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Ochratoxin A contamination of the autochthonous dry-cured meat product "Slavonski Kulen" during a six-month production process

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ABSTRACT

The aim of the study was to investigate OTA contamination of "Slavonski Kulen" during a six-month production process. Raw meat & spices ($n = 7$) used with the production and final products with either intact or damaged casings ($n = 99$) were sampled on the production day 30, 60, 90, 120, 150 and 180, and analysed for OTA presence using an enzyme-linked immunosorbent assay (ELISA) and high performance liquid chromatography with fluorescence detection (HPLC-FLD). No statistically significant differences ($p > 0.05$) were observed among the intact samples on any above-given production day, the maximal OTA value attributable to raw material contamination thereby being 3.18 μg/kg. As for the damaged samples, the mean OTA concentration established on the production day 180 was 7.92 ± 6.50 μg/kg, while the maximal observed OTA value equalled to 17.0 μg/kg. The results demonstrated that casing damaging witnessed during a long-term dry-cured meat products' ripening can result in OTA entry from the surface into the product, causing a significant OTA contamination.

1. Introduction

Ochratoxin A (OTA) is a secondary fungal metabolite produced by various *Aspergillus* and *Penicillium* species, primarily *Aspergillus ochraceus* and *Penicillium verrucosum*, and can often be found in various foodstuffs. Due to its well-evidenced carcinogenic, nephrotoxic, teratogenic and other toxic effects on different organisms (Creppy, 1999; Pfohl-Leszkowicz & Manderville, 2007), OTA has been classified by the International Agency for Research on Cancer into the 2B Group of possible human carcinogens (IARC, 1993), thus representing an issue of concern for human health. Among farmed animals, pigs are known to be particularly sensitive to OTA, with their exposure resulting in the accumulation of this toxin in animal tissues and subsequently also in final meat products owing to an indirect transmission from animals fed on contaminated feed (Curtui, Gareis, Usleber, & Martlbauer, 2001; Gareis & Scheuer, 2000; Malagutti, Zannotti, Scampini, & Sciaraffia, 2005; Perši, Pleadin, Kovačević, Scortichini, & Milone, 2014; Pleadin et al., 2013).

Many studies have reported that, on top of indirect contamination (*carryover* effect), OTA may enter dry-cured meat products through another pathway, that is to say, from the product surface overgrown with OTA-producing moulds during the ripening process (Asefa et al., 2011; Battilani, Formeti, Toscani, & Virgili, 2010; Dall'Asta et al., 2010; Pleadin et al., 2015). Environmental conditions needed for mycotoxin production are generally more restrictive than those necessary for mould growth. Rodríguez, Rodríguez, Martín, Nuñez, and Córdoba (2012) pointed out that the analysis of mycotoxin production dynamics may be of great benefit for an early detection of toxigenic moulds before any toxins are actually produced.

As oppose to some EU countries and despite of the State Programme declared by the Ministry of Agriculture that stipulates the detection of OTA residues in offal (kidney and liver) sampled from pigs delivered to slaughterhouses, Croatia has insofar failed to define the maximal residue limit (MRL) or to propose the guideline OTA value for meat and final meat products. The maximal recommended levels of OTA in products intended for animal feeding are laid down under the Commission Recommendation 2006/576/EC (EC, 2006), whereas the EU Commission Regulation (No 105/2010) brings the opinion that the establishment of maximal OTA levels in meat products, edible offal and blood-based products does not appear to be necessary for the public health protection (Pleadin et al., 2013).

Dry-cured meat products traditionally produced in Croatia, in particular Croatian traditional fermented pork sausages, are best

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represented by “Slavonski Kulen”. This autochthonous meat product is produced according to traditional procedures followed by many Croatian households and represents a mixture of the highest-quality (offal-free) minced pork cleaned of connective tissue, damaged parts, blood vessels and hard pork fatback, into which salt and spices such as red paprika (hot and sweet) and garlic are added. The minced meat is then stuffed into a pig's appendix (*intestinum caecum*) (Babić et al., 2011; Kovačević, 2001; Kovačević, Mastanjević, Šubarić, Jerković, & Marijanović, 2010). After equalizing the stuffing (conditioning) temperature, the sausages are smoked, fermented, dried and left to months-long ripening. This high-quality meat product is characterized by specific organoleptic properties (taste and odour), which come as a result of technological processes (drying and smoking), as well as a result of a long ripening process (Jerković et al., 2010; Kovačević, 2014; Pleadin et al., 2015).

OTA is a mycotoxin of particular concern when it comes to dry-cured meat products, since the appropriate temperature and moisture surrounding the storage of such products may facilitate OTA production (Amézqueta, Peñas, Arbizu, & DeCertain, 2009). In “Slavonski Kulen”, OTA may also be present due to the natural contamination of feed the pigs providing raw meat are fed on (Perši et al., 2014; Pleadin et al., 2013) or due to the activity of certain moulds of *Aspergillus* and *Penicillium* genera, which may spontaneously grow on the surface of this product during its long ripening period (Frece, Markov, & Kovačević, 2010).

Data have revealed that OTA represents a moderately stable molecule resistant to high temperature, able to survive most of the food processing procedures (at least to some extent), so that it may likely be present in final meat products and prove itself difficult to remove (Moss, 1996). Food processing may involve boiling, baking, frying, roasting, fermentation and so forth, the degree to which OTA is destroyed along their line thereby depending on parameters such as pH, temperature and the ingredients present; within this frame, food processing procedures utilizing the highest temperatures have been proven as the most efficient (Pleadin et al., 2014). Studies have shown that some technological processes are capable of reducing OTA levels, but not of eliminating the toxin completely (O'Brien & Dietrich, 2005; Bullerman & Bianchini, 2007; Pleadin et al., 2014). When it comes to the contamination with toxigenic moulds and the production and accumulation of secondary toxic metabolites, the critical points are water activity ($a_w < 0.9$), cracks developed on product surfaces and the surrounding temperature ($T < 20$ °C) (Asefa et al., 2011).

The aim of this study was to investigate into the possibility of OTA contamination of the traditional Croatian fermented sausage termed “Slavonski Kulen” by virtue of analysing raw materials used in its production and the final products during a six-month ripening taking place under controlled and characteristic environmental conditions. Given that during the production and storage product casings can easily be damaged, within this study frame both products having an intact and products having a damaged (cracked) casing were analysed.

2. Materials and methods

2.1. The production process

“Slavonski Kulen” was prepared according to traditional recipes and traditional technology described in the literature (Babić et al., 2011; Kovačević, 2014). The highest-quality meat (pork thighs, backs and shoulder blades) were cut into stripes (30 × 10 × 3 cm), ground through a plate having holes measuring 8–12 mm in diameter, spiked with salt (2%), red paprika (1%), hot paprika (0.7%) and garlic (0.2%), and stuffed into a pig's appendix. The production of the Sausage took place in a small rural household. Raw “Slavonski Kulen” was smoked, fermented and dried (until the production day 30) and then left to ripen in dark chambers. The average ripening temperature was 12–16 °C, while the relative humidity ranged from 70 to 80%. The ripening process lasted for 6 months.

Some casings were damaged at 4–5 spots using a knife; the damage was inflicted on a surface area of 0.5 cm^2, i.e. on roughly 0.5–1% of the product surface. The casings were damaged after smoking that took place on the production day 15.

2.2. Samples and preparation

In total, 33 samples of “Slavonski Kulen” were produced. All raw materials ($n = 7$), that is to say, spices and raw meat (pork fatback ($n = 1$), pork ($n = 1$), garlic ($n = 2$), salt ($n = 1$), red paprika ($n = 1$) and hot paprika ($n = 1$)) used with the production were firstly analysed for OTA concentration. Sampling of “Slavonski Kulen” ($n = 3$) took place on the production day 30, 60, 90, 120, 150 and 180. Out of each reference (casing-intact) and damaged (cracked-casing) sample, weighing 1.59–2.13 kg, three separate portions were randomly cut out (to the right, to the left and in the middle of the sample), giving the total sample pool of $n = 99$, then homogenized and subjected to analyses (Table 1). Each sample portion was analysed for its basic physicochemical properties (on the production days 30–180) in triplicate and for OTA levels (on the production days 0–180) in duplicate.

Homogenization was achieved using a Grindomix GM 200 device (Retsch, Germany). Homogenized samples were prepared according to ISO 3100-1:1975 and stored at +4 °C or −20 °C pending analysis (of either physicochemical properties or OTA concentration).

2.3. Chemicals and reagents

OTA standard used for sample fortification and validation of the implemented analytical methods was obtained from Acros Organics (Geel, Belgium). Standard solutions were prepared as an aqueous stock and working solution in concentrations of 20,000 μg/L (stock), 200 μg/L (sample fortification) and 50 μg/L (validation process) and stored at +4 °C pending analyses.

ELISA made use of a Ridascreen OTA ELISA kit provided by R-Biopharm (Darmstadt, Germany). Each kit contains a micro-titre plate with 96 wells coated with OTA antibodies, OTA standard aqueous solutions (0, 0.05, 0.10, 0.30, 0.90, and 1.80 μg/L), peroxidase-conjugated OTA, substrate/chromogen (tetramethylbenzidine), the stop reagent (1 N-sulphuric acid), the dilution buffer and the washing buffer (10 mM-phosphate buffer, pH = 7.4).

PBS-buffer (pH = 7.4 ± 0.1) used for the preparation of samples subjected to HPLC-FLD analyses was prepared by dissolving NaCl (8.0 g), Na_2HPO_4 (1.16 g), KH_2PO_4 (0.2 g) and KCl (0.2 g) in demineralised water (MilliQ system, Millipore, Milford, USA), so as to obtain 1 L of buffer in total. As for the OTA elution, methanol/acetic acid solution (98:2, v/v) was used. The mobile phase used with HPLC-FLD method consisted of acetonitrile/water/isopropanol/acetic acid solution represented in the ratio of 46:46:6:2.

All other chemicals and solvents used with physicochemical analyses and for OTA determination were of an analytical or HPLC grade, respectively.

2.4. Determination of physicochemical properties

Physicochemical properties were determined using standard or internally validated analytical methods: ISO 1442:1997 (water), ISO 936:1998 (ash), ISO 1443:1973 (fat) and ISO 937:1978 (protein). The