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Aflatoxin M1 in Milk and Milk Products in Jordan and Methods for its Reduction: A Preliminary Study

Khalaf S. Al-Delaimy[1*] and Iman F. Mahmoud[2]

[1]Department of Clinical Nutrition and Dietetics, Faculty of Pharmacy and Medical Sciences, University of Petra, Amman, Jordan.
[2]Department of Nutrition and Food Technology, The University of Jordan, Amman, Jordan.

Authors' contributions

This work was carried out in collaboration between both authors. Author KSAD designed the study, finalized the manuscript and oversaw the laboratory analyses. Author IFM performed the laboratory analyses, managed literature searchers. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: The aim of this study was to assess Aflatoxin M1 (AFM1) levels in liquid and powdered milk and other dairy products in Jordan using ELISA technique and 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 AFM1 content. Anti-aflatoxin treatments of fermentation, high heat, garlic (*Allium sativum*), black cumin (*Nigella sativum*) and

**Corresponding author: E-mail: kaldelaimy@uop.edu.jo;*

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 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, and 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 by 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. In the early months of 1960 at total of 100,000 turkey poultries died in mysterious circumstances in the United Kingdom. Thorough investigations showed that the turkeys died from acute liver necrosis and bile duct hyperplasia after they consumed infected groundnuts [1].

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

Although the potency of AFM1 is one tenth that of AFB1, AFM1 still represents a potential carcinogen for humans [2]. The contamination of dairy products with AFM1 has been recognized as a significant human health hazard, particularly for babies, since milk is their basic food.

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

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

2. MATERIALS AND METHODS

2.1 Sampling and Sample Preparation

A total of 22 samples of raw (n=3), pasteurized (n=4) and powdered milk (n=3); infant formula (n=3); yoghurt (n=3); labaneh (low moisture sour yoghurt produced by straining yoghurt by clean cloth to remove 50% of the water) (n=3); and jameed (traditional dry yoghurt) (n=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) of each of liquid milk samples and reconstituted powdered milk, infant formula and jameed were cooled to 10ºC 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). 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 AFMI1 standards provided by the same source (R-Biopharm AG, Dermstadt, Germany).

The ELISA assay is based on the affinities of the monoclonal or polyclonal antibodies against AFM1. ELISA is a relatively 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 investigated by many and compared to other methods, such as HPLC and found to be a more sensitive and reliable method than HPLC [16]. The AFM1 ELISA kit has a quantitation range of 5-100 ppt with high reliability but lower levels are detected as the lowest concentration that could be differentiated from zero.

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). The color intensity is measured photometrically at 450 mm and is inversely proportional to the AFM1 concentration in the sample up to 80.

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

Absorbance % = (Absorbance of standard (or sample)÷ Absorbance of zero standard)× 100

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

2.3 Reduction of AFM1

Testing for AFM1 reduction was carried out with high heat treatment, fermentation, and the addition of water extracts of garlic, black cumin and carrots added to milk samples before fermentation. AFM1 was artificially added at initial levels of 7.5 - 12.5 ng/ml milk and mixed vigorously for 5 min. by vortex mixer. Five equal portions of milk samples, each sample was containing 3 ml were used in each reduction AFM1 experiment.

2.3.1 Effect of heat treatment

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

2.3.2 Effect of fermentation

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

2.3.3 Effect of plant extracts during milk fermentation

Water extracts of garlic (*Alium sativum*), black cumin (*Nigella sativum*) and carrots (*Daucus carota sativus*) were used for an attempt to detoxifyAFM1 by these extracts. Garlic extracts were prepared according to the methods used by Al-Delaimy and Ali; Yin and Change [17,18]. Milk samples with AFM1 were prepared as described above and designated as G0, G1, G2, G3 and G4. Ten percent (10%) of garlic extracts was added to each of 4 milk samples (except the control G0) before fermentation at 42ºC and incubated for 3, 6, 24 and 48 hrs, respectively. Samples were assayed for AFM1 by ELISA technique to observe the AFM1 reduction effect of garlic extract.

Black cumin seed extracts were prepared as was described by Garcia et al. [19] and experimented with similar procedure as was for garlic water extract.

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

3. RESULTS AND DISCUSSION

Standard curve (Fig. 1) of the absorbency % against known AFM1 concentrations was plotted for the determination of AFM1 level by ELISA technique in all samples in this study.

Table 1 shows AFM1 content in milk and dairy products samples collected from different locations in Amman, Jordan by using the 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, and 14 ppt were detected in 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.

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, 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 extremely high levels of 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. There was variability in AFM1 levels in the samples were collected and this reflects different production and hygienic practices that requires further investigation and

research. This might be more relevant for local products of labenah and jameed that are more unique to the country of Jordan than other countries and have established procedures for production [29,30].

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 72ºC for 15 seconds, while boiling at 100ºC 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 (121ºC for 15 min) which decreased AFM1 from 12.5 ppt to 5.5 ppt. The autoclaving may have partially destroyed the toxin.

Previous studies [31-34] also pointed out that pasteurization, boiling and sterilization did not reduce AFM1 concentration significantly. Deveci and Sezgin [35] on the other hand reported a reduction of 16% of AFM1 concentration after pasteurization of cow's milk contaminated artificially with 1.5 µg/L of the toxin. A recent study from India confirmed the inability of boiling to reduce AFM1 in several local milk types [36]. AFM1 in this study and other studies is considered a heat stable toxin.

The effect of fermentation time on the reduction of AFM1 in milk with artificially added toxin is presented in Fig. 3. Progressive reduction of AFM1 from 11.5 ppt before fermentation and as the time of fermentation increased to 6 hrs it was reduced to 10 ppt (a 13% reduction), reduced to 9 ppt after 24 hrs (a 22% reduction) and reduced to 7.5 ppt after 48 hrs (a 35% reduction). Partial reduction of AFM1 by milk fermentation was also reported by other workers. The reduction ranged between 12% [37] and up to 97% of total initial AFM1 (600 ppb) added before fermentation of milk to yoghurt. One explanation of the effect of fermentation on AFM1 is the presence of Lactobacillus spp in milk that binds to the toxin [38]. Abbes et al. [38] reported that *Lactobacillus rhamnosus* at a level of Log 8/ml in reconstituted milk could remove AFM1 after 24hrs at 30ºC in phosphate buffer saline. *Lactobacillus rhamnosus* has more ability to remove the toxin than *Lactobacillus plantarum*. Similar to our finding, Galvano et al. [39] also found that AFM1 in yoghurt were low. A possible explanation is that low levels of AFM1 in yoghurt samples as well as in jameed are due to the effect of lactic acid formation by lactic acid bacteria acting as an anti-AFM1 during fermentation processes [39].

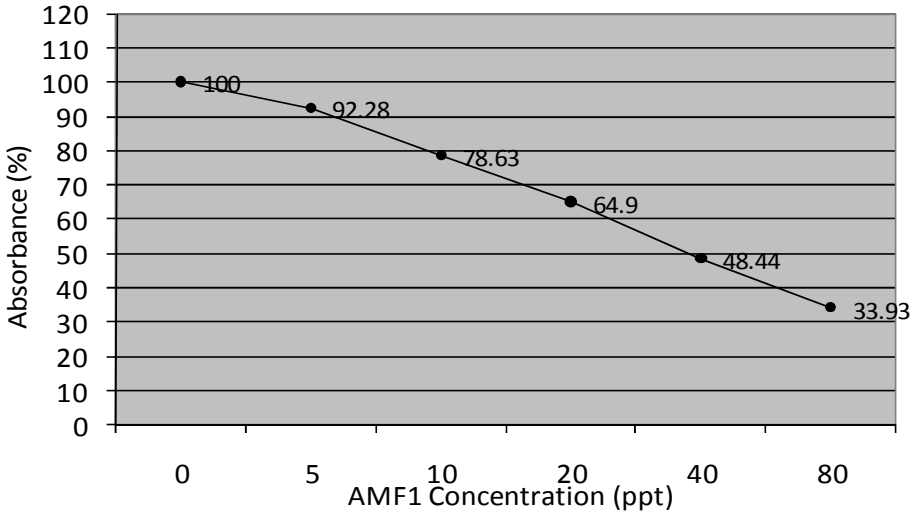

Fig. 1. Standard curve of aflatoxin M1 contents in six standard concentrations

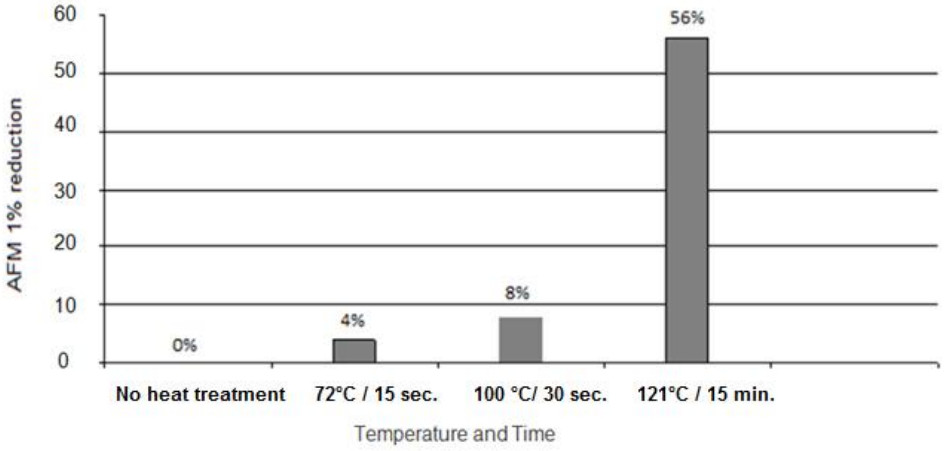

Fig. 2. The effect of different heat treatments on the detoxification of aflatoxin M1 in milk measured by percentage reduction for each temperature starting with 12.5 ppt level with no heat treatment and then increasing to pasteurization temperature (72ºC), boiling temperature (100ºC) and autoclaving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

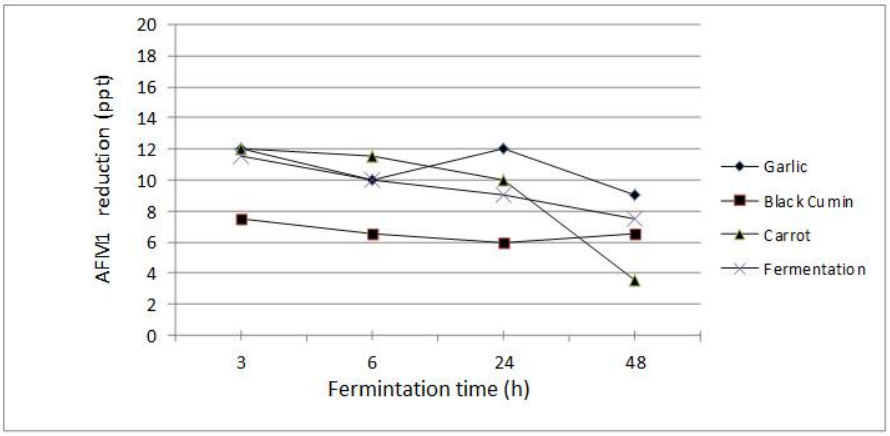

Fig. 3. The effect of fermentation time alone, and fermentation plus garlic, black cumin or carrot water extracts on the reduction of an initial aflatoxin M1 concentration of 12 ppt with garlic, 7.5 ppt with black cumin, 12.5 ppt with carrot and 11.5 ppt with fermentation alone (as control)

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

No.	Dairy product	AFM1 (ppt)	No.	Dairy product	AFM1 (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

The effect of 10% of each garlic, black cumin and carrot water extracts on the reduction of AFM1 levels during milk fermentation to yoghurt was studied (Fig. 3). Garlic extracts reduced AFM1 from 12 ppt to 9 ppt, a 25% reduction, after a fermentation period of 48 hrs (Fig. 3). Garlic extract was reported to significantly reduce the AFB1 [40]. Limited data exists on the effect of garlic on the reduction of AFM1 content in milk and/or during fermentation. The effect of black cumin on the reduction of AFM1 was found to be slightly less than that of garlic extract and at around 20% after fermentation periods of 48 hrs (Fig. 3). This reduction may be partially attributed to the effect of the interaction between fermentation activities and black cumin extract content or the fact that the baseline levels at the start of fermentation was low at only 7.5 ppt. Lower anti-aflatoxin M1 activity with the addition of both garlic and black cumin water extracts compared to the effect of fermentation process alone was observed. It may be speculated that those extracts may have caused metabolic interferences with AFM1 reduction activities. Garlic for example contain alicin, sulfur containing compound as the active ingredient agent. Carrot water extracts seems to be more effective on the reduction of AFM1 than any of garlic and black cumin extracts (Fig. 3). The initial level of AFM1 (12.5 ppt) added to milk before fermentation was reduced to 3.5 ppt (a reduction of 72%) after 48 hrs. Less reduction (8% and 20%) was observed after fermentation periods of 6 hrs and 24 hrs, respectively. It is possible to indicate that the carotenoids compounds in carrots as antioxidants may interact and bind the AFM1 configuration. In agreement with this finding, a study in Egypt [41] showed that adding 5% and 20% carrot juice to yoghurt resulted in a reduction of 20% and 70% of the initial concentration of AFM1, respectively.

This is an important and significant finding that requires further investigation about the potential for carrot juice as an effective anti-aflatoxin M1 (AFM1 reduction).

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.). They were less than the maximum European standard for AFM1 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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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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