was
164 plotted for the determination of AFM₁ level by ELISA technique in all samples in this study.
165 This standard curve is nearly the same as the standard curve given by the ELISA
166 manufacturing company of AFM₁ kit.



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Figure 1: Standard curve of aflatoxin M₁ contents in six standard concentrations.

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Table 1 shows AFM1 content in milk and dairy products samples collected from different location in Amman, Jordan by using ELISA technique. The highest levels were found in 2 labaneh samples purchased from local brands (25.8 and 22 ppt of AFM1). Levels of AFM1 of 19, 15.5, 14, ppt were detected in a yoghurt, raw milk, and jameed samples respectively. No AFM1 was detected in imported infant formula samples (Guigoz and Similac) which may reflect the effective standards and regulations exercised by manufacturers. In addition, samples of each of pasteurized milk (University of Jordan Dairy Production) and labaneh (Baladna) also did not have detectable levels of AFM1. These AFM1 levels found in milk and dairy products in Jordan are considered low and within the European maximum limit of 50 ppt. Most or all tolerance level of AFM1 in different countries use the maximum tolerance levels of either European countries levels of 50 ppt, or USA levels of 500 ppt but there are also some countries with country specific levels that are in between these two levels.

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Table 1: Aflatoxin M₁ contents (ppt) of different dairy products collected in Amman.

No.	Dairy product	AFM ₁ (ppt)	No.	Dairy product	AFM ₁ (ppt)
1	Raw milk	2.0	12	Infant formula	0.5
2	Raw milk	15.5	13	Infant formula	0.0
3	Raw milk	9.8	14	Yoghurt	19.0
4	Pasteurized milk	0.0	15	Yoghurt	2.0
5	Pasteurized milk	8.2	16	Yoghurt	12.4
6	Pasteurized milk	6.8	17	Labaneh	25.8
7	Pasteurized milk	6.1	18	Labaneh	22.0
8	Powder milk	5.2	19	Labaneh	0.0
9	Powder milk	7.8	20	Jameed	14.0
10	Powder milk	3.5	21	Jameed	13.0
11	Infant formula	0.0	22	Jameed	8.0

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The AFM₁ levels in samples from Jordan were lower than the levels in milk samples consumed in Kuwait [20, 21], Iran [22], Turkey [23], Syria [24] and Pakistan [25]. Approximately 82% of the dairy samples from our study had detectable AFM1 (with 85% in milk samples). A study in Turkey found that 55% of raw milk, 25% of ultra-heat treated milk,

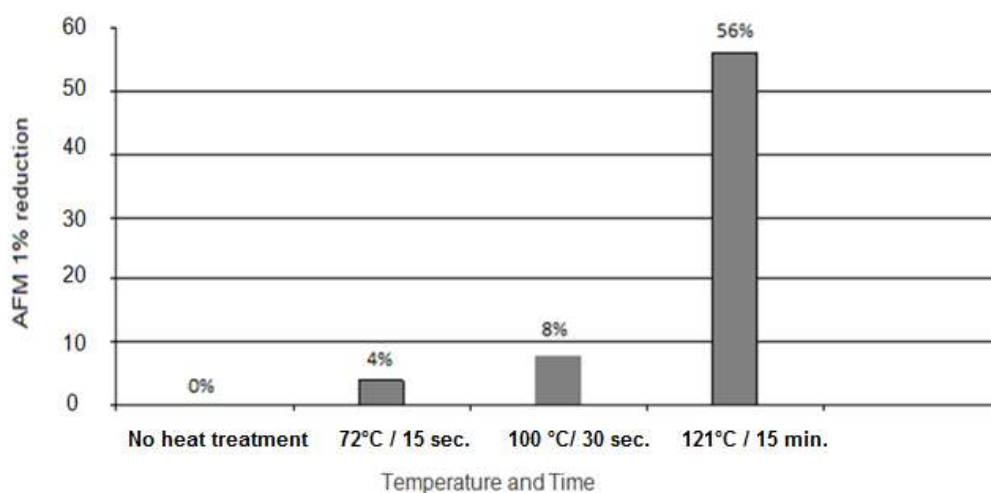
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20% white pickled milk, and 20% of yoghurt samples were contaminated with AFM1 above the acceptable Turkish legal limit (250 ng/kg) [10]. In India, for example, AFM1 was found to contaminate up to 99% of milk samples tested and reaching an extremely high levels of as high as 48000 ppt [26]. In Thailand the levels reached 6600 ppt [27]. In China, using the ELISA assay method to determine the occurrence of AFM1 in milk, it was found that 48.1% of milk samples and 4.5% of yoghurt samples had detectable levels of AFM1. These levels were found to be within the current regulatory limit in China [28]. These results indicate that the health risks associated with AFM1 in China are relatively low. In Japan AFM1 was detected in 99.5% of the tested samples, although the levels were very low (ranging between 1-29 ppt) [9]. In our study similar AFM1 levels in milk and dairy products were found as in Japan. It is believed that the higher levels of AFM1 in milk and dairy products in some countries are probably due to poor hygienic and storage conditions with high humidity. These factors are favorable for the growth of *Aspergillus flavus* and *Aspergillus parasiticus* in animal feeds.

Reduction of AFM1 by high heat treatments (Fig. 2) shows no significant effect after pasteurization and boiling treatments. There was a decrease in AFM1 from 12.5 ppt to 11.5 ppt after pasteurization at 72°C for 15 seconds, while boiling at 100°C for 30 seconds had no effect by decreasing AFM1 from 12.5 ppt to only 12 ppt. A Significant decrease in AFM1 (nearly 56% reduction) was observed after sterilization treatment using the autoclave (121°C for 15 min) which decreased AFM1 from 12.5 ppt to 5.5 ppt. The autoclaving may have partially destroy the chemical formulation of the toxin.

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213 **Figure 2: The effect of different heat treatments on the detoxification of aflatoxin M1 in**
 214 **milk measured by percentage reduction for each temperature starting with 12.5 ppt**
 215 **level with no heat treatment and then increasing to pasteurization temperature (72 °C),**
 216 **boiling temperature (100 °C) and autoclaving (121 °C for 15 minutes).**

217

218 Previous studies [29, 30, 31, 32] also pointed out that pasteurization, boiling and sterilization
 219 did not reduce AFM1 concentration significantly. Deveci and Sezgin [33] on the other hand

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220 reported a reduction of 16% of AFM1 concentration after pasteurization of cow's milk
221 contaminated artificially with 1.5 µg/L of the toxin. A recent study from India confirmed the
222 inability of boiling to reduce AFM1 in several local milk types [34]. AFM1 in this study and
223 other studies is considered a heat stable toxin.

224 The effect of fermentation time on the reduction of AFM1 in milk with artificially added toxin
225 is presented in Figure 3. Progressive reduction of AFM1 from 11.5 ppt before fermentation
226 and as the time of fermentation increased to 6 hrs it was reduced to 10 ppt (a 13%
227 reduction), reduced to 9 ppt after 24 hrs (a 22% reduction) and reduced to 7.5 ppt after 48
228 hrs (a 35% reduction). Partial reduction of AFM1 by milk fermentation was also reported by
229 other workers. The reduction ranged between 12% [35] and up to 97% of total initial AFM1
230 (600 ppb) added before fermentation of milk to yoghurt. One explanation of the effect of
231 fermentation on AFM1 is the presence of *Lactobacillus spp* in milk that binds to the toxin
232 [36]. Abbes et al. [36] reported that *Lactobacillus rhamnosus* at a level of Log 8/ml in
233 reconstituted milk could remove AFM1 after 24hrs at 30 °C in phosphate buffer saline.
234 *Lactobacillus rhamnosus* has more ability to remove the toxin than *Lactobacillus plantarum*.
235 Similar to our finding, Galvano et al. [37] also found that AFM1 in yoghurt were low. A
236 possible explanation is that the low levels of AFM1 in yoghurt samples as well as in jameed
237 (defatted dried skim yoghurt) are due to the effect of lactic acid formation by lactic acid
238 bacteria acting as an anti-AFM1 during fermentation processes [37]. Results of yoghurt
239 AFM1 content from our study and that of others supports this explanation.

240 The effect of 10% of each garlic, black cumin and carrot water extracts on the reduction of
241 AFM1 levels during milk fermentation to yoghurt was studied (Fig. 3). Garlic extracts reduced
242 AFM1 from 12 ppt to 9 ppt, a 25% reduction, after fermentation period of 48 hrs (Fig. 3).
243 Garlic extract was reported to significantly reduce the AFB1 [38]. Limited data exists on the
244 effect of garlic on the reduction of AFM1 content in milk and/or during fermentation. The
245 effect of black cumin on the reduction of AFM1 was found to be slightly less than that of
246 garlic extract and at around 20% after fermentation periods of 48 hrs (Fig. 3). This reduction
247 may be partially attributed to the effect of the interaction between fermentation activities and
248 black cumin extract content or the fact that the baseline levels at the start of fermentation
249 was low at only 7.5 ppt. Lower anti-aflatoxin M1 activity with the addition of both garlic and
250 black cumin water extracts compared to the effect of fermentation process alone was
251 observed. It may be speculated that those extracts may have caused metabolic
252 interferences with AFM1 reduction activities. Garlic for example contain alicin, sulfur
253 containing compound as the active ingredient agent. Carrot water extracts seems to be
254 more effective on the reduction of AFM1 than any of garlic and black cumin extracts (Fig. 3).
255 The initial level of AFM1 (12.5 ppt) added to milk before fermentation was reduced to 3.5 ppt
256 (a reduction of 72%) after 48 hrs. Less reduction (8% and 20%) was observed after
257 fermentation periods of 6 hrs and 24 hrs, respectively. It is possible to indicate that the
258 carotenoids compounds in carrots as antioxidants may interact and bind the AFM₁
259 configuration. In agreement with this finding, a study in Egypt [39] showed that adding 5%
260 and 20% carrot juice to yoghurt resulted in a reduction of 20% and 70% of the initial
261 concentration of AFM1, respectively.
262 This is an important and significant finding that requires further investigation about the
263 potential for carrot juice as an effective anti-aflatoxin M1 (AFM1 reduction).
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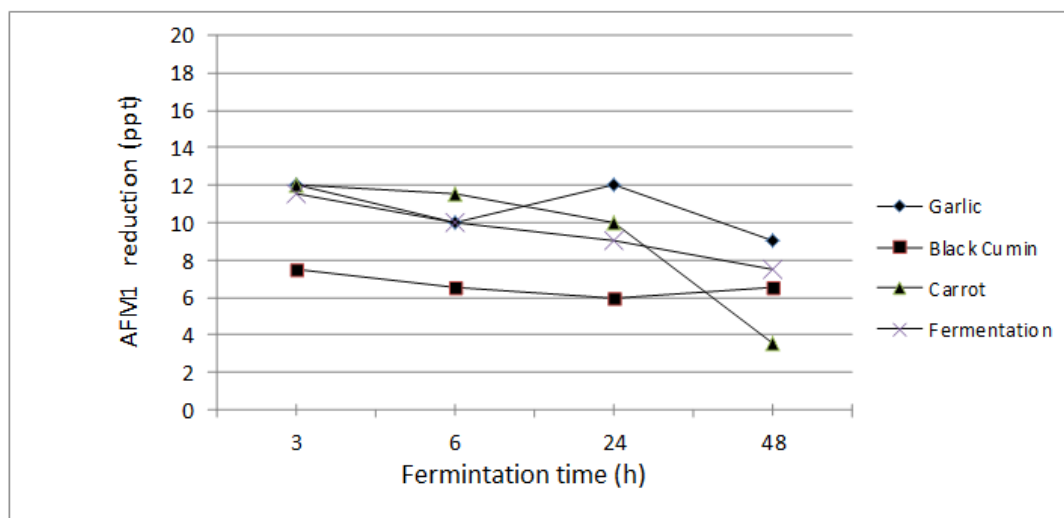


Figure 3: The effect of fermentation time alone, and fermentation plus garlic, black cumin or carrot water extracts on the detoxification of an initial aflatoxin M₁ concentration of 12 ppt with garlic, 7.5 ppt with black cumin, 12.5 ppt with carrot and 11.5 ppt with fermentation alone (as control).

4. CONCLUSION

The AFM1 levels in milk and milk products samples collected in Amman, Jordan were considered to be low (ranging between 25.8-nill ppt.). and within the European standard maximum level of 50 ppt. They were less than the maximum European standard of 50 ppt. Fermentation of milk does decrease AFM1 levels, but carrot water extract added to the fermentation process seemed to have the highest impact on AFM1 levels after 48 hrs and clearly exceeds that of other extracts of garlic or black cumin. This finding from this preliminary study deserves further studies to determine its use in dairy food technology, especially in areas and countries where very high levels of AFM1 have been found in milk and dairy products.

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285 **COMPETING INTERESTS**

286

287 None

288

289 **AUTHORS' CONTRIBUTIONS**

290

291 “ ‘KA’ designed the study, finalized the manuscript and oversaw the laboratory analyses.
292 ‘IM’ performed the laboratory analyses, managed literature searchers and wrote the first
293 draft of the manuscript. All authors read and approved the final manuscript.”

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295 **CONSENT (WHERE EVER APPLICABLE)**

296

297 Not Applicable

298

299 **ETHICAL APPROVAL (WHERE EVER APPLICABLE)**

300

301 Not Applicable

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