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## DETECTION OF MYCOTOXINS IN RAW MILK, TRADITIONAL YOGURT AND BUTTERMILK IN MOROCCO BY ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO HIGH RESOLUTION MASS SPECTROMETRY (UPLC/HRMS)

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#### ABSTRACT

Given the unavoidable presence of mycotoxins in agricultural products and their potential harm to humans and animals, it is crucial to detect and remove them from the food supply. Mycotoxins are toxic compounds produced by certain fungi, posing serious risks to food safety and health. Dairy products like raw milk, traditional yogurt, and buttermilk are especially prone to contamination due to their production and storage conditions. This summary examines the frequency, types, and effects of mycotoxin contamination in these dairy items. In this research, 60 samples of raw milk, traditional yogurt, and buttermilk (20 each) were collected from traditional dairies in the Temara region of Morocco. These samples were analyzed for mycotoxins using high-resolution mass spectrometry coupled with liquid chromatography (LC-HRMS). The findings revealed that 76% of raw milk samples were contaminated with AFM1, with additional contaminants including AFB1 (41%), AFB2 (24%), AFG1 (13%), AFG2 (9%), OTA (18%), and ZEA (16%). Traditional yogurt samples showed levels of contamination, with AFM1 at 75%, AFB1 (68%), AFB2 (42%), AFG1 (35%), AFG2 (14%), OTA (13%), and ZEA (12%). Buttermilk samples had contamination rates higher than yogurt, with AFM1 (83%), AFB1 (27%), AFB2 (53%), AFG1 (59%), AFG2 (18%), OTA (24%), and ZEA (22%).

Key Words: Mycotoxins, raw milk, traditional yogurt, buttermilk, UPLC/HRMS.

#### **INTRODUCTION**

Mycotoxins are toxic compounds produced by certain species of mold, mainly from the genus *Aspergillus*, *Penicillium*, and *Fusarium*. These toxins can contaminate a wide range of food products, including cereals, nuts, and livestock feed (Richard, J. L. 2007). When dairy cows consume feed contaminated with mycotoxins, these toxins can end up in the milk, posing risks to human and animal health (Pitt, 2009).

Among the mycotoxins of greatest concern to the dairy sector, aflatoxin M1 is particularly noteworthy. It is a metabolite of

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aflatoxin B1, produced by the molds *Aspergillus flavus* and *Aspergillus parasiticus*. When a cow ingests aflatoxin B1 in its diet, this toxin is metabolized in the liver and converted to aflatoxin M1, which is then excreted in the milk. Aflatoxin M1 is stable for pasteurization, which means it can persist in processed dairy products (Bryden, 2012).

In addition to aflatoxins, other mycotoxins such as zearalenone, fumonisins, trichothecenes (such as deoxynivalenol), and ochratoxin A may also be present in cattle feed. Although these toxins are less frequently detected in milk than aflatoxin M1, their presence poses similar food safety concerns (Fink-Gremmels, J. 2008).

The detection of mycotoxins in milk requires sensitive and precise techniques due to the low concentrations and complexity of the High-resolution milk matrix. mass spectrometry coupled liquid to chromatography (LC-HRMS) has become an essential technique in the field of mycotoxin control due to its accuracy, sensitivity, and ability to analyze several compounds simultaneously, thus guaranteeing improved food safety and compliance with regulatory standards (Rubert, 2012).

The present work aimed to assess the presence of some mycotoxins in samples of raw milk and traditional yoghurt purchased during the spring of 2024 in the region of Temara in Morocco and to assess their concentrations in order to evaluate the health risks for consumers.

## MATERIALS AND METHODS

#### Sampling:

To investigate the presence of mycotoxins and their levels in raw bovine milk, traditional fermented yoghurt (Raib) and buttermilk (Lben), which are marketed to traditional mini dairies in the Temara region. A total of 60 samples (20 samples of each matrix). The samples were collected randomly between March and June 2024. The samples were kept frozen at -20°C until they were analyzed.

#### **Extraction:**

Milk and yoghurt samples were prepared using the modified QuEChERS method (Manav ÖG 2019). Briefly, 10 g of samples were placed in a 50 ml polypropylene tube, then 10 ml of acetonitrile containing 1% (v/v) formic acid was transferred to the tube and the mixture vortexed for 30 seconds. After adding 5.7 g sodium chloride, 6 g sodium sulfate, and 4.2 g sodium acetate to the mixture, the tube was immediately agitated for 1 min. The sample was centrifuged at 5,000 rpm for 5.5 minutes. Next, 7 ml of the resulting extract was transferred to a 15 ml polypropylene tube with 300 mg magnesium sulfate, 0.5 g diatomaceous earth, and 150 mg C18. The mixture was vortexed for 1 min, followed by centrifugation at 5,000 rpm for 5.5 min. After the clean-up process, 1,000 µL of the extract was transferred to a vial for UHPLC-Q-Orbitrap analysis.

#### UHPLC-Q-Orbitrap HRMS analysis

Quantitative and qualitative mycotoxin profiles were obtained following the protocol described by Izzo et al. (2022). Chromatographic separation of mycotoxins was carried out using an Ace C18 150 mm 2.1 mm 3 µ column. The volume of injection was 5 µL, and the mobile phase was composed as follows: phase A (H<sub>2</sub>O in 0.1% HCOOH + 5 mM NH<sub>4</sub>HCO<sub>2</sub>) and phase B (methanol in 0.1% HCOOH + 5 mM  $NH_4HCO_2$ ). The analytes were separated at a flow rate of 0.2 mL/min. The linear chromatographic gradient was set as follows: 0 to 0.6 min, 15% B; 0.6 to 2.6 min, up to 80% B; 2.6 to 5.7 min, 100% B; 5.7 to 7.7 min, up to 15% B. Finally, the gradient was maintained for 2.3 min at 15% B for column re-equilibration. Running time was 10 minutes in total.

Mass spectrometry analysis was carried out using an Orbitrap Exploris 120; the parameters of the ion source were 310°C for capillary temperature, 305°C for evaporation temperature, 2.8 kV for spray voltage, sheath gas pressure of 35 arbitrary units, and auxiliary gas of 10 arbitrary units. In addition to the precursor ions, two confirmation ions per compound were monitored with a maximum mass error of 2 ppm (the difference between practical and theoretical mass) to ensure accurate identification in accordance with regulations. The calibration of the mass spectrometers was done regularly for three days and before each sequence. Table 1 shows elemental composition, retention time, collision energy, theoretical and measured mass, precise mass error, and confirming ions. Data processing was carried out on TraceFinder 5.1.

#### Statistical analysis:

The Excel version 2016 software package was used. Data are presented as follows: mean, standard deviation (SD), and range (minimum to maximum).

#### RESULTS

Analyst	Retention Time (min)	Elemental Composition	Adduct Ion	Theoretical Mass (m/z)	Measured Mass (m/z)	Accuracy (Δ ppm)	Collision Energy (eV)	Product Ior	ns Ion (m/z)
ZAN	3,5	C18H24O5	[M-H]-	317,1395	317,1391	1,1	-32	131,0501	175,0399
AFM1	3.7	C17H12O7	[M+H]+	329,0656	329,0651	1,4	40	273,0754	229,0491
AFG1	3.8	C17H12O7	[M+H]+	329,0656	329,0655	0,2	40	243,0647	200,0464
AFG2	4.0	C17H12O7	[M+H]+	331,0812	331,0803	2,7	37	245,0800	313,0701
AFB2	7.8	C17H14O6	[M+H]+	315,0863	315,0866	-1,0	36	259,0595	287,0906
AFB1	8.3	C17H12O6	[M+H]+	313,0707	313,0701	1,8	36	285,0749	269,0437
ОТА	8.5	C20H18NO6Cl	[M+H]+	404,0895	404,0890	1,3	16	358,0830	341,0566

Table 1: Chromatographic retention time and Q-orbitrap HRMS parameters for 7 target Mycotoxins

Table 2: Mycotoxins concentration (ng/L) in Raw Milk

Parameters	AFM1	AFB1	AFB2	AFG1	AFG2	Ochratoxins A	Zearalenone
Mean	7,7	0,6	0,8	1,2	2,4	1,8	1,2
S.D	21,1	0,4	0,7	1,1	1,3	1,8	0,8
Minimum	0	0	0	0	0	0	0
Maximum	108,5	2,5	4,1	5,8	6,9	6,2	4,1
Percent of positive samples	76	41	24	13	9	18	16
Exceeding EM	4	0	0	0	0	0	0
Exceeding EC	4	0	0	0	0	0	0
Exceeding US FDA	4	0	0	0	0	0	0

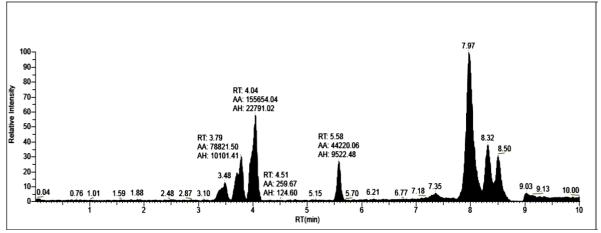
AFM1 maximum limit: Moroccan regularisation (MR) 50 ng/L, European Commission 2006 (EC) 50 ng/L, US FDA: US FDA, (2011), 500 ng/L FDA: Food and Drug Administration

Parameters	AFM1	AFB1	AFB2	AFG1	AFG2	Ochratoxin A	Zearalenone
Mean	11,9	0,7	0,7	1,4	2,4	1,7	1,3
S.D	18,6	0,7	0,8	1,2	1,3	1,1	1
Minimum	0	0	0	0	0	0	0
Maximum	98,7	3,7	5,1	7,1	5,8	4,8	5,6
Percent of positive samples	75	68	42	35	14	13	12

**Table 3:** Mycotoxins concentration (ng/L) in traditional yogurt (Raib)

 Table 4: Mycotoxins concentration (ng/L) in traditional Buttermilk (Lben)

Parameters	AFM1	AFB1	AFB2	AFG1	AFG2	Ochratoxins A	Zearalenone
Mean	6,7	0,5	0,6	2,1	1,9	1,7	1,3
S.D	18,2	0,5	0,4	0,6	0,8	0,9	0,7
Minimum	0	0	0	0	0	0	0
Maximum	85,3	3,8	2,9	3,2	4,1	4,8	5,3
Percent of positive samples	83	27	53	59	18	24	22



**Figure 1.** Example of chromatogram of a raw milk matrix enriched with 50 µg/L of Mycotoxin AFM1, AFB1, AFB2, AFG1, AFG2, OTA, and ZEA.

#### DISCUSSION

Seven mycotoxins were detected and quantified: AFM1, AFB1, AFB2, AFG1, AFG2, OTA, and ZEA in raw milk, yoghurt, and buttermilk samples is summarized in tables (2, 3, 4).

#### • Raw milk

The results presented in Table 2 show that 76% of raw milk samples were contaminated with

AFM1, while 24% of milk samples analyzed were below the detection limit of our method. AFM1 levels ranged from 0 to 108.5 ng/L, with an overall mean concentration of  $7.7 \pm 21.1$  ng/L. The results also showed contamination of samples by other mycotoxins AFB1 (41%), AFB2 (24%), AFG1 (13%), AFG (9%), OTA (18%), and ZEA (16%).

#### •Traditional yoghurt

Analysis of the traditional yoghurt samples also revealed contamination by the mycotoxins AFM1 (75%), AFB1 (68%), AFB2 (42%), AFG1 (35%), AFG2 (14%), OTA (13%), and ZEA (12%); this was higher than that found in the raw milk samples.

#### •Buttermilk

The results of the analysis of buttermilk samples also indicated contamination by the mycotoxins AFM1 (83%), AFB1 (27%), AFB2 (53%), AFG1 (59%), AFG2 (18%), OTA (24%), and ZEA (22%), with positivity rates close to those of traditional yoghurt samples.

In Morocco, while dairy products like milk, yoghurt, buttermilk, and cheese are commonly consumed, only three studies have investigated mycotoxins in pasteurized milk. Zinédine et al. (2007) found that 88.8% of pasteurized milk samples were contaminated with AFM1, with concentrations ranging from 0.001 to 0.117 µg/L. Marnissi et al. (2012) reported that 27% of raw milk samples contained AFM1, with levels ranging from 0.010 to 0.100  $\mu$ g/L and an average concentration of 0.043 µg/L in positive samples. More recently, Alahlah et al. (2020) observed that AFM1 was present in 100% of powdered milk samples and 35% of UHT milk samples, with average concentrations of 0.0255 μg/kg and 0.0148 μg/kg, respectively.

In Jordan, Herzallah (2009) noted the presence of AFB1, AFB2, AFG1, AFG2, and AFM2 in milk, each with an occurrence rate of 8.3%. Most research on mycotoxins in milk primarily focuses on AFM1, the principal hydroxylated aflatoxin metabolite found in the milk of dairy cows consuming AFB1-contaminated feed. Stoloff (1997) and Battacone *et al.* (2003) found that AFM1, which can appear in animal milk within 12 to 24 hours of AFB1 ingestion, can remain detectable for up to 3 days after the last exposure to the toxin.

The OTA is classified as carcinogenic by The International Agency for Research on Cancer. Exposure to OTA has been linked to specific endemic kidney diseases in the Balkans, known as Balkan Endemic Nephropathy (BEN) and Urinary Tract Tumors (UTT). Therefore, the tolerable weekly intake is 120 ng/kg body weight (PTWI), which has been recommended by the European Commission.

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Research from Italy, Sweden, Norway, France, and China reported OTA levels ranging from 5 to 84.1 ng/L, which are generally low enough that adults are unlikely to exceed the PTWI (Sørensen & Elbæk, 2005; Pattono et al., 2011; Breitholtz-Emanuelsson et al., 1993; Skaug, 1999; Boudra et al., 2007; Elzupir et al., 2009; Huang et al., 2014). Nonetheless, if cows consume high amounts of OTA, exceptions might occur (Gonzalez-Osnaya et al., ~2008). Additionally, abrupt changes in feed or high-protein concentrates can impact the rumen microorganisms' ability to break down OTA (Fink-Gremmels, 2008; Skaug, 1999). In Sudan, an OTA contamination level of 2730 ng/L was observed (Elzupir et al., 2009). Despite typically low OTA levels in milk, these concentrations could be significant for high consumers, particularly children. Another study indicated that young children consuming large quantities of milk might exceed the Tolerable Daily Intake (TDI) of 5 ng/kg bw/day (Skaug 1999). Moreover, children's diets might include other OTA sources.

Due to their toxic properties, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has set a Provisional Maximum Tolerable Daily Intake (PMTDI) of 0.5 mg/kg body weight for ZEA and its metabolites. Analyses of approximately 400 milk samples from Hungary, Egypt, the UK, and China have revealed ZEA, ZAN, and α-ZAL concentrations of up to 12.5 mg/kg (Sandor, 1984; El-Hoshy, 1999; SCOOP, 2003; Huang et al., 2014; Xia et al., 2009). In a worst-case scenario, where ZEA levels reach 12.5 mg/kg (El-Hoshy, 1999), an individual weighing between 50 and 70 kg would need to consume 2 to 2.8 liters of milk daily to exceed the PMTDI. Therefore, ZEA exposure through milk is not deemed a significant health risk. However, the toxicity of ZEA metabolites should be considered; for instance,  $\alpha$ -ZEL is three times more estrogenic than ZEA (Mirocha, Pathre, and Robison, 1981); in milk, a maximum concentration of 73.5 ng/kg was reported by Huang *et al.* (2014) in China.

Exposure of consumers to mycotoxins can occur through the ingestion of contaminated crops (such as cereals) or the ingestion of animal products (like dairies) from animals that have consumed contaminated feed (Capriotti *et al.*, 2012). Chronic exposure to mycotoxins is linked to various health issues, including genotoxicity, immune suppression, carcinogenicity, hepatotoxicity, nephrotoxicity, estrogenic effects (Anfossi *et al.*, 2010).

Mycotoxin contamination in milk and livestock presents, in addition to public health risk, an economic loss due to reduced productivity (Bryden, animal 2012). Evidence of mycotoxins in feed and some studies indicating their presence in the plasma of dairy cows (Winkler et al., 2014) suggest these toxins might be transferred into milk. While rumen flora is expected to protect against certain mycotoxins such as aflatoxin B1, deoxynivalenol, ochratoxin A and zearalenone by converting them into less harmful substances others like patulin and fumonisins can bypass this defence (Fink-Gremmels, unchanged 2008). Furthermore, the efficacy of the rumen barrier can be compromised by factors such as animal health conditions, diet, or feed contamination with high concentrations of mycotoxins (Pattono et al., 2011).

In conclusion, the presence of the mycotoxins AFB1, AFB2, AFG1, AFG2, AFM1, OTA, and ZEA in raw milk, traditional yoghurt, and buttermilk with high levels of positivity may present a risk to consumers and indicates that feedstuffs are contaminated with these mycotoxins. Because of these results, we need to study the relationship between mycotoxin levels in feed and dairy during all seasons of the year in this region.

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# الكشف عن السموم الفطرية في الحليب الخام، الزبادي التقليدي واللبن في المغرب عن طريق تحليل الطيف الكتلي عالي الدقة (UPLC/HRMS) كروماتو غرافي سائل فائق الأداء مقترن بمقياس

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نظراً لوجود السموم الفطرية في المنتجات الزراعية وضررها المحتمل على البشر والحيوانات، فمن الضروري اكتشافها وإز التها من الإمدادات الغذائية. السموم الفطرية هي مركبات سامة تنتجها بعض الفطريات، مما يشكل مخاطر جسيمة على سلامة الأغذية والصحة. منتجات الألبان مثل الحليب الخام واللبن الزبادي التقليدي واللبن الزبادي معرضة بشكل خاص للتلوث بسبب ظروف إنتاجها وتخزينها. يتناول هذا الملخص تكرار وأنواع وآثار التلوث بالسموم الفطرية في مدعم عند عبد معن المحتمل على البلن الزبادي التقليدي واللبن الزبادي معرضة بشكل خاص للتلوث بسبب ظروف إنتاجها وتخزينها. يتناول هذا الملخص تكرار وأنواع وآثار التلوث بالسموم الفطرية في هذه المنتجات. في هذا البحث، تم جمع ٢٠ عينة من الحليب الخام واللبن واللبن التقليدي (٢٠ لكل منهما) من منتجات الألبان التقليدية في منطقة تمارة بالمغرب. تم تحليل هذه العينات بحنًا عن السموم الفطرية والأفلاتوكسينات باستخدام قياس الطيف الكتلي عالي الدقة مقترنًا بالكروماتو غرافيا السائل عالية السموم الفطرية والأفلاتوكسينات باستخدام قياس الطيف الكتلي عالي الدقة مقترنًا بالكروماتوغرافيا السائل عالية إضافية بما في ذلك (١٢٨). كشفت النتائج أن ٢٢٪ من عينات الحليب الخام كانت ملوثة بـ ١٢٢٨، مع ملوثات الموم الفطرية والأفلاتوكسينات باستخدام قياس الطيف الكتلي عالي الدقة مقترنًا بالكروماتو غرافيا السائل عالية إضافية بما في ذلك (١٢٨). كشفت النتائج أن ٢٢٪ من عينات الحليب الخام كانت ملوثة بـ ١٢٢٨، مع ملوثات الدقية إضافية بما في ذلك (١٢٨). أملهرت عينات الزبادي التقليدية مستويات من التلوث، مع ١٢٢٨، (١٢٣). أظهرت عينات الزبادي التقليدية مستويات من التلوث، مع ٢٢٨٨، (١٢٨)، ورادي ٢٢٪)، ورزيا ٢٦٪). أظهرت عينات الزبادي التقليدية مستويات من التلوث، مع ٢٢٨، (١٢٥)، ٢٢٪)، ورزيا ٢٠٪). أظهرت عينات الزبادي التقليدية مستويات من النلوث، مع ٢٢٨، (١٢٨) عاليه، من ٢٢٠٪)، ورزيا ٢٢٪). أظهرت همان الزبادي التقليدية مستويات من التلوث، مع ٢٢٨، (١٢٨)، ٢٢٠٪)، ورريا ٢٠٪). أظهرت عينات الزبادي التقليدية مستويات من التلوث، مع ٢٢٨، (١٢٥)، ٢٦٤٨، (٢٠٪)، رزيا ٢٠٪)، معري مع (٢٦٤)، معدلات تلوث مشابية الزبادي، مع (٢٦٤)، (٢٤٤) حجم، (٢٤٤) حجم، دورة 2٤٤)، معدلات تلوث مشابية الزبادي، مع (٢٤٤)، (٢٤٤)، (٢٣٤)، ٢٤٤)، (٢٤٤)، معدلات ماري معدلات تلوث مشابيهة الزباديي، مع (٢٤٤)، (٢٤٤)، (٢٤٤)، م

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