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Natural occurrence of aflatoxin B_1, ochratoxin A and citrinin in Croatian fermented meat products

Highlights

Game and semi-dry sausages and fermented dry-meat products were analyzed for mould and mycotoxin contamination.

The isolated fungi were of *Aspergillus* and *Penicillium* genera.

All of the analyzed meat products were predominantly OTA-contaminated.

Some of the samples were co-contaminated with AFB_1, OTA and CIT.

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Natural occurrence of aflatoxin B_1, ochratoxin A and citrinin in Croatian fermented meat products

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34 **Abstract**
35 When domestic animals are exposed to mycotoxins, significant amounts of the latter shall be
36 carried over into animal products such as milk, eggs and meat. This study was carried out in
37 order to determine the possible presence of aflatoxin B_1 (AFB_1), ochratoxin A (OTA) and
38 citrinin (CIT) in game sausages (n=15), semi-dry sausages (n=25) and fermented dry-meat
39 products (n=50), randomly taken from individual producers and the Croatian market. AFB_1
40 and OTA were quantified using ELISA, while CIT was quantified using HPLC- fluorescence
41 detector. Out of 90 samples, the fungi most frequently isolated from dry-cured meat products
42 were of *Penicillium* species, while *Aspergillus* was isolated from only one sample. As much
43 as 68.88% of the samples were positive for mycotoxins. Finally, the analysis of different types
44 of meat products resulted in OTA identification in 64.44%, CIT identification in 4.44% and
45 AFB_1 identification in 10% of the samples. The maximum OTA concentrations established in
46 the commercial sausage samples equalled to 7.83 μg/kg, while that of AFB_1 amounted to 3.0
47 μg/kg. Generally, although OTA was detected in all three types of products in different
48 percentage shares, mutual differences were not statistically significant ($P>0.05$).
49

50 *Keywords:* Aflatoxin B_1, Ochratoxin A, Citrinin, Fermented meat products, Croatia
51
52

53 **1. Introduction**
54

55 The presence of mycotoxins in food and feed depends on many biological factors,
56 such as the region of origin, season, humidity and temperature, and the conditions under
57 which the crops are harvested, stored and processed. When not controlled, these toxins can be
58 transferred onto animals and humans through the ingestion of contaminated feed and food
59 (Dashti, Al-Hamli, Alomirah, Al-Zenki, Abbas, & Sawaya, 2009). Given the fact that it has
60 been proven that in cows fed on contaminated feed aflatoxin B_1 gets to be converted into
61 aflatoxin M_1, known as the "milk toxin" and subsequently excreted into the milk of lactating
62 cows, concerns about the entry of mycotoxins into the food chain through meat, eggs, milk
63 and dairy products have been raised (carryover effects) (Allcroft, & Carnaghan, 1963;
64 Kamkar, 2006; Markov, Frece, Čvek, Lovrić, & Delaš, 2010; Iha, Barbosa, & Okada, 2011).
65 Therefore, maximum allowed or at least maximum recommended levels set for mycotoxins in
66 meats and/or other animal products would perhaps be of value.

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67 However, in some European countries regulatory values or recommendations have
68 been established only for aflatoxins and ochratoxin A, and only for some foodstuffs of an
69 animal origin, while for other mycotoxins the risk management has been based on the control
70 of contamination of food of a vegetal origin intended for both human and animal
71 consumption.

72 Long-term research done in Croatia has indicated that mycotoxins frequently
73 contaminate cereals (Pleadin, Sokolović, Perši, Zadravec, Jaki, & Vulić, 2012; Pleadin,
74 Vahčić, Perši, Ševelj, Markov, & Frece, 2013), so that a systematic control of mycotoxins in
75 food and feed is necessary in order to avoid negative health effects, as well as economic
76 losses that might arise in the agricultural sector on the grounds of the aforementioned. In
77 order to assess the risk for animals and consequently also humans, the Croatian Food Agency
78 conducted the study entitled "A study of incidence of mycotoxins in feedstuffs and feed
79 mixtures in Croatia" (HAH, 2012). The obtained results indicate frequent contamination with
80 the analyzed mycotoxins, but, when it comes to aflatoxin B_1, at concentrations much lower
81 than the maximum allowable concentrations defined under the Ordinance on undesirable
82 substances in animal feed (OG 80/2010). Other mycotoxins (zearalenone, fumonisins,
83 deoxynivalenol) were also analyzed; the determined concentrations were below those
84 recommended by the European Commission.

85 Recommendations for continuous monitoring and analysis of raw materials and feed
86 mixtures were issued under specific projects, as well as under the National residua monitoring
87 plan and the National plan of feed inspections and monitoring. The analysis of specific animal
88 feeds should be focused on mycotoxins to which these animals are particularly vulnerable.

89 Among various mycotoxins, aflatoxins are the most commonly documented, while
90 ochratoxin A is an important food-borne contaminant causing nephrotoxicity and has been
91 suspected to play a role in Balkan endemic nephropathy (Mally, Hard, & Dekant, 2007).
92 Ochratoxin A is mainly produced by *Aspergillus ochraceus* (Duarte, Pena, & Lino, 2010)**,**
93 citrinin by *Penicillium citrinum,* while *P. verrucosum* produces both of the toxins (Sweeney &
94 Dobson, 1998). Because *P. verrucosum* is one of the major producers of ochratoxin A in
95 cereals, it is not surprising that both mycotoxins often occur together, although citrinin
96 presence has been reported much less frequently.

97 Although citrinin-producing fungal strains have been isolated from dry-cured meat
98 products suggesting that the presence of citrinin in such products is to be expected, no data on
99 citrinin content in dry-cured meat products can be found across literature. While maximum
100 allowed levels (MRLs) for various mycotoxins have been set for a number of foodstuffs and

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101 feedstuffs, the occurrence of citrinin has insofar failed to be regulated either under these or
102 any other Regulations enforced by the European Union (EU). No MRLs for citrinin in food
103 and feed have been reported by the Food and Agriculture Organization (FAO), as well (FAO,
104 2004). However, given that it co-occurs with ochratoxin A, citrinin might also be controlled
105 indirectly, that is to say, within the legal frame already set for ochratoxin A.

106 Therefore, the aim of this study was to monitor the natural occurrence of moulds and
107 mycotoxins - aflatoxin B_1 (AFB_1), ochratoxin A (OTA) and citrinin (CIT) - in different types
108 of fermented meat products coming from Croatian individual producers and the meat industry.
109 For the sake of AFB_1 and OTA determination, competitive enzyme-linked immunosorbent
110 assay (ELISA) was validated and implemented, whereas the concentration of CIT was
111 determined using high performance liquid chromatography with fluorescence detection
112 (HPLC-FLD).

113

114

115 **2. Materials and Methods**

116

117 **2.1 Samples**

118 The experiment involved 90 samples of fermented meat products, divided into three groups:
119 game sausages (n=15), semi-dry sausages (n=25) and fermented dry-meat products (n=50).
120 Each sample was collected from a different individual producer or Croatian meat industrial
121 facilities. The analyses were performed on samples of game sausages made from rabbit, wild
122 boar, deer, roe deer and mixed (wild boar, deer and domestic pig) meat, as well as on samples
123 of semi-dry sausages which included the grill sausage, the Kranjska sausage, the Slavonian
124 sausage, the Zagorje sausage, the homemade garlic sausage and the samples of domestic and
125 industrial dry-meat products (domestic Slavonian sausage, homemade mixed (pork and beef)
126 sausage, homemade dry Zagorje sausage, winter sausage, Srijemska sausage, Čajna sausage,
127 Kulenova Seka, Panona, Milanska sausage, prosciutto, Buđola sausage).
128 The samples were prepared according to the traditional methods commonly used in Croatia.
129 All samples were cut into small pieces, homogenized in a kitchen blender (Moulinex) and
130 stored at 4 ºC prior to mould and mycotoxin isolation and identification.

131

132 **2.2 Isolation and identification of moulds**

133 Ten grams of each sample were diluted using 90 ml of sterile saline solution (0.7%-NaCl,
134 0.05% Tween 80) and homogenized for 15 min using a laboratory shaker. Decimal dilutions

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135 of the sample were further prepared in accordance with the governmental regulation HRN EN
136 ISO 6887-1: 2004. The presence of moulds was detected in full line with the procedures set
137 under the HRN ISO 13681:2001 standard.
138 Morphologically different mould colonies grown on Sabouraud agar (Biolife, Italy) after a 5-
139 day cultivation at 25 °C, were examined under 100/1.25 magnification using an Olympus
140 microscope and the immersion oil (Samson, Noonim, Meijer, Houbraken, Frisvad, & Varga
141 2007).
142
143 **2.3 Determination of AFB_1 and OTA**
144
145 *Extraction procedures*
146 AFB_1 analyses: To 100 g of the homogenized samples, 10 mL of citric acid solution (20%)
147 was added, mixed thoroughly and then supplemented with 20 g of diatomaceous earth. After
148 the addition of 200 mL of dichlormethane, the sample was stirred and left to be shaken for 30
149 minutes. In the subsequent course, the sample was filtrated, collected into a flask containing
150 10 g of Na_2SO_4 and filtrated again. Two mL of the filtrate were evaporated to dryness and
151 reconstituted in 5 mL of 100%-methanol. In the next step, 25 mL of the phosphate buffered
152 saline (pH 7.4) were added so as to obtain the total sample volume of 30 mL. The latter
153 volume was applied on an immunoaffinity column Aflaprep® R-Biopharm (Darmstadt,
154 Germany). One mL of the filtrate was evaporated, with the residues being subsequently
155 dissolved in 1,440 µL of methanol and 360 µL of the distilled water.
156 OTA analyses: To 1 g of a sample, 0.5 mL of 1 M-H_3PO_4 and 3 mL of ethyl acetate were
157 added and mixed vigorously. After the centrifugation (1 min, 2000 rpm) at the room
158 temperature, the supernatant (ethyl acetate) was transferred and 3 mL of ethyl acetate were
159 added again. After mixing and centrifugation, ethyl acetate layers were combined and 3 mL of
160 0.65 M-$NaHCO_3$ were added, vortexed and continued to be mixed for another 15 min. After
161 the centrifugation (5 min, 2000 rpm), 1 mL of the lower aqueous phase was transferred and
162 heated in a water bath for 3 min at 100 ºC. The content was then shaken and cooled; after
163 cooling, 4 mL of the distilled water were added. Aliquots were diluted using 0.13 M-$NaHCO_3$
164 (1+1 mL).
165
166 *ELISA-based analyses*
167 Competitive ELISA tests were performed using Ridascreen® AFB_1 and OTA ELISA kits
168 provided by R-Biopharm (Darmstadt, Germany). Each kit contains a microtiter plate with 96

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169 wells coated with capture antibodies, six standard AFB_1/OTA solutions, peroxidase-
170 conjugated AFB_1/OTA, the substrate/chromogen (urea peroxide/tetramethylbenzidine), a stop
171 reagent (1 N-sulphuric acid), the dilution buffer and the washing buffer (10 mM-phosphate
172 buffer, pH=7.4). The analyses were conducted as described in the package insert provided by
173 the manufacturer and made use of a ChemWell 2910 auto-analyzer (Awareness Technology,
174 Inc., USA). Each sample was analysed in duplicate. After the completion of the above-
175 described procedures and the addition of 100 µL of the stop reagent, absorbances were
176 measured at 450 nm. Concentrations of both analytes were calculated from six-point
177 calibration curves and corrected for recovery values.
178
179 *Validation of the ELISA assay*
180 OTA and AFB_1 standards used for sample fortification within the frame of the validation
181 process were obtained from Sigma-Aldrich (Steinheim, Germany). Standard solutions
182 employed for both analytes and with both methods were prepared as an aqueous stock and
183 working solution in concentrations of 10,000 ng/mL and 10 ng/mL, respectively, and were
184 stored at +4 ºC until analyses. All other chemicals and solvents were of an analytical and
185 HPLC grade, respectively.
186 For both analytical parameters, the limit of detection (LOD) and the limit of quantification
187 (LOQ) were calculated from the mean value of ten control meat product sample runs plus
188 two- and ten-fold standard deviation, respectively. The recoveries were determined at four
189 different levels (six replicates per concentration level) by virtue of spiking the control samples
190 with the standard working solution correspondent to the assessed content levels. As regards
191 the determination of an intermediate precision, the same steps were repeated on two
192 additional occasions within a month using two different ELISA kit lots, but under the same
193 analytical conditions.
194
195 **2.4 Determination of CIT**
196
197 *Extraction and purification*
198 The procedures used with CIT extraction were based on the proposed Vicam CitriTest HPLC
199 Instruction Manual. Ten g of the ground sample were placed into a blender jar and
200 supplemented with 50 ml of 70%-methanol. The blender jar was covered and mixed at high
201 speed for a minute. The extract was then poured onto a fluted filter paper. The filtrate was
202 collected into a clean vessel. Aliquots of a 1-mL filtered extract were transferred into another

203 clean vessel. The extract was diluted with 49 mL of 10 mM-phosphoric acid and mixed. The
204 diluted extract was then poured into a clean vessel through a microfiber filter.
205 Column chromatography: Ten mL of the extracted sample were passed through the column, 5
206 ml of 10 mM-phosphoric acid was passed through the immunoaffinity columns to washed any
207 remaining impurities and analyte than was eluated with 1 mL of the eluting solution
208 (methanol:10 mM phosphoric acid, 70:30). The eluate was collected into a glass cuvette. The
209 cuvette was vortexed and the sample was injected into a HPLC.
210
211 *HPLC–FLD conditions*
212 The analysis made use of HPLC equipped with a FLD detector, produced by Shimadzu
213 (Tokyo, Japan), with the employed columns being Waters Sunfire c18, Waters, USA (4.6 x 20
214 mm, 2.5µm) and the corresponding guard column. The final solution was analyzed under the
215 following conditions: mobile phase- water : 0.1%-phosphoric acid : acetonitrile (60:40); flow
216 rate 1.0 mL /min; FLD detector's wavelength set at 350 nm-excitation and 500 nm- emission;
217 sample injection volume 50 µL; total running time 5 min.
218
219 *Validation of the applied method*
220 The performance characteristics descriptive of the method in use were established by virtue of
221 single-laboratory validation procedures. To that effect, standard CIT solution (Sigma-Aldrich,
222 Steinheim, Germany), CIT-free sausages and sausages spiked with this mycotoxin were used.
223 The method was evaluated for its linearity, selectivity, trueness, precision, LOD & LOQ and
224 ruggedness. The linearity was studied within 0.002-0.1 mg/L range. The calibration curve was
225 plotted at six levels based on independent replicates. The trueness of the method was
226 investigated based on the mean recoveries obtained for the spiked samples' replicates at each
227 of the studied levels. The precision estimated under repeatability conditions and within the
228 reproducibility range, was expressed as a relative standard deviation descriptive of the spiked
229 samples' replicates at each of the studied levels. LOD and LOQ were established using
230 standard solutions. The selectivity was determined based on the profile of chromatograms
231 obtained for standard CIT solution, CIT-free and CIT-spiked sausage.
232 The ruggedness of the method was tested by virtue of registering small changes witnessed
233 within the extraction times (0.5 min and 5 min).
234
235 **2.5 Statistical analysis**
236

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237 Statistical analysis was carried out using the statistical package STATISTICA 10.0 for
238 Windows (Stat-Soft, Inc, Tulsa, OK, USA) and embraced only data on mycotoxin- positive
239 samples. The effects of game meat and the type of game sausages on OTA concentrations
240 were assessed using two factor-Analysis of Variance (ANOVA). Differences in OTA
241 concentrations across five types of semi-dry sausages and seven types of dry-meat products
242 were checked using Student's t-test. The differences were considered to be significant
243 whenever the P-value was proven to be <0.05.
244
245
246 **3. Results & Discussion**
247
248 Based on the results of the nation-wide survey conducted by the Croatian Food
249 Agency (HAH, 2011) on a representative adult population sample, the consumption of
250 fermented meat products witnessed in Croatia amounts to 52.23 g/day (consumers only).
251 Whereas it has been proven that the presence of moulds and mycotoxins in foodstuffs (meat,
252 eggs, milk and dairy products) is a justified cause for concern, the purpose of this study was to
253 determine the concentration of AFB_1, OTA and CIT in fermented meat products' samples.
254 Keeping in mind that meat products are not the only source of mycotoxins that can be found
255 in a daily diet (but also nuts, spices, dried fruits, wine, coffee), total dietary mycotoxin intake
256 could likely be underestimated.
257 The initial quantitative screening method used to determine the presence of AFB_1 and
258 OTA in the investigated samples was ELISA assay. LOD and LOQ estimated for the method
259 were 0.65 μg/kg and 1.01 μg/kg for AFB_1, and 0.51 μg/kg and 0.89 μg/kg for OTA,
260 respectively. The validation procedure resulted in acceptable mean recoveries of 83.8% for
261 AFB_1 and 86.1% for OTA, as well as in acceptable mean intermediate precision of 82.5% for
262 AFB_1 and 85.5% for OTA; the same goes for the variation coefficients (CV<10%) (Table 1).
263 Given the satisfactory validation results, ELISA was applied for the determination of AFB_1
264 and OTA in the analyzed meat product samples.
265 One of the main reasons for non-existence of CIT-targeted legislation, seen
266 worldwide, is either a lack of suitable analytical methods for its routine determination or CIT
267 instability in foodstuffs (Xu, Wang, Lee, Jia & Sung, 2003; Xu, Jia, Gu & Sung, 2006; EFSA
268 2012). To date, HPLC has been successfully applied for the analysis of CIT in grains, fungal
269 cultures, cheese, feeds, dietary supplement RMR and biological fluids (Wu, Kuo, Lee, Hsu &
270 Pan 2011; EFSA 2012), where LODs as low as 0.1 μg/kg can be achieved. ELISA has been

271 reported to be employed with CIT detection in wheat, barley, maize, RMR, and other grain,
272 with the pertaining LODs of 2 to 15,000 μg/kg (Hartl & Stenzel, 2007; Kononenko & Burkin,
273 2007; Li, Wang, Zheng & Guo, 2010). The results of the validation of methodology employed
274 with CIT presence analyses are shown in Table 2. The estimated LOD was 0.5 μg/kg, while
275 the LOQ equalled to 1.0 μg/kg.

276 The data presented in this study show that mycotoxins were detected in 64.44% of the
277 90 samples under analysis. In all of the contaminated samples (64.44%), OTA was revealed to
278 be the predominant contaminant, while 10% of the samples were contaminated with AFB_1
279 and only 5.55% with CIT in concentrations above the LOQ. Mixed (OTA, CIT & AFB_1)
280 contamination was detected in three samples (3.33%) only (Table 3).

281 Mycotoxin concentrations determined across the three groups of fermented meat
282 products are shown in Tables 4-6.

283 The results obtained with game sausages under study are shown in Tables 3 and 4.
284 OTA was detected in 93.33% of game sausage samples, with its concentration ranging from
285 <0.05 to 3.07 μg/kg. In addition to OTA, CIT and AFB_1 were found in one sample of wild
286 boar sausage in concentrations of 1.0 μg/kg and 1.5 μg/kg, respectively. OTA in the mean
287 concentration of 2.84 μg/kg was found in wild boar sausages, while the samples of rabbit
288 sausages harboured OTA in the mean concentration of 2.27 μg/kg. Mixed sausages made
289 from wild boar, deer and domestic pigs were shown to contain OTA in the mean
290 concentration of 2.16 μg/kg. Deer and roe deer sausages were contaminated with OTA in
291 concentrations below 2.00 μg/kg. ANOVA showed significant differences in OTA
292 concentrations across various types of game sausages ($P<0.05$).

293 In the samples of game sausages, moulds in the outer and inner part were not
294 detected. However, it is not clear whether OTA contamination occurred due to a carryover
295 effect or probably due to an environmental contamination, given that during the winter season
296 wild animals are more exposed to plants which may be contaminated with mycotoxins. Such
297 contamination may also have occurred due to inadequate hygienic conditions in the
298 manufacturing rooms. All samples were homemade. Game, as a representative of wildlife, is
299 considered to be a suitable bio-indicator of environmental pollution. Game has a freedom to
300 choose on what to feed, the feeding thereby being dependent on seasonal availability of
301 certain types of food. Game feeds on a large territory and mainly lives much longer than
302 domestic animals, whose nutrition is uniform and controlled.

303 In Croatia, fermented meat products have traditionally been manufactured in rural
304 households and on family farms, so that many varieties of the same product are present on the

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305 market. Traditional fermented sausages are mostly made from pork, pork and beef and pork
306 back fat, with the addition of salt and specific spice mixture (ground black pepper, minced red
307 pepper, garlic). The mixture is filled into natural swine casings, smoked and ripened at lower
308 temperatures (Kozačinski, Zdolec, Hadžiosmanović, Cvrtila, Filipović & Majić, 2006; Babić
309 et al., 2011).

310 The data presented in Tables 3 and 5 show that, out of 25 samples of semi-dry
311 sausages, even 84% were positive for OTA. AFB_1 was detected in 2 (8%) and CIT in only one
312 (4%) sample. Contamination of semi-dry sausages with all three mycotoxins was not proven.
313 The highest level of OTA was determined in the samples of Kranjska sausage (3.28 μg/kg),
314 while the mould of *Penicillium* genus and OTA in the highest mean concentration of 2.15
315 μg/kg were detected in the Slavonian sausage. In semi-dry sausages, mean concentrations of
316 OTA ranged from 0.79 to 2.15 μg/kg.

317 Out of 5 types of semi-dry sausages under analysis, only Kranjska sausage, homemade
318 garlic sausage and the Slavonian sausage did not statistically significantly differ in their mean
319 OTA concentrations ($P>0.05$).

320 In Europe and Croatia, moulds of the *Penicillium* genus are used as typical starter cultures for
321 dry sausage production. These species are surface-inoculated because they are thought to
322 improve sausage aroma, texture and appearance. Whenever a mould growth is considered
323 desirable, the manufacturing process must run at the strict temperature and under the
324 controlled humidity. Moulds in fermented meat products may not only affect their
325 organoleptic properties, but also produce mycotoxins and pose as a potential health hazard to
326 consumers. Therefore, the exposure of human consumers may also be the result of mycotoxin
327 synthesis during the product ripening stage. Indeed, several studies have shown that mould
328 species belonging to *Penicillium* and *Aspergillus* genera can be isolated from meat products
329 such as ripened sausages or dry-cured ham (Rodríguez, Rodríguez, Martín, Nuñez &
330 Córdoba, 2012a; Iacumin et al., 2009; Tabuc, Bailly, Bailly, Querin, & Guerre, 2004; Asefa et
331 al., 2011). This specific mycoflora enables the attainment of the desirable aroma and flavour
332 of the product, but is usually complex and composed of many fungal species, out of which
333 several may be toxigenic, at least *in vitro*. Therefore, contamination with a toxigenic strain
334 may lead to mycotoxin synthesis and its accumulation in the final product (Bailly, Tabuc,
335 Querin, & Guerre, 2005). In this study, moulds were found in only 4 (4.44%) samples of
336 semi-dry sausages and dry-meat products (Table 3). The reason behind the low number of
337 mould-contaminated samples could also be the fact that industrially-made sausages were
338 unanimously vacuumed and randomly picked from local shops. In addition, bearing in mind

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339 that domestic sausage-making procedures are not standardised and are uncontrolled, it is
340 reasonable to assume that moulds possibly grown on a sausage casing surface could easily be
341 washed off while rinsing the sausage prior to its use.

342 The data presented in Table 3 show that mould contamination was found in an outer
343 layer of one sample of the winter salami and Prosciutto. The number of Croatian meat plants
344 inoculating mould spores on sausage surfaces within the frame of the winter salami
345 production boils down to one, while other meat plants do not resort to this method. Also,
346 contamination was found in an inner part of one sample of mixed (pork and beef) homemade
347 sausage and Slavonian sausage. Although moulds were isolated, mycotoxin concentrations
348 found in these samples were below the LOQ, which is in agreement with the research output
349 of many authors, who have stated that growth of moulds does not necessarily indicate the
350 presence of a corresponding mycotoxin (Mateo, Gil-Serna, Patino, & Jiménez, 2011;
351 Rodríguez, Rodríguez, Luque, Martín, & Córdoba, 2012b). In 27 samples of dry-meat
352 products, no mycotoxin contamination was found. OTA was detected in 54% samples of dry-
353 meat products, in the mean concentration range of 0.84 to 3.51 μg/kg. OTA was found in both
354 homemade and commercial samples, although at different levels. The maximum OTA
355 concentration in winter salami was 7.83 μg/kg, while in prosciutto it amounted to 1.03 μg/kg
356 (Table 6). The mean concentration of OTA in the winter salami was significantly different
357 from that found in all other types of dry-meat products ($P<0.05$).

358 These data are in agreement with those recently reported by Dall'Asta et al. (2010),
359 who investigated the occurrence of OTA in dry sausages from northern Italy. CIT was
360 detected in salami (n=3) purchased only from the market, while AFB_1 was detected in 2
361 homemade and 4 commercial samples. Two out of seven winter salami samples contained
362 CIT at concentrations of 1.0 and 1.3 μg/kg, respectively. Whenever CIT was found in a
363 sample, it always co-occurred with OTA. In two dry sausages (winter salami and Čajna
364 salami) CIT was found in combination with OTA and AFB_1 (Table 3). The highest AFB_1
365 concentrations of 2.7 and 3.0 μg/kg were found in the samples of winter and Čajna salami,
366 respectively. The fact that commercial sausage samples (winter and Čajna salami) contained
367 the highest mycotoxin concentrations, underlines the importance of hygienic environmental
368 control in the ripening plants.

369 The results obtained in a total of 90 samples showed the risk of AFB_1 and CIT
370 contamination of fermented meat products to be minimal, generally due to the low rate of
371 carryover of the examined mycotoxins to the edible tissues, given that the primary target of
372 AFB_1 is the liver, while that of CIT is the kidney. In muscles, only low levels of AFB_1 and

373 CIT can be found, often below the detection limits of the methods used (Beaver et al., 1990;
374 Bintvihok, Thiengnin, Doi, & Kumagai, 2002; Abramson, Mills, Marquardt, & Frohlich,
375 1997). This can be explained by a highly intense AFB_1 liver metabolism and weak CIT
376 absorption after an oral administration, combined with its quick elimination through urine and
377 faeces (Hirano, Adachi, Bintvihok, Ishibashi, & Kumazawa, 1992; Stubblefield, Honstead, &
378 Shotwell, 1991; Phillips, Berndt, & Hayes, 1979). Moreover, the use of spices contaminated
379 with toxigenic mould strains may also represent a source of secondary mycotoxin
380 contamination of the final product (Refai, Niazi, Aziz, & Khafaga, 2003). The data presented
381 in this study show OTA to be the dominant contaminating mycotoxin. Although OTA was
382 detected in all three types of products in different percentage shares, the differences were not
383 statistically significant (P>0.05).

384 Among farmed animals, pigs are known to be particularly sensitive to OTA
385 accumulation, with OTA residues resting in several edible organs (predominantly kidneys, but
386 also liver, muscle and fat) (Gareis & Wolf, 2000; Lusky, Tesch, & Gobel, 1993; Pietri,
387 Bertuzzi, Gualla, & Piva, 2006). Therefore, the consumption of meat contaminated with OTA
388 has also been suspected to represent a source of exposure for humans (JECFA, 2001). Neither
389 the European Commission nor Croatia has set the maximum allowed and the maximum
390 recommended levels for mycotoxins in meat or other animal products. However, some
391 countries have enforced OTA MRLs, for example Denmark (of 10 μg/kg in pig kidney),
392 Estonia (of 10 μg/kg in pig liver), Romania (of 5 μg/kg in pig kidney, liver and meat) and
393 Slovakia (of 5 μg/kg in meat and milk). Other countries have developed guidelines for
394 recommended maximum OTA levels, for example Italy (pig meat and derived products, 1
395 μg/kg) (Duarte et al., 2010).

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398 **4. Conclusions**

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400 Although EFSA (EFSA-Q-2003-039, 2004) has outlined that the real risk of mycotoxin
401 contamination of meat is negligible, the results obtained within the frame of this study show
402 that, even though 96% of the samples were mould-free, the samples of all three types of meat
403 products contain mycotoxins. This may be attributed to contamination arising on the grounds
404 of carryover effect or the addition of spices contaminated with mycotoxins. If there is a risk of
405 mycotoxin residua presence in animal products such as meat, eggs, milk and milk products,
406 the situation in the field should be constantly monitored so as to protect public health. We

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407 should always keep in mind that mycotoxins can be a problem, particularly since there is no
408 sensory mycotoxin contamination warning. Therefore, further research on mycotoxin
409 occurrence in meat and meat products is necessary.

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412 **5. References**

413

414 Abramson, D., Mills, J. T., Marquardt, R. R., & Frohlich, A. A. (1997). Mycotoxins in fungal
415 contaminated samples of animal feed from western Canada. *Canadian Journal of Veterinary*
416 *Research*, 61, 49–52.

417

418 Allcroft, R., & Carnaghan, R. B. A. (1963). Groundnut toxicity: An examination for toxin in
419 human food products from animals fed toxic groundnut meal. *Veterinary Research, 75,* 259–
420 263.

421

422 Asefa, D. T., Kure, C. F., Gjerde, R. O., Langsrud, S., Omer, M. K., Nesbakken, T., et al.
423 (2011). A HACCP plan for mycotoxigenic hazards associated with dry-cured meat production
424 processes. *Food Control, 22*, 831-837.

425

426 Babić, I., Markov, K., Kovačević, D., Trontel, A., Slavica, A., Đugum, .J, Čvek, D., Svetec,
427 I.K., Posavec, S. & Frece, J. (2011). Identification and characterization of potential
428 autochthonous starter cultures from a Croatian "brand" product "slavonski kulen". *Meat*
429 *Science, 88,* 517-524.

430

431 Bailly, J. D., Tabuc, C., Querin, A., & Guerre, P. (2005). Production and stability of patulin,
432 ochratoxin A, citrinin and cyclopiazonic acid on dry cured ham. *Journal of Food*
433 *Protection,68*, 1516–1520.

434

435 Beaver, R. W., Wilson, D. M., James, M. A., Haydon, K. D., Colvin, B. M., Sangster, L. T.,
436 Pikul, A. H., & Groopman, J. D. (1990). Distribution of aflatoxins in tissues of growing pigs
437 fed an aflatoxin contaminated diet amended with a high affinity aluminosilicate sorbent.
438 *Veterinary and Human Toxicology*, *32(1),* 16–18.

439

ACCEPTED MANUSCRIPT

440 Bintvihok, A., Thiengnin, S., Doi, K., & Kumagai, S. (2002). Residues of aflatoxins in the
441 liver, muscle and eggs of domestic fowls. *Journal of Veterinary Medical Science, 64,*1037–
442 1039.
443
444 Dall'Asta, C., Galaverna, G., Bertuzzi, T., Moseriti, A., Pietri A., Dossena, A., & Marchelli
445 R. (2010). Occurrence of ochratoxin A in raw ham muscle, salami and dry-cured ham from
446 pigs fed with contaminated diet. *Food Chemistry, 120*, 978–983.
447
448 Dashti, B, Al-Hamli, S., Alomirah, H., Al-Zenki, S., Abbas, A.B., & Sawaya, W. (2009).
449 Levels of aflatoxin M1 in milk, cheese consumed in Kuwait and occurrence of total aflatoxin
450 in local and imported animal feed. *Food Control, 20,* 686-690.
451
452 Duarte, S.C., Pena, A., & Lino, C.M. (2010). Ochratoxin A in Portugal: A Review to Assess
453 Human Exposure. *Toxins, 2(6),* 1225–1249.
454
455 EFSA-Q-2003-039, R. N. (2004). Opinion of the scientific panel on contaminants in food
456 chain on a request from the commission related to ochratoxin A (OTA) as undesirable
457 substance in animal feed. In EFSA (European Food Safety Authority). *The EFSA Journal,*
458 *101,* 1–36.
459
460 EFSA (European Food Safety Authority), (2012). Scientific Opinion on the risks for public
461 and animal health related to the presence of citrinin in food and feed. *The EFSA Journal*
462 *10(3)*, 260, 11pp
463
464 FAO (Food and Agriculture Organization), 2004. Worldwide regulations for mycotoxins in food and
465 feed in 2003. FAO Food and Nutrition paper 81. Food and Agriculture organization of the United
466 Nations, Rome, Italy.
467
468 Gareis, M., & Wolff, J. (2000). Relevance of mycotoxin contaminated feed for farming
469 animals and carry over of mycotoxins in food of animal origin. *Mycoses, 43*, 79-83.
470
471 HAH (Croatian Food Agency), (2011) National Food Consumption survey, unpublished data
472
473 HAH (Croatian Food Agency), (2012) Mycotoxins in Feed. *Scientific opinion, adopted by*
474 *HAH Scientific Committee 23 October 2012 http://www.hah.hr/english/panels.php*

ACCEPTED MANUSCRIPT

475

476 Hartl, A., & Stenzel, W.R. (2007). Development of a method for the determination of citrinin
477 in barley, rye and wheat by solid phase extraction on aminopropyl columns and HPLC-FLD.
478 *Mycotoxin Research, 23,* 127-131.
479
480 Hirano, K., Adachi, Y., Bintvihok, A., Ishibashi, S., & Kumazawa, N. H. (1992). An
481 improved method for extraction and cleanup of aflatoxin B1 from liver. *Journal of Veterinary*
482 *Medicine and Science, 54,* 567.
483
484 HRN ISO 13681:2001 Meat and meat products -- Enumeration of yeasts and moulds --
485 Colony-count technique (ISO 13681:1995)
486
487 HRN ISO 6887-1: 2004 Microbiology of food and animal feeding stuffs -- Preparation of test
488 samples, initial suspension and decimal dilutions for microbiological examination -- Part 1:
489 General rules for the preparation of the initial suspension and decimal dilutions (ISO 6887-
490 1:1999; EN ISO 6887-1:1999)
491
492 Iacumin, L., Chiesa, L., Boscolo, D., Manzano, M., Cantoni, C., Orlic, S., et al. (2009).
493 Moulds and ochratoxin A on surfaces of artisanal and industrial dry sausages. *Food*
494 *Microbiology, 26(1),* 65–70.
495
496 Iha, M.H., Barbosa, C.B., & Okada, I.A. (2011). Occurrence of aflatoxin M1 in dairy products
497 in Brazil. *Food Control, 22 (129)*, 1971-1974.
498
499 JECFA. (2001). Safety evaluation of certain mycotoxins in food. Fifty-six report. WHO
500 Technical Report Series, Geneva, 47.
501
502 Kamkar, A. (2006): A study of the occurrence of aflatoxin M1 in Iranian Feta cheese. *Food*
503 *Control 17*, 768-775.
504
505 Kononenko. G.P., & Burkin. A.A. (2007). Immunoenzyme method for the determination of
506 citrinin. *Journal of Analytical Chemistry, 62,* 691-696.
507

ACCEPTED MANUSCRIPT

508 Kozačinski, L., Zdolec, N., Hadžiosmanović, M., Cvrtila, Ž., Filipović, I., & Majić, T. (2006).
509 Microbial flora of the Croatian traditionally fermented sausage. *Archiv für*
510 *Lebensmittelhygiene, 57 (9/10),* 141-147.
511
512 Li Y-N., Wang Y-Y., Zheng Y-Q., & Guo Y-H. (2010). Preparation and Characterization of
513 The HighSpecificity Monoclonal Antibodies Against Citrinin. *Progress in Biochemistry and*
514 *Biophysics, 37,* 1248-1253.
515
516 Lusky, K., Tesch, D., & Gobel, R. (1993). Influence of the mycotoxin ochratoxin A on animal
517 health and formation of residues in pigs and different types of sausages derived from these
518 animals. *Archiv Fur Lebensmittelhygiene, 44*, 131-134.
519
520 Mally, A., Hard, G.C., & Dekant, W. (2007). Ochratoxin A as a potential etiologic factor in
521 endemic nephropathy: Lessons from toxicity studies in rats. *Food and Chemical Toxicology,*
522 *45 (11)*, 2254-2260.
523
524 Markov, K., Frece, J., Čvek, D., Lovrić, N., & Delaš, F. (2010). Aflatoxin M1 in raw milk and
525 binding of aflatoxin by lactic acid bacteria. *Mljekarstvo, 60(4),* 244 – 251.
526
527 Mateo, E.M., Gil-Serna, J., Patino, B., & Jiménez, M. (2011). Aflatoxins and ochratoxin A in
528 stored barley grain in Spain and impact of PCR-based strategies to assess the occurrence of
529 aflatoxigenic and ochratoxigenic Aspergillus spp. *International Journal of Food*
530 *Microbiology, 149(2),* 118-26.
531
532 Ordinance on undesirable substances in animal feed (2010). Pravilnik o nepoželjnim tvarima
533 u hrani za životinje N.N. 80/2010.
534
535 Phillips, R. D., Berndt, W. O., & Hayes, A. W. (1979). Distribution and excretion of (14C)
536 citrinin in rats. *Toxicology, 12,* 285–298.
537
538 Pietri, A., Bertuzzi, T., Gualla, A., & Piva, G. (2006). Occurrence of ochratoxin a in raw ham
539 muscles and in pork products from Northern Italy. *Italian Journal of Food Science, 18(1),* 99–
540 106.
541

ACCEPTED MANUSCRIPT

542 Pleadin, J., Sokolović, M., Perši, N., Zadravec, M., Jaki, V., & Vulić, A. (2012).
543 Contamination of maize with deoxynivalenol and zearalenone in Croatia. *Food control*, *28*
544 *(1)*, 94-98.
545
546 Pleadin, J., Vahčić, N., Perši, N., Ševelj, D., Markov K., & Frece, J. (2013). Fusarium
547 mycotoxins' occurrence in cereals harvested from Croatian fields. *Food control*, *32, (1)*, 49-
548 54.
549
550 Refai, M.K., Niazi, Z.M., Aziz, N.H., **&** Khafaga, N.E. (2003). Incidence of aflatoxin B1 in
551 the Egyptian cured meat basterma and control by gamma-irradiation. *Nahrung, 47(6),* 377-
552 382.
553
554 Rodríguez, A., Rodríguez, M.,Martín, A.,Nuñez, F., & Córdoba, J. J. (2012a). Evaluation of
555 hazard of aflatoxin B1, ochratoxin A and patulin production in dry-cured ham and early
556 detection of producing moulds by qPCR. *Food Control, 27*, 118-126.
557
558 Rodríguez, A., Rodríguez, M., Luque, M.I., Martín, A, & Córdoba, J.J. (2012b). Real-time
559 PCR assays for detection and quantification of aflatoxin-producing molds in foods. *Food*
560 *Microbiology, 31(1),* 89-99.
561
562 Samson, R.A., Noonim, P., Meijer, M., Houbraken, J., Frisvad, J.C., & Varga, J. (2007).
563 Diagnostic tools to identify black aspergilla. *Studies in Mycology, 59,* 129–145.
564
565 Stubblefield, R. D., Honstead, J. P., & Shotwell, O. L. (1991). An analytical survey
566 ofaflatoxins in tissues from swine grown in regions reporting 1988 aflatoxin contaminated
567 corn. *Journal of the Association of Official Analytical Chemists, 74,* 897.
568
569 Sweeney, M.J., **&** Dobson, A.D. (1998). Mycotoxin production by Aspergillus, Fusarium and
570 Penicillium species. *International Journal of Food Microbiology, 43(3),* 141-158.
571
572 Tabuc, C., Bailly, J. D., Bailly, S., Querin, A., & Guerre, P. (2004). Toxigenic potential of
573 fungal mycoflora isolated from dry cured meat products: preliminary study. *Revue de*
574 *Médecine Vétérinaire, 156*, 287-291.
575

576 Wu, C.L., Kuo, Y.H., Lee, C.L., Hsu, Y.W., & Pan T,M. (2011). Synchronous high-
577 performance liquid chromatography with a photodiode array detector and mass spectrometry
578 for the determination of citrinin, monascin, ankaflavin, and the lactone and acid forms of
579 monacolin K in red mold rice. *Journal of the Association of Official Analytical Chemists*
580 *International, 94,* 179-190.
581

582 Xu, B.J., Wang, Q.J., Lee, JH, Jia, X.Q., & Sung, C.K. (2003). HPLC analysis of citrinin in
583 red yeast rice. *Food Science and Biotechnology, 12,* 376-380.
584

585 Xu, B.J., Jia, X.Q., Gu, L.J., & Sung, C.K. (2006). Review on the qualitative and
586 quantitative analysis mycotoxin citrinin. *Food Control, 17,* 271-285.

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Table 1: The results of the validation of methodology applied with OTA and AFB_1 detection

Analyte	LOD	LOQ	Spiked concentration (µg/kg)	Recovery (%)	CV (%)	Intermediate precision (%)	CV (%)
AFB_1	0.65	1.01	1.5	78.7	4.5	76.8	6.3
			2.0	82.6	5.6	80.3	7.2
			2.5	85.3	6.1	82.6	8.4
			3.0	88.7	7.2	90.4	9.5
OTA	0.51	0.89	1.0	80.6	3.9	81.3	5.1
			1.5	84.6	4.5	82.5	7.7
			2.0	87.9	5.9	85.7	9.1
			2.5	91.4	6.7	92.5	9.8

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Table 2: The results of the validation of methodology applied with CIT detection

Parameter		Obtained result	
Linearity			k=0.9998
Trueness			93.5%
Precision		RSD	9.39%
LOD			0.5 µg/kg
LOQ			1 µg/kg
Selectivity			satisfactory
Rugedness (extraction time)	0.5 min	RSD	20.5%
	5 min		19.8%

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Table 3: The occurrence of moulds, OTA, CIT and AFB_1 in fermented meat product

	Game sausages	Semi-dry sausages	Dry meat products	Total
No. of samples	15	25	50	90
Positive (%)	93.33	84	54	64.44
Positive / total				
OTA	14/15	21/25	23/50	58/90
CIT	1/15	1/25	3/50	3/50
AFB_1	1/15	2/25	6/50	6/50
OTA + CIT	1/15	1/25	3/50	3/50
OTA+CIT+AFB_1	1/15	0/25	2/50	2/50
Moulds	nd	1/25[a]	3/50[b,c,d]	4/90

nd – not detected

[a] Slavonian sausage – *Penicillium* sp. was isolated

[b] Winter salami - *Penicillium* sp. was isolated

[c] Prosciutto - *Penicillium* sp. was isolated

[d] Homemade mixed sausage (pork and beef) - *Penicillium* sp. and *Aspergillus* sp. were isolated

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Table 4: Concentrations of OTA, CIT and AFB_1 in game sausages

Samples of sausages	No. of samples	Range of value (µg/kg)		
		OTA	CIT	AFB_1
Rabbit	3	2.21 – 2.37	<1.0	<1.0
Wild boar	3	2.70 – 3.07	<1.0 - 1.0	<1.0 – 1.5
Deer	3	1.86 – 2.03	<1.0	<1.0
Roe deer	3	<0.05 – 1.37	<1.0	<1.0
Mixed sausage (wild boar, deer and pork)	3	1.55 – 2.71	<1.0	<1.0

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Table 5: Concentrations of OTA, CIT and AFB_1 in semi-dry sausages

Samples of sausages	No. of samples	Range of value (µg/kg)		
		OTA	CIT	AFB_1
Grill sausage	7	1.51 – 1.86	<1.0	<1.0 - 1.1
Kranjska sausage	4	<0.05 – 3.28	<1.0	<1.0
Slavonian sausage	4	2.03 – 2.31	<1.0	<1.0
Zagorska sausage	5	1.23 – 1.62	<1.0	<1.0 – 1.0
Homemade garlic sausage	5	<0.05 – 2.12	<1.0	<1.0

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Table 6: Concentrations of OTA, CIT and AFB_1 in dry-meat products

Samples of sausages	No. of samples	Range of value (µg/kg)		
		OTA	**CIT**	**AFB_1**
Domestic slavonian sausage	3	2.03 – 2.31	<1.0	<1.0 - 1.2
Homemade mixed sausage (pork and beef)	5	<0.05 – 4.05	<1.0	<1.0
Homemade dry Zagorje sausage	5	1.53 – 2.83	<1.0	<1.0 – 1.1
Winter salami	7.	<0.05 – 7.83	<1.0 – 1.3	<1.0 – 2.7
Srijemska salami	6	<0.05 – 3.28	<1.0	<1.0
Čajna salami	5	<0.05 – 4.71	<1.0 – 1.0	<1.0 – 3.0
kulenova seka	5	<0.05 – 1.68	<1.0	<1.0
Panona	3	<0.05	<1.0	<1.0
Milanska	3	<0.05	<1.0	<1.0
Prosciutto	3.	<0.05 – 1.49	<1.0	<1.0 – 1.7
Buđola	5	<0.05	<1.0	<1.0

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