A survey on the aflatoxin M1 occurrence in raw milk and dairy products from water buffalo in South China

Ling Guo, Yanyan Wang, Peng Fei, Jianxin Liu, Daxi Ren

Abstract

The objective of this study was to investigate the levels of aflatoxin M1 (AFM1) concentration in raw milk and dairy products from water buffalo in South China. 136 raw milk and 86 dairy products samples were collected randomly from buffalo dairy pastures and supermarket in Guangxi, Guizhou, Yunnan and Guangdong province during winter season. AFM1 levels were determined using the enzyme linked immunosorbent assay (ELISA) method and verified by high performance liquid chromatography (HPLC). AFM1 was detected in 85 (62.5%) of raw buffalo milk samples, and the content ranged between 4 and 243 ng/kg, among them, 8 (5.9%) samples were over the European Regulation (50 ng/kg). For the dairy products, AFM1 was found in 64 (74.4%) samples, and the content ranged between 4 and 235 ng/kg. AFM1 occurrence (100%) and content (43.1 ng/kg) in cheese was significantly higher than that in high temperature short time (HTST) milk, ultra heat treated (UHT) milk and yogurt. None of the raw milk and dairy products of AFM1 content was over the China standard (500 ng/kg). Therefore, AFM1 contamination in buffalo raw milk and dairy products was not represented a serious public health problem in south China.

1. Introduction

Aflatoxins are a group of compounds with the fundamental structure of bifuran (the basic structural to come into being toxicity) and coumarin (the basic structural to result in carcinogenesis) and can be divided into four aflatoxin groups, including aflatoxin B1, B2, G1, and G2 (Ketney, Santini, & Oancea, 2017; Mahmoud Fashandi, Abbasi, & Mousavi Khanehghah, 2018). Among the aflatoxin groups, AFM1 is the hydroxylated metabolite of aflatoxin B1 (AFB1) that can be present in the milk and milk products of animals that are fed with AFB1-contaminated feed (Bahrami, Shahbazi, & Nikousefat, 2016; Gizachew, Szonyi, Tegegne, Hanson, & Grace, 2016; Xiong, Xiong, Zhou, Liu, & Park, & Khobragade, 2016). Ansari et al. (2019) assessed AFM1 contamination of pasteurized milk in Maragheh city of northwestern Iran. Nile et al. (2016) analyzed the AFM1 contamination in milk from some species (buffalo, cow, goat, and sheep) in different districts of Maharashtra, India. Bellio et al. (2016) evaluated the AFM1 contamination in cow milk in northern Italy over a three-year period (2012–2014). The presence of AFM1 in raw bulk milk was investigated in the milk-producing basin of northwestern France in 2003 (Boudra et al., 2007). In addition, the AFM1 level in milk and dairy products have also been researched in China (Wang et al., 2017; Xiong, Xiong, et al., 2018; Zheng et al., 2013). AFM1 was also detected in different dairy products, including milk, yogurt, cheese, butter and laban in Qatar (Hassan, Thani, Atia, Meer, & Balmas, 2018). In addition, AFM1 was also found in breast milk, eight (6.4%) and twenty-two (32.8%) breast milk samples were detected AFM1 in Pakistan and Portuguese, respectively (Bogalho et al., 2018; Khan, Ismail, Gong, Akhtar, & Hussain, 2018).

Buffalo milk is the second largest milk all over the world. The safety of buffalo milk is related with many people. In recent years, some studies covering the safety and quality aspects of water buffalo and their products have been reported. Murru, Peruzy, Carlo, Mercogliano, & Fraulo, (2018) evaluated Listeria monocytogenes survival during the...
manufacture of water buffalo Mozzarella after *Listeria monocytogenes* were used to contaminate raw milk. Some researchers found that the quality or function of buffalo milk products (kefir or cheese) were similar or higher than cow milk (Dimitreli, Exarhopoulos, Antoniou, Zotos, & Bampidis, 2017; Gul, Atalar, Mortas, & Dervisoglu, 2018; Rafiq et al., 2018). However, there are few studies of the AFM1 contamination in buffalo milk and dairy products. AFM1 contamination in buffalo milk or dairy products had been reported in some countries such as Pakistan, Italy and Turkey (De Roma, Rossini, Ritienni, Gallo, & Esposito, 2017; Kara & Ince, 2014; Nile et al., 2016).

AFM1 content in food is related to public health and safety closely, however, few researchers reported the AFM1 content in buffalo raw milk and dairy products in China. Therefore, the aim of this study was to survey the occurrence and content of AFM1 in buffalo raw milk and dairy products from four mainly buffalo raised provinces (Guangxi, Guizhou, Yunnan, and Guangdong) of south China in the winter.

# 2. Materials and methods

## 2.1. Reagents and chemicals

AFM1 ELISA kits were purchased from Suwei Microbiology Reagent co. LTD (Jiangsu, China). Acetonitrile, petroleum ether and so on were purchased from Tianli Chemical Reagent co. LTD (Tianjin, China). All the chemicals used in this study were all of high performance liquid chromatography (HPLC) grade unless otherwise specified and purified water was obtained by a Milli-Q water system (Millipore, MA, USA).

## 2.2. Sample collection

A total of 136 raw buffalo milk samples were collected from 35 pastures during October 2016 and March 2017 from south China, which included 45 raw buffalo milk samples from 15 pastures in Guangxi province, 36 raw buffalo milk samples from 9 pastures in Guizhou, 35 raw buffalo milk samples from 7 pastures in Yunnan and 20 raw buffalo milk samples from 4 pastures in Guangdong. Approximated 1000 mL of all day milk samples were collected from milk tanks with refrigeration system in pasture, samples were frozen in the refrigerator (−20 °C) until analysis. Totally 86 buffalo dairy products were collected from supermarket during the October 2016 and March 2017 from 4 provinces. The buffalo dairy products samples include the HTST milk samples (n = 16), the UHT milk samples (n = 26), the yogurt samples (n = 27) and the cheese samples (n = 17), some samples were collected from the same brand within different date of production. Samples were sealed and stored at −20 °C before analyzed.

## 2.3. Sample preparation

Raw buffalo milk and buffalo dairy products samples were prepared for the quantitative test according to the methods described by El Khoury, Atoui, and Yaghi (2011) with minor adjusted.

### 2.3.1. Liquid milk

The liquid milk samples (HTST and UHT milk) were centrifuged at 3500 g for 10 min below 10 °C (Shanghai Centrifuge Institute Co., Ltd., Shanghai, China). The upper creamy layer was removed completely through drinking straw, the lower fluid was used for AFM1 analysis.

### 2.3.2. Yogurt samples

The yogurt was diluted three times with purified water. Five grams of sample diluent and 20 mL of acetonitrile were added to a 100 mL triangular flask. The mixture was extracted after shaking for 5 min. Suspension was centrifuged at 4000 g for 10 min and 1 mL of the supernatants were evaporated at 50 °C under N2 gas using termovap sample concentrator (NanBei instruments co. LTD, Zhengzhou, China). The residue was redissolved in the mixture containing 750 μL sample diluent and 0.5 mL petroleum ether. The mixture was centrifuged at 4000 g for 15 min. The lower phases were used for the AFM1 quantitative test.

### 2.3.3. Cheese samples

The treatment of cheese samples is similar to that of yogurt samples. The difference was that 20 mL of acetonitrile was replaced by 15 mL of samples extract in the 100 mL triangular flask and the residue was redissolved in 1 mL sample diluent and 1 mL petroleum ether. Then, the cheese samples were used for the AFM1 analysis.

## 2.4. ELISA screening method for analysis of AFM1

The qualitative analysis of AFM1 in the milk and milk products samples was performed using competitive enzyme immunoassay with an ELISA plate reader (Thermo Fisher Company, MA, USA). According to the manufacturer’s instructions, ELISA test procedure was carried out with the AFM1 Elisa kit. Firstly, the required reagents and microwell plate were stored at 20 °C for at least 30 min. Liquid reagent were shook well before using. About 250 μL of washing buffer was added into separate wells for rinsing. The washing buffer was poured off after 40 s, the microwell plate was patted dry on the absorbent paper. This rinsing step was repeated twice. 50 μL of the AFM1 standard solutions and prepared samples were added to the separate wells and incubated for 30 min at 37 °C in the dark. Then, 50 μL of the enzyme conjugate was added to the well, mixed gently by manually shaking the plate and incubated for 15 min at room temperature in the dark. Next, the reaction liquid in the wells was poured out and the wells were washed for five times with 250 μL of washing buffer. Then, 50 μL of the substrate and 50 μL of chromogen solution were added to the wells and the mixture was mixed gently through shaking the plate and incubated at 37 °C in the dark for 15 min. Finally, 50 μL of the stop reagent solution was added into the wells for stopping the reaction. The plate was shaken gently for mixing well. The absorbance value of each well was measured at 450 nm using the spectrophotometer ELISA plate reader. The AFM1 content was calculated according to the standard curve.

## 2.5. HPLC confirmatory method for analysis of AFM1

HPLC also used to confirm the results of AFM1 by ELISA, detail information of machine and chemicals, and procedure were similar with previous study from our lab (Xiong, Wang, Ma, & Liu, 2013). Determination of AFM1 levels was performed using a HPLC system (Palo Alto, CA). Separation of AFM1 was achieved with a CAPCELL PAK MGII-C18 (150 mm × 2.1 mm i.d., 1.8 mm) column (Shiseido, Tokyo, Japan). 0.1% formic acid/acetonitrile/methanol (v/v/v, 56/22/22) was used as the mobile phase at a flow rate of 0.3 mL/min. The limit of detection (LOD) and limit of quantification (LOQ) were set at 5 ng/kg and 10 ng/kg, respectively.

## 2.6. Statistical analysis

The study adopted statistical methods to evaluate the incidence of AFM1 in samples, reported as mean and standard deviation (SD). And the data were statistically analyzed by a one way analysis of variance (ANOVA) using SPSS version 19.0 (SPSS, Inc., Chicago, IL, US). The level of confidence required for significance was set at P ≤ 0.05.

# 3. Results and discussion

ELISA and HPLC were applied for the quantification of AFM1 levels in buffalo raw milk and dairy products. The incidence and concentration of AFB1 in raw buffalo milk in south China were shown in Table 1. For 136 raw buffalo milk samples, 62.5% (n = 85) samples were positive for AFM1. Within these samples, the AFM1 content in 40 (29.6%) was less than 10 ng/kg, in 37 samples was between 10 and 50 ng/kg,
and in 8 samples (5.9%) ranged between 50 and 500 ng/kg. The overall mean level of AFM1 in raw buffalo milk samples was 37.4 ± 18.7 ng/kg. The minimum and maximum of AFM1 concentration in raw buffalo milk were 4 and 243 ng/kg, respectively. Totally, 8 samples were at a level greater than the legal limit of 50 ng/kg set by EC (2006) for liquid milk, no sample above the legal limit of 500 ng/L set by the US FDA (1996, p. 219) or China (MoH, 2011).

Recently, AFM1 contamination in milk was becoming a serious public health problem, which cause some surveys reported in the world. These announced that the incidence of AFM1 in buffalo milk were 7.2%, 50%, 27%, 38.7%, and 52% in Southern Italy, India, Afyonkarahisar of Turkey, Ahvaz of Iran, and Ismailia of Egypt, respectively (De Roma et al., 2017; Kara & Ince, 2014; Motawee, Bauer, & McMahon, 2009; Nile et al., 2016; Rahimi, Bonyadian, Rafie, & Kazemeini, 2010). A study on raw cow and buffalo milk samples in Shush city of Iran showed that AFM1 was detected in 90 of 120 samples (123 ng/L) was significantly higher than in other seasons in South China around the YRD region (Xiong et al., 2013), as the inadequate storage conditions and long storage time. In this study, most of samples were collected during the winter season, the AFM1 in raw buffalo milk (42.4 ng/L) and dairy products (27.6 ng/L) was lower. Differences between the occurrences of AFM1 in these studies might be the difference of geographical conditions, climate, seasonal variations, or feed systems (Bahrami et al., 2016; Ertas, Gonulalan, Yildirim, & Karadal, 2011; Xiong et al., 2018). Another reason could attribute to the AFM1 presence in milk has caused the worldwide attention to pasture manager, method such as using Solis Mos to remove AFB1 in the feeds was popular in pasture to control AFM1 now (Xiong, Wang, Zhou, & Liu, 2018).

As shown in Table 2, the incidence of AFM1 in buffalo milk products in Southern China also exhibited a higher positive rate. In total, 74.4% (64 of the 86) buffalo milk products samples were positive for AFM1. The incidence of AFM1 in HTST milk, UHT milk, Yogurt and Cheese was 75%, 76.9%, 55.5% and 100% respectively. Among all the positive buffalo milk products samples, 43% samples (37 of the 86) contained AFM1 at levels of less than 10 ng/kg, 26.7% samples (23 of the 86) at levels of 10–50 ng/kg, and 4 samples over 50 ng/kg. Many researchers had reported the AFM1 content and incidences in dairy products, results were varied significantly between countries and regions. Some of them found high AFM1 content in dairy products. According to a research in Pakistan, 37.5% of milk, 12.5% of powdered milk, 20% of flavored milk, 20% of yogurt and 16% of flavored milk samples from summer were found exceeded the recommended levels of AFM1 (50 ng/kg, EU permissible limit), as compared to 38.1%, 37.1%, 15.6%, 21.4%, 27.7% and 40% samples of milk, UHT milk, powdered milk, flavored milk, yogurt and flavored yogurt from winter (Iqbal, Asi, & Malik, 2017). In another study, AFM1 was found in 43 (86%) of raw milk samples, in 38 (63%) of cheese, in 28 (56%) of yoghurt out of 210 analyzed samples in Turkey and the range of AFM1 contamination was 1–378 ng/kg (Ertas et al., 2011). Some other studies found lower aflatoxin contamination in dairy products. Researchers in Qatar detected AFM1 in 85%, 76%, 85%, 67% and 76% of the milk, yogurt, cheese, butter and laban samples, none of the tested samples presented AFM1 levels above the EU maximum limits of 50 ng/L or kg for milk, yogurt and butter, and 250 ng/kg for cheese (Hassan et al., 2018). Sakuma, Kamata, Sugita-Konishi, and Kawakami (2011) showed that AFM1 was not detected in 60 samples of cheese in Japan. AFM1 was detected in 48% and 42% of the milk and dairy samples, and in all

<table>
<thead>
<tr>
<th>Location</th>
<th>Samples</th>
<th>Pastures</th>
<th>Positive (n/%)</th>
<th>Frequency distribution of AFM1 concentration (ng/kg)</th>
<th>AFM1 concentration (ng/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mean ± SD</td>
<td>Min</td>
</tr>
<tr>
<td>Guangxi</td>
<td>45</td>
<td>15</td>
<td>30/66.7%</td>
<td>16 10 4 5 243</td>
<td>48.5 ± 20.5a</td>
</tr>
<tr>
<td>Guizhou</td>
<td>36</td>
<td>9</td>
<td>22/61.1%</td>
<td>10 9 3 4 172</td>
<td>40.4 ± 24.5b</td>
</tr>
<tr>
<td>Yunnan</td>
<td>35</td>
<td>7</td>
<td>19/54.3%</td>
<td>8 10 1 4</td>
<td>36.2 ± 15.3c</td>
</tr>
<tr>
<td>Guangdong</td>
<td>20</td>
<td>4</td>
<td>14/70.0%</td>
<td>6 8 0 5</td>
<td>26.1 ± 15.7d</td>
</tr>
<tr>
<td>Total</td>
<td>136</td>
<td>35</td>
<td>85/62.5%</td>
<td>40 37 8</td>
<td>37.4 ± 18.7</td>
</tr>
</tbody>
</table>

**Means within a column with the same superscript letter were not significantly different (P < 0.05).**
Lactic acid bacteria have the ability to reduce the level of AFM1 in AFM1 content in yogurt lower than liquid milk and indicated that some samples. Similar with our study, researchers in Lebanese also found that cow milk products have also been conducted (Li et al., 2017; Xiong more and more investigation of AFM1 contamination in cow milk and Yaman, 2008). In this study, lowest AFM1 content was found in yogurt However, researchers in Turkey found the 38% marketed cheese AFM1 levels higher than EU permissible limits (Dashti et al., 2009). In Kuwait, only 2.5% of the tested cheese samples were contaminated with AFM1 levels higher than EU permissible limits (Yapar, Elmalı, Kart, & Yaman, 2008). In this study, lowest AFM1 content was found in yogurt samples. Similar with our study, researchers in Lebanese also found that AFM1 content in yogurt lower than liquid milk and indicated that some Lactic acid bacteria have the ability to reduce the level of AFM1 in yogurt (El Khoury et al., 2011).

With the gradual upgrading and expansion of Chinese dairy market, more and more investigation of AFM1 contamination in cow milk and cow milk products have also been conducted (Li et al., 2017; Xiong et al., 2018; Zheng et al., 2017). A recent research showed that the occurrence of AFM1 in dairy products samples (UHT milk samples and pasteurized milk samples) from central China was approximately 73.6% of 242 samples (Xiong et al., 2018). The result of Li et al. (2017) showed that AFM1 contamination was detected in 267 of the 5650 raw milk sample analysed in the major milk-producing areas of China (including Hebei, Heilongjiang, Henan, Inner Mongolia, Shandong, and Xinjiang provinces). Zheng et al. (2017) showed that AFM1 levels in raw cow milk from Southern, Northern, Northeast, and Western regions of China during the four seasons from 2013 to 2015 were detected in 21.5% of 1550 samples. Results of this study indicated that water buffalo milk AFM1 level were below the maximum limit of China (500 ng/kg) and different from AFM1 levels of UHT and pasteurized milk, raw milk and raw cow milk determined by Xiong et al. (2018), Li et al. (2017), and Zheng et al. (2017). In brief, the AFM1 contamination in buffalo raw milk and dairy products was not represented a serious public health problem in south China.

As shown in Table 3, many counties had set the Max limited of AFM1 in milk and dairy products. The Max limited of AFM1 in milk and dairy products in EU and some country is 50 ng/kg, in USA and some Asia country include China is 500 ng/kg. Some countries such as Italy, Iran and Turkey set the different Max limited in cheese (250–500 ng/kg) and milk powder (500 ng/kg), Bazile set 5000 ng/kg in milk powder and 2500 ng/kg in cheese (ANVISA-Agencia Nacional de Vigilância Sanitaria, 2011; CAC 2001; EU 2006). Accoring to Table 3, AFM1 in few buffalo milk and cheese samples is over 50 ng/kg in EU country such as Italy and Turkey. The ratio of AFM1 in buffalo milk and dairy product sample over 50 ng/kg was 15.8%, 16%, 8% and 5.4% in Pakistan, India, Iran and China (Hussain, Anwar, Asi, Munawar, & Kashif, 2010; Kamkar et al., 2014; Nile et al., 2016; Rahimi et al., 2010). The highest of ratio over 50 ng/kg (48%) was found in Egypt, however, only 2% of milk sample was over 250 ng/kg (Motawee et al., 2009). Therefore, AFM1 content in buffalo milk and dairy products is at a safe level in most countries.

Aflatoxins are highly toxic compounds and agro-food products, cereals, spices, and milk potentially were contaminated with different types of aflatoxins, especially AFM1, which is frequently found in milk and milk products (Ismail et al., 2018; Ketey et al., 2017). A high AFM1 pollution in milk and milk products creates a severe health threat due to the fact that milk and milk products are consumed extensively by peoples from all around the world (Grace, Kiarie, Kirino, Lindahl, & Ahlborg, 2018). AFM1 is likely to cause cancer and some children could be stunted due to AFM1 exposure from milk (Mahdavi, Niknazar, Arehosseini, & Vahed Jabbari, 2010). Therefore, some measures were took to reduce the AFM1 contamination in milk and milk products in processes, such as thermal processing, concentration and drying to impair some minor effects on AFM1 in milk, salting to decreased the AFM1 levels in cheese and ferment to reduce the AFM1 levels in yogurt (Barukčić, Bilandžić, Markov, Jakopovic, & Božanić, 2018; Campagnollo et al., 2016). In general, researches indicate that aflatoxins do not pose a threat to consumer’s health due to the levels of AFM1 below the regulatory limits of each country (Campagnollo et al., 2016). According to this result (the overall mean level of AFM1 in the raw buffalo milk and buffalo dairy products samples was 37.4 ± 18.7 ng/kg and 27.6 ± 10.7 ng/kg, respectively), it can be calculated that the AFM1 of daily intakes is approximately 8.3–11.3 ng if the consumer drinks 300 g of buffalo milk daily. However, if the high contaminated dairy products were ingested in the long term, the incidence of cases will be greatly improved and the contamination of mycotoxins may become one of the most serious public health problems (Campagnollo et al., 2016).

### 4. Conclusions

In summary, the occurrence of AFM1 of in buffalo raw milk and dairy products was relatively higher compared to European countries. However, the content of AFM1 in buffalo raw milk and dairy products was in a safety level for the Chinese Regulation. Only 5.9% of samples over the European standard (50 ng/kg), none of the samples was over the China max limited level (500 ng/kg). Therefore, AFM1

<table>
<thead>
<tr>
<th>Country*</th>
<th>AFM1 Maximum limits (ng/kg) in different countries and content in buffalo milk and dairy products in different researches.</th>
</tr>
</thead>
<tbody>
<tr>
<td>USA</td>
<td>50 500</td>
</tr>
<tr>
<td>EU</td>
<td>50 50</td>
</tr>
<tr>
<td>Italy</td>
<td>50 250 (soft cheese); 450 (hard cheese)</td>
</tr>
<tr>
<td>Turkey</td>
<td>50 250 (cheese)</td>
</tr>
<tr>
<td>Pakistan</td>
<td>50 50 15.8%; 17.0%</td>
</tr>
<tr>
<td>India</td>
<td>50 50 16%</td>
</tr>
<tr>
<td>Iran</td>
<td>100 20 (butter and butter milk); 250 (cheese); 500 (milk powder) 8%</td>
</tr>
<tr>
<td>China</td>
<td>50 500 5.4%</td>
</tr>
<tr>
<td>Egypt</td>
<td>50 50 48%</td>
</tr>
</tbody>
</table>

*AFM1 limited standard or reference from different countries or area: USA, US FDA (1996); EU, European Commission (2001) and European Commission (2006); Turkey, Codex Alimentarius Commission (2001); Iran, Atabak et al. (2015); Pakistan, Iqbal et al. (2011); China, MoH, (2011).
contamination in buffalo raw milk and dairy products was not represented a serious public health problem in south China.

Conflicts of interest

The authors declare no conflict of interest.

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Food Control, 34(2), 356–361.

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