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Mini Review

Selection and application of autochthonous functional starter cultures in traditional Croatian fermented sausages

[1*]Zdolec, N., [1]Dobranić, V., [2]Horvatić, A. and [3]Vučinić, S.

[1]University of Zagreb, Faculty of Veterinary Medicine, Department of Food Hygiene, Technology and Safety. Heinzelova 55, 10000 Zagreb, Croatia
[2]Ruđer Bošković Institute, Bijenička cesta 54, 10000 Zagreb, Croatia
[3]Croatian academy of sciences and arts, Zrinski trg 11, 10000 Zagreb, Croatia

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Abstract

Microbiota of the traditional fermented sausages is a reach source of potential starter cultures for artisan or industrial applications. Lactic acid bacteria (LAB) and coagulase-negative cocci (CNC) are the main microbial groups involved in the meat fermentation and routinely implemented in sausage production as starters, in order to upgrade product's safety and quality. The strain selection is based on technological and safety properties which should be tested by phenotypic and genotypic methods. Further characterization by proteomic approaches could give us an additional knowledge on metabolic activity of the culture under specific conditions and enable the optimization of the fermentation process. Although dry fermented sausages are microbiologically stabile products, some specific hazards could occur. Due to the spreading of antimicrobial resistance, a risk potential of commensal bacteria such as LAB and CNC is still questionable. Thus competitive and protective functional starter cultures contribute to the reduction or elimination of microbiological and toxicological risks.

Introduction

Regional food specialties in Croatia, such as dry fermented meat products have been traditionally manufactured in rural households and family farms. In general, these are traditional products that should be recognized as autochthonous Croatian products. Only some of these (e.g. dry fermented sausage "kulen") are produced under control in rural manufacturing facilities and then put on the market (Kozačinski *et al*., 2008a). Since natural production is rather "extensive" than standardized and constant, there are efforts to improve hygienic and technological conditions thorough legislation and practice. The fact that traditional sensorial features of sausages can't be achieved in industrial scale production, gives the chance to private producers or small and medium enterprises (SMEs) for protection of autochthonous foods. However, it is expected that some kind of fermented sausage (e.g. kulen) has the same standard quality in all facilities related to equipment, raw materials, additives, technological parameters (smoking, ripening chambers). In the Northwest part of Croatia, the traditional fermented sausages are manufactured from pork, beef, pork back fat with the addition of salt and specific spice mixture (ground black pepper, minced red pepper, garlic), filled in natural swine casings, smoked and ripened at lower temperatures (Kozačinski *et al*., 2006a). There are also efforts for protection of traditional horsemeat dry fermented sausages (Šimić and Mioković, 2008; Alagić *et al*., 2008; Alagić *et al*., 2011a; Alagić *et al*., 2011b). The east part of Croatia is known for traditional Slavonian homemade dry sausage and kulen which are recognized in specific peppery flavor. Adriatic part of Croatia is more specialized in traditional dry cured meat products such as Dalmatian smoked ham or Istrian ham.

Fermentation is one of the oldest processes of meat preservation, which depends on the biological activity of indigenous microbiota (Ross *et al*., 2002; Hutkins, 2006). Knowledge about the role of microorganisms in the food fermentation dates long ago, so that individual strains, considered fit from the technological and hygienic point of view, have been introduced in the production of fermented foodstuffs in form of starter cultures. In case of fermented sausages, these are lactic acid bacteria and coagulase-negative cocci, prevailingly species of the genus *Lactobacillus*, *Pediococcus* and *Staphylococcus* (Hutkins, 2006). Naturally fermented sausages with autochthonous "wild" microbial

*Corresponding author.
Email: *nzdolec@vef.hr*

populations are rich source of potential starter cultures with favorable technological and hygienic properties for food applications. For selection of potential functional starter cultures, the strains must be phenotypically and genotypically characterized, including technological, safety and probiotic features (Ammor and Mayo, 2007). However, results of *in vitro* studies at given laboratory conditions could be promising, but performance of the culture in real food fermentation may not be relevant. In this review, we summarize Croatian recent findings related to traditional fermented sausages from microbiological point of view with particular emphasis to selecting/excluding criteria for autochthonous starter cultures.

Microbial studies of Croatian traditional fermented sausages

Microbiological succession during the maturation of dry fermented sausages is a complex and constant process. It is dependent on many factors such as initial contamination, intrinsic and extrinsic hurdles - salt, pH, water activity, nitrites, casings, temperature and moisture (Zdolec, 2007). Process of acidification and desiccation are favorable for stabilizing microbial associations constituted mainly from lactic acid bacteria and micrococci-staphylococci group. Recent studies conducted on Croatian fermented sausages from microbiology point of view were related to monitoring of indigenous microbial changes during the ripening (Kozačinski *et al*., 2006a; Zdolec *et al*., 2007a; Alagić *et al*., 2008), determination of autochthonous microbiota (Kozačinski *et al*., 2006a; Cvrtila *et al*., 2008; Zdolec *et al*., 2009; Frece *et al*., 2010a; Babić *et al*., 2011), selection of potential protective strains/cultures (Zdolec *et al*., 2009; Frece *et al*., 2010), artificially inoculation of sausages with foodborne pathogens (Hadžiosmanović *et al*., 2005; Drosinos *et al*., 2006; Čaklovica *et al*., 2006; Kozačinski *et al*., 2006b; Zdolec *et a*l., 2007a; Zdolec *et al*., 2007b; Zdolec *et al*., 2007c; Zdolec *et al*., 2008a), application of starter cultures, protective cultures or bacteriocins (Zdolec, 2007; Zdolec *et al*., 2008b; Zdolec *et al*., 2008c; Zdolec *et al*., 2008d; Medić *et al*., 2009; Nežak *et al*., 2011) and determination of antimicrobial resistance (Zdolec *et al*., 2011).

Identification of indigenous microbiota

Identification of indigenous microbiota was performed by means of culture-dependent as well as culture-independent methods (Table 1). In general, good correlation is found for traditional and molecular methods of identification for main LAB and CNC species in different European fermented sausages. However, molecular identification showed that phenotypic methods are unable to identify some lactic acid bacteria/coagulase-negative cocci (LAB/CNC) species (Iacumin *et al*., 2006), suggesting that culture-independent methods should be the first choice for studying bacterial diversity and ecology of fermented sausages (Rantsiou and Cocolin, 2006). Biochemical and molecular identification of intrinsic LAB or CNC in different types of Croatian sausages showed the domination of *Lactobacillus plantarum* and S*taphylococcus xylosus*. Those species are also found as dominant in other Mediterranean countries (Iacumin *et al*., 2006; Drosinos *et al*., 2007; Albano *et al*., 2009). One recent study (Babić *et al*., 2011) reported *Leuconostoc mesenteroides* as one of dominant LAB in Croatian dry sausage "kulen", which implicates even more the need for introducing competitive technologically suitable starter cultures, because that species is heterofermentative and can cause sensorial deviations of sausages.

Table 1 Results of determination of indigenous microbiota of different types of traditional Croatian fermented sausages

Strains selected	Sausage type	Methods of characterization	Reference
Lb. plantarum *Lb. delbrueckii* *Ln. mesenteroides* *Lb. acidophilus* *S. xylosus* *S. warneri* *S. lentus* *S. auricularis* *C. famata* *Penicillium* sp., *Aspergillus* sp.	Kulen	API 50 CHL API STAPH API 20C AUX RAPD PCR	Frece *et al*., 2010b Babić *et al*., 2011
Lb. plantarum *Lb. brevis* *Lb. curvatus* *Lb. pentosus* *Lb. fermentum* *Ln. mesenteroides* *Lc. lactis* *P. pentosaceus* *S. xylosus* *S. capitis* *S. camosus* *S. saprophyticus*	Artisan traditional fermented sausage from North-West Croatia	API 50 CHL API STAPH PCR	Kozačinski *et al*., 2006a Kozačinski *et al*., 2008b Cvrtila *et al*., 2008
Lb. plantarum *Lb. fermentum* *Lb. pentosus* *Lb. delbrueckii* *W. viridescens* *W. confusa*	Artisan horse-meat dry sausage	API 50 CHL	Alagić *et al*., 2011a Alagić *et al*., 2011b
Lb. curvatus *Lb. brevis* *Ln. mesenteroides*	Homemade traditional fermented sausage from North-West Croatia	API 50 CHL	Zdolec *et al*., 2007a
Lb. acidophilus *S. xylosus*	Homemade traditional fermented sausage from North Croatia	API 50 CHL API STAPH	Frece *et al*., 2010d
Lc.lactis subsp. *lactis* *C. zeylanoides*	Horsemeat dry sausage	API 50 CHL API 20C AUX	Markov *et al*., 2010
S. xylosus *S. warneri* *S. lentus* *S. auricularis*	Kulen	API STAPH	Frece *et al*., 2010c
Lb. fermentum *Lb. curvatus* *Lc. lactis* subsp. *lactis* *S. xylosus* *S. sciuri*	Homemade Slavonian sausage	API 50 CHL API STAPH	Dobranić *et al*., 2011

**Lb.=Lactobacillus*; *Ln.=Leuconostoc*; *C.=Candida*; *S.=Staphylococcus*; *P.=Pediococcus*; *W.=Weisella*; *Lc.=Lactococcus*

Antimicrobial activity of LAB strains/starter cultures

Most researches related to Croatian fermented sausages during last decade was focused on antimicrobial activity of selected lactic acid bacteria and their application as protective starter cultures, as shown in Table 2. Antimicrobial activity of LAB is expressed through synthesized organic acids (lactic, acetic), hydrogen peroxide, carbon dioxide, enzymes, reuterin, dyacethil and bacteriocins (Holzapfel *et*

Table 2 Results of testing antimicrobial activity of selected strains from traditional Croatian fermented sausages

Strains tested for antimicrobial activity	Indicator microorganism	Strains with strongest antimicrobial activity	Test methods	Reference
Lb. paracasei *Lb. casei* *Lb. brevis* *Lb. curvatus* *Lb. fermentum*	*L. monocytogenes* *L. welshimeri* *Y. enterocolitica* *P. aeruginosa* *S. aureus* *Salmonella* spp.	*Lb. paracasei* *Lb. brevis*	Agar spot test Agar well diffusion test	Zdolec *et al.*, 2009
Lb. plantarum *Lb. fermentum* *Lb. pentosus* *Lb. delbrueckii* *W. viridescens* *W. confusa*	*L. monocytogenes*	*Lb. plantarum*	Agar spot test Agar well diffusion test	Alagić *et al.*, 2011b
Lb. curvatus *Lb. brevis* *Ln. mesenteroides*	*L. monocytogenes*	*Ln. mesenteroides*	Agar spot test Agar well diffusion test	Zdolec *et al.*, 2007b
Lb. plantarum *Lb. delbrueckii* *Ln. mesenteroides* *Lb. acidophilus*	*E. coli* *S. aureus* *L. monocytogenes* *Salmonella* Typhimurium	*Lb. plantarum* *Lb. delbrueckii* *Ln. mesenteroides*	Turbidimetric assay	Frece *et al.*, 2010b Babić *et al.*, 2011
Lb. acidophilus	*E. coli* *S. aureus* *L. monocytogenes* *Salmonella* spp.	*Lb. acidophilus KA1*	Turbidimetric assay	Frece *et al.*, 2010d
Lc. lactis subsp. *lactis*	*E. coli* *S. aureus* *L. monocytogenes* *Salmonella* spp.	*Lc. lactis* subsp. *lactis 5K1*	Turbidimetric assay	Markov *et al.*, 2010
S. xylosus *S. warneri* *S. lentus* *S. auricularis*	*E. coli* *S. aureus* *L. monocytogenes* *Salmonella* spp.	*S. xylosus* *S. warneri* *S. lentus*	Disk diffusion Turbidimetric assay	Frece *et al.*, 2010c

**Lb.=Lactobacillus*; *Ln.=Leucoonostoc*; *S.=Staphylococcus*; *W.=Weisella*; *Lc.=Lactococcus*

al., 1995). Creating higher quantities of hydrogen peroxide, CO_2, dyacethil or acetic acid in fermented meat products isn't desirable from technological reasons despite the possible antimicrobial activity. However, the spectrum of LAB activity will depend on genetic-phenotypic characteristics of species, i.e. on ability of synthesizing products with antimicrobial properties in given conditions. *In vitro* studies of LAB antimicrobial properties showed promising results due inhibiting main food-borne pathogens. Many studies have reported the antimicrobial effect of LAB toward the bacteria *Listeria monocytogenes*, *Escherichia coli*, *Salmonella* spp., *Clostridium* spp., *Staphylococcus aureus* and others (Leroy *et al*., 2006). Inhibition of *L. monocytogenes*, *L. welshimeri*, *Salmonella Enteritidis*, *Salmonella* spp. and *Yersinia enterocolitica* was shown under laboratory conditions (Zdolec *et al*., 2009), but also in fermented sausages for *Listeria*, enterobacteria, enterococci, staphylococci and yeasts (Zdolec *et al*., 2007c; Zdolec *et al*., 2008d). Such cognitions nowadays lead to development of an increasing number of the new functional starter cultures comprised of species with known metabolic profile and proved antimicrobial effect.

Results observed indicate a strong protective potential of autochthonous LAB, as well as their favorable technological properties for industrial implementation. Pilot production of sausages with introduced new protective functional starter cultures or natural protective agents (bacteriocins) showed a significant improvement of microbial quality and sensorial features of final products (Zdolec *et al*., 2007c; Zdolec *et al*., 2008d). Thus, strong antilisterial activity of *Lactobacillus sakei* cultures was found during the ripening, despite unusually high levels of experimentally inoculated pathogen. However, natural ripening process was also able to reduce *Listeria* bellow limits of detection which is important finding regarding regular product's safety. Furthermore, *Lb. sakei* culture reduced enterococci, yeasts and total viable count during the ripening that could partially influence the sensorial features of final products. Upgrading of safety and quality by applying (non)bacteriocinogenic (non)autochtonous strains as starter cultures was reported in different kinds of fermented sausages and other fermented meat products all over the world (Liu *et al*., 2010; Laukova et al., 2010; Sriphochanart and Skolpap, 2010; Bonomo *et al*., 2011; Vatanyoopaisarn *et al*., 2011; Jaworska *et al*., 2011). Bacteriocin-based conservation strategy in food production is intensively tested during last decades, but using bacteriocins as additives is still questionable mainly due their un-stability in reach proteolytic matrix such as fermented sausages. However, most studies showed that sausages produced with bacteriocinogenic cultures are preferable than non-inoculated products or inoculated with non-bacteriocinogenic culture. A part of this product's improving should be attributed to bacteriocins produced *in situ* (Zdolec *et al*., 2007c; Liu *et al*., 2010; Laukova *et al*., 2010).

Potential hazards related to LAB and CNS

Selection of potential starter cultures for fermented sausages production should consider the potential hazards which are mainly related to presence of transferable antibiotic resistance markers in lactic acid bacteria or coagulase-negative cocci (Bernardeau *et al*., 2008; Talon and Leroy, 2011). It would be of particular importance to monitor the presence of same markers in animal LAB and/or CNC, because animals would be the first link of spreading antibiotic resistance through agri-food chain. Preventing the occurrence or reducing the number of resistant commensal bacteria in animals should be the prerequisite for easier selection of potential functional starter cultures from food (Zdolec, 2012).

Preliminary testing of antimicrobial susceptibility of LAB isolated from Croatian traditionally fermented sausages revealed in selection of several (multi) resistant strains (Zdolec *et al*., 2011). Recently, we have studied the resistance in CNC from sausages by means of qualitative-quantitative E-test and PCR method to detect *mec*A, *tet*M, *tet*K and *erm*B genes (Zdolec *et al*., in press). Preliminary results showed low prevalence of (multi)resistant strains,

but majority of them were resistant to erythromycin and tetracycline.

Proteomic approach

Technological expectations of potential starter culture should also rely on –omics characterisation of the strain and influence of sausage production conditions on the enzymatic expression of culture applied. For this purpose we preliminary screened proteomic profiles of two CNS strains from two spontaneously fermented sausages using 2-DE gels combined with mass spectrometry identification. Strains were identified to the genus level by PCR as staphylococci and by biochemical tests as *S. epidermidis* (Dobranić *et al*., in press). Proteomic analysis revealed differential expression among 31 proteins between two strains of different origin. Since *S. epidermidis* is opportunistic pathogen and even frequently found in spontaneously fermented sausages (Marty *et al*., 2012), the proteomic profiling could be a useful tool in epidemiological studies. However, proteomic approach is particularly important for studying protein-related succession during the maturation of fermented meat products (Gašo-Sokač *et al*., 2011), and assessment the influence of starter cultures.

Conclusion

Constant and recognizable quality of traditional fermented sausages is one of the main consumer's demands. However, many varieties of the same product are present on the market, which is result of different "standard procedures" applied. Selection of raw materials, additives, casings and microclimatic conditions should be standardized and employed in all production facilities for each sausage type. One of the leading factors in developing of product's uniformity is starter culture activity. Dominant autochthonous strains in LAB or CNC population which are isolated from the specific fermented sausage should be considered as potential starter cultures for the same product. These strains are most adapted to specific intrinsic ecology of specified fermented sausage and technological procedures implemented. However, domination of some LAB and CNC species/strains could result in products of lower quality and/or safety. For this reason selection procedure of potential starters should involve all potentially "risky" criteria such as toxigenicity, acquired transmissible antimicrobial resistance or technologically unacceptable pathways (production of gases, acetic acid etc.). For this purpose and for the specified sausage type, the proteomic approach could provide a deep insight into the real functionality of specific starter cultures in meat fermentation.

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