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Survey of the enniatins and beauvericin in raw and UHT cow's milk in Poland

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Abstract

Introduction: The enniatins A, A1, B and B1 (ENNs) and beauvericin (BEA) are structurally related compounds produced by *Fusarium* species. They occur as contaminants in cereals, such as wheat, barley and maize. They are called "emerging mycotoxins", because they have been reported in feed and food and their toxic effects are not fully known. Data on their levels in food (especially in milk) are limited. The study aimed to evaluate the occurrence of ENNs and BEA in milk. **Material and Methods:** A total of 103 bovine milk samples (76 of raw milk and 27 of UHT milk) were collected from different parts of Poland and analysed using liquid chromatography–tandem mass spectrometry. **Results:** Among the 76 raw milk samples, 31 (41%) and 15 (20%) samples were contaminated with ENN B and with BEA, respectively. No contamination with other enniatins was found. The highest concentration of BEA was found in raw milk and was 6.17 μg kg^{-1}. Out of the 27 samples of UHT milk, 16 (59%) were contaminated with ENN B at concentrations ranging from 0.157 μg kg^{-1} to 0.587 μg kg^{-1} (limit of quantification (LOQ) 0.098 μg kg^{-1}). Beauvericin was detected in 9 UHT milk samples (33%) at concentrations ranging from 0.101 μg kg^{-1} to 1.934 μg kg^{-1} (LOQ 0.095 μg kg^{-1}). **Conclusion:** This study demonstrated constant but low milk contamination in Poland with ENN B and BEA. The analysis of milk samples revealed that the emerging mycotoxins ENN B and BEA were measured in trace amounts. It does not suggest any immediate risk to milk consumers; however, it is unknown whether long-term exposure to low levels of toxins may be harmful.

Keywords: enniatins, beauvericin, milk, LC-MS/MS.

Introduction

Emerging mycotoxins were defined as compounds which are neither routinely determined nor yet regulated by food law. While their detection is not attempted as part of standard food or feed quality control, the evidence of their incidence is growing (11). These toxins are becoming significant points of interest as new compounds such as fusaproliferin (FP), beauvericin (BEA), enniatins (ENNs) and moniliformin (MON) present in food and feed and produced by the most common grain-contaminating fungi, which are *Fusarium* spp. (15). Enniatins A, A1, B and B1 and BEA are often found in different food commodities; hence, investigations of their presence were published (19, 30). Enniatins are structurally related mycotoxins representing a large group of cyclic hexadepsipeptides. Beauvericin is a cyclic hexadepsipeptide that consists of alternating D-hydroxy-isovaleryl-(2-hydroxy-3-methylbutanoic acid) and N-methylphenylalanine moieties (Fig. 1 and Table 1).

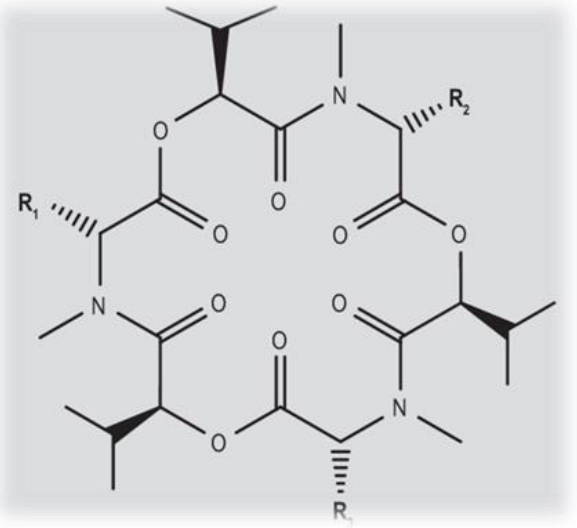


Fig. 1. The chemical structures of enniatins A, A1, B, B1 and beauvericin

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Table 1. Structural formula of enniatins (ENNs) and beauvericin (BEA)

Compound	Side chain R_1	Side chain R_2	Side chain R_3
BEA	phenylmethyl	phenylmethyl	phenylmethyl
ENN A	sec-butyl	sec-butyl	sec-butyl
ENN A1	sec-butyl	iso-propyl	sec-butyl
ENN B	iso-propyl	iso-propyl	iso-propyl
ENN B1	iso-propyl	sec-butyl	iso-propyl

The wide range of the biological activity of these compounds is associated with their ionophoric behaviour. All cyclodepsipeptides (*e.g.* ENNs and BEA) have antibacterial, insecticidal, antifungal, herbicidal and even antibiotic properties, which may lend themselves to the development of new drugs. On the other hand, their potential cytotoxic activity may affect the central nervous system. They also show apoptotic and immunosuppressive effects (8, 25, 30).

The contents of ENNs and BEA in food are not regulated by legislation. A comprehensive scientific opinion on their presence was published by the European Food Safety Authority (EFSA) in 2014 (7). Occurrence data reported to the EFSA by 12 European countries on samples taken between 2000 and 2013 show a high co-occurence of four ENNs (A, A1, B and B1) and BEA in cereal grains. The EFSA opinion concluded that acute exposure to ENNs such as ENN B does not indicate a risk to human health (7). Chronic exposure may nevertheless give concern because the inadequate toxicity data do not make a risk assessment possible. It was emphasised that the primary source of these metabolites are plant materials and that transfer to food of animal origin is limited.

No concentration limits for BEA and ENNs in food have been established, although the EFSA has assessed their presence in feed at high levels (up to mg kg^{-1} or ppm) (7). Furthermore, it should be pointed out that the exposure of ruminant livestock animals to ENNs or BEA has not been accurately estimated because the data on their concentrations in feed are insufficient, and the actual exposure to emerging mycotoxins is probably much higher (18).

A study was conducted on the presence of ENNs and BEA in fish feed and the possible transfer to fish and filleted fish tissue (20). Although all feeds were contaminated with BEA, ENN B and ENN B1, none of the tested mycotoxins was detected in whole fish or fillets. Therefore, no transfer of the parent compound from the feed to the animal-derived food commodity was noticed. This evidence suggests no risk for human consumption. However, there may still be some concern that molecules of these compounds may be metabolised and deposited in organs at concentrations below those found in recent investigations (12, 20, 31).

Interestingly, there is little information about the levels of these substances in food of animal origin, *e.g.* milk. Cow's milk is essential in a healthy and balanced diet, especially for children, the largest group of consumers (22). These mycotoxins, present as feed contaminants, can be excreted in the biological fluids and milk of animals or humans (1), as parent substances or as metabolites (18). Mycotoxins are not eliminated by mechanised milk processing such as pasteurisation or sterilisation. Therefore, it is essential to keep mycotoxins in milk under tolerable levels (9).

When present in silage or other feed materials, ENNs and BEA can have antibacterial effects and modify the rumen microflora, which may reduce the detoxification properties of the rumen and facilitate the passage of the mycotoxins the tissue environments of the animal's body where they may bioaccumulate as well as into the milk. This may negatively impact line processes during the production of cottage cheese, hard cheese, or beverages such as kefir or yoghurt, such that ENNs and BEA may remain as contaminants in the finished food products. Rubert *et al.* (29) and Braun *et al.* (2) presented scientific data on human milk which included frequent detection of emerging mycotoxins.

Although many studies have been conducted to examine the carry-over of mycotoxins or their metabolites from different feed matrices to ruminant milk, few documented occurrences of ENNs and BEA in cow's milk are in the literature. González-Jartín *et al.* (9) developed a new method for the simultaneous analysis of regulated, modified and emerging mycotoxins from species of the *Aspergillus*, *Alternaria*, *Fusarium* and *Penicillium* genera in milk *via* a technique of liquid chromatography coupled with mass spectrometry (LC-MS). For provision of more extensive data, this study aimed to determine ENN and BEA concentration levels in raw and processed cow's milk. To the best of the authors' knowledge, it is the first report presenting levels of ENNs and BEA in animal milk in Eastern Europe.

Material and Methods

Sampling. The study used 76 raw milk and 27 UHT milk samples collected from various parts of the country and sent to the Reference Laboratory for Mycotoxin Analysis at the National Veterinary Research Institute, Pulawy, Poland (Fig. 2). The raw milk was collected by the District Veterinary Inspectorates as part of their programme to control chemical residues in food of animal origin. Samples of UHT milk were purchased at retailers in different parts of Poland. Milk samples were kept frozen below −16°C until the day of analysis.

Reagents. Acetonitrile, methanol, ammonium acetate, octadecyl sorbent C18, acetic acid (99.5%) and formic acid (99.5%) (all ULC/MS Optigrade®) were provided by J.T. Baker (Philipsburg, NJ, USA). Purified water was obtained from a Milli-Q Advantage system (MilliporeSigma, Burlington, MA, USA). Standards of ENN A, A1, B and B2 and BEA were purchased from Sigma-Aldrich (Schnelldorf, Germany). All standards were kept in the conditions recommended by the supplier. Stock standard solutions of ENNs and BEA were prepared in methanol and stored in the dark at ≤−16°C for a maximum of 12 months. The stock

standard solutions were used to prepare a working standard solution containing all of the analytes at the concentration of 1.25 ng mL^{-1} BEA and ENNs and stored in the dark at ≤6°C for a maximum of six months.

Sample preparation. Details of the method have been published elsewhere (23). A 5 g mass of each milk sample was weighed into a Falcon tube. For the spiked sample, 25 µL of a mixed working standard solution of ENNs and BEA at 1 µg mL^{-1} was added. A 10 mL aliquot of the extraction solvent (acetonitrile:water:formic acid, 79:20:1; v/v/v) was added to each sample. The samples were shaken for 1 min on a vortex mixer and centrifuged at 4,000 rpm for 15 min at 4°C. Then 10 g of Na_2SO_4 was added to each sample, and the solution was shaken for 1 min on a vortex mixer and centrifuged at 4,000 rpm for 15 min at 4°C. Subsequently, all of the extract was transferred to a glass test tube with 300 mg $MgSO_4$ and centrifuged again at 4,000 rpm for 15 min at 4°C. Next, 1 mL of extract was evaporated to dryness (N_2, 40 ± 5°C). The dry residue was dissolved in mobile phase A and mobile phase B in the proportions of 1:1 (v/v). The extracts were transferred to vials for liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis.

Instrumental parameters. The LC-MS/MS parameters were based on a previously described multianalyte method with some modifications (29). Briefly, the system consisted of a Shimadzu Nexera X2 ultra-high performance liquid chromatograph (Shimadzu, Kyoto, Japan) coupled with an 8050 triple quadruple mass spectrometer (Shimadzu), which was operated in positive electrospray mode (ESI+). LabSolution software (version 5.60 SP2) was used for control and data analysis (Shimadzu).

The experiments were conducted in the positive electrospray mode using a Kinetex Biphenyl column, 100 × 2.1 mm, 2.6 µm particle size (Phenomenex, Torrance, CA, USA) with a guard cartridge of the same material operated at 40°C. A mixture of methanol and 0.1% acetic acid in a 10 mM ammonium acetate solution in the proportions of 5:95 (v/v) was used as mobile phase A, and a mixture of methanol and 0.1% acetic acid in the ammonium acetate solution in a ratio of 95:5 (v/v) was mobile phase B. The gradient of the mobile phase was 0% of A from 0 to 2 min; 20% of A from 2 to 4.1 min; 40% of A from 4.1 to 9 min, held to 13 min; and 0% of A from 13 to 13.1 min, held to 16 min. The flow rate was 0.3 mL min^{-1}, and the injection volume was 5 µL. The mass spectrometer working parameters were optimised as heating gas flow of 8 L min^{-1}, nebulising gas flow of 2 L min^{-1}, drying gas flow of 8 L min^{-1}, desolvation line temperature of 240°C, interface temperature of 300°C, and resolution Q1 and Q3 unit. Two multiple reaction monitoring (MRM) transitions for each analyte were monitored (Table 2).

Data evaluation. LabSolution was used for peak integration and data processing. The analyte identification was performed according to the SANTE/12089/2016 Guidance document on the identification of mycotoxins in food and feed (5). The identification criteria were comparison of analyte peak retention time in samples with the peak of the calibration standards, the retention time of the internal standard (within the tolerance range ± 0.05 min), selection of at least two characteristic fragmentary ions and of their ion ratio (within ± 30% (relative) of an average of calibration standards from the same sequence), and the peaks having a signal-to-noise ratio (S/N) = 10.

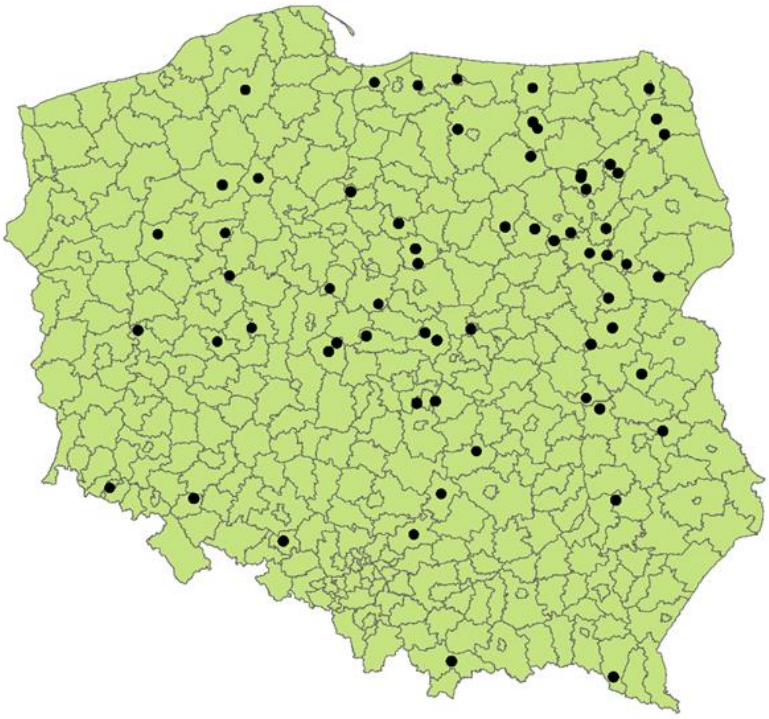


Fig. 2. Locations of collections of the milk samples for analysis

Table 2. Optimised tandem mass spectrometry parameters

Analyte	Retention time (min)	Precursor ion (m/z)	Ion species	Product ion[a] (m/z)	C.E.[b] (V)	Q1 pre bias (V)	Q3 pre bias (V)	Dwell time (ms)
Enniatin A	11.66	699.30	$(M+H)^+$	699.3/682.3 699.3/210.2 699.3/100.1	−18 −31 −55	−32 −32 −32	−36 −23 −21	16
Enniatin A1	11.44	685.30	$(M+H)^+$	685.3/668.3 685.3/210.1 685.3/100.1	−20 −29 −55	−32 −32 −32	−34 −23 −19	16
Enniatin B	11.04	657.30	$(M+H)^+$	657.3/640.3 657.3/196.2 657.3/86.1	−18 −32 −55	−30 −30 −30	−34 −21 −18	16
Enniatin B1	11.24	671.30	$(M+H)^+$	671.3/654.2 671.3/196.1 671.3/210.1	−18 −32 −29	−30 −30 −30	−34 −22 −23	16
Beauvericin	12.26	801.30	$(M+NH_4)^+$	801/134 801/244 801/784	−55 −34 −20	−22 −22 −22	−27 −18 −30	33

[a] – confirmation/quantitation; [b] C.E. – collision energy

Table 3. Validation results for the determination of enniatins and beavericin in fresh and UHT milk

Parameters		Raw milk			UHT milk		
Working range		0.15–50 µg kg^{-1}			0.15–50 µg kg^{-1}		
LOD (µg kg^{-1})	ENN A	0.098			0.099		
	ENN A1	0.097			0.096		
	ENN B	0.098			0.098		
	ENN B1	0.088			0.089		
	BEA	0.095			0.095		
LOQ (µg kg^{-1})	ENN A	0.126			0.126		
	ENN A1	0.125			0.128		
	ENN B	0.128			0.124		
	ENN B1	0.129			0.130		
	BEA	0.101			0.099		
Spiking level (µg kg^{-1})		2.5	5.0	7.5	2.5	5.0	7.5
Recoveries (%)	ENN A	93	83	84	98	72	77
	ENN A1	81	91	88	99	78	76
	ENN B	87	96	90	81	82	84
	ENN B1	90	93	79	80	88	85
	BEA	81	92	91	82	79	91
Repeatability (RSD_r %)(n = 6)	ENN A	5.2	3.4	3.7	5.2	3.4	3.7
	ENN A1	6.8	5.8	4.3	4.4	3.6	4.9
	ENN B	5.1	6.5	4.6	4.9	7.3	7.1
	ENN B1	7.2	7.3	6.7	5.8	6.1	6.9
	BEA	5.5	8.0	7.6	3.8	5.5	7.5
Within-lab reproducibility (RSD_{wR} %) (n = 18)	ENN A	10.1	14.8	11.9	13.5	7.8	16.9
	ENN A1	12.4	17.5	16.9	11.9	15.8	6.9
	ENN B	8.6	15.2	15.3	8.7	13.0	12.2
	ENN B1	12.5	14.1	12.4	9.3	11.3	8.3
	BEA	9.8	9.7	11.0	4.9	12.0	8.9
Uncertainty expanded (U(y)) (µg kg^{-1})	ENN A	$u_c(y) = 0.55$; k = 2, U(y) = 1.10			$u_c(y) = 1.10$; k = 2, U(y) = 2.20		
	ENN A1	$u_c(y) = 0.66$; k = 2, U(y) = 1.32			$u_c(y) = 0.78$; k = 2, U(y) = 1.56		
	ENN B	$u_c(y) = 0.50$; k = 2, U(y) = 1.00			$u_c(y) = 0.47$; k = 2, U(y) = 0.94		
	ENN B1	$u_c(y) = 0.53$; k = 2, U(y) = 1.06			$u_c(y) = 0.60$; k = 2, U(y) = 1.20		
	BEA	$u_c(y) = 0.55$; k = 2, U(y) = 1.10			$u_c(y) = 0.59$; k = 2, U(y) = 1.18		

LOD – limit of detection; LOQ – limit of quantification; ENN – enniatin; BEA – beauvericin; RSD_r – relative standard deviation for repeatability; RSD_{wR} – relative deviation for within-lab reproducibility; $u_c(y)$ – combined uncertainty

Validation. As stipulated by European Commission Decision 2002/657/EC (3) concerning the performance of analytical methods and their validation and the characteristics of the method, the following parameters were established: linearity (working range), the limit of detection (LOD), the limit of quantification (LOQ), recovery, repeatability (coefficient of variation – CV), within-laboratory reproducibility (CV) and expanded uncertainty with the use of the MUkit Measurement Uncertainty Kit 1.0.3.7 (combined standard uncertainty for a spiking level of 5 µg kg^{-1}) (21). The specificity was checked by analysing 20 different pseudo-blank feed samples to evaluate possible interferences. The limit of detection and LOQ were also calculated based on an S/N ratio of the first sample of matrix-matched calibration curves (LOD S/N = 3, LOQ S/N = 10). A matrix-matched calibration curve for each mycotoxin was established using six concentration levels in a range from 0.15 µg kg^{-1} to 50 µg kg^{-1}. The recovery, repeatability (CV) and within-laboratory reproducibility (CV) were calculated based on the results from analysis of samples of raw and UHT milk (n = 18) spiked with ENNs and BEA at the levels 2.5, 5.0 and 7.5 µg kg^{-1} and processed through the appropriate extraction procedure described above. All characteristics and parameters of the method which were integral to its validation are shown in Table 3.

Results

Beauvericin and ENNs are analysed by LC-MS using different ionisation techniques, such as thermospray (33), ESI (15), or atmospheric pressure chemical ionisation (11). Triple quadrupole mass spectrometer detectors using an ESI source in the positive ionisation mode were used as the mass analyser in the method presented. Based on the validation results obtained (Table 3), the proposed procedure is suitable for the quantification of ENNs and BEA and meets the criteria of Commission Decision 2002/657/EC (3) and the SANTE/12089/2016 guidance document (5). The method's sensitivity was assessed by the LOD and LOQ, which ranged from 0.088 to 0.099 µg kg^{-1} and from 0,099 to 0.130 µg kg^{-1}, respectively (Fig. 3).

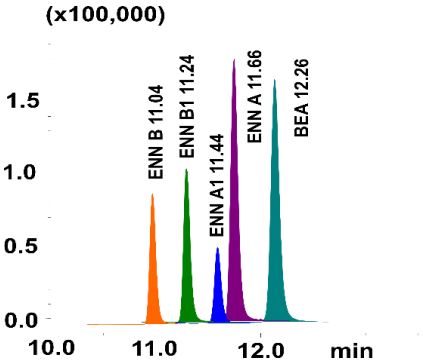


Fig. 3. The LC-MS/MS chromatogram of a spiked milk sample of enniatins (ENNs) and beauvericin (BEA) at a concentration of LOQ level

The trueness of the method, expressed as recovery of analytes, was evaluated at three spiking levels (Table 3). The repeatability evaluation showed a relative standard deviation (RSD) lower than 10% for three spiking levels as far as precision was concerned. In contrast, RSDs lower than 20% were obtained in the reproducibility studies. This method meets the general criteria for toxin analysis for all compounds determined (5, 6).

Of the ENNs, only ENN B was detected in the tested milk samples. However, in more than half of the samples, whether raw or UHT, BEA was also found. The higher occurrence was noticed for ENN B (31 out of 76 raw milk samples – 41% of samples, and 16 out of 27 UHT milk samples – 59% of samples). For BEA, 15 out of 76 survey samples (20%) and 9 out of 27 UHT milk samples (33%) were contaminated.

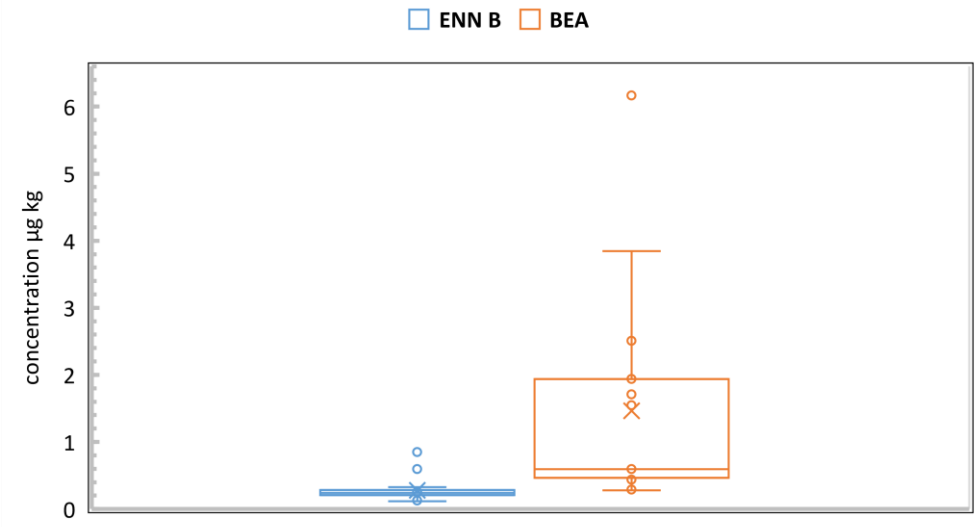


Fig. 4. Concentrations of enniatin B (ENN B) and beauvericin (BEA) in raw milk

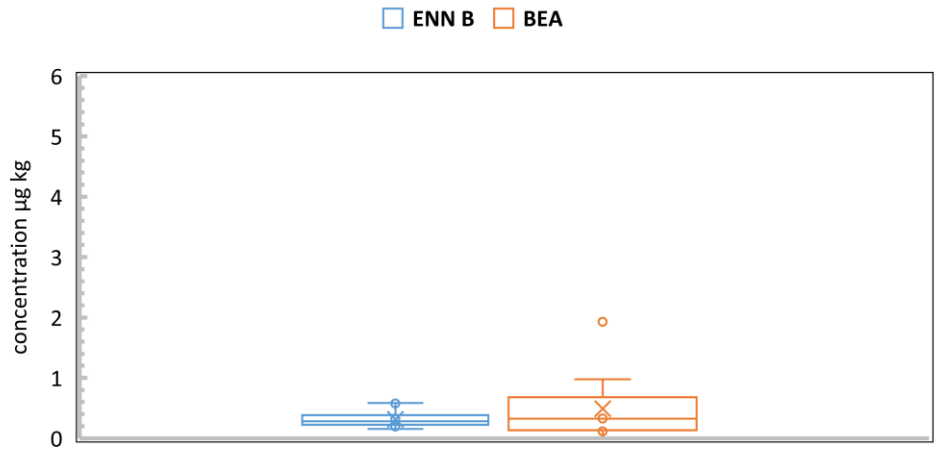


Fig. 5. Concentrations of enniatin B (ENN B) and beauvericin (BEA) in UHT milk

The concentrations of detected analytes were very low and were within the working range of the method of 0.15 µg kg^{-1}–50 µg kg^{-1}. For raw milk samples, the highest concentration was observed for BEA at 6.16 µg kg^{-1}, that of ENN B peaking at 0.85 µg kg^{-1}. For UHT milk, the highest concentration was again one of BEA at 1.93 µg kg^{-1}, and 0.59 µg kg^{-1} was the highest ENN B concentration. This study shows similar ENNs and BEA concentrations in raw and UHT milk, with slightly higher toxin concentrations having been obtained in raw milk. The milk samples' concentrations of ENN B and BEA are presented in summarised form below in the boxplots (Figs 4 and 5).

Discussion

The contamination of feed with *Fusarium* toxins is high, and BEA and ENNs are regularly found in cereal and cereal products (17, 32, 35, 37). Additionally, they can be found in many raw materials, especially maize samples. An important aspect of the research to understand *Fusarium* hazards is the study of ENNs and BEA with co-occurring mycotoxins and their interactions, especially moniliformin (MON) and deoxynivalenol (DON). Beauvericin and ENNs may also bioaccumulate because of their lipophilic nature (33); therefore, their toxicokinetics and their possible residues in animal tissues and food of animal origin should be investigated.

However, there are limited data available in the literature on ENNs and BEA in food of animal origin. The latest EFSA report indicated that a risk assessment for dietary exposure to beauvericin and enniatins was not possible because there was a lack of data regarding acute and chronic toxicity and genotoxicity (7). The carry-over of these substances into sheep's milk was previously reported despite sheep in general, being considered the most resistant ruminants to mycotoxins. In a study by Piątkowska *et al.* (24), only one ENN, ENN B, was detected in raw sheep's milk. These authors detected low levels of ENN B in 18 out of 20 samples (90%) of sheep's milk with an average concentration of 7.8 ± 1.7 µg kg^{-1}. The results are similar to those obtained in the presented work.

There is little data on the prevalence of ENN and BEA in cow's milk. One study (9) found the frequent occurrence of low levels of all ENNs and BEA in this matrix. Enniatins and BEA were found in 31 milk samples from different farms in Portugal. The concentrations detected in milk above the LOQ for these toxins were within the calibration ranges: 3.12–200 µg kg^{-1} for BEA and 0.78–200 µg kg^{-1} for ENNs. The LOQ for BEA was 1.95 µg kg^{-1}, for ENN A 0.08 µg kg^{-1}, for ENN A1 0.37 µg kg^{-1}, for ENN B 0.27 µg kg^{-1} and for ENN B1 0.24 µg kg^{-1}. A 67% proportion of the milk samples was contaminated with ENN A and ENN A1, ENN B was detected in 58% of the samples, ENN B1 in 45%, and BEA in as much as 90%. The highest concentration in a milk sample was measured for ENN A and was 4.76 µg kg^{-1}. Unlike the result of the research by González-Jartín *et al.* (9), no ENN A, A1 or B1 was detected in Polish milk, and only ENN B and BEA were noted. The determined mycotoxins were in a similar concentration range in the present study to the range determined by those researchers.

The detected concentrations of ENNs and BEA in our study were within the same concentration range regardless of the type of milk tested (raw or UHT). The results showed that ENN B and BEA were present in both milk materials above the determined limits of quantification: ENN B was at 0.128 µg kg^{-1} concentration in raw milk and 0.124 µg kg^{-1} in UHT milk, and BEA was at 0.128 µg kg^{-1} concentration in raw milk and 0.124 µg kg^{-1} in UHT milk. Detection of ENN B was successful in 41% of raw milk samples and 59% of UHT milk samples. The BEA mycotoxin was found in 20% of raw milk samples, with a highest concentration of 6.165 µg kg^{-1}, and detected in 33% of UHT milk samples. The detected concentrations of these toxins were within the working range of the method.

In the case of other foods of animal origin, based on the available scientific information, researchers

determined that the levels of ENNs and BEA in the muscles or eggs of turkeys and broilers are low. Analysis of 479 Finnish samples of whole eggs and egg yolk showed that the occurrence of BEA and ENN B and ENN B1 was widespread. However, in most cases the contaminations were trace amounts (<limit of quantification) (16).

Because of the potential transfer of these toxins to milk, the milk test results may be correlated with the levels of ENNs and BEA in the feed for dairy cows. Many publications point to the presence of the above analytes in silage. The levels largely depend on the raw materials used for silage production , conditions of the ensiling process, and pH (36).

The research published in 2019 by Panasiuk (23) showed that BEA and ENNs were present in almost all grass and maize silage samples. Beauvericin was the most commonly detected, with a presence in 108 samples (87%) and average and maximum concentrations of 35.8 μg kg^{-1} and 1,309 μg kg^{-1}, respectively. Enniatins were the most prevalent toxins in the investigated silage, identified as ENN A in 66%, ENN A1 in 71%, ENN B in 89% and ENN B1 in 78% of the tested samples. These results for BEA and the four ENNs are similar to those reported by other authors (4, 10, 14, 26, 27, 34), who noted that the most frequently detected toxin was ENN B (51%), at an average concentration of 393 μg kg^{-1}.

The most commonly found emerging mycotoxins reported in the literature were ENN B and BEA. There has been one study to date on the degree of transfer of ENNs and BEA to bovine milk (9), and there is no scientific data on their chronic toxicity. Therefore, if high concentrations of these compounds occurred in feed for ruminants, their possible transfer to milk could not be ruled out.

It is also worth noting that most publications on mycotoxin determination in milk focus on the aflatoxin M1 residue problem (28), because this is a quite well-known hazard. This is due to the proven toxicity of aflatoxins and the consequent introduction of a limit for them in feed and milk. However, in moderate climates these residues do not occur in practice, as indicated by the results of official surveys of feed and milk. Studies show that many other compounds are present in milk, including unregulated mycotoxins which have the potential to affect human health (13, 35, 37).

No international limits for emerging mycotoxins in milk have been established in legislation, and the consequences of long-term exposure to low concentrations of xenobiotics are unknown, so it is necessary to pay special attention to controlling contamination levels and limiting human exposure to these compounds . Existing data on the biological activity of BEA and ENNs indicate the possible toxicity of these compounds and the need for more chronic toxicity studies.

In summary, based on the available data, it is possible for ENNs and BEA to be transferred from feed into milk, but their concentration in food of animal origin is low. This study determined emerging mycotoxins like ENNs and BEA only in trace amounts; nevertheless, such contamination cannot be completely dismissed as a concern because the potential harm of long-term exposure to low levels of toxins is unknown. The ENNS and BEA concentrations in UHT milk are lower than in fresh milk, which is probably because the product comprises a wider mixture of milk batches of different origins. However, this does not affect the overall picture of the levels of these compounds in milk, which, according to current knowledge, is not one illustrating any risk to consumers. Existing data on the biological activity of BEA and ENNs indicate the possible toxicity of these compounds and the need for more chronic toxicity studies. Since no international limits for emerging mycotoxins in food have been established, it is necessary to be more vigilant for the occurrence of ENNs and BEA in food of animal origin.

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Animal Rights Statement: None required.

References

1. Braun D., Ezekiel C.N., Marko D., Warth B.: Exposure to Mycotoxin-Mixtures via Breast Milk: An Ultra-Sensitive LC-MS/MS Biomonitoring Approach. Front Chem 2020, 8, 423, doi: 10.3389/fchem.2020.00423.
2. Braun D., Schernhammer E., Marko D., Warth B.: Longitudinal assessment of mycotoxin co-exposures in exclusively breastfed infants. Environ Int 2020, 142, 105845, doi: 10.1016/j.envint.2020.105845.
3. Commission of the European Communities: Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. OJEC L 2002, 221, 45, 17/08/2002, 1–29.
4. Dagnac T., Latorre A., Fernández Lorenzo B., Llompart M.: Validation and application of a liquid chromatography-tandem mass spectrometry based method for the assessment of the co-occurrence of mycotoxins in maize silages from dairy farms in NW Spain. Food Addit Contam Part A 2016, 33, 1850–1863, doi: 10.1080/19440049.2016.1243806.
5. Directorate-General for Health and Food Safety of the European Commission: SANTE/12089/2016 Guidance document on identification of mycotoxins in food and feed. DG SANTE, Brussels, https://food.ec.europa.eu/system/files/2017-05/cs_contaminants_sampling_guid-doc-ident-mycotoxins.pdf.
6. European Commission: Commission Regulation (EU) No 519/2014 of 16 May 2014 amending regulation (EC) No 401/2006 as regards methods of sampling of large lots, spices and food supplements, performance criteria for T-2, HT-2 toxin and citrinin and screening methods of analysis. OJEU L 2014, 147, 57, 17/05/2014, 29–43.
7. European Food Safety Authority Panel on Contaminants in the Food Chain (CONTAM), Benford D., Ceccatelli S., Cottrill B., DiNovi M., Dogliotti E., Edler L., Farmer P., Fürst P.,

Hoogenboom L., Knutsen H.K., Lundebye A.-K., Metzler M., Nebbia C.S., O'Keeffe M., Rietjens I. (until 2 May 2014), Schrenk D., Silano V., van Loveren H., Vleminckx C., Wester P.: Scientific Opinion on the risks to human and animal health related to the presence of beauvericin and enniatins in food and feed 1. EFSA J 2014, 12, 3802, doi: 10.2903/j.efsa.2014.3802.

8. Fináková S., Proksa B., Šturdíková M.: Biosynthesis and biological activity of enniatins. Pharmazie 2007, 62, 563–568, doi: 10.1691/ph.2007.8.7600.
9. González-Jartín J.M., Rodríguez-Cañás I., Alfonso A., Sainz M.J., Vieytes M.R., Gomes A., Ramos I., Botana L.M.: Multianalyte method for the determination of regulated, emerging and modified mycotoxins in milk: QuEChERS extraction followed by UHPLC–MS/MS analysis. Food Chem 2021, 356, 129647, doi: 10.1016/J.FOODCHEM.2021.129647.
10. Gromadzka K., Blaszczyk L., Chelkowski J., Waskiewicz A.: Occurrence of Mycotoxigenic Fusarium Species and Competitive Fungi on Preharvest Maize Ear Rot in Poland. Toxins 2019, 11, 224, doi: 10.3390/toxins11040224.
11. Gruber-Dorninger C., Novak B., Nagl V., Berthiller F.: Emerging Mycotoxins: Beyond Traditionally Determined Food Contaminants. J Agric Food Chem 2017, 65, 33, 7052–7070, doi: 10.1021/acs.jafc.6b03413.
12. Ivanova L., Fæste C.K., Van Pamel E., Daeseleire E., Callebaut A., Uhlig S.: Presence of Enniatin B and Its Hepatic Metabolites in Plasma and Liver Samples from Broilers and Eggs from Laying Hens. World Mycotoxin J 2014, 7, 167–175, doi: 10.3920/WMJ2013.1609.
13. Izzo L., Rodríguez-Carrasco Y., Tolosa J., Graziani G., Gaspari A., Ritieni A.: Target Analysis and Retrospective Screening of Mycotoxins and Pharmacologically Active Substances in Milk Using an Ultra-High-Performance Liquid Chromatography/High-Resolution Mass Spectrometry Approach. J Dairy Sci 2020, 103, 1250–1260, doi: 10.3168/JDS.2019-17277.
14. Jajić I., Dudaš T., Krstović S., Krska R., Sulyok M., Bagi F., Savić Z., Guljaš D., Stankov A.: Emerging Fusarium Mycotoxins Fusaproliferin, Beauvericin, Enniatins, and Moniliformin in Serbian Maize. Toxins 2019, 11, 357, doi: 10.3390/toxins11060357.
15. Jestoi M.: Emerging *Fusarium*-mycotoxins fusaproliferin, beauvericin, enniatins, and moniliformin–A review. Crit Rev Food Sci Nutr 2008, 48, 21–49, doi: 10.1080/10408390601062021.
16. Jestoi M., Rokka M., Järvenpää E., Peltonen K.: Determination of *Fusarium* mycotoxins beauvericin and enniatins (A, A1, B, B1) in eggs of laying hens using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Food Chem 2009, 115, 1120–1127, doi: 10.1016/j.foodchem.2008.12.105.
17. Jestoi M., Rokka M., Yli-Mattila T., Parikka P., Rizzo A., Peltonen K.: Presence and concentrations of the *Fusarium*-related mycotoxins beauvericin, enniatins and moniliformin in Finnish grain samples. Food Addit Contam 2004, 21, 794–802, doi: 10.1080/02652030410001713906.
18. Křížová L., Dadáková K., Dvořáčková M., Kašparovský T.: Feedborne Mycotoxins Beauvericin and Enniatins and Livestock Animals. Toxins 2021, 13, 32, doi: 10.3390/toxins13010032.
19. Luz C., Saladino F., Luciano F.B., Mañes J., Meca G.: Occurrence, toxicity, bioaccessibility and mitigation strategies of beauvericin, a minor *Fusarium* mycotoxin. Food Chem Toxicol 2017, 107, 430–439, doi: 10.1016/j.fct.2017.07.032.
20. Nácher-Mestre J., Beltrán E., Strachan F., Dick J.R., Pérez-Sánchez J., Berntssen M.H.G., Tocher D.R.: No transfer of the non-regulated mycotoxins, beauvericin and enniatins, from feeds to farmed fish reared on plant-based diets. Food Chem 2020, 323, 126773, doi: 10.1016/j.foodchem.2020.126773.
21. Näykki T., Virtanen A., Leito I.: Software support for the Nordtest method of measurement uncertainty evaluation. Accred Qual Assur 2012, 17, 603–612, doi: 10.1007/s00769-012-0932-0.
22. Ojuri O.T., Ezekiel C.N., Eskola M.K., Šarkanj B., Babalola A.D., Sulyok M., Hajšlová J., Elliott C.T., Krska R.: Mycotoxin co-exposures in infants and young children consuming household- and industrially-processed complementary foods in Nigeria and risk management advice. Food Control 2019, 98, 312–322, doi: 10.1016/j.foodcont.2018.11.049.
23. Panasiuk L., Jedziniak P., Pietruszka K., Piatkowska M., Bocian L.: Frequency and levels of regulated and emerging mycotoxins in silage in Poland. Mycotoxin Res 2019, 35, 17–25, doi: 10.1007/s12550-018-0327-0.
24. Piątkowska M., Sulyok M., Pietruszka K., Panasiuk Ł.: Pilot study for the presence of fungal metabolites in sheep milk from first spring milking. J Vet Res 2018, 62, 167–172, doi: 10.2478/jvetres-2018-0026.
25. Prosperini A., Berrada H., Ruiz M.J., Caloni F., Coccini T., Spicer L.J., Perego M.C., Lafranconi A.: A Review of the Mycotoxin Enniatin B. Front Public Heal 2017, 5, 1–11; doi: 10.3389/fpubh.2017.00304.
26. Rasmussen R.R., Storm I.M.L.D., Rasmussen P.H., Smedsgaard J., Nielsen K.F.: Multi-mycotoxin analysis of maize silage by LC-MS/MS. Anal Bioanal Chem 2010, 397, 765–776, doi: 10.1007/s00216-010-3545-7.
27. Reisinger N., Schürer-Waldheim S., Mayer E., Debevere S., Antonissen G., Sulyok M., Nagl V.: Mycotoxin Occurrence in Maize Silage—A Neglected Risk for Bovine Gut Health? Toxins 2019, 11, 1–18, doi: 10.3390/toxins11100577.
28. Rodríguez-Carrasco Y., Izzo L., Gaspari A., Graziani G., Mañes J., Ritieni A.: Simultaneous Determination of AFB1 and AFM1 in Milk Samples by Ultra High Performance Liquid Chromatography Coupled to Quadrupole Orbitrap Mass Spectrometry. Beverages 2018, 4, 2, 43, doi: 10.3390/BEVERAGES4020043.
29. Rubert J., León N., Sáez C., Martins C.P.B., Godula M., Yusà V., Mañes J., Soriano J.M., Soler C.: Evaluation of mycotoxins and their metabolites in human breast milk using liquid chromatography coupled to high resolution mass spectrometry. Anal Chim Acta 2014, 820, 39–46, doi: 10.1016/j.aca.2014.02.009.
30. Santini A., Meca G., Uhlig S., Ritieni A.: Fusaproliferin, beauvericin and enniatins: occurrence in food – a review. World Mycotoxin J 2012, 5, 71–81, doi: 10.3920/WMJ2011.1331.
31. Serrano A., Capriotti A., Cavaliere C., Piovesana S., Samperi R., Ventura S., Laganà A.: Development of a Rapid LC-MS/MS Method for the Determination of Emerging *Fusarium* mycotoxins Enniatins and Beauvericin in Human Biological Fluids. Toxins 2015, 7, 3554–3571, doi: 10.3390/toxins7093554.
32. Svingen T., Lund Hansen N., Taxvig C., Vinggaard A.M., Jensen U., Have Rasmussen P.: Enniatin B and beauvericin are common in Danish cereals and show high hepatotoxicity on a high-content imaging platform. Environ Toxicol 2017, 32, 1658–1664, doi: 10.1002/tox.22367.
33. Thakur R.A., Smith J.S.: Liquid Chromatography/Thermospray/ Mass Spectrometry Analysis of Beauvericin. J Agric Food Chem 1997, 45, 1234–1239, doi: 10.1021/jf960506u.
34. Tolosa J., Rodríguez-Carrasco Y., Ferrer E., Mañes J.: Identification and Quantification of Enniatins and Beauvericin in Animal Feeds and Their Ingredients by LC-QTRAP/MS/MS. Metabolites 2019, 9, 33, doi: 10.3390/metabo9020033.
35. Urbaniak M., Waśkiewicz A., Stępień Ł.: Fusarium Cyclodepsipeptide Mycotoxins: Chemistry, Biosynthesis, and Occurrence. Toxins 2020, 12, 765, doi: 10.3390/toxins12120765.
36. Wambacq E., Vanhoutte I., Audenaert K., De Gelder L., Haesaert G.: Occurrence, prevention and remediation of toxigenic fungi and mycotoxins in silage: a review. J Sci Food Agric 2016, 96, 2284–2302, doi: 10.1002/jsfa.7565.
37. Wu Q., Patocka J., Nepovimova E., Kuca K.: A Review on the Synthesis and Bioactivity Aspects of Beauvericin, a *Fusarium* Mycotoxin. Front Pharmacol 2018, 9, 1338, doi: 10.3389/fphar.2018.01338.