Enterococcus spp. isolated from meat, meat products and the manufacturing environment

Ines Lačanin[1], Marta Dušková[1,5], Josef Kameník[2], Ondrej Šedo[3,4], Zbyněk Zdráhal[3,4], Renáta Karpíšková[1,5]

[1]Department of Milk Hygiene and Technology
[2]Department of Gastronomy
Faculty of Veterinary Hygiene and Ecology
University of Veterinary and Pharmaceutical Sciences Brno
[3]Research Group Proteomics
Central European Institute of Technology (CEITEC)
[4]National Centre for Biomolecular Research
Faculty of Science
Masaryk University
[5]Veterinary Research Institute
Brno, Czech Republic

Abstract

Enterococcus spp. is the most controversial group of lactic acid bacteria that have been ascribed a beneficial or detrimental role in food and feed. The aim of our study was to monitor the occurrence of *Enterococcus* spp. in collected samples of meat and meat products. Enterococci originated from 34 meat and meat product samples collected from several producers in the Czech Republic, and a number of swab samples (n = 7) were also taken from the environment whit which meat comes into contact during manufacturing. Identification of isolates was performed by MALDI-TOF MS profiling. Totally 58 *Enterococcus* spp. included *E. faecalis, E. faecium, E. gilvus, E. thailandicus, E. divriesei, E. hermanniensis, E. hirae, E. casseliflavus* and *E. durans* were identified.

Enterococci, environment, MALDI-TOF MS, meat

Introduction

The genus *Enterococcus* is the most controversial and one of the largest groups belonging to the lactic acid bacteria (LAB) group. A gram-positive homofermentative cocci, occurring singly or in pairs can be found in a variety of habitats including humans and animals. From the taxonomic point of view, enterococci have been reviewed several times and today consist of more than 50 species of which *Enterococcus faecalis* and *Enterococcus faecium* are the most important (Giraffa 2003 and Khan et al. 2010). These bacteria play an important role in food and feed fermentation and nowadays these strains are frequently used as probiotics. They are considered as potential cholesterol-lowering agents, for treatment of gastrointestinal diseases and for immune regulation (Franz et al. 2011). With their ability to produce enterocins (class II of bacteriocins) *Enterococcus* strains can provide natural preservation of dairy products and a hurdle to the growth of other microorganisms (antimicrobial activity against spoilage or pathogenic bacteria such as *Clostridium* spp., *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus*). On the other hand, enterococci can also have a negative influence. They can cause diseases as opportune pathogens and also they carry enzymes involved in the production of biogenic amines (Fouquié Moreno et al. 2006 and Franz et al. 2011). Enterococci also possess efficient gene transfer mechanisms. They are able to exchange genes of antibiotic resistance in various environments with a wide range of bacteria including their own species, other pathogens such as *Staphylococcus aureus* and *Listeria* spp., and non-pathogenic species (Pesaveto et al. 2014).

Address for correspondence:
Ines Lačanin, mag.nur.
Department of Milk Hygiene and Technology
Faculty of Veterinary Hygiene and Ecology
University of Veterinary and Pharmaceutical Sciences Brno
Palackého tř. 1946/1, 612 42 Brno, Czech Republic

Phone: +420 541 562 728
E-mail: ilacanin@vfu.cz
www.maso-international.cz

The aim of this study was to monitor the occurrence of *Enterococcus* spp. in collected samples of meat and meat products.

Materials and Methods

The enterococci originated from 41 samples collected from several producers in the Czech Republic. A total of 34 samples were collected consisting of samples and swab samples of meat and meat products, while additional samples ($n = 7$) were taken from the environment with which meat comes into the contact during manufacturing: 6 swab samples and one sample of spices taken randomly from one producer. The origin and frequency of the collected samples is presented in Table 1.

Table 1.The origin and number of enterococci isolates

	Commodity	Origin of the samples*	No. of positive samples	No. of suspected isolates
Environment	Weighing scale	b	1	1
	Hydrolysate	a	1	1
	Case	b	1	1
	Brine	a, b	3	6
	Spices	g	1	3
	Vysočina salami	a, c, e, f, h	24	32
	Mixed meat for the preparation of Vysočina salami	a	1	2
	Vienna frankfurters	d	1	2
Meat and meat products	Smoked pork ham with brine	a	3	4
	Pork shoulder	a	1	1
	Raw pork ham after injection	b	1	1
	Raw pork ham after tumbling	b	1	1
	KrásenskyUherák salami	a	1	2
	Hot pepper salami	a	1	1
Total			41	58

* a – h: factories from which the samples were taken

Basic processing of the samples was carried out immediately after sampling according to the ISO 7218 and ISO 6887-1 Standards. The amount of 25 g of meat sample was diluted in 225 ml of MRS broth (Oxoid, England). Environmental surfaces and meat were swabbed with a sterile swab (area 100 cm^2) and placed in a tube with MRS broth. Different dilutions were made from which 200 µl was aseptically spread on MRS agar (Oxoid, England) and cultivated aerobically at 30 °C for 72 hours. All colonies from each sample that showed different morphological characteristics were selected and purified for further characterization. The cultures were also tested for the presence of catalase and oxidase (Erba Lachema, Czech Republic).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used for the identification of isolates. Only isolates with negative catalase and oxidase tests (Erba Lachema, Czech Republic) were used for MALDI-TOF MS analysis and were prepared according to a standard protocol (Freiwald and Sauer 2009). Mass spectra measurements were carried out using an Ultraflex III instrument (Bruker Daltonik, Bremen, Germany) operated in the linear positive ion mode using FlexControl 3.0 software. Mass spectra were processed using Flex Analysis (version 3.0; Bruker Daltonik) and BioTyper software (version 3.0; Bruker Daltonik). The identification results were expressed by BioTyper log(scores) indicating the similarity of the unknown MALDI-TOF MS profile to available database entries. A BioTyper log(score) greater than 2.00 indicates a highly confident identification at the species level. A BioTyper log(score) between 1.70 and 1.99 means identification at the species level with lower confidence.

Results and Discussion

In this study the occurrence and identification of *Enterococcus* spp. were investigated with MALDI-TOF MS. MALDI-TOF MS was used in this work as the method of choice for the rapid and reliable identification of already suspected LAB that can be found in selected

samples. A total of 58 suspected enterococci isolates were obtained from 41 collected samples (Table 1). Identification to the species with high confidence (with Biotyper log(score) greater than 2.00) was obtained for 50% of the isolates. The identification outputs were, in most cases, in concordance with MALDI-TOF MS-based cluster analyses (Plate VI, Fig. 1). Identification by MALDI-TOF MS showed the presence of *E. faecalis* and *E. faecium*, which are the two most common *Enterococcus* species appearing in food and foodstuff, as the most abundant isolates (71%) in the analyzed samples.

In summary, of the identified *Enterococcus* spp., 26 were identified as *E. faecalis* and 15 as *E. faecium,* while the remaining isolates were identified as *E. gilvus* (n = 6), *E. thailandicus* (n = 6) and only one from each as: *E. divriesei, E. hermanniensis, E. hirae, E. casseliflavus* and *E. durans* (Plate VI, Fig. 1).

Meat is a commodity of high nutritional value. Its high water, protein and other water-soluble constituent content makes this foodstuff a perfect medium for the growth of microorganisms, involving either initial microflora or microorganisms that can cause its spoilage (Fernandes 2009). They may occur either inside or on the surface of the product.

The MALDI-TOF MS is recognized as one of the bacterial chemotaxonomic methods that is widely applied for the identification and typing of microorganisms due to its simplicity, reliability and high specificity (Mazzeo et al. 2006; Carbonnelle et al. 2011; Šedo et al. 2011; Doan et al. 2012 and Mimica et al. 2013). In their study, Santos et al. (2015) tested and verified that MALDI-TOF MS can be used for the identification of *Enterococcus* spp. The results obtained by MALDI-TOF MS showed that more than half of the analyzed samples (n = 27; 66%) contained strains of *E. faecalis* and *E. faecium,* two of the enterococci occurring most frequently in food and foodstuffs. Despite significant increase in the use of MALDI-TOF MS in microbiological laboratories, the identification output of this method is still not accepted for definite and fully reliable characterization of bacterial species. Nevertheless, several studies, including the present one, indicate that, prospectively, MALDI-TOF MS may become a new "gold standard" of microbial identification for both food and clinical isolates (Mazzeo et al. 2006; Carbonnelle et al. 2011; Doan et al. 2012 and Mimica et al. 2013).

According to the study by Pesaveto et al. (2014) the predominant *Enterococccus* species isolated from raw meat samples were also *E. faecalis* and *E. faecium.* In retail meat samples from Canada, Aslam et al. (2012) found a higher prevalence of *E. faecalis* (86%) and just 2% of *E. faecium.* In Germany Peters et al. (2003) reported a dominance of *E. faecalis* (72%) and only 13% of *E. faecium* in samples of sausages, ham and minced meat. Comparing the results of our study and other studies, it can be seen that of *Enterococcus* spp., *E. faecalis* is more abundant than *E. fecium* in samples of tested meat and meat products and the manufacturing environment.

Enterococci are LABs that play an important role in the environment, food and clinical microbiology. They are regular inhabitants of the gastrointestinal tract (GIT) of humans and warm-blooded animals and indicators of fecal contamination, but as well they occur in soil, surface waters, plants, vegetables and insects (Fouquié Moreno et al. 2006 and Pieniz et al. 2014). Their presence in the GIT of animals may lead to the contamination of raw meat at the time of slaughtering (Fouquié Moreno et al. 2006 and Franz et al. 2011). In food, however, *Enterococcus* spp. do not always appear due to fecal contamination. There are many factors affecting the effectivity of enterococci in every food system. Their persistence in the external environment, their ability to grow at low and high temperatures (from 10 to 45 °C), extreme pH (4.6 – 9.9) and salinity (6.5%), their resistance to pasteurization temperatures and sanitizing agents, and their adaptability to various substances, imply their occurrence as an important part of food microflora in heat-treated products and products manufactured from raw materials (Fouquié Moreno

et al. 2006 and Fisher and Phillips 2009). In fact, they are added deliberately to some kinds of meat product during the production process as starter cultures to extend shelf life and/or to improve their organoleptic properties (texture, smell, taste, color) and also because of their ability to produce enterocins to inhibit the multiplication of pathogenic bacteria (Fouquié Moreno et al. 2006 and Pesaveto et al. 2014).

The majority of the analyzed samples collected in this study that contained enterococci were samples of the meat product Vysočina salami. This product, one of the most popular and widespread in the Czech Republic, is a hot smoked dry sausage, a-non-fermented salami. It is prepared from pork meat or beef and pork back fat. It is a heat-treated product with a maximum water activity of 0.93, a minimum content of pure muscle proteins of 13% and a maximum fat content of 50% (Dušková et al. 2015). Unless enterococci were added as starter cultures (information that is unavailable to us), their presence in the analyzed samples indicates that they must have originated from the environment (inadequate cleaning of equipment) or from the raw meat used in production. This hypothesis is further supported by our findings of enterococci in a sample of spices used for the production of Vysočina salami and in swabs from various parts of the manufacturing environment, from which enterococci could easily have been transformed to the processed product during the manufacturing process. Their resistance to conditions during the production process (temperature, pH, salt) and multiplication capability may be the reason for their presence in the salami.

The role of these bacteria in human diseases and their involvement in biogenic amine production raise concerns about their safety and may cause a failure in using these bacteria as starter cultures or probiotics in the food industry. Other issues arise from antibiotic resistance and in particular the multiresistance of these bacteria (Franz et al. 2011). On the other hand, these bacteria indicate possible fecal contamination which fact must be taken into account. However, according to the European Union legislation, there are no limits for the concentration of enterococci in food and foodstuffs.

Conclusions

Our study reveals the appearance of *Enterococcus* spp. mostly in Vysočina salami samples and also in the environment related to their production, as well as in the selected meat products. The fact that the prevalence of enterococci is high in the tested samples may be attributed to their resistance to heat, acid, salt and harsh conditions during food processing. Their benefits to the properties of meat products should be considered carefully in the light of the possible health risk.

Acknowledgments

This study was carried out with the support of the Proteomics Core Facility of CEITEC – Central European Institute of Technology, ID number CZ.1.05/1.1.00/02.0068, financed from the European Regional Development Fund and project of Ministry of Agriculture RO0516.

References

Aslam M, Diarra MS, Checkley S, Bohaychuk V, Masson L 2012: Characterization of antimicrobial resistance and virulence genes in *Enterococcus* spp. isolated from retail meats in Alberta, Canada. Int J Food Microbiol **156**: 222-230

Carbonnelle E, Mesquita C, Bille E, Day N, Dauphin B, Beretti JL, Ferroni A, Gutmann L, Nassif X 2011: MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory. Clin Biochem **44**: 104-109

Doan NTL, Van Hoorde K, Cnockaert M, de Brandt E, Aerts M, Le Thanh B, Vandamme P 2012: Validation of MALDI-TOF MS for rapid classification and identification of lactic acid bacteria, with a focus on isolates from traditional fermented foods in Northern Vietnam. Lett Appl Microbiol **55**: 265-273

Dušková M, Kameník J, Šedo O, Zdráhal Z, Saláková A, Karpíšková R, Lačanin I 2015: Survival and growth of lactic acid bacteria in hot smoked dry sausages (non-fermented salami) with and without sensory deviations. Food Cont **50**: 804-808

Foilquié Moreno MR, Sarantinopoulos P, Tsakalidou E, De Vuyst L 2006: The role and application of enterococci in food and health. Int J Food Microbiol **106**: 1-24

Fernandes R 2009: Microbiology Handbook - Meat Products. Leatherhead Food Research and RSC Publishing, Cambridge: 174 p

Fisher K, Philips C 2009: The ecology, epidemiology and virulence of *Enterococcus*. Microbiol **155**: 1749-1757

Freiwald A, Sauer S 2009: Phylogenetic classification and identification of bacteria by mass spectrometry. Nat Protoc **4**: 732-742

Franz CMAP, Huch M, Abriouel H, Holzapfel W, Gálvez A 2011: Enterococci as probiotics and their implication in food safety. Int J Food Microbiol **151**: 125-140

Giraffa G 2003: Functionality of enterococci in dairy products. Int J Food Microbiol **88**: 215-222

International Standard ISO 6887-1 1999: Microbiology of food and animal feeding stuffs - Preparation of tests samples, initial suspension and decimal dilutions for microbiological examination - Part 1: General rules for the preparation of the initial suspension and decimal dilutions. Czech Office for Standards, Metrology and Testing: 12 p

International Standard ISO 7218 2007: Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations. Czech Office for Standards, Metrology and Testing: 68 p

Khan H, Flint S, Yu PL 2010: Enterocins in food preservation. Int J Food Microbiol **141**: 1-10

Mazzeo MF, Sorrentino A, Gaita M, Cacace G, Di Stasio M, Facchiano A, Comi G, Malorni A, Siciliano RA 2006: Matrix-assisted laser desorption ionization-time of flight mass spectrometry for the discrimination of foodborne microorganisms. Appl Environ Microb **72**: 1180-1189

Mimica MJ, Martino MDV, Pasternak J 2013: MALDI-TOF MS in the clinical microbiology laboratory. J Bras Patol Med Lab **49**: 256-259

Peters J, Mac K, Wichmann-Schauer H, Klein L, Ellerbroek G 2003: Species distribution and antibiotic resistance patterns of enterococci isolated from food of animal origin in Germany. Int J Food Microbiol **88**: 311-314

Pieniz S, Andreazza R, Anghinoni T, Camargo F, Brandelli A 2014: Probiotic potential, antimicrobial and antioxidant activities of *Enterococcus durans* strain LAB18s. Food Cont **37**: 251-256

Pesaveto G, Calonico C, Ducci B, Magnanini A, Lo Nostro A 2014: Prevalence and antibiotic resistance of *Enterococcus* spp. isolated from retail cheese, ready-to-eat salads, ham, and raw meat. Food Microbiol **41**: 1-7

Santos T, Capelo JL, Santos HM, Oliveira I, Marinho C, Gonçalves A, Araújo JE, Poeta P, Igrejas G 2015: Use of MALDI-TOF mass spectrometry fingerprinting to characterize *Enterococcus* spp. and *Escherichia coli* isolates. J Proteomics **127**: 321-331

Šedo O, Sedláček I, Zdráhal Z 2011: Sample preparation methods for MALDI-MS profiling of bacteria. Mass Spectrom Rev **30**: 471-434

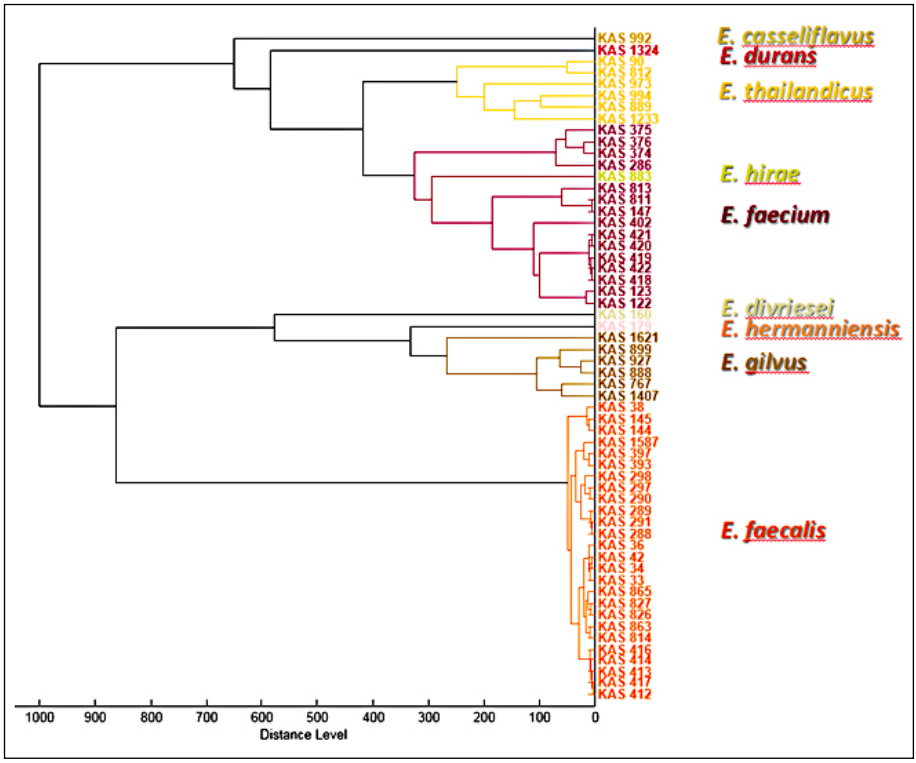

Fig. 1. The dendrogram of totally 58 enterococci created on the basis of cluster analysis of MALDI-TOF mass spectra