Chemical composition and occurrence of mycotoxins in traditional meat products from the households of Bosnia and Herzegovina

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SUMMARY

The aim of this study was to investigate chemical composition and mycotoxins occurrence in traditional meat products (TMP) from the households of Bosnia and Herzegovina. During the period 2015-2016, in total 55 traditional meat products were sampled, of which 25 pork sausages, 15 mixed sausages produced from pork and beef, 7 pancetta and 8 samples of prosciutto. Basic chemical and fatty acids composition was determined by use of ISO methods, whereas mycotoxins aflatoxin B_1 (AFB_1) and ochratoxin A (OTA) levels were determined using immunoassay ELISA methods. The results showed variations in chemical parameters among the TMPs with significantly higher ($p < 0.05$) content of stearic acid and lower content of linoleic acid in the mixed meat sausages than in TMPs produced completely of pork. OTA was determined in 7 samples of different products with maximal level of 6.20 µg/kg, whereas AFB_1 was determined in only one pork sausage with concentration slightly higher than the method limit of quantification (1.91 µg/kg). Mycotoxin contamination of TMP, especially by OTA, suggesting that to avoid such contamination, meat and meat products on households should be produced and processed under standardized and well-controlled conditions.

Key words: *chemical composition, aflatoxin B_1, ochratoxin A, traditional meat products, households, Bosnia and Herzegovina*

INTRODUCTION

Many European countries have a long tradition of production of autochthonous meat products and their frequent consumption. These products, traditionally produced by rural households and family farm estates, are mostly made from pork and less as products of different mixed meats. From the nutritional point of view, traditional meat products (TMP) are an important source of proteins of high biological value. However, it is known that such products present some negative health aspects as a consequence of their high animal fat content and because of their possible contamination due to the lack of standardised quality and technology of production under various hygienic conditions of the household production. Most of these products are produced for private use, while only a smaller part of these products are placed on the market under controlled conditions. In the production of TMPs it is necessary to abide by the basic norms of production that affect the properties of the final products and to give importance

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to the quality and safety of used raw materials and additives (Frece et al., 2010).

Since meat and meat products are rich in fat, especially saturated fatty acids, consumers today are advised to consume moderate quantities of these products (Valsta et al., 2005; Fernández et al., 2007), while producers are trying to influence the fatty acids profile in order to get them closer to nutritionally acceptable values (Valencia et al., 2006; Pelser et al., 2007; Pleadin et al., 2014a). It is known show that nutritional composition of dry-cured TMP in terms of fatty acid composition is affected by many factors, from breed selection, feeding and farming of animals to technological processes and parameters implemented during production (Jiménez-Colmenero et al., 2001; Siciliano et al., 2013; Pleadin et al., 2015a). Studies showed that fatty acid composition of meat is on average about 40% of saturated (SFA), 40% of monounsaturated (MUFA) and about 2-25% of polyunsaturated (PUFA) fatty acids, wherein the most common unsaturated fatty acid of all kinds of meat and meat products is oleic acid (C18:1) (Woods and Fearon, 2009; Pleadin et al., 2014a; Pleadin et al., 2015a; Barbir et al., 2014).

Commercially available TMPs produced in households under uncontrolled conditions, represent a potential source of mycotoxins and pose a hazard to human health (Pleadin et al., 2015b). In addition, meat products can also contribute to human mycotoxins' intake as a result of indirect transfer from farm animals exposed to naturally contaminated grains and feed (carry-over effects), or due to direct contamination of the products with moulds or naturally contaminated spice mixtures used in meat production (Pleadin et al., 2013; Perši et al., 2014). It is known that moulds belonging to Penicillium spp. and Aspergillus spp. are often producers of highly toxic aflatoxin B1 (AFB1) and ochratoxin A (OTA), which can be detected in meat products such as ripened sausages or dry-cured hams as the mould's secondary products on the surface of meat products (Dall'Asta et al., 2010; Rodríguez et al., 2012; Markov et al., 2013; Pleadin et al., 2015b). Industrial processing of meat products, such as heating, salting, drying and storage, have no significant effect on the reduction of these mycotoxins in the final meat product (Raters and Matissek, 2008; Amézqueta et al., 2009; Pleadin et al., 2014b).

Given the lack of data of TMP produced in Bosnia and Herzegovina, the aim of the study was to determine basic nutritional parameters and fatty acids composition of four different types of TMP sampled from different households of Bosnia and Herzegovina during two years, and to investigate occurrence of mycotoxins AFB1 and OTA as frequent contaminants of these type of products.

MATERIALS AND METHODS

Sampling and sample preparation

The study included a total of 55 samples of TMPs of which 25 pork sausages, 15 mixed meat sausages (pork and beef), 7 pancetta and 8 prosciutto samples. The meat products were randomly sampled during two years period (2015-2016) from the households located in the southern and southwestern parts of Bosnia and Herzegovina (surroundings of Livno, Tomislavgrad, Kupres, Mostar and Posušje). The products were prepared according to the original recipes of each rural household from the first and second category meat (offal-free) using production technology generally characteristic for these products (Pleadin et al., 2013).

Samples were homogenized for 15 s at 5000 - 6000 rpm on homogenizer Grindomix GM 200 (Retch, Germany) and then stored in a plastic container filled to the top. All steps of samples preparation were performed in accordance with standard method (ISO, 1991). After determination of water content, they were stored at 4 °C until determination of other basic chemical properties, fatty acid composition and mycotoxins.

Reagents and reference materials

ELISA kits used for AFB1 (Aflatoxin B1; Art. No. R1211) and OTA (Ochratoxin A; Art. No. R1311) determinations were provided by R-Biopharm (Darmstadt, Germany). Each kit contains a micro-titre plate with 96 wells coated with AFB1/OTA antibodies, AFB1 methanol/water standard solutions (0, 1, 5, 10, 20, and 50 µg/L) or OTA aqueous standard solutions (0, 50, 100, 300, 900, and 1,800 ng/L), peroxidase-conjugated AFB1/OTA, substrate/chromogen solutions, stop reagents, and dilution and washing buffers.

Hexane and methanol used in the analysis of fatty acids were of HPLC grade (JT Baker Derventer, Netherland), while all the other chemicals were of analytical grade (Kemika, Zagreb, Croatia). Ultra-pure water with electrolytic conductivity of ≤ 0.05 S/cm was obtained using Milipore Direct-Q 3 UV (Merck, Darmstadt, Germany).

Standard solution of fatty acids methyl esters (37 fatty acids), concentration of 10 mg/mL, was prepared by resolving standard SupelcoTM 37 Component FAME Mix (Bellefonte, Pennsylvania, SAD) in hexan. Prepared solution was stored in a freezer at -20 °C and used for identification of fatty acid methyl esters with each analysis. Certified reference material (CRM) with certified values of water content, total protein and fat content, T0149 (FAPAS, England) was used for verification of the analytical methods. In addition, CRM with assigned content of seven individual fatty acids, BCR 163 (Institute for Reference Materials and Measurements, Belgium)

was also used in analyses of fatty acids composition to compare the obtained values with values certified by the manufacturer.

Determination of basic chemical parameters

Water content was determined gravimetrically (ISO, 1997) using universal oven Memmert UF75 plus (Schwabach, Germany) at 103 °C. Ratio of total proteins was determined by the Kjeldahl method (ISO, 1978) with the use of block for digestion Unit 8 Basic (Foss, Sweden) and automatic device for distillation and titration Vapodest 50s Gerhardt (Königswinter, Germany). Ash content was obtained according to standard method (ISO, 1998) by burning the samples at 550 °C in a furnace LV9/11/P320 (Nobertherm, Germany) and total fat by Soxhlet method (ISO, 1973), which involves followed by fat extraction with petroleum ether using the Soxtherm 2000 Automatic device (Gerhardt, Germany) after acid hydrolyses of samples. Carbohydrate content was determined by calculation, based on the determination of water, ash, total protein and fat content. Results of the analysis are expressed as the mean of two parallel determinations, in percentage (%) of weight, with an accuracy of 0.01%.

Determination of fatty acids methyl esters

Methyl esters of fatty acids were prepared from the extracted fat according to EN ISO 12966-2:2011, which includes dissolving glycerides in isooctane and transesterification using metanolic potassium hydroxide solution. Methyl esters were analyzed by gas chromatography (GC) according to standard method (ISO, 2015) using gas chromatographer 7890BA (Agilent Technologies, USA) with the capillary column HP88 of 100 m length, internal capillary diameter 0.25 mm and thickness of stationary phase of 0.20 µm (Agilent Technologies, USA). Applied conditions of gas chromatography were described earlier by Pleadin et al. (2016). Fatty acids methyl esters were identified by comparison with retention times of 37 fatty acids methyl esters of the standard mixture analyzed under the same conditions. Along with samples and standard, CRM was also prepared and analysed in each analysis samples. Results are expressed as a percentage (%) of particular fatty acid in total fatty acids.

Determination of mycotoxins

Sample preparation for determination of AFB1 and OTA for all types of meat products is described in details by Pleadin et al. (2015b). A competitive ELISA test was performed according to the instructions of AFB1 and OTA ELISA kits manufacturer using an auto analyzer ChemWell 2910 (Awareness Technology, Inc., USA). The instrument provides the performance of the ELISA test, construction of calibration curves and calculation of final concentrations, together with statistical data processing. AFB1 and OTA concentrations (µg/kg) were calculated based on calibration curves using mathematical interpolation and multiplication by the sample's dilution factor. Final concentrations were calculated based on the average recovery values obtained for each mycotoxin and the implemented method.

Data analysis

Statistical analysis was performed using computer program SPSS 20.0 (SPSS Inc., USA). Results are expressed as mean±SD. Shapiro Wilks test was conducted to determine whether the results of the analyzed parameters have a normal distribution ($p > 0.05$). To evaluate the difference in values of basic chemical parametrs and fatty acids between the groups of products, one way ANOVA and Kruskal Wallis test were used, with level of significance defined at $p < 0.05$.

RESULTS AND DISCUSSION

The results of analysis of basic chemical composition of different types of TMPs produced on the households of Bosnia and Herzegovina are presented in Table 1.

Water content ranged from 24.25±1.89% in pork sausages to 43.56±2.37% in pancetta. The share of total fat ranged from a minimum value in prosciutto (21.39±2.01%) to the maximum in mixed sausages (36.72±3.14%). The lowest proteins content was in pancetta (20.12±1.55%) and the highest in prosciutto (36.19±1.28%). Basic chemical properties (except for ash and carbohydrate content) mostly show significant differences ($p < 0.05$) in composition of the analysed products pointing to variations in their quality. Such variations can be attributed to differences in the amount of

Table 1 Basic chemical composition of traditional meat products from Bosnia and Herzegovina

Parameter	Mean value ± SD (%)			
	Pork sausages	Mixed sausages*	Pancetta	Prosciutto
Water	24.25±1.89^{c,d}	25.41±2.12^{c,d}	43.56±2.37^{a,b,d}	35.36±2.68^{a,b,c}
Total fat	35.55±3.18^{c,d}	36.72±3.14^{c,d}	29.42±3.18^{a,b,d}	21.39±2.01^{a,b,c}
Total proteins	33.78±3.22^{c}	32.18±3.74^{c}	20.12±1.55^{a,b,d}	36.19±1.28^{c}
Ash	5.37±0.33	5.25±0.27	6.77±1.02	7.01±0.56
Carbohydrate	0.89±0.24	0.45±0.21	< 0.10	< 0.10

** Made of mixed beef and pork*
Significant differences ($p < 0.05$): ^{a}vs. pork sausages; ^{b}vs. mixed sausages; ^{c}vs. pancetta; ^{d}vs. prosciutto

added backfat and choosing more or less fatty meat by individual manufacturers. However, determined values of the chemical composition are characteristic and comparable with the data published earlier for the similar types of TMPs from the other countries of this European part in which in general the similar technological procedures are applied (Pavičić, 2004; Pleadin et al., 2014b).

The main fatty acids present in dry-fermented sausages and dry-cured hams are MUFA (41-59%), SFA (30-45%) and PUFA (9-18%). The most prevalent MUFA are oleic (C18:1n9) (38-42%) and palmitoleic (C16:1n7) (2-3%) fatty acid. The principal SFA are palmitic (C16:0) (23-24%) and stearic (C18:0) (10 -15%) acids (Fernández et al., 2007; Casaburi et al., 2007; Visessanguan et al., 2006; Pleadin et al., 2014a; Pleadin et al., 2015a). The main PUFA component is linoleic acid (C18:2n-6) with shares of up to 6-16%, generally lower in dry-cured hams (7-10%) in comparison to dry-fermented sausages (10-16%) (Moretti et al., 2004; Jurado et al., 2008; Jiménez-Colmenero et al., 2010; Olivares et al., 2011).

The average fatty acid composition determined in TMPs in this study is shown in Table 2.

Table 2 Fatty acid composition of traditional meat products from Bosnia and Herzegovina

Fatty acids	Mass fractions of fatty acids* (%)			
	Pork sausages	Mixed sausages**	Pancetta	Prosciutto
C10:0	0.10±0.01	0.09±0.02	0.08±0.02	0.10±0.02
C12:0	0.09±0.01	0.07±0.01	0.09±0.02	0.08±0.04
C14:0	1.34±0.27	1.50±0.38	1.36±0.19	1.40±0.21
C15:0	n.d.	n.d.	0.09±0.02	n.d.
C16:0	23.12±2.15^{b}	25.15±2.67^{a,c,d}	23.82±1.69^{b}	23.73±1.78^{b}
C17:0	0.29±0.04	0.15±0.08^{c}	0.64±0.10b	0.34±0.06
C18:0	14.15±0.38^{b}	15.23±1.15^{a,c,d}	14.66±2.22^{b}	13.81±1.03^{b}
C20:0	0.47±0.09^{b}	0.08±0.07a	0.25±0.08	0.31±0.07
∑ SFA	**39.56±0.42^{b}**	**42.27±0.63^{a,c,d}**	**40.99±0.54^{b}**	**39.77±0.46^{b}**
C14:1	0.07±0.02	n.d.	0.06±0.01	n.d.
C16:1	2.39±0.37	2.59±0.55	2.21±0.64	2.33±0.88
C18:1n9t	0.42±0.10	0.56±0.38	0.36±0.10	0.35±0.08
C18:1n9c	44.31±4.31^{b}	46.07±3.89^{a,c}	43.71±3.64^{b}	45.40±3.47
C20:1	0.22±0.02	n.d.	0.46±0.12^{d}	0.06±0.02^{c}
C22:1n9	0.24±0.03	n.d.	n.d.	0.19±0.06
∑ MUFA	**47.65±0.81^{b}**	**49.22±1.61^{a,c,d}**	**46.80±0.90^{b}**	**48.33±0.91^{b}**
C18:2n6c	11.27±2.16^{b}	7.17±1.76^{a,c,d}	11.04±2.17^{b}	10.65±1.89^{b}
C18:2n6t	0.08±0.02	0.07±0.03	n.d.	n.d.
C18:3n6	0.25±0.08	0.09±0.04	0.12±0.08	0.19±0.13
C20:2n6	0.10±0.03	0.06±0.02	n.d.	n.d.
∑ PUFA n-6	**11.70±0.57^{b}**	**7.39±0.46^{a,c,d}**	**11.16±1.13^{b}**	**10.84±1.01^{b}**
C18:3n3	1.08±0.32	1.11±0.12	1.02±0.12	1.04±0.10
∑ PUFA n-3	**1.08±0.32**	**1.11±0.12**	**1.02±0.12**	**1.04±0.10**

** Mass fraction of fatty acid is expressed as the total proportion of fatty acids, mean ± SD*
***Made of mixed beef and pork*
n.d. - not detected; limit of detection (LOD) = 0.05%
Significant differences ($p < 0.05$): ^{a}vs. pork sausages; ^{b}vs. mixed sausages; ^{c}vs. pancetta; ^{d}vs. prosciutto

Oleic acid (C18:1n9c) was found in the highest amounts, ranging from 43.71±3.64% in pancetta to 46.07±3.89% in mixed sausages. Palmitic acid (C16:0) was the major saturated fatty acid found, from 23.12±2.15% in pork sausages to 25.15±2.67% in mixed sausages, followed by stearic acid (C18:0) from 13.81±1.03% in prosciutto to 15.23±1.15% in mixed sausages, respectively. Linoleic acid (C18:2n6c) ranged from 7.17±1.76% in mixed sausages to 11.27±2.16% in pork sausages. Araujo de Vizcarrondo et al. (1998) observed that the beef cuts presented a predominant presence of oleic acid (36.21%), palmitic acid (25.67%) and stearic acid (20.97%). The authors determined that oleic and palmitic acids are present in pork meat at 42.83 and 24.15%, respectively, with lower quantities of stearic and higher amounts of linoleic acid in pork than in beef. The mentioned results are in accordance with results of this study, as we also determined a higher content of stearic acid and a lower content of linoleic acid in mixed meat sausages than in TMPs produced completely of pork.

The observed lower ratios of linoleic acid in mixed sausages of pork and beef in comparison to completely pork products are in accordance with earlier observations that pointed to lower content of this fatty acid in beef in comparison to pork (Nieto and Ros, 2012). Namely, in ruminants linoleic acid (C18:2 n–6) and α-linolenic acid

(C18:3 n-3) being degraded in the rumen by microbial biohydrogenation (70–95% and 85-100%, respectively) into MUFA and SFA and only a small proportion, around 10% of dietary consumption, is available for incorporation into tissue lipids. By that reason, beef contain lower content of linoleic acid, compared with pork meat. Beef muscle also contains significant proportions of long chain (C20-22) PUFAs which are formed from C18:2n–6 and C18:3 n–3 by the action of Δ5 and Δ6 desaturase and elongase enzymes. It is known that rumen microorganisms hydrogenate a substantial proportion of PUFA diet, resulting in high levels of SFA for deposition in muscle tissue. Consequently beef meat is characterised by a low relationship between fatty acids PUFA and SFA (ratio P/S), which increases the risk of cardiovascular problems and other diseases (Nieto and Ros, 2012).

Share of fatty acids groups for all products was in the descending order; MUFA > SFA > PUFA. MUFA were the major constituents of all analysed TMPs. A higher MUFA content was determined in mixed sausages (49.22%) in comparison to TMPs made of pork (46.80-48.33%). Obtained MUFA content can be comparable with data on different dry-cured meat products reported in the literature: 42% (Muguerza et al., 2004), 51% (De Campos et al., 2007), 44% (Rubio et al., 2007) and 47% (Del Nobile et al., 2009). Mixed sausages resulted with the highest SFA portion (42.27%), generally higher than that of pork meat products (39.56-40.99%). Similar trends for PUFA and SFA were showed in the mentioned studies, as well as in this study.

Nutritional ratios of n-6/n-3, PUFA/SFA and MUFA/SFA of analysed TMPs are shown in Table 3.

Table 3 Values of nutritional ratios of traditional meat products from Bosnia and Herzegovina

Meat product	Nutritional ratio*		
	n-6/n-3	PUFA/SFA	MUFA/SFA
Pork sausages	10.83^{b}	0.32^{b}	1.20
Mixed sausages**	6.66^{a,c,d}	0.20^{a,c,d}	1.16
Pancetta	10.94^{b}	0.30^{b}	1.14
Prosciutto	10.42^{b}	0.30^{b}	1.22

** The PUFA/SFA values above 0.4-0.5 and of n-6/n-3 below 4 are recommended (Wood et al., 2004; Wood et al., 2008)*
***Made of mixed beef and pork*
Significant differences ($p < 0.05$): ^{a}vs. pork sausages; ^{b}vs. mixed sausages; ^{c}vs. pancetta; ^{d}vs. prosciutto

Higher n-6/n-3 ratio was determined for pork products (10.42-10.94), while the ratio value for the mixed sausages was lower (6.66).These results are in line with Enser et al. (1996), who pointed to higher n-6/n-3 ratio for pork in comparison to beef. Also, PUFA/SFA ratio showed a lower value in pork sausages (0.20) than in pork products (0.30-0.32), as the consequence of lower PUFA n-6 and higher SFA content determined in mixed sausages. Values of MUFA/SFA ratio were similar for all the products, ranging from minimal 1.14 in pancetta to maximal 1.22 determined in prosciutto. MUFA/SFA ratio values were not statistically significantly different (p=0.89) between pork and pork/beef products (mixed sausages).

Regarding the occurrence of mycotoxins as important parameters of food safety, earlier studies performed in European countries have shown that mycotoxin contamination of dry-cured meat products is quite common, and, therefore, it should be monitored (Chiavaro et al., 2002; Pietri et al., 2006; Markov et al., 2013; Pleadin et al., 2015b). While mycotoxins occur in meat primarily as a result of an indirect transfer from naturally contaminated feed, in case of meat products the occurrence is often influenced by the recipe used with their production. Many studies evidenced that the presence of mycotoxins is influenced by the origin of meat and the use of edible tissues, blood and spices that can be contaminated by mycotoxins, as well as by the production and storage conditions particularly for dry-cured meat products which are overgrown by the moulds (Asefa et al., 2011; Pleadin et al., 2013; Perši et al., 2014).

In this study, mycotoxin analyses of TMPs shown that AFB1 was determined in only one sample of pork sausage (1.91 µg/kg), which represents the concentration slightly higher than the analytical method limit of quantification (LOQ = 1.5 µg/kg), whereas it was not detected in any sample of mixed sausages, pancetta and prosciutto. Analyses of OTA shown that three of 25 samples of pork sausages were contaminated with this mycotoxin with concentrations in range from 3.71 to 6.20 µg/kg. Per two samples of pancetta and prosciutto had OTA concentrations in range from 2.62 to 5.84 µg/kg and from 2.11 to 4.47 µg/kg, respectively, whereas in mixed sausages there were no detectable OTA.

Maximal levels (MLs) for AFB1 and total aflatoxins in food are laid down under the Commission Regulation (EC) No 1881/2006 amended by the Commission Regulation (EU) No 165/2010 and are permitted to be not more than 2 and 4 µg/kg, respectively. Although European Union (EU) has the most extensive regulations governing AFB1 presence in various types of food and feed, the definition of the MLs or maximal recommended levels for specific meat products is still not defined. In the Regulation 1881/2006/EC, the Commission of the European Communities defined OTA MLs in different food, allowing thereby no OTA presence in meat and meat products. The level of 1 µg/kg is chosen as maximal recommended OTA level currently defined for pork products by some EU countries (i.e. in Italy) (Rodríguez et al., 2012).

Among the analysed TMPs Rodríguez et al. (2012) observed the highest mean OTA concentration (6.20 µg/kg) in pork sausages, slightly lower in pancetta (5.84 µg/kg) and in prosciutto (4.47 µg/kg), whereas OTA was not

determined only among mixed sausages, which argues in favour of our findings. In the study by Pleadin et al. (2015b), performed on TMPs from Croatian households and markets, the maximal OTA level in the fermented sausages and hams was around 5 times (5.10 µg/kg) to 10 times (9.95 µg/kg) higher than mentioned maximal recommended level (1 µg/kg), whereas AFB1 levels were not significantly higher than the applied method limit of quantification. The level of TMPs contamination observed in this study can be compared with the results of the mentioned studies, both in terms of OTA and AFB1.

Findings of this study could be explained with the fact that during the ripening period surface of different TMPs is typically overgrown with moulds that mainly belong to *Aspergillus* spp. and *Penicillium* spp. (Comi et al., 2004; Dall'Asta et al., 2010; Rodríguez et al., 2012; Markov et al., 2013). Such growth is generally appreciated because of the enzymatic activities that contribute to the development of the flavour specific to these products (Ockerman et al., 2000). On the other hand, any extensive and uncontrolled microbial growth may facilitate development of an off-flavour and cause a direct mycotoxin contamination of the product (Dall'Asta et al., 2010). Literature data indicate that OTA contamination of meat products is frequently the consequence of *Penicillium nordicum* and *Penicillium verrucosum* colonisation (Spotti et al., 2001; Battilani et al., 2007; Iacumin et al., 2009).

Besides a possible direct contamination of the products due to improper manufacturing or/and storage conditions, OTA contamination observed in this study could also be the consequence of the indirect contamination that comes as a result of contaminated feed used during animal fattening or use of contaminated spices in production process. Therefore, in order to avoid mycotoxin contamination, meat and meat products should be produced and processed under standardized and well-controlled conditions with control of each phase of the production and storage chain.

CONCLUSIONS

The results of study showed variations of parameters of chemical composition per type of meat product with higher content of stearic acid and lower content of linoleic acid in mixed meat sausages than in TMPs produced completely of pork. The results also pointed towards to quite common mycotoxin contamination of TMPs, especially that by OTA, pointing that these products should be produced and storage under standardized and well-controlled conditions to prevent possible mycotoxins contamination. Further researches are needed to investigate the source of TMPs mycotoxin contamination and the conditions of their production and storage, as well as to implement measures to reduce such contamination and to prevent hazard to consumers.

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