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MYCOPOPULATIONS AND OCHRATOXIN A – POTENTIAL CONTAMINANTS OF *PETROVSKÁ KLOBÁSA*

ABSTRACT: *Petrovská klobása* is traditionally produced dry fermented sausage from the area of Bački Petrovac (Vojvodina Province, Serbia) that has been protected with designation of origin (PDO) according to Serbian legislation. Contamination of this kind of sausage casings by different mould species often occur during the production process, mainly during the ripening and storage. The aim of this study was to isolate and identify moulds that contaminate ingredients used for *Petrovská klobása* production and its casings during different phases of ripening and storage. Sampling was done during the production process and after 2, 6, 9, 11, 14, 34, 65, 90, 120, 217 and 270 days. Total mould counts in components ranged from 1.60 (mechanically mixed filling) to 4.14 (red hot paprika powder) $\log_{10}$ CFU/g, while the number of moulds isolated from sausage casing surfaces ranged from 0.01 (C_3 sausage, 217th day) to 1.60 (C_1 sausage, 270th day) $\log_{10}$ CFU/cm^2. After total mould counts were determined, isolates were identified and classified in five genera for components (*Penicillium* – 7 species; *Fusarium* – 2 species; *Aspergillus* – 1 species; *Alternaria* – 1 species; *Verticilium* – 1 species) and 3 genera for casings surfaces (*Penicllium* – 3 species; *Aspergillus* – 1 species; *Eurotium* – 1 species). It was appointed that 83.33% of isolated species are potential producers of toxic metabolites.

The analyses of ingredients and sausages on the presence of ochratoxin A, following the ELISA method, gave the negative results.

KEY WORDS: fermented sausage, mould contamination, ochratoxin A

INTRODUCTION

Fermented dry sausage is defined as a mixture of seasoned, raw meat, fat, salt and various spices, which is stuffed into casings, subjected to fermentation and then allowed to dry (Leroy et al., 2006; Ammor and Mayo, 2007). Distinctive environmental and climatic conditions, as well as cultural and social backgrounds of the populations in different geographical regions, determine a great variety of fermented sausages, produced in European coun-

tries (Ikonić et al., 2010). *Petrovská klobása* is one of the most representive Serbian fermented sausages. It is a traditional dry fermented sausage, produced in small household enterprisses in the municipality of Bački Petrovac (Vojvodina province, Serbia). It is produced from pork meat and fat, with addition of spices, salt and sugar. The traditional procedure exludes the addition of any aditives and starter cultures (Petrović et al., 2010; Jokanović et al., 2010; Tasić et al., 2010). Due to its unique sensory profile and recognizable quality, *Petrovská klobása* has been protected with designation of origin (PDO) according to Serbian legislation (Petrović et al., 2007).

Mould growth on traditional dry fermented sausages can be observed during ripening and storage. It can be a quality problem, because if extensive growth has occurred, sausages may become spoiled due to visible mould colonies on the surface and off-flavors they produce. Moreover, mould growth may represent a health risk because some of the fungal species associated to meat products, such as *Penicillium*, *Aspergillus* and *Fusarium* species, are able to produce mycotoxins (Frisvad and Thrane, 2002). Mycotoxins are extracellular metabolites and they diffuse into substrate after synthesis in fungal cells, so removing of mouldy sausage casings before consumption wouldn't remove the toxins. Mould growth on fermented sausage is therefore an important issue, as it may present a significant economic problem, as well as risk for human health (Papagianni et al., 2007; Castellari et al., 2010).

The most effective way to prevent contamination of fermented sausages with mycotoxins is to avoid growth of mycotoxigenic fungi (Muñoz et al., 2010). The first step so would be isolation of moulds from raw material and moulds that contaminate fermented sausages in different phases of ripening and storage, followed by identification of possible toxigenic species among the isolates.

The aim of this work was to isolate and identify mould contamination of the raw material used for dry fermented *Petrovská klobása* production, and its surface mycobiota in different periods of ripening and storage. Also, all components used for the sausage production and sausages sampled in different phases of ripening and storage, were tested on the presence of ochratoxin A.

MATERIALS AND METHODS

In the present study, mycological and mycotoxicological examinations of *Petrovská klobása* during processing, ripening and storage were carried out.

Sausage production: Sausages were produced in December 2009, in Bački Petrovac, from cold pork meat and fat, salt, red hot paprika powder, garlic and caraway, using traditional procedure. The filling was prepared by two different methods – in the first case, the components were mixed manually, and in the second case they were mixed using mixing machine. The first filling was divided in two parts – one was stuffed into pig natural casings (samples C_1) and other was stuffed into plastic casings (samples C_2). The second filling was stuffed in plastic casings (samples C_3).

Ripening and storage: Produced sausages were left to ripen in chambers in Bački Petrovac (1^{st}-11^{th} day) and Kucura (11^{th}-65^{th} day). On the 65^{th} day they were moved and stored in an industrial facility in Novi Sad.

Mycological investigations: In order to evaluate mould contamination, a sow carcass was swabbed after barking, covering four locations at the carcass (ham, back, belly and jowl). Pig natural casings were also swabbed. Examined parameter was total mould count per square centimeter, expressed as $\log_{10}$ CFU/cm^2. Wet swabs were placed in tubes containing 9 ml of sterile saline solution, a series of decimal dilutions were prepared and 1 ml of each dilution was placed in sterile Petri dish in duplicate. Plates were subsequently poured with 12-15 ml of warm (45 ± 1°C) Sabouraud-maltose agar containing 2% of chloramphenicol. Medium was mixed well with inoculum; the mixture was allowed to solidify and incubated at the 25°C during 7 days. Sampling of the two fillings and the components (chopped meat, salt, garlic, caraway and paprika) was carried out parallel with manufacturing process, and total mould count per gram was determined, using dilution method by Koch (Harrigan, 1998). Inoculated Petri dishes were incubated at the 25°C during 7 days. Results were expressed as $\log_{10}$ CFU/g. Sampling of sausage casings surfaces was taken on 2^{nd}, 6^{th}, 9^{th}, 11^{th}, 14^{th}, 34^{th}, 65^{th}, 90^{th}, 120^{th}, 217^{th} and 270^{th} day of ripening and storage. The surfaces of sausage casings were swabbed using wet swabs, and isolation and identification of moulds were performed as described above.

The identification of isolated fungal species was performed according to the patterns described by Ellis (1971), Nelson et al. (1983), Samson and Frisvad (2004) and Samson et al. (2004).

Ochratoxin A analyses: All sampled components and sausages, which were stored frozen until the analyses were carried out, were tested on the presence of ochratoxin A using ELISA method. Screening method for analysis was done using Neogen Veratox® testing kits with limits of detection of 1 µg/kg (ppb) for ochratoxin A. The test itself is a competitive direct enzyme-linked immunosorbent assay (CD-ELISA). Free mycotoxins in the samples and controls are allowed to compete with enzyme-labelled mycotoxins (conjugates) for the antibody binding sites. After a wash step, substrate is added, which reacts with the bound conjugate to produce blue color. More blue color means less mycotoxin. The test is read in a microwell reader (Thermolabsystem, Thermo, Finland) to yield optical densities. The optical densities of the controls form the standard curve, and the sample optical densities are plotted against the curve to calculate the exact concentration of mycotoxin (Kokić et al., 2009).

RESULTS AND DISCUSSION

Results for total mould counts per cm^2 (TMC/ cm^2) of carcass and casings surfaces, expressed as $\log_{10}$ CFU/cm^2, are shown in Table 1. Total mould count determined in the swab samples of belly, jowl and natural pig casings was very low (in range -0.3-0.51 $\log_{10}$ CFU/cm^2), while the mould contamination of ham and back did not occur.

Tab. 1 – Total mould count per cm^2 of carcass and casings surfaces [log_{10} CFU/cm^2]

	Swab	TMC/cm^2
Carcass	Ham	0
	Back	0
	Belly	0.44 ± 0.01
	Jowl	-0.30 ± 0.06
Casings	Natural pig casing	0.51 ± 0.02

Results for total mould count per gram of sausage components (TMC/g), expressed as log_{10} CFU/g, are presented in Table 2. As it can be seen, all components used for sausage production were contaminated by moulds. The highest mould contamination of spices was detected in the sample of red hot paprika powder, followed by caraway, garlic and salt. As expected, manually mixed filling contamination was considerably higher than contamination of mechanically mixed filling.

Tab. 2 – Total mould count per gram of sausage components [log_{10} CFU/g]

Component	TMC/g
Chopped meat	2.71 ± 0.002
Salt	2.32 ± 0.002
Garlic	2.65 ±0.004
Red hot paprika powder	4.14 ± 0.001
Caraway	3.90 ±0.001
Manually mixed filling	2.34 ±0.002
Mechanically mixed filling	1.60 ± 0.020

Table 3 and Table 4 show the results obtained from identification of isolated moulds. Mould species isolated from the swab samples are listed in Table 3, and results for mould species isolated from the components and two sausage fillings are presented in Table 4. Tables also present the moiety of species in mycopopulations isolated from specific sample.

Tab. 3 – Fungal species isolated from carcass and natural casings

Swab	Species	Isolated (%)
Carcass – belly	*Penicillium spinulosum*	100
Carcass – back	*Verticilium lecanii*	100
Natural pig casings	*Penicillium aurantiogriseum*	100

Tab. 4 – Fungal species isolated from components used for *Petrovská klobása* production

Component	Species	Isolated (%)
Chopped meat	*Penicillium roqueforti*	100
Salt	*Penicillium janthinellum*	100
Garlic	*Fusarium subglutinans*	100
Red hot paprika powder	*Aspergillus flavus* *Penicillium aurantiogriseum*	33.33 66.67
Caraway	*Alternaria citri* *Penicillium glabrum* *Penicillium italicum*	10 70 20
Manually mixed filling	*Fusarium sporotrichioides* *Fusarium subglutinans* *Penicillium roqueforti* *Penicillium aurantiogriseum*	50 30 10 10
Mechanically mixed filling	*Penicillium decumbens*	100

Isolated moulds belong to 5 genera and 12 different species. Genera are *Aspergillus, Alternaria, Fusarium*, *Penicillium* and *Verticilium,* and species are: *Aspergillus flavus* Link*, Alternaria citri* Ellis & Pierce apud Pierce, *Fusarium sporotrichioides* Sherb.*, F. subglutinans* (Wollenw. & Reink.) Nelson, Toussoun & Marass, *Penicillium spinulosum* Thom*, P. aurantiogriseum* Dierckx*, P. janthinellum* Biourge*, P. glabrum* (Wehmer) Westling*, P. roqueforti* Thom, *P. italicum* Wehmer*, P. decumbens* Thom and *Verticilium lecanii* (Zimm.) Viégas. *Penicillium* genus was presented with 7 different species, which makes 58.33% of all isolated species. Carcass belly, natural pig casings, four components (chopped meat, salt, paprika, caraway) and both fillings were contaminated with some of *Penicillium* species, which makes 66.67% of all tested samples. The most abundant was *P. aurantiogriseum,* which was isolated from 25% of tested samples.

Fusarium genus was presented with two species and it was isolated from two different samples (16.67%). Garlic was significantly contaminated with *Fusarium subglutinans*, while both isolated species – *F.subglutinans* and *F. sporotrichioides*, were isolated from manually mixed filling.

Aspergillus, *Alternaria* and *Verticillium* genera were presented with one species each. *Aspergillus flavus* was isolated from paprika powder, *Alternaria citri* from caraway and *Verticilium lecanii* from carcass back swab. Picture 1 shows the presence of different mould genera in mycopopulations isolated from raw material used for *Petrovská klobása* production.

Comparing total mould counts isolated from components with maximum allowed count according to Rule Book for Microbiological Accuracy of Foodstuffs on the Market (Official Journal FRY, 26/93, 53/95, 46/02) and results obtained by several authors (Dimić et al., 2008; Hashem and Alamri, 2010), it can be concluded that meat and spices used for *Petrovská klobása* production were of acceptable quality. However, some of the isolated species, such as *Penicillium aurantiogriseum*, *Aspergillus flavus, Fusarium subglutinans, F.sporotrichioides* etc., are reported to be toxigenic (Ožegović and

Pepeljnjak, 1995; Samson et al., 2004; Marasas et al., 1984), so they posses a high risk for human health.

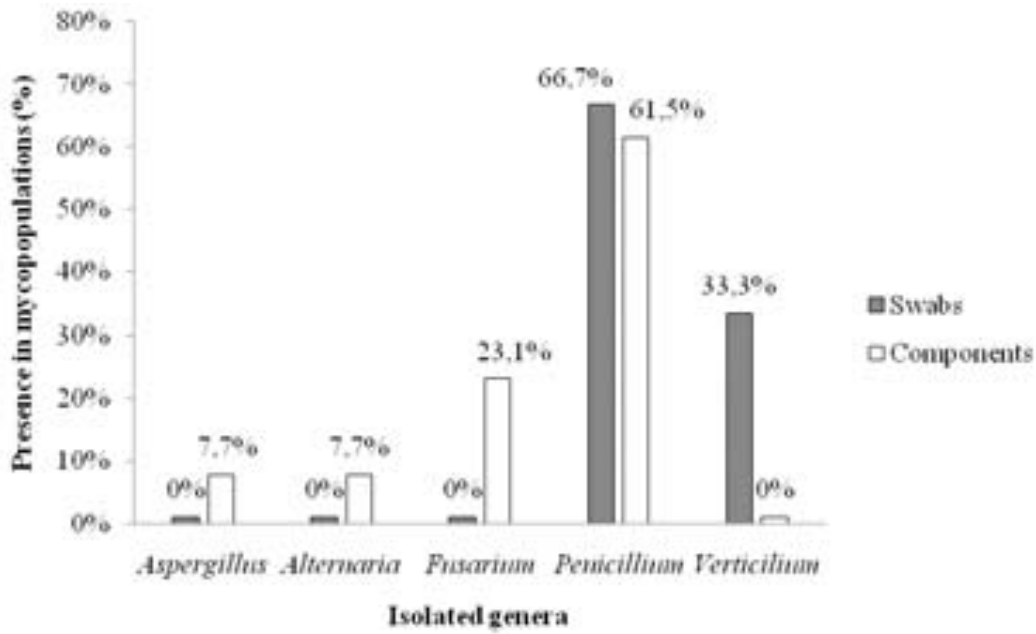

Fig. 1 – Presence of different mould genera in mycopopulations isolated from raw material used for *Petrovská klobása* production.

As has been reported by a number of authors (Škrinjar 2008; Vesković-Moračanin et al., 2009; Iacumin et al., 2009), mould contamination of fermented sausage casings often occur during the production process, mainly during the ripening and storage. Therefore, the surface of sausages C_1, C_2 and C_3 were swabbed in a few different periods of ripening and storage, in order to examine the presence of moulds on the sausage casings and identified possible toxigenic species.

During the first 90 days of ripening and storage, none of the tested samples was contaminated by moulds. The first mould growth was noticed on the 120^{th} day on the casings of sausage C_1. On the 217^{th} day, the moulds were isolated from the surface of C_1 and C_3 sausages. On the 270^{th} day, all three types of sausages were contaminated by moulds. Results for total mould counts isolated from different sausages on different days of ripening and storage, species and percentage of species in isolated mycopopulations, are shown in Table 5.

The moulds isolated from sausage casings belong to three genera: *Eurotium, Penicillium* and *Aspergillus. Eurotium* and *Aspergillus* genera were presented with one species each – *E.herbariorum* (Wiggers) Link and *A. versicolor* (Vuill.) Tiraboschi, and *Penicillium* with three – *P. aurantiogriseum* Dierckx, *P.chrysogenum* Thom and *P.olsonii* Bainier and Sartory.

Tab. 5 – Total mould count per cm^2 [log_{10} CFU/cm^2] and fungal species isolated from *Petrovská klobása* casings during ripening and storage

Sausage	Day of sampling	TMC/cm^2	Species	Isolated (%)
C_1	120	0.69 ± 0.01	*Eurotium herbariorum*	100
C_1	217	0.95 ± 0.01	*Eurotium herbariorum*	100
C_3	217	0.01 ± 0.01	*Penicillium olsonii*	100
C_1	270	1.60 ± 0.002	*Eurotium herbariorum* *Penicillium aurantiogriseum* *Penicillium chrysogenum* *Aspergillus versicolor*	37.5 25 25 12.5
C_2	270	0.65 ± 0.01	*Penicillium chrysogenum*	100
C_3	270	0.78 ± 0.005	*Penicillium aurantiogriseum* *Penicillium chrysogenum*	50 50

In the mycopopulations isolated from sausage C_1 casings, the species *Eurotium herbariorum* dominated. This species was the only contaminant of sausage C_1 casings after 120 and 217 days of ripening and storage, and makes 37.5% of total mould count isolated from this sausage casings on the 270th day. *Penicillium aurantiogriseum* makes 25% of isolated moulds on the last day of sampling, as well as *P.chrysogenum*, while the species *Aspergillus versicolor* makes 12.5% in isolated mycopopulations.

The mould growth on the sausage C_2 casings was detected only on the last day of sampling, and it was contaminated with one species, *P. chrysogenum*.

Swabs of sausage C_3 casings showed the presence of moulds on the 217th and on the 270th day of ripening and storage. In the first case, it was just one species, *Penicillium olsonii*, while the mycopopulations isolated after 270 days consisted of two species, *P.aurantiogriseum* and *P.chrysogenum*, with equal moiety.

The 83.33% of isolated species has been reported (Samson et al., 2004; Marasas et al., 1984) as potentially toxigenic, with the ability to synthesize different types and amounts of mycotoxins, under specific conditions. Considering their possible harmful effect on people's health, this percentage can be regarded as very dangerous.

The tests of components and *Petrovská klobása* samples on the presence of ochratoxin A, gave the negative results. It can be concluded that biosynthesis of this toxin didn't occur, even some of the isolated moulds are potentially toxigenic. Other explanation of the negative results, considering samples of sausages, might be the possible interaction between mycotoxigenic fungi and some lactic acid bacteria presented in sausage filling. It has been reported by a number of authors lately that some lactic acid bacteria can inhibit the biosynthesis of mycotoxins, but they are also capable of binding, detoxifying and/or degrading already synthesized toxins (Schnürer and Magnusson, 2005; Shetty and Jespersen, 2005; Dallé et al., 2010).

CONCLUSION

This study has identified mould species that cause contamination of traditional dry fermented sausage, *Petrovská klobása*. Isolated moulds can originate from contaminated raw material used for sausage production, or could come from environment, facilities and/or handling, as secondary contamination. A high percentage, 83.33%, of the species isolated at high frequencies are capable of producing mycotoxins. All tested samples of *Petrovská klobása* were ochratoxin A free, however, the presence of potential toxigenic moulds posses a high risk of the product contamination with mycotoxins. Care therefore has to be taken during production and storage, and a raw material of high quality has to be used.

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МИКОПОПУЛАЦИЈЕ И ОХРАТОКСИН А – МОГУЋИ КОНТАМИНЕНТИ ПЕТРОВАЧКЕ КОБАСИЦЕ (*PETROVSKÁ KLOBÁSA*)

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Резиме

Petrovská klobása је на традиционалан начин произведена сува ферментисана кобасица из околине Бачког Петровца (Војводина, Србија), која је заштићена ознаком географског порекла, према закону Републике Србије. Контаминација ове врсте кобасица различитим врстама плесни се веома често дешава током процеса производње, посебно током зрења и складиштења. Циљ овог рада је био да се изолују и идентификују плесни које контаминирају састојке коришћене у производњи Петровачке кобасице и њен омотач, у различитим фазама зрења и складиштења. Узорковање је обављено током производње и након 2, 6, 9, 11, 14, 34, 65, 90, 120, 217 и 270 дана. Након одређивања укупног броја плесни, изолати су идентификовани и сврстани у пет родова, изолованих из компонената (*Penicillium* – 7 врста; *Fusarium* – 2 врсте; *Aspergillus* – 1 врста; *Alternaria* – 1 врста; *Verticilium* – 1 врста) и три рода изолованих са површине омотача (*Penicllium* – 3 врсте; *Aspergillus* – 1 врста; *Eurotium* – 1 врста). Показано је да 83.33% изолованих врста спада у потенцијалне произвођаче токсичних метаболита.

Анализом састојака и готових кобасица на присуство охратоксина А, коришћењем ELISA методе, добијени су негативни резултати.