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Autochthonous functional starter cultures and mycotoxins in "SLAVONSKI KULEN"

UDC: 637.523 (497.54)

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Summary

The aim of this work was to investigate possible presence of mycotoxins from "Slavonski kulen", produced in rural households. The presence of mycotoxins was determined in 6 of 8 analyzed samples. Mycotoxins concentrations were 0.02 to 1.6 ng for ochratoxin A (OTA) and 0.1 to 231 ng for aflatoxin B1 (AFB1) and were defined not only on the surface layer, but also in the centre of "Slavonski kulen". Also, the microbial population of the traditional "Slavonski kulen" was identified and subjected to technological and functional characterization in order to select potential autochthonous functional starter cultures. Dominant microflora was lactic acid bacteria (LAB), and from the surface 6 of 8 analyzed "Slavonski kulen" were isolated molds from the genera *Penicillium* sp. and *Aspergillus* sp. All lactobacilli isolates produced a significant amount of lactic acid and showed antimicrobial activity against pathogenic test microorganisms.

Keywords: Slavonski kulen, autochthonous microbial populations, starter culture, mycotoxins

Introduction

Aflatoxins are toxic metabolites produced by fungi, e.g., *Aspergillus flavus* and *Aspergillus parasiticus*, growing on cereals, nuts, legumes, fruits and other susceptible crops. The results of their toxicity range from gastroenteritis to cancer. The presence of mycotoxins in food and feed depends on many biological factors, such as region, season, humidity, and temperature, as well as the conditions under which crops are harvested, stored and processed. When not controlled, these toxins can be transferred to animals and humans trough the ingestion of contaminated feed and food (Dashti et al., 2009). Endemic nephropathy (EN) is a fatal human kidney disease that occurs in the eastern part of the Croatian Brodsko-posavska County. In the 1970s, the aetiology of the

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disease was believed to be related to exposure to mycotoxin ochratoxin A (OTA) (Krogh, 1974). High incidence of otherwise rare urothelial tumours observed later in the same endemic region was also associated with this toxin (Čeović et al., 1991; Miletić-Medvedev, 2005). Studies on laboratory and domestic animals have shown that OTA is nephrotoxic, carcinogenic, genotoxic, and immunotoxic (IPCS, 2001). The International Agency for Research on Cancer (IARC) has classified OTA as Group 2B carcinogens (possible human carcinogen).

There is a wide variety of traditional sausages and meat products in Croatia and the most famous is "Slavonski kulen". Traditional fermented sausages are manufactured without addition of starter cultures in small-scale processing units. Thus the fermentation of these products only relies on the indigenous microbial flora whose composition is variable and the growth promoted by the environmental conditions (Cocolin et al., 2001). Nowadays, the need for safe products with standard and desirable technological properties has resulted in the use of starter cultures for the production of the dry fermented sausages, to control the fermentation and ripening process, inhibiting the growth of other undesirable microorganisms (Drosinos et al., 2005). Generally, starter cultures consist of lactic acid bacteria, Gram-positive catalase-positive cocci (*Staphylococcus*, *Kocuria*), yeasts and moulds, depending on the sausage type (Drosinos et al., 2007).

Lactic acid bacteria (LAB) play an important role in meat preservation and fermentation processes and are considered technologically fundamental. They are able to decrease pH by lactic acid production, produce bacteriocins to prevent the growth of pathogenic and spoilage microorganisms, provide diversity by the modification of raw material to obtain new sensory properties, improve the safety, the stability and the shelf life of meat products (Fontana et al., 2005; Frece et al., 2005a; Frece et al., 2009) and they also contribute to the development of flavour, colour and texture. Several studies suggested that *Staphylococcus* species, rather than LAB, play an important role in the development of sensory properties (flavour, texture, colour) of fermented sausages by reduction of nitrates, proteolytic and lipolytic activities (Mauriello et al., 2004; Olesen et al., 2004). Moreover, the ability of CNS to produce antimicrobial compounds may improve safety and shelf-life of sausages (Simonova et al., 2006).

The main challenge in developing starter cultures is to improve safety, but also to preserve the typical sensory quality of traditional sausages (Talon et al., 2008). The most promising microorganisms for starter cultures are those which are selected from autochthonous microflora since they are well adapted to the meat environment and to the specific manufacturing process and are capable of dominating the microbiota of the product due to their specific metabolic capabilities.

Nowadays, increasing number of studies is focused on the isolation and identification of autochthonous functional starter cultures, with the aim of

developing new functional meat products, which will be recognized and labelled as autochthonous due to the influence of climate and vegetation of the region in which they are produced (Frece, 2007). Examples include microorganisms that generate aroma compounds, health-promoting molecules, bacteriocins or other antimicrobials, contribute to cured meat colour, possess probiotic qualities, or lack negative properties such as the production of biogenic amines and toxic compounds (Leroy et al., 2006).
The aim of this study was to investigate possible presence of mycotoxins from the "Slavonski kulen", identify and characterize naturally present microbial population, especially lactic acid bacteria on the basis of their important technological properties in order to select potential autochthonous functional starter cultures.

Materials and Methods

Classical microbiological and biochemical (API) methods for isolation and identification of microorganisms were shown in Table 1.

Table 1. Classical microbiological and biochemical (API) methods for isolation and identification of microorganisms

Microorganisms	Method	Nutrient media	Incubation condition	API test
Staphylococcus spp.	ISO 6888-1:1999 [a]	BP (Merck)	37 ºC 48 hours	API STAPH V4.1
Lactic acid bacteria	ISO 13721:1995 [b]	MRS agar (Biolife)	30 ºC 48 - 72 hours	API 50 CHL V5.1
Yeasts and moulds	ISO 13681:1995 [c]	Sabouraud agar (Biolife)	25 ºC 48 - 72 hours	API 20 C AUX V4.0 Yeasts

[a]Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of coagulase-positive staphylococci (Staphylococcus aureus and other species) -- Part 1: Technique using Baird-Parker agar medium
[b]Meat and meat products -- Enumeration of lactic acid bacteria -- Colony-count technique at 30 degrees C
[c]Meat and meat products -- Enumeration of yeasts and moulds -- Colony-count technique

Technological characterization of lactic acid bacteria

Lactic acid bacteria species isolates were subjected to technological characterization.

Effect of NaCl and temperature on microbial growth and proteolytic activity

Effect of 5 % NaCl, temperature (12 ºC, 18 ºC and 22 ºC) and proteolytic activity of LAB species isolates were tested according to Bonomo et al., 2008.

Determination of lactic acid production by HPLC

The ability of lactic acid bacteria to produce lactic acid was measured according to Trontel, et al. (2010) and expressed in g/l.
Sample pretreatment and analysis of glucose and product concentrations in it using high-pressure liquid chromatography (HPLC) was performed as described previously (Trontel et al., 2010). Glucose, lactic acid, acetate and ethanol were obtained from the Sigma-Aldrich (Bellefonte, USA). H_3PO_4 (85 % v/v) (Sigma-Aldrich, Hamburg, Germany) was used to prepare the mobile phase (0.1 % v/v H_3PO_4), and deionized water with conductivity < 1 μS was used to prepare the mobile phase and standard solutions. The experimental set-up consists of a Shimadzu Class-VP LC-10A$_{VP}$ system (Shimadzu, Kyoto, Japan). The piston pump (LC-10AD$_{VP}$) delivered the mobile phase at 0.5 mL min^{-1}. The substrate and product were separated using a Supelcogel^{TM} C-610H (30cm x 7,8 mm ID, 9μm) analytical column with a Supelcogel^{TM} H (5 cm x 4.6 mm ID, 9 μm) guard column (both supplied by Sigma-Aldrich; Hamburg, Germany), and detected by a refractive index detector (RID-10A).

Antimicrobial activity

Antimicrobial activity of lactic acid bacteria isolates from the "Slavonski kulen" was tested by turbidimetric method (Frece, 2007; Leboš et al., 2008).
The results were expressed as positive (+) on antimicrobial activity if LAB isolates were inhibit the growth of test microorganisms (during 72 hours of cultivation). Test microorganisms used were following: (i) *Staphylococcus aureus* 3048, (ii) *Escherichia coli* 3014, (iii) *Salmonella typhimurium* 3064 and (iv) *Listeria monocytogenes* ATCC 23074 (all from the Collection of microorganisms of Laboratory for General Microbiology and Food Microbiology, Faculty of Food Technology and Biotechnology, University of Zagreb, Croatia). Test microorganisms were grown at 37 ºC for 74 h in Nutrient broth (beef extract 3g/l; peptone 5 g/l; Biolife, Milano, Italy).

Sensitivity to oxgall bile

Resistance to bile was tested according to Gilliland and Walker (1990). Brain Heart Infusion broth (BHI, Becton and Dickinson) was prepared by the addition of 1 % (w/v) oxgall (Becton and Dickinson). The volume 50 μl of an 18 h culture of each strain was added to 5 ml of BHI broth with oxgall. After incubation at 37 ºC for 24 h, the bacterial growth of strains was measured using a spectrophotometer (Helios ε, "Unicam", USA) at 600 nm. Numbers of viable cells were estimated at 0 h and after 24 h of incubation on MSA agar (Simonova et al., 2006).

Determination of aflatoxin B1 and ochratoxin A by ELISA (Enzyme Linked immunosorbent assay) method

Aflatoxin B1 was determined using Immunolab Aflatoxin B1 kit, cat. No. AB1-E01, and ochratoxin A using the Neogen kit - Veratox 8610th. The results were reading on a microplate reader (Tecan, Sunrise), at the absorbance of 450 nm for AFB1, and at 650 nm for ochratoxin A.

Statistical analysis

All experiments were carried out in triplicate. The results are expressed as mean ± S.D. (standard deviation). The SAS statistical computer package was used to analyze the experimental data (SAS Institute, Cary, NC, USA).

Results and discussion

Since on area six of eight samples of "Slavonski kulen" were isolated fungi from the genera *Aspergillus* sp. and *Penicillium* sp. (Table 3), the potential presence of ochratoxin A and aflatoxin B1, were measured by ELISA method (Table 3). Sources of mycotoxins in the samples may be the mold, or spices added to the "Slavonski kulen". Sampling was conducted according to the Regulation on sampling and analysis methods for official control of the amount of mycotoxins in food (NN 45/08), and samples were taken at a depth of 1 and 2 cm measured from the surface and from the mid of the "Slavonski kulen", to see how deep mycotoxins can penetrate into the kulen (Table 3). In six of eight samples of the "Slavonski kulen" from which were isolated molds it was proven the presence of AFB1 from 0.1 to 231 ng and ochratoxin A from 0.02 to 1.6 ng. From the results of research, it can be concluded that the isolated *Aspergillus* sp. and *Penicillium* sp. can synthesize mycotoxins, since in the samples from which were not isolated molds, were not detected mycotoxins (Table 3). It is interesting that ochratoxin A was found in the middle of "Slavonski kulen", while AFB1 was found only on the surface and in a depth of 1 cm. Furthermore, in a sample of "Slavonski kulen" from which were isolated both moulds *Aspergillus* sp. and *Penicillium* sp., it was evidenced lower concentration of ochratoxin A and AFB1, compared to samples from which were isolated pure cultures of moulds (Table 3). This can be explained that the *Penicillium* sp. and *Aspergillus* sp. act antagonistically, inhibiting the growth and production of mycotoxins. Our results are in agreement with the results of other authors (Mandić et al., 2007), who also determine ochratoxin A in the value of 3.8 ng, and AFB1 in the value of 1.33 ng in dry fermented sausages. It should be noted that the permissible amount of ochratoxin A in meat products are not regulated by any legislation at EU level or in Croatia. Classical microbiological and biochemical (API) methods (Table 1) were used for identification of microorganisms isolated from a traditionally

produced "Slavonski kulen". The results of microbiological analysis showed that the dominant microflora in the samples of the "Slavonski kulen" were lactic acid bacteria (Table 2). Lactic acid bacteria counts were from 3.0 ± 2.1 to 9.23 ± 1.7 $\log_{10}$ CFU/g (Table 2).

Table 2. Technological characteristics of LAB species isolates and some of the selection criteria for starter cultures

Technological characteristics and selection criteria	**LAB species**				
	L. plantarum	***L. delbrueckii***	***Leuconostoc mesenteroides***	***L. brevis***	***Lactococcus lactis***
$\log_{10}$ CFU/g samples of "Slavonski kulen"	4.70 ± 1.2	3.0 ± 2.1	9.23 ± 1.7	8.30 ± 1.2	8.0 ± 1.5
Growth in the presence 5 % NaCl	+	+	+	+	+
Growth at 12 ºC	+	-	+	+	+
Growth at 18 ºC	+	+	+	+	+
Growth at 22 ºC	+	+	+	+	+
Proteolytic activity	+	+	+	+	+
Homofermentative species	+	+	-	-	+
Heterofermentative species	-	-	+	+	-
Catalase test	+	+	-	+	+
Concentration of lactic acid (g/l)	21.96 ± 1.1	26.95 ± 0.6	14.76 ± 0.4	16.26 ± 0.3	18.76 ± 0.2
pH of the medium	3.65 ± 1.3	3.25 ± 2.3	3.95 ± 2.5	3.37 ± 2.1	3.45 ± 1.5
$\log_{10}$ CFU/g with 1 % oxgall (viability)	6.3 ± 0.9	5.2 ± 2.3	5.8 ± 1.5	5.02 ± 1.7	5.25 ± 2.2
Antimicrobial activity	+	+	+	+	+

The results of identification showed that dominant LAB species isolated in our study were *Lactobacillus plantarum, Leuconostoc mesenteroides*, *Lactobacillus delbrueckii, Lactobacillus brevis and Lactococcus lactis* (Table 2). Furthermore, the growth rate at different temperatures (2-4 to 24 °C), the tolerance of salt concentrations of 2-10 (max 15) %, and of pH in the range 4.2-6.0 are limiting factors affecting the persistence and competitiveness of the starter culture over the entire fermentation and ripening process (Ammor and Mayo, 2007). Thus, technological characterization; the ability of the isolates to grow at 12, 18 and 22 °C and in the presence of 5 % NaCl was tested (Table 2). In our study, all LAB isolates were shown good technological characteristics, because they able to grow in the presence of 5 % NaCl and at 18 and 22 °C (Table 2). Furthermore, all LAB isolates, except *Lactobacillus acidophilus* 7K2, also grew at 12 °C. Technologically relevant properties such as proteolytic activity of LAB isolates were determined in this study. Lactic acid bacteria usually do not possess strong proteolytic properties, although a degree of peptidase activity has been observed for some meat strains (Leroy et al., 2006). According to the results obtained in this study, all LAB isolates showed proteolytic activity (Table 2). Our study confirmed results of previous study

(Bonomo et al., 2008) reporting that most of lactic acid bacteria (71 %) showed a middle-low proteolytic activity and a small group (29 %) had a higher ability. The results of our study showed that the most of LAB isolates were homofermentative (Table 2), so they do not produce gas from glucose. Heterofermentative LAB are not suitable for sausage production because the formation of large amounts of carbon dioxide leads to holes of different sizes in the product. In addition these LAB produce acetic acid that causes a pungent off-flavour (Ammor and Mayo, 2007). Furthermore, all LAB species isolated in our study, except *Leuconostoc mesenteroides* 6K1, were catalase positive (Table 2). Catalase activity is one of the desired properties for starter cultures used in food fermentation as it can prevent rancidity and colour defects in the products due to the effect of hydrogen peroxide (Lücke, 1985).

The production of lactic acid, which results in a decrease of the pH, contributes to the development of texture, colour and acid taste, prevents the growth of pathogenic and spoilage microorganisms and thus improving safety and stability of the meat products. The ability of LAB isolates to produce lactic acid is shown in Table 2. The results showed that all isolated lactobacilli produced a significant amount of lactic acid between 14.76 ± 0.4 and 26.95 ± 0.6 g/l. The production of lactic acid by LAB isolates resulted in pH reduction in the substrate between 3 and 4. These results are in agreement with the results obtained by Bonomo et al. (2008) who reported that 65 % of LAB strains had good activity, while other strains showed a high acidifying capability with maximum decrease in pH of 2.15. Since one of the most important characteristics of functional starter cultures is inhibition of pathogens, in this paper, antimicrobial activity of the isolated potential starter cultures against pathogenic microorganisms from food was investigated. The presence of the most frequently found pathogens, e.g., *S. aureus*, *L. monocytogenes*, *E. coli* and *Salmonella* spp. can be controlled by a combination of low pH, competitive exclusion with starter cultures and/or bacteriocin production (Frece et al. 2010; Markov et al., 2009; Simonova et al., 2006). The main antimicrobial effect responsible for safety is evidently the rate of acidification of the raw meat (Lücke, 2000). Nevertheless, certain antimicrobials such as bacteriocins may also play a role, in particular in slightly acidified products or to eliminate undesirable microorganisms that display acid tolerance (Leroy et al., 2006). Preliminary study results, obtained by disk-diffusion method, showed that all LAB species displayed high antimicrobial activity against pathogenic test microorganisms and the inhibition zones were from 20 ± 1.7 to 24 ± 2.3 mm (the results not shown). Therefore, the growth of the pathogenic test microorganisms in the presence of the isolates was followed by turbidimetric method. The results obtained by turbidimetric method showed that all LAB isolates displayed significant inhibition of the growth of all investigated pathogenic test microorganisms during 72 hours of cultivation (Table 2). Percentage of growth inhibition of *E. coli*, *S. aureus*, *Salmonella* spp. and *L. monocytogenes* in the presence of LAB isolates after 72 hours was 69.71-85.34 %, 59.29-86.89 %, 74.91-88.59 % and 57.91-82.39 %, respectively (the results not shown).

In order to act as a probiotic in the gastrointestinal tract the bacteria must be able to survive the acidic conditions of the stomach and resist the bile acids at the beginning of the small intestine (Erkkilä and Petäjä, 2000; Frece et al., 2005a; Frece et al., 2009; Frece et al., 2005b). Therefore, we investigated resistance of lactic acid bacteria isolates to 1 % oxgall bile at pH 2.5. The results of our study showed that among LAB isolates *Lactobacillus plantarum* 1K and *Lactobacillus delbrueckii* 2K were particularly resistant to bile since they displayed growth in the presence of 1 % bile at pH 2.5 (Table 2). The results obtained in this study are in agreement with previous studies, which underlined how *Lactobacillus plantarum* isolated from fermented sausages was the most resistant to bile salts (Pennacchia et al., 2004). Moreover, Vinderola and Reinheimer (2003) reported the resistance of commercial and collection probiotic strains of *Lactobacillus delbrueckii* to bile, which is in accordance with the results of our study. In our study, the most sensitive lactobacilli isolates were *Leuconostoc mesenteroides* 6K1 and *Lactobacillus acidophilus* 7K2 since their number was reduced in the presence of 1 % bile at pH 2.5.

Table 3. Concentrations of aflatoxin B1 and ochratoxin A (ng) in samples of "Slavonski kulen"

Samples of "Slavonski kulen" and genera of moulds	**Ochratoxin A (ng)**	**AFB1 (ng)**
K1 *Penicillium* sp.isolated		
1 cm deep	1.2 ± 1.2	0.1 ± 0.2
2 cm deep	1.0 ± 1.5	< 0.1 ± 0.2
center	1.0 ± 1.4	< 0.1 ± 0.3
K2 *Aspergillus* sp. isolated		
1 cm deep	1.49 ± 1.7	0.3 ± 1.2
2 cm deep	1.39 ± 2.2	< 0.1 ± 0.2
center	1.06 ± 1.2	< 0.1 ± 0.2
K3 moulds were not isolated		
1 cm deep	< 0.05 ± 0.1	< 0.1 ± 0.2
2 cm deep	< 0.05 ± 0.2	< 0.1 ± 0.1
center	< 0.05 ± 0.2	< 0.1 ± 0.2
K4 moulds were not isolated		
1 cm deep	< 0.05 ± 0.2	< 0.1 ± 0.3
2 cm deep	< 0.05 ± 0.1	< 0.1 ± 0.3
center	< 0.05 ± 0.2	< 0.1 ± 0.2
K5 *Aspergillus* sp. isolated		
1 cm deep	1.5 ± 0.2	0.475 ± 1.2
2 cm deep	1.6 ± 1.5	< 0.1 ± 0.2
center	1.5 ± 1.2	< 0.1 ± 0.2
K6 *Aspergillus* sp. + *Penicillium* sp. isolated		
1 cm deep	1.09 ± 0.4	0.1 ± 1.1
2 cm deep	0.94 ± 1.2	< 0.1 ± 0.2
center	0.94 ± 0.7	< 0.1 ± 0.2
K7 *Aspergillus* sp. isolated		
1 cm deep	0.02 ± 0.3	150 ± 2.2
2 cm deep	0.02 ± 0.5	125 ± 3.2
center	0.01 ± 0.1	110 ± 1.2
K8 *Penicillium* sp. isolated		
1 cm deep	0.4 ± 0.5	231 ± 1.2
2 cm deep	0.4 ± 0.2	210 ± 1.4
center	0.2 ± 0.2	190 ± 1.1

Conclusions

Lactobacillus species isolated in this study could be considered as a potential autochthonous functional starter cultures since they are natural microflora of the "Slavonski kulen" and possess desirable technological and probiotic characteristics regarding the growth capability at different temperatures and salt concentrations, proteolytic activities, production of lactic acid, antimicrobial activity against pathogenic microorganisms and tolerance to bile salts. This work can be considered as preliminary study in developing an autochthonous functional starter culture with the aim of improving safety of the product by inhibiting the growth of undesirable microorganisms and preserving typical sensory quality of the product. Further studies should be carried out to detailed phenotypic, genotypic and physiological characterization of isolated strains of LAB, with the purpose of creating a "bank" of indigenous functional starter cultures for fermented meat products.

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