

Differences in the mycoflora of dry-fermented sausages casings before and after six months of refrigerated storage



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Abstract

During the curing period of dry-fermented sausages, the surface mycoflora changes according to the environmental conditions and intrinsic factors, such as nutrient content, water activity, pH value and the presence of antimicrobial substances (starter cultures). In this study, 25 samples of dry-fermented sausages from Croatian family small holdings were examined, the casings peeled off and examined for mycobiota before and after six months of refrigerated storage in plastic bags, at +4 °C in darkness, at a low humidity and evaporation rate. For mycobiota identification, traditional (macroscopic and microscopic) and molecular (Polymerase Chain Reaction, PCR) methods were combined. At the beginning

of the study (after sausage curing), the prevalent isolated species were from the genera *Penicillium* (74%), *Aspergillus* 14% and *Mucor* (12%). After the six-month period, a different mould structure was observed, with the dominant isolation of *Aspergillus* genus (64%), followed by the genera *Penicillium* (34%) and *Mucor* (2%). In comparison with the literature data, it can be concluded that the casing mycoflora on dry-fermented sausages stored in the refrigerator changed in the same way as if they not been refrigerated, though the process of mycoflora change was slower with refrigeration.

Key words: mycoflora; surface moulds; dry-fermented sausages; casings; refrigerated storage

Introduction

During curing, the surface of dry-fermented sausages becomes overgrown with moulds whose spores originate

mostly from the curing environment. The presence of moulds on the surface of these products contributes to the development

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of the positive characteristics typical of the final products, since they retain moisture and contribute to the degradation of fat and proteins due to their lipolytic and proteolytic enzymatic activities (Toldra, 1998; Ockerman et al., 2000; Bruna et al., 2003). Moreover, the presence of these moulds results in anti-oxidising effects, contributes to better colour and sensory characteristics, prevents excessive surface drying and protects against pathogenic microorganisms (Scolari et al., 2003; Ludemann et al., 2004). However, under adverse environmental conditions, certain mould species can produce mycotoxins, which have carcinogenic, immunologic and allergic effects, and are particularly relevant in the case of food consumed by humans (Comi and Lacumin, 2013; Pleadin et al., 2015a; Zadavec et al., 2019). The negative effects of moulds on the appearance, smell, taste and nutritional value of dry-fermented meat products can be caused by the overgrowth of unwanted spoilage moulds (Samson et al., 2002; Scholte et al., 2002; Asefa et al., 2009; Lešić et al., 2020).

The important factors affecting mould growth on the surface of dry-fermented sausages can be summarised in the following categories: (i) intrinsic factors, including nutrient content, water activity, pH value and the presence of antimicrobial substances (starter culture); (ii) extrinsic factors, related to the storage environment, including storage temperature, gas composition and relative humidity (Hamad, 2012). In general, moulds that cover sausage surfaces can grow at water activities in the range of 0.70–0.95, temperature from -2 to 40 °C, and in an atmosphere with 1% O₂. However, differences in these factors can have an effect on their growth dominance. *Penicillium* and *Mucor* species prefer lower temperatures for growth than *Aspergillus* species, and *Mucor* species grow at higher water activity values ($a_w > 0.9$) while *Penicillium* and

Aspergillus grow at a_w levels from 0.8 to 0.9. Moreover, *Aspergillus* species, especially *Teleomorphs* (*Eurotium*-type), and *Mucor* species can grow in an atmosphere of 1–2% O₂ (Pitt and Hotchkiss, 2009; Morin-Sardin et al., 2016).

According to the literature (Castellari et al., 2010; Sonjak et al., 2011; Zadavec et al., 2020) the predominant mould genus to overgrow and cover the surface of sausages is *Penicillium*, followed by *Aspergillus* and *Mucor*. Sausages ripening in the winter period are more suitable for *Penicillium* and *Mucor* species growth, than for *Aspergillus* species, which prefer higher temperatures, especially teleomorphs. On the surface of dry-fermented sausages ripening over a longer period of time (up to six months), such as Kulen and Kulenova seka (traditional pepper sausages), after ripening, *Aspergillus* becomes predominant, followed by the *Penicillium* and *Mucor* genera (Pleadin et al., 2017; Zadavec et al., 2020).

The aim of this study was to examine the mycobiota changes of the surfaces of Croatian domestic dry-fermented sausage casings at the end of the curing period (right after production) and after six months of refrigerated storage.

Materials and Methods

Samples

A total of 25 pairs of domestic dry-fermented sausages were sampled from small family holdings in central and northern Croatia. The domestic sausages were produced according to traditional recipes of pork meat of the first and the second category and fat, with the addition of table salt, sweet and hot red paprika powdered seasoning, ground pepper and garlic, and stuffed into casings (Kovačević, 2018). Prior to stuffing, the meat and fat were mechanically minced. After stuffing, the sausages underwent drying and curing over a period of two to

two and a half months using the smoking process.

The sampled sausage pairs were peeled and one-half of each pairs was tested for mycobiota at the beginning of the study (following the end of the curing period, right after their production and sampling, without refrigerated storage), while the second half of the pair was analysed after six months of refrigerated storage in plastic bags at 4–8 °C in darkness, at a low evaporation rate.

Mould identification

Peeled casings were placed in Petri dishes with 18%-glycerol (DG-18, Merck, Germany) and incubated for seven days in darkness at 25 ± 1 °C. For the purpose of species identification, the identified genera were sub-cultivated in malt extract agar (MEA, Difco International) and Czapek yeast extract agar (CYA, Difco International) and incubated for seven days at 25 ± 1 °C in darkness. The mould isolates were identified to the species level based on macroscopic and microscopic morphological traits. For the determination of the micro-morphological traits, slides were prepared from the MEA medium, using lactophenol cotton blue (LPCB) as the mounting medium. Slides were analysed under a light microscope (AX10 type, Zeiss, Germany) at 400–1000 × magnification. All isolates were identified according to Pitt and Hocking, (2009) and Samson et al. (2019).

Molecular identification of the mould isolates was performed to verify the results of the traditional identification methods. Deoxyribonucleic acid (DNA) was extracted from about 100 µg of mould colonies using the Dneasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The primers specific for beta-tubulin (benA)—Bt2a (5'-GGTAACCAAATCG-GTGCTGCTTTC-3') and Bt2b (5'-ACCCT-CAGTGTAGTGACCCTTGGC-3'), and for calmodulin (CaM) loci—Cmd5 (5'-

CCGAGTACAAGGARGCCTTC-3') and Cmd6 (5'-CCGATRGAGGTCATRACGT-GG-3') were selected for polymerase chain reaction (PCR) amplification. Each 25 µL-PCR reaction mix contained 12.5 µL 2× PCR buffer (HotStarTaq Plus MasterMix Kit, Qiagen), 2.5 µL 10× Coral Load, 0.4 µM each primer, nuclease-free water and 1 µL DNA extract. The procedure was performed under the following cycling conditions: 95 °C for 5 min, followed by 40 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 60 s, concluding with the final extension at 72 °C for 10 min. PCR products were checked using gel electrophoresis in 1.5%-agarose gel stained with GelStar nucleic acid stain (Lonza, Switzerland) and visualised using UV trans-illumination. All PCR products of an adequate size were purified prior to sequencing using either an ExoSAP-IT PCR clean up reagent (Affymetrix, Santa Clara, CA, USA), or a QIAquick Gel Extraction Kit (Qiagen). Purified samples were sent to Macrogen Inc. (Amsterdam, Netherlands) for paired-end sequencing. The obtained sequences were aligned using Lasergene SeqManPro DNASTAR 13 (Madison, WI, USA). The edited sequences were compared with those available from the CBS-KNAW Fungal Biodiversity Centre database (<http://www.cbs.knaw.nl>) and GenBank using the BLAST algorithm (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Statistical analysis

Statistical analysis was performed using SPSS Statistics software v. 22 (IBM, NY, USA). The statistical significance of the difference in the number of mould isolates was determined using Mann-Whitney U test, with statistical significance set at $P \leq 0.05$.

Results and Discussion

The curing time of dry-cured meat products in different ripening stages is

correlated with the level of humidity and water activity, which directly affects the presence of mycobiota (Castellari et al., 2010). Earlier studies reported that the surface of dry-fermented sausages are predominantly overgrown by the *Penicillium* genus, followed by *Aspergillus* and *Mucor*, among which *P. nalgoviense*, *P. commune* and *P. solitum* are generally the most common mould species, followed by *Aspergillus* anamorphs, and the rarest isolated species *M. racemosus* (Asefa et al., 2009; Castellari et al., 2010; Sonjak et al., 2011; Pleadin et al., 2017; Zadavec et al., 2020).

However, the relevant literature provides no data as to the differences between mould communities on the surface of dry-fermented sausages before and after a period of refrigerated storage. The hypothesis of this study was that the mycoflora community on dry-fermented sausage casings would not change under constant conditions of darkness, low temperature (4–8 °C) and storage in plastic

bags that prevent evaporation. Isolated mould species from the surface of dry-fermented sausage casings before and after six months of refrigerated storage obtained in this study are presented in Figures 1 and 2.

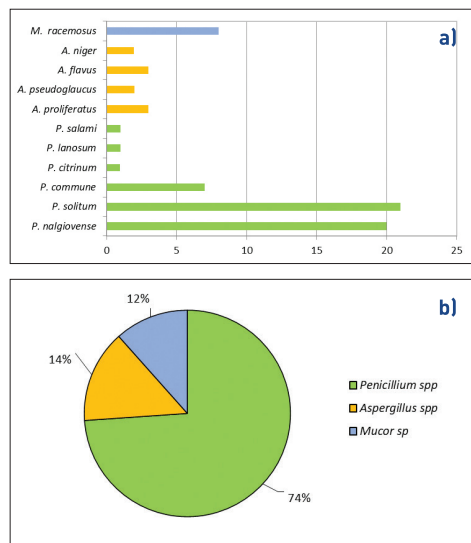


Figure 1. (a) number of isolated species from dry-fermented sausage casings, and (a) shares of each isolated genus at the beginning of the study (without refrigerated storage)

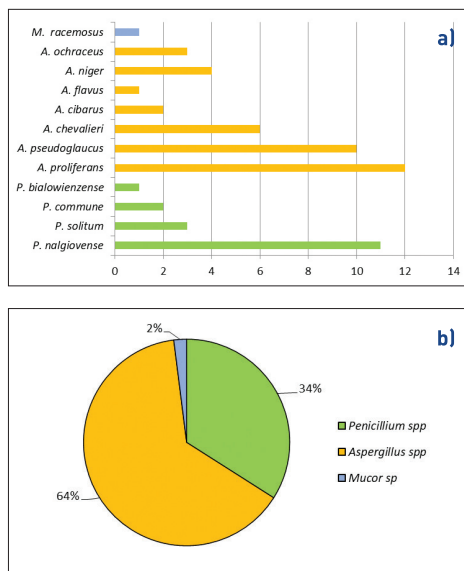


Figure 2. (a) Number of isolated species from the casings of dry-fermented sausages, (b) shares of each isolated genus after six months of refrigerated storage

No significant differences were observed in the total number of isolated species before and after the six-month refrigeration period ($P=0.976$). At the beginning of the study, the prevalent species were from the genus *Penicillium* (74%), with six species, where the most often isolated species were *P. nalgoviense* (21) and *P. solitum* (20), whereas *Aspergillus* species accounted for a lower percentage (14%) with only four species, *A. flavus*, *A. niger*, *A. proliferans* and *A. pseudoglaucus*. The *Mucor* genus was isolated in 12% of samples, with *M. racemosus* as the only species.

After the six-month refrigeration period, a different mould structure was

obtained. The *Aspergillus* genus was predominant (64%), followed by the genera *Penicillium* (34%) and *Mucor* (2%). The most isolated *Aspergillus* species were the teleomorphs *A. cibarus*, *A. chevalieri*, *A. pseudoglaucus* and *A. proliferans*, followed by the anamorphs *A. ochraceus*, *A. niger* and *A. flavus*. The most isolated *Penicillium* species were *P. nalgoviense*, *P. commune*, *P. solitum* and *P. bialowienzense*, while *Mucor racemosus* was the only representative of the *Mucor* genus. It was observed that *A. ochraceus*, *A. cibarus*, *A. chevalieri* and *P. bialowienzense* were not isolated at the beginning of the study, but were only identified after the refrigeration period.

The results from the beginning of the present study are in line with previous studies that also reported that the surface of dry-fermented sausages is predominantly overgrown by the *Penicillium* genus, followed by *Aspergillus* and *Mucor* (Castellari et al., 2010; Pleadin et al., 2017; Zadravec et al., 2020; Lešić et al., 2020). At the beginning of the study, six *Penicillium* species and four *Aspergillus* species were isolated, which is also in line with Asefa et al. (2009), Comi and Iacumin, (2013) and Pleadin et al. (2017), who reported more *Penicillium* than *Aspergillus* species on the surface of short ripening dry-cured meat products.

It is important to point out that among the *Aspergillus* species, the potentially mycotoxigenic species *A. niger* (ochratoxin A producer) and *A. flavus* (aflatoxin B₁ producer) (Pleadin et al., 2014; Pleadin et al., 2015b; Lešić et al., 2020), were isolated at the beginning of the study, as also reported in other studies (Asefa et al., 2009; Sonjak et al., 2011; Comi and Iacumin, 2013; Pleadin et al., 2017; Rodrigues et al., 2019). However, no mycotoxin-producing *Penicillium* species were isolated in this study. After six months of refrigerated storage, the mycotoxigenic species *A.*

ochraceus was isolated in addition to *A. niger* and *A. flavus*. It is worth mentioning that moulds produce mycotoxins when they are triggered by the environmental conditions, which could also refer to the stressful low temperatures implemented in this study (Mannaa and Kim, 2017).

The results after six months of refrigerated storage are comparable with reports by Comi and Iacumin (2013), Pleadin et al. (2017) and Zadravec et al. (2020) on the surface of dry-cured meat products ripening over a one-year period. In those studies, the most prevalent species were also *Aspergillus* species, especially teleomorphs, followed by *Penicillium* and *Mucor*. These observations were explained by the long period of ripening including a hot summer period, which is preferred by *Aspergillus* species, as opposed to *Penicillium* species that favour lower temperatures and a colder climate (Pitt and Hocking, 2009; Zadravec et al., 2020).

The explanation of the results presented here can be found in the fact that ascospores of *Aspergillus* teleomorphs (*A. cibarus*, *A. chevalieri*, *A. pseudoglaucus*, *A. proliferans*) can survive in a wide range of temperatures (4–43 °C), and a very low water activity (0.71) for up to 120 days. Despite the fact that *Penicillium* spores remain vital at lower temperatures (even under 0 °C) than *Aspergillus* spores, *Penicillium* species require a higher water activity for spore vitality (0.8) (Pitt and Hocking, 2009). *Mucor* spores are vital at low temperatures, even at -4 °C, but require a higher water activity of 0.86 (Pitt and Hocking, 2009; Marin-Sardin et al., 2016). Additionally, *Aspergillus* species, especially teleomorphs, *P. solitum*, *P. commune*, *P. nalgoviensis* and *Mucor racemosus* (isolated in the present study) remain vital in an atmosphere of 1–2% O₂ (Pitt and Hocking 2009; Schirmer et al., 2020).

An interesting observation is that *A. ochraceus*, *A. cibarus*, *A. chevalieri* and

P. bialowienzense were isolated after six months of refrigerated storage, but not at the beginning of the study. These results can be explained by the delicate nature of isolation, where primary isolation moulds overgrow the Petri dish, and some dominant moulds can overgrow others. In that case, some mould species may be overlooked during identification.

Conclusions

It has been assumed that a decrease of the temperature will stop natural processes or remedy them; however, this study refutes that claim. The mycoflora structure on dry-fermented sausages changes with the time of refrigeration just as in the natural environment. During storage, even at low temperatures, moulds use water and oxygen for survival, while water activity and oxygen concentration decreases. A similar process occurs during the ripening of dry-cured meat products, when was activity decreases over time, greatly affects mould survival on a surface. Therefore, lowering the temperature only slows but does not stop natural processes. A limitation of this study is that dry-fermented sausages were not stored whole with the filling and casing due to possible spoilage, which may have affected water activity. It can be concluded that the mycoflora would most likely have changed in the same way if the sausages had not been refrigerated, only these change would have occurred faster. Therefore, a future study should analyse the impact of refrigerated storage on the presence of mycoflora over a timeframe of months.

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Razlike u mikoflori na ovitcima trajnih fermentiranih kobasica prije i šest mjeseci nakon skladištenja u hladnjaku

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Tijekom razdoblja zrenja trajnih fermentiranih kobasica mikoflora koja obrasta i prekriva njihovu površinu mijenja se u ovisnosti o uvjetima okoliša i "unutarnjim čimbenicima", koji uključuju sadržaj hranjivih sastojaka, aktivitet vode, pH vrijednost i prisutnost antimikrobnih tvari (starter kulture). U ovom je istraživanju uzorkovano dvadeset i pet parova trajnih fermentiranih kobasica s malih hrvatskih obiteljskih poljoprivrednih gospodarstava, odljušten je njihov ovitak te je ispitivana mikoflora prije i nakon skladištenja u hladnjaku tijekom šest mjeseci, u plastičnim vrećicama, na +4 °C, u tami, pri maloj vlažnosti i brzini isparavanja. Za identifikaciju plijesni kombinirane su tradicionalne (makroskopske i mikroskopske) i molekularne (lančana reakcija polimeraze,

PCR) metode. Na početku istraživanja (nakon sazrijevanja i uzimanja uzoraka kobasica) prevladavale su izolirane vrste iz roda *Penicillium* (74 %), *Aspergillus* 14 % i *Mucor* (12 %). Nakon razdoblja od šest mjeseci uočena je drugačija struktura plijesni, pri čemu je pretežno izoliran rod *Aspergillus* (64 %), a zatim *Penicillium* (34 %) i *Mucor* (2 %). Sagledavajući literaturne podatke može se zaključiti da se mikoflora na ovitcima trajnih fermentiranih kobasica pohranjenih u hladnjaku mijenjala na isti način kao što bi se i mijenjala bez pohranjene u hladnjaku, jedino što bi proces promjena mikoflore vremenski trajao kraće.

Ključne riječi: mikoflora, površinske plijesni, trajne fermentirane kobasice, ovitci, pohrana u hladnjaku