

Tanja D. ŽUGIĆ-PETROVIĆ^{1*},
Dragana M. STANISAVLJEVIĆ¹,
Predrag D. ILIĆ¹, Katarina G. MLADENOVIĆ²,
Mirjana Ž. MURUZOVIĆ²,
Sunčica D. KOČIĆ-TANACKOV³, Ljiljana R. ČOMIĆ²

¹ College of Agriculture and Food Technology, Department of Food Science and Technology
Ćirila i Metodija 1, Prokuplje 18400, Serbia

² University of Kragujevac, Faculty of Science, Department of Biology and Ecology
Radoja Domanovića 12, Kragujevac 34000, Serbia

³ University of Novi Sad, Faculty of Technology, Department of Food Preservation Engineering
Bulevar cara Lazara 1, Novi Sad 21000, Serbia

EFFECT OF WATER ACTIVITY ON THE RADIAL GROWTH OF FUNGI ISOLATED FROM DRY-CURED SHEEP HAM, *IN VITRO* (SERBIA)

ABSTRACT: In the Western Balkans, traditional dry-cured sheep ham called Pastrma or Stelja is produced. Dry-cured sheep ham from Sjenica is produced in a very complex manner, and a prerequisite for its production is the sanitary safety of raw materials in accordance with veterinary and sanitary regulations. Isolation and preliminary identification of fungi from sheep ham were carried out in this study, as well as *in vitro* testing of the effects of water activity (a_w) on the growth of isolated fungi. Fungi were isolated from 9 samples of dry-cured sheep meat taken from three households in the area of Sjenica in two productive years (2015 and 2016). Species of genus *Penicillium* were isolated as dominant in all the investigated samples. Water activity was tested on MY50GF agar from the series of malt extract yeast extract glucose fructose agar. Water activity was set to values of 0.87, 0.89, and 0.97. The results of the research showed that the growth of fungal colonies is under the direct influence of water activity. Fungi grew fastest at water activity of 0.97, and the highest growth of all tested species was recorded after 3 (*A. niger*), 7 (*P. patulum*), and 10 days (*A. nidulans*).

KEYWORDS: sheep ham, fungi, water activity, growth

INTRODUCTION

Dry-cured sheep meat – Pastrma or Stelja (in Turkish pastyrma or bastyrma) is a characteristic product of the Western Balkans (Stamenković and

* Corresponding author. E-mail: tanja.zugicpetrovic@yahoo.com

Dević, 2006). It is produced in the traditional way from whole carcasses of animals aged 1–6 years, using the meat of fattened male castrates and barren sheep (Stamenković and Dević, 2006).

Sjenica sheep Pastrma (Serbia) is prepared by cutting the whole carcass from sternum to pelvis, removing the head, spinal cord and internal organs. Only kidneys with surrounding fat are left on the carcass while leg muscles are removed for ham production. Such boneless meat salted and dry-cured is called Stelja (Stojković et al., 2015). Air-drying and ripening take place during the winter period of 4 to 6 months, whereby meat develops its characteristic microbiota that defines its organoleptic properties and quality.

Complex production method and the fermentation of the product influence the richness of microbiota which develops in dry-cured ham from Sjenica. It is mostly composed of lactic acid bacteria and coagulase-negative staphylococci, but some fungi can be found as a part of the microbiota as well. Composition of microbial population depends on microorganisms found in the meat or in the environment during the production process to the product itself (Žugić-Petrović et al., 2016). Dried sheep meat is characterized by low water activity (a_w) and high concentrations of salt, which are good conditions for the development of xerophilic fungi (Sonjak et al., 2011).

Fungi play an important role in the production of dried meat products; their growth on the surface is often desirable so they are often added as a starter in the production of some meat products (Canel et al., 2013). Fungi directly affect the product's quality, and can be responsible for the formation of specific taste and aroma of dried meat, due to their lipolytic and proteolytic activity (Ludemann et al., 2004; Scolari et al., 2003). Proteolytic changes occurring in dry-cured sheep ham can lead to an increase in free amino acids, which serve as precursors of volatile compounds (Martin et al., 2004). Fungi form a barrier on the surface of smoked meat which prevents the penetration of light and oxygen into deeper layers of the product thus making it more stable (Sonjak et al., 2011).

Diversity of fungi species growing on the surface of Stelja depends on the quality of raw materials and hygienic quality of the production environment. Dry-cured meat products are characterized by long and complicated production process with each stage of the production process requiring specific physico-chemical conditions that may have a positive impact on the growth of certain fungi. Some conditions may determine and accelerate the development of the dominant fungi species (Scolari et al., 2003).

The aim of this research was to isolate and preliminary identify the fungi from dry-cured sheep meat, as well as to investigate the impact of water activity on their growth. Fungi were isolated from the samples of dry-cured sheep meat – Sjenica sheep ham (Western Serbia), produced with the traditional method at three different households, under identical microclimate conditions and from the meat of Pramenka – autochthonous sheep species from Sjenica and Pešter.

MATERIALS AND METHODS

Samples of sheep ham

Nine samples of sheep ham were taken from three producers (A, B, and V) in the territory of Sjenica (Western Serbia) during two production years (2015 and 2016). All samples were produced in the traditional manner without the addition of starters and under the identical microclimate conditions from the meat of Pramenka – autochthonous sheep species from Sjenica and Pešter.

In the first production year (2015) 3 samples were taken from the producer A. Six samples of sheep ham in total were taken in the second production year (2016), 3 from each household of B and V. Sampling of dry-cured sheep meat was conducted according to the regulations of general and specific food hygiene requirements at any stage of production, processing and transport (*Official Gazette RS*, 72/2010).

Isolation and identification of fungi

Isolation of fungi from the surface of the sheep ham was conducted using dichloran 18% glycerol agar (DG18 agar) (Merck, Darmstadt). Surface of the tested sheep ham was leaned against DG18 agar surface and kept for 30 seconds in order to transmit fungal spores from the sheep ham to the surface of the substrate. Then the substrate was incubated at 25 °C for 5 days. After that, the colonies for which, based on the macromorphological properties, it was assumed to belong to genera *Penicillium*, *Aspergillus*, and *Eurotium*, were subcultured onto the Czapek Yeast Extract Agar (CYA) (Merck, Darmstadt). Inoculated mediums were incubated for 7 days at 25 °C. Isolates for which it was presumed to belong to the genus *Mucor* were grown on Malt Extract Agar (MEA) (Merck, Darmstadt), for 7 days at 25 °C. Obtained pure cultures of fungi were identified according to the keys for determination (colony diameter, color and texture, microscopic characteristics – hyphae and conidiophore appearance, size and shape of vesicles, metulae, phialides, and conidia) described in literature by Klich (2002), Samson et al. (2004), Samson and Frisvard (2004), and Pitt and Hocking (2009). Isolated and identified fungal cultures were kept on Sabouraud Maltose Agar (SMA) (Torlak, Beograd, Serbia) at 4 °C as part of the collection of the Laboratory for Food Microbiology at the Faculty of Technology, University of Novi Sad, Serbia.

Investigation of the effect of water activity on the growth of fungi

The effect of different water activity values (0.87 a_w , 0.89 a_w , and 0.97 a_w) on the fungal growth was tested for the following fungal cultures: *Penicillium corylophilum*, *Penicillium carneum*, *Penicillium patulum*, *Aspergillus nidulans*,

Aspergillus niger, *Eurotium herbariorum* and *Mucor racemosus* isolated from dry-cured sheep ham from Sjenica (Western Serbia).

Substrates with different a_w values were prepared using Malt extract yeast extract glucose 50% agar (MY50G) substrates (Pitt and Hocking, 1985). The substrates with different a_w values were prepared by adding different concentration of glucose to the basic medium MY50G (20% for 0.87 a_w , 50% for 0.89 a_w and 60% for 0.97 a_w) (Beuchat and Hocking, 1990). Water activity values of prepared substrates were tested by using Meter group INC 40515 device, in three repetitions. Media are poured into Petri dishes (ϕ 90 mm TER) and centrally inoculated with mature spores of seven-day fungi cultures (incubated at 25 °C, on SMA).

Colony growth was monitored during 15 days at 25 °C on third, fifth, seventh, tenth, twelfth and fifteenth day when the diameter of the colonies was measured using a ruler in three repetitions.

RESULTS AND DISSCUSION

Species of genus *Penicillium* were isolated as dominant in all the investigated samples in two production years (2015 and 2016) (Table 1).

Table 1. Isolated species of fungi from the surface of sheep ham

Mould species	A (2015)	B (2016)	V (2016)
	Frequency of isolation (%)		
<i>Penicillium carneum</i>	18	0	33
<i>Penicillium caseifulvum</i>	6	0	13
<i>Penicillium confertum</i>	2	30	7
<i>Penicillium corylophilum</i>	0	6	0
<i>Penicillium crustosum</i>	0	0	2
<i>Penicillium polonicum</i>	8	40	25
<i>Penicillium rugulosum</i>	30	0	0
<i>Penicillium solitum</i>	30	0	16
<i>Aspergillus nidulans</i>	0	2	0
<i>Aspergillus niger</i>	2	0	0
<i>Aspergillus penicillioides</i>	2	0	2
<i>Eurotium chevalieri</i>	0	0	2
<i>Eurotium herbariorum</i>	2	4	0
<i>Mucor racemosus</i>	0	13	0
<i>Mucor plumbeus</i>	0	5	0

Three producers in two production years A (2015); B (2016); V (2016).

Macromorphological characteristics of some of the isolated types of fungi are shown in Figure 1 and 2. *M. racemosus* formed wavy, white colonies on the SMA. Colonies of *E. chevalieri* were yellow-ocher with an elevated center in the middle and irregular edges. *A. niger* formed a compact white mycelium with a thick layer of dark brown to black conical heads. Colonies of *P. corylophilum* had dark-green fasciculus texture (conidiophore bundles) with pronounced coremias in the central part and with the presence of a colorless exudate. *P. carneum* formed fluffy, pistachio green colonies with concentric circles and radial folds on CYA. Colonies of *P. polonicum* were fluffy, blue-green in color, with a reddish-brown pigment in the base.

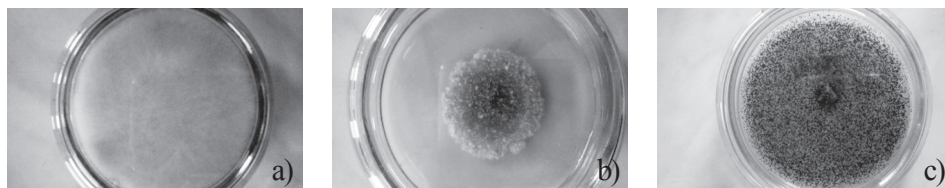


Figure 1. *M. racemosus* on SMA (a), *E. chevalieri* on CYA (b), *A. niger* on CYA (c)

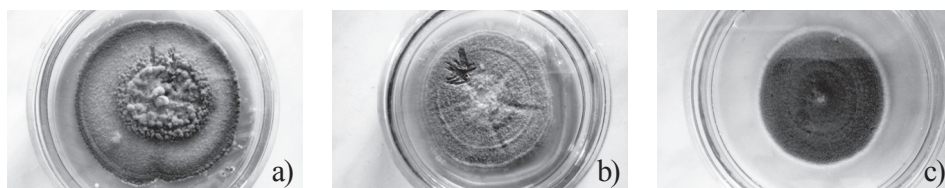


Figure 2. *P. corylophilum* on CYA (a), *P. carneum* on CYA (b), *P. polonicum* on CYA (c)

The obtained results are consistent with studies of other authors. Sonjak et al. (2011) in their work highlighted the importance of *Penicillium* genus as the largest part of the surface microbiota in all the studied dry meat products. *Penicillium* species have also been confirmed in a large percentage (88.3%) in the Norwegian smoked meat products, where *Penicillium nalgiovense* was the dominant species (Asefa et al., 2009). Toledano et al. (2011) proved the good potential of *P. nalgiovense* as a starter culture which was isolated from the ham.

Microbiota isolated from San Daniele dry ham was largely comprised of 2 genus *Penicillium* spp. and *Aspergillus* spp., which were found during the ripening process (Comi et al., 2013).

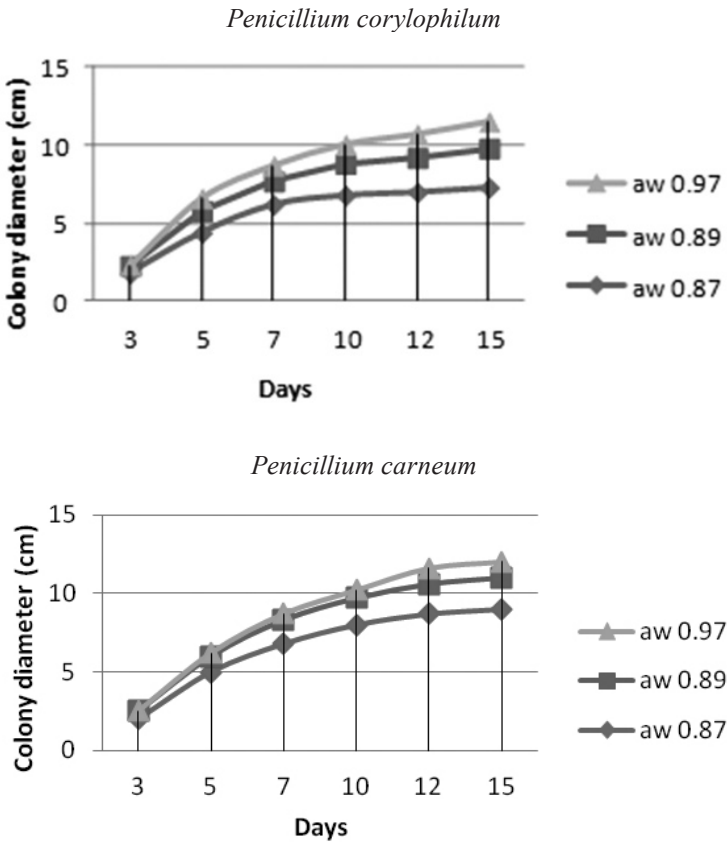
Among the 65 analyzed dry hams, *Aspergillus* and *Penicillium* were the dominant genera (Rojas et al., 1991). *Aspergillus* spp. primarily consisted of *Aspergillus glaucus*, *Aspergillus fumigatus*, *Aspergillus niger*, and *Aspergillus flavus* (Rojas et al., 1991). *Aspergillus* species were present in all samples of Sjenica dry-cured sheep ham. *E. herbariorum* and *M. racemosus* were isolated to a lesser extent. In the research of fungi from Istria ham, Comi et al. (2004) identified five genera, where *Eurotium* spp., *Aspergillus* spp. and *Penicillium*

spp. were most frequently isolated from ham samples. *Eurotium* strains isolated from Nebrodi hams, *E. herbariorum*, *Eurotium rubrum* and *Eurotium cristatum* have been detected (Berni et al., 2012).

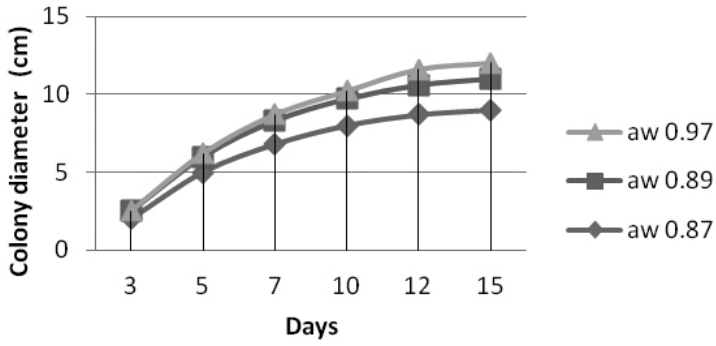
Growth of fungi is influenced by a variety of environmental or intrinsic factors, such as the composition of the product, pH, and temperature. Water availability is probably the single most important environmental factor affecting germination, growth, and establishment on nutrient-rich substrates of fungi (Dantigny et al., 2005).

Temperature and a_w are the most important factors that determine the ability of fungi to grow in meat products (Dantigny et al., 2005).

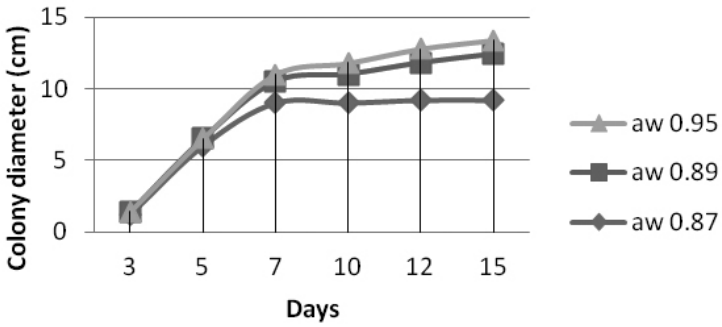
The effect of a_w to isolated strains of fungi *P. corylophilum*, *P. carneum*, *P. patulum*, *A. nidulans*, *A. niger*, *E. herbariorum* and *M. racemosus* are shown in Figure 3.



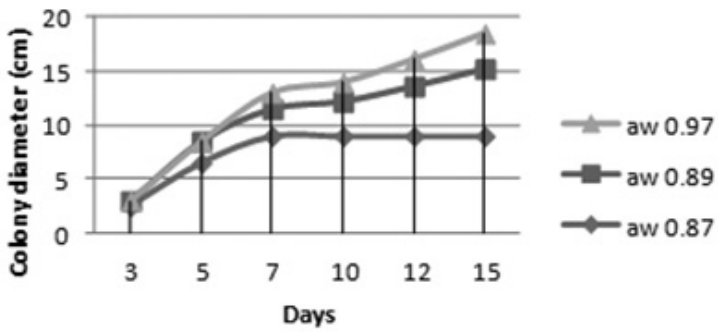
Penicillium patulum



Aspergillus nidulans



Aspergillus niger



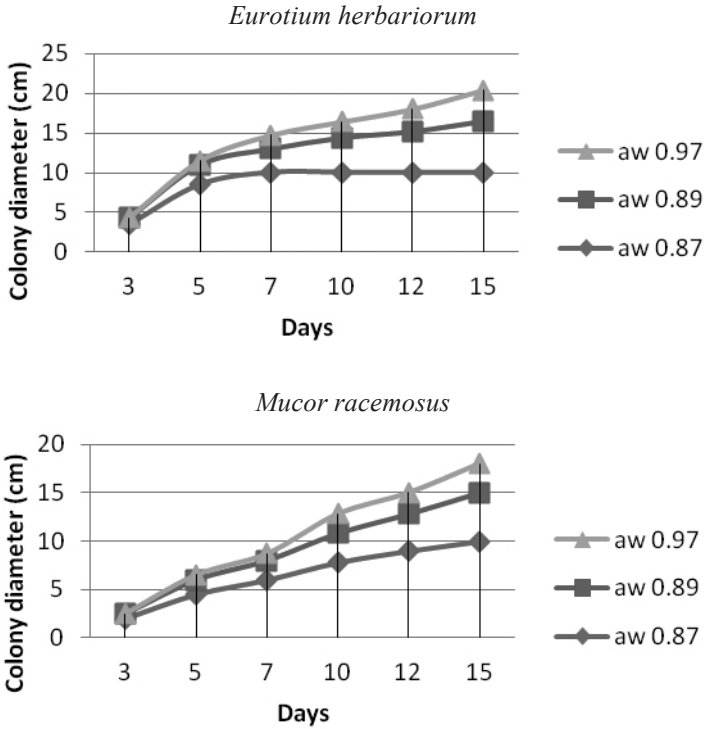


Figure 3. Colony diameters of tested fungi on medium with different a_w values

Table 2. Mean value and SD of colony diameters on medium with different a_w values after 15 days of growth

Fungi	a_w 0.97%	a_w 0.89	a_w 0.87
<i>P. corylophilum</i>	5.58±2.10	1.43±0.71	1.04±0.60
<i>P. carneum</i>	6.58±2.67	1.43±0.57	0.51±0.41
<i>P. patulum</i>	7.26±3.22	1.66±1.17	0.55±0.46
<i>A. nidulans</i>	7.26±3.22	1.66±1.7	0.55±0.46
<i>A. niger</i>	7.5±2.64	3.16±2.0	1.51±1.32
<i>E. herbariorum</i>	8.66±2.6	3.73±2.0	1.81±1.44
<i>M. racemosus</i>	6.55±2.98	2.63±1.63	1.40±1.16

The growth of fungi isolated from the dry-cured sheep meat was under the direct influence of water activity (Table 2). All isolates grew well at the water activity of 0.97. The maximum growth of *P. patulum*, *A. niger*, and *E. herbariorum* was reached in 7 days, while the isolates of *P. corylophilum*, *P. carneum*, and *M. racemosus* reached their maximum after 15 days.

Growth reduction of the isolated fungi at lower a_w values was more noticeable (Table 2). Thus, in the majority of fungal isolates germination of spores was completely inhibited the first 5 (*A. nidulans*, *P. patulum*) and 3 days (*P. corylophilum*, *P. carneum*, *A. niger* and *M. racemosus*) at the value of a of 0.87 (Figure 3).

The effect of 0.89 a_w influenced the growth of the isolates less when compared to the growth at 0.87 a_w . At 0.89 a_w in the first few days of growth the diameter of the colonies was observed to be 0.2 (*P. patulum*) to 1.5 cm (*E. herbariorum*). After 15 days of incubation colony diameter of *P. corylophilum*, *P. carneum*, and *P. patulum* isolates varied from 2 (*P. carneum*) to 3.2 cm (*P. patulum*) (Figure 3).

The results show that the growth of *E. herbariorum* was least affected by a_w value. The size of colonies in all tested a_w had the same value after 12 and 15 days of incubation (Figure 3).

Gibson et al. (1994) studied the effect of a_w on the growth of fungi by using ten different water activities (a_w) between 0.995 and 0.810, adjusted with equal mixtures of glucose and fructose at 30 °C to *Aspergillus* genus *flavi* (*A. flavus*, *A. oryzae*, *A. parasiticus* and *A. nomius*). In their paper they predicted the growth of colonies and proved that the colony diameter for each different a_w value changed about 3mm on the average. The diameters of the colonies were between 7.8 (10th day) to 10 cm (15th day). Gibson et al. (1994) pointed out that the minimum water activity for the growth of *A. flavus* ranged from 0.81 to 0.95.

CONCLUSION

Dominant species of fungi were isolated and identified by using samples of dry-cured sheep ham from Western Balkans. They include strains from the genera *Penicillium*, *Aspergillus*, *Eurotium*, and *Mucor* (*P. corylophilum*, *P. carneum*, *P. patulum*, *A.s nidulans*, *A. niger*, *E. herbariorum*, and *M. racemosus*). The growth of fungi isolated from the dry-cured sheep meat was under the direct influence of water activity as one of the most important intrinsic growth factors. In the tested range of 0.87 to 0.97 a_w , the growth of the tested fungi and colony diameter decreased as a_w decreased.

REFERENCES

- Asefa DT, Gjerde RO, Sidhu MS, Langsrud S, Kure CF, Nesbakken T, Skaar I (2009): Moulds contaminants on Norwegian dry-cured meat products. *Int. J. Food Microbiol.* 128: 435–439.
- Berni E, Cacchioli C, Diaferia C (2012): *Characterization of surface mycoflora in Nebrodi hams*. Options Méditerranéennes, A no. 101, 7th International Symposium on the Mediterranean Pig.

- Beuchat, LR, Hocking AD (1990): Some considerations when analyzing foods for the presence of xerophilic fungi. *J. Food Prot.* 53: 109–116.
- Canel RS, Wagner JR, Stenglein SA, Ludemann V (2013): Indigenous filamentous fungi on the surface of Argentinean dry fermented sausages produced in Colonia Caroya (Cordoba). *Int. J. Food Microbiol.* 164: 81–86.
- Comi G, Iacumin L (2013): Ecology of moulds during the pre-ripening and ripening of San 409 Daniele dry-cured ham. *Food Res. Int.* 54: 1113–1119.
- Comi G, Orlic S, Redzepovic S, Urso R, Iacumin L (2004): Moulds isolated from Istrian dried ham at the pre-ripening and ripening level. *Int. J. Food Microbiol.* 96: 29–34.
- Dantigny P, Guilmarat A, Bensoussan M (2005): Basis of predictive mycology. *Int. J. Food Microbiol.* 100: 187–196.
- Gibson AM, Baranyi J, Pitt JI, Eyles MJ, Roberts TA (1994): Predicting fungal growth: the effect of water activity on *Aspergillus flavus* and related species. *Int. J. Food Microbiol.* 23: 419–431.
- Klich AM (2002): Identification of common *Aspergillus* species. Utrecht, Netherlands: CBS, Fungal Biodiversity Centre.
- Ludemann V, Pose G, Pollio ML, Segura J (2004): Determination of growth characteristics and lipolytic and proteolytic activities of *Penicillium* strains isolated from Argentinean salami. *Int. J. Food Microbiol.* 96: 13–18.
- Martín A, Córdoba JJ, Núñez F, Benito MJ, Asensio MA (2004): Contribution of a selected fungal population to proteolysis on dry-cured ham. *Int. J. Food Microbiol.* 94: 55–66.
- Pitt IJ, Hocking DA (1985): New species of fungi from Indonesian dried fish. *Mycotaxon.* 22: 197–208.
- Pitt IJ, Hocking DA (2009): *Fungi and Food Spoilage*. 3rd ed. Springer Science, Business Media, LLC., Dordrecht – Heidelberg – London – New York.
- Rojas FJ, Jodral M, Gosalvez F, Pozo R (1991): Mycoflora and toxigenic *Aspergillus flavus* in Spanish dry-cured ham. *Int. J. Food Microbiol.* 13: 249–255.
- Samson RA, Frisvad JC (2004): *Penicillium* subgenus *Penicillium*: new taxonomic schemes, mycotoxins and other extrolites. *Stud. Mycol.* 49: 1–174.
- Samson RA, Hoekstra ES, Frisvad JC (2004): *Introduction to Food and Airborne Fungi*. 7th edition. Centraalbureau voor Schimmelcultures. Utrecht, Netherlands. 389 pp.
- Scolari G, Sarra PG, Baldini P (2003): Mikrobiologija suhega mesa. In: Z. Bem, J. Adamic, B. Zlender, S. Smole Mozina, L. Gasperlin (Eds.): *Mikrobiologija zivil zivalskega izvora*. Biotehniška fakulteta, Oddelek za živilstvo, Ljubljana, pp. 351–362.
- Službeni glasnik Republike Srbije* (72/2010): Pravilnik o opštim i posebnim uslovima higijene hrane u bilo kojoj fazi proizvodnje, prerade i prometa. Available: <http://www.overa.rs/pravilnik-o-opstim-i-posebnim-uslovima-higijene-hrane-u-bilo-kojoj-fazi-proizvodnje-prerade-i-prometa.html>.
- Sonjak S, Ličen M, Frisvad JC, Gunde-Cimerman N (2011): The mycobiota of three dry-cured meat products from Slovenia. *Food Microbiol.* 28: 373–376.
- Stamenković T, Dević B (2006): Senzorska svojstva ovčije stelje. *Tehnologija mesa.* 47: 115–122.
- Stojković S, Grabež V, Bjelanović M, Mandić S, Vučić G, Martinović A, Thauland Håseth T, Velemir A, Egelanddal B (2015): Production process and quality of two different dry-cured sheep hams from Western Balkan countries. *Food Sci. Technol.* 64: 1217–1224.

- Toledano A, Jordano R, López C, Medina LM (2011): Proteolytic activity of lactic acid bacteria strains and fungal biota for potential use as starter cultures in dry-cured ham. *J. Food Prot.* 74: 826–829.
- Žugić Petrović T, Muruzović M, Mladenović K, Ilić P, Kocić Tanackov S, Čomić Lj (2016): Characterization of coagulase-negative staphylococci isolated from dried meat of sheep carcasse – Sjenica sheep prosciutto. *Vet. J. Republic Srpska*. DOI: 10.7251/VETJ1601026Z.

УТИЦАЈ АКТИВНОСТИ ВОДЕ НА РАДИЈАЛНИ РАСТ ПЛЕСНИ ИЗОЛОВАНИХ ИЗ ОВЧИЈЕ СТЕЉЕ, *IN VITRO* (СРБИЈА)

Тања Д. ЖУГИЋ ПЕТРОВИЋ¹, Драгана М. СТАНИСАВЉЕВИЋ¹, Предраг Д. ИЛИЋ¹, Катарина Г. МЛАДЕНОВИЋ², Мирјана Ж. МУРУЗОВИЋ², Сунчица Д. КОЦИЋ ТАНАЦКОВ³, Љиљана Р. ЧОМИЋ²

¹ Висока пољопривредно-прехранбена школа – Прокупље

Прехранбена технологија

Тирила и Методија 1, Прокупље 18400, Србија

² Универзитет у Крагујевцу

Природно-математички факултет

Департаман за биологију и екологију

Радоја Домановића 12, Крагујевац 34000, Србија

³ Универзитет у Новом Саду, Технолошки факултет

Катедра за инжењерство конзервиране хране

Булевар цара Лазара 1, Нови Сад 21000, Србија

РЕЗИМЕ: На западном Балкану производи се традиционално сушено овчије месо која се назива пастрма или стеља. Овчија стеља са подручја Сјенице (Западна Србија) производи се на веома сложен начин, а предуслов за производњу меса је хигијенска сигурност сировина која испуњава ветеринарске и санитарне услове производње. У раду је вршена изолација и прелиминарна категоризација, као и испитивање *in vitro* ефеката активности воде (a_w) на раст плесни из сувог меса овчијег трупа (овчија стеља). За потребе истраживања коришћено је девет узорака овчијег сувог меса узетих из три домаћинства у две производне године (2015, 2016) с подручја Сјенице. Иzolовано је и идентификовано седам различитих врста плесни и то: *Penicillium corylophilum*, *Penicillium carneum*, *Penicillium patulum*, *Aspergillus nidulans*, *Aspergillus niger*, *Eurotium herbariorum* и *Mucor racemosus*. Активност воде истраживана је на MY50GF агару из серије подлога Малт екстракт квасац екстракт глукоза фруктоза агара. Активност воде је подешена на вредности од 0,87; 0,89 и 0,97. Резултати истраживања су показали да је раст колонија под директним утицајем активности воде. Плесни су најбрже расле при активности воде од 0,97 a_w , при чему је код свих испитиваних врста највећи пораст забележен између 3 (*A. Niger*), 7 (*P. patulum*) и 10 дана (*A. nidulans*).

КЉУЧНЕ РЕЧИ: овчија стеља, плесни, активност воде, брзина раста