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IDENTIFICATION OF *STAPHYLOCOCCUS* SPP. ISOLATED FROM FOOD BY TWO METHODS

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ABSTRACT

One hundred and ninety-three staphylococcal strains were isolated from samples of alaska pollock meat (*Theragra chalcogramma*), atlantic mackerel meat (*Scomber scombrus*), atlantic herring meat (*Clupea harengus*), and femoral muscle samples of the wild pheasants (*Phasianus colchicus*), wild rabbits (*Oryctolagus cuniculus*) and bryndza cheese. Phenotypic manifestations typical of individual staphylococcal species were detected in these isolates. Species identification was also performed by matrix-assisted-laser-desorption-ionization-mass-spectrometry on the basis of which 5 species of staphylococci were determined: *S. aureus, S. epidermidis, S. capitis, S. haemolyticus, S. warneri.* Subsequently, the presence of the *16S rDNA* gene was confirmed by PCR assay; a specific sequence for *S. aureus* species; putative transcriptional regulator gene *serp0107*, a specific sequence of the *sodA* gene was used to identify *S. epidermidis* and to identify *S. warneri, S. haemolyticus, S. capitis* species. When comparing the results of identification by PCR assay and matrix-assisted-laser-desorption-ionization-mass-spectrometry, the identification at the genus level was identical in all cases (100%). However, the discrepancies in results were confirmed at the species level, where congruence for identification of isolates by PCR assay and matrix-assisted-laser-desorption-ionization-mass-spectrometry was only 80%. For these isolates, the score value ranged from 1.953 to 2.564.

Keywords: MALDI-TOF-MS, meat, PCR, bryndza cheese, staphylococci

INTRODUCTION

Staphylococci often occur in food samples, whether as a desirable or contaminating microflora **(Iacumin *et al.*, 2006; Martín *et al.*, 2006)**. In particular, the group of coagulase-negative staphylococci (CoNS) includes some species that have GRAS status (generally considered safe). These types of staphylococci are involved in the development of the organoleptic features of the final products. Some CoNSs are used as starter cultures to produce dry fermented sausage and cheese due to their aromatic and pigmentary abilities **(Corbière Morot-Bizot *et al.*, 2007)**. Although CoNS is generally considered to be a bacterium that has a positive role in the production of some fermented foods **(Irlinger, 2008)**, the identification of certain risk factors in CoNS strains, as well as the existence of nosocomial and urinary tract infections related to *Staphylococcus epidermidis* and *Staphylococcus saprophyticus*, raised questions regarding the presumption of safety of species belonging to this group **(Zell *et al.*, 2008)**.

Von Eiff *et al.* (2006) suggest that the occurrence and pathogenic potential of some CoNS species may correspond to emerging pathogen in food. The identified risk factors correspond to virulence factors **(Zell *et al.*, 2008)**, antibiotic resistance **(Regecová *et al.*, 2014)**, the ability to produce biogenic amines **(Martín *et al.*, 2006)** and biofilm formation on the surface of equipment used in the food industry **(Silva *et al.*, 2017)**. Among the potential pathogens of the genus *Staphylococcus* spp. we classify mainly *Staphylococcus epidermidis*, *Staphylococcus capitis*, *Staphylococcus haemolyticus* and *Staphylococcus warneri*, which inhabit human skin. Although infection with these staphylococci is rare compared to *Staphylococcus aureus*, at endangered hosts, the risk of developing staphylococcal infection increases **(Stollberger *et al.*, 2006)**.

At the same time, it is generally accepted that the food chain has been recognized as one of the major routes of transmission of potential pathogens as well as antibiotic-resistant bacteria between the human and animal populations. Staphylococci, belonging to this group of bacteria, can be present in many different types of additives used in the food industry due to their high resistance and multi-application ability **(Pesavento *et al.*, 2014)**. The identification and differentiation of food-related staphylococci is essential to ensure quality and is the basis for ensuring the availability of safe and healthy food for consumers **(Drozenová and Petráš, 2000; Wenning *et al.*, 2014)**.

Traditional methods used to identify and classify bacteria, which are based on the analysis of morphological, physiological and biochemical traits or genetic approaches (DNA-DNA or RNA-DNA hybridization, determination of G+C content in DNA), are currently supplemented by sequence analysis of small subunits of rRNA by PCR methods **(Bencúrová *et al.*, 2013)**. However, it is necessary to shorten the analysis time, in the species identification of microorganisms (including bacteria of the genus *Staphylococcus* spp.) without any former knowledge of genetic targets.

One such method is matrix-assisted laser desorption ionization time-of-flight-mass-spectrometry (MALDI-TOF-MS). Over the last few years, MALDI-TOF-MS tools for microbiological identification of potential pathogens and foodborne pathogens have been improved. These commercial, easy-to-use MALDI-TOF-MS devices contain their own algorithms and databases. Many studies report fast, cost-effective, and accurate performance of MALDI-TOF-MS systems **(Clark *et al.*, 2013; Croxatto *et al.*, 2012)**. However, data for comparison among commercial MALDI-TOF-MS devices are limited, especially for Gram-positive cocci isolated from food **(Deak *et al.*, 2015)**.

Therefore, the aim of this study is to identify selected species of the genus *Staphylococcus* spp. based on phenotypic expression and PCR to be used as common methods for the identification of staphylococci. At the same time, the MALDI-TOF-MS method is used to identify food isolates of staphylococci. However, the accuracy of this method may vary compared to the identification of clinical isolates of staphylococci identified by this method in clinical studies.

MATERIAL AND METHODS

Sampling

Individual strains were isolated from 5 samples of alaska pollock meat (*Theragra chalcogramma*) originating in FAO fishing zone 61 (China), 5 samples of atlantic mackerel meat (*Scomber scombrus*) originating in FAO 27 fishing zone (Ireland), 5 samples of atlantic herring meat (*Clupea harengus*) fishing effort originating in FAO 27 fishing zone (Norway), from 3 samples of the thigh muscle of the wild pheasants (*Phasianus colchicus*), 4 samples of wild rabbits (*Oryctolagus cuniculus*) originating from a hunt in the region of East Slovakia and 6 samples from meat of carcasses of chickens after combined cooling in a local poultry slaughterhouse. Strains were also isolated from 3 samples of bryndza cheese from purchased in the regular shopping branch. Sampling, preparation of test samples, initial suspensions and decimal dilutions for microbiological examination were carried out according to the requirements of **ISO 6887-2 (2017)**, **ISO 6887-3 (2017)** and **ISO 6887-5 (2020)**.

Isolation of strains and detection of their phenotypic features

Staphylococci were isolated from food samples according to **ISO 6888-1/A1 (2003)** and subsequently staphylococcal colonies from the surface of agar medium by Baird-Parker (OXOID, Hampshire, UK) were individually inoculated on the surface of Columbia blood agar and after 24 hours incubation at 37°C, the presence and type of hemolysis as well as the formation of yellow pigment were evaluated. They were then inoculated from blood agar into test tubes with 3 ml of Brain Heart Infusion Broth (BHI broth; OXOID, Hampsire, UK).
After 18-24 hours of incubation at 37°C, 0.1 ml of the multiplied broth culture of the test strains was added to 1 ml of reconstituted freeze-dried rabbit plasma in a test tube (Staphylo PK test, IMUNA, Šarišské Michal'any, SR). The inoculated plasma was incubated at 37°C. Formation of coagulum was considered as positive reaction. Results were read after 1, 2, 3, 6 and 24 hours. Each isolate propagated in BHI broth was also inoculated onto the surface of DNase agar (OXOID, Hampsire, UK) containing DNA. DNase-producing colonies hydrolyzed the DNA contained in the agar. After 24 hours of incubation at 37°C, the surface of the medium was flooded and acidified with 1 N hydrochloric acid, the DNAprecipitated, the medium became clear zones appeared around DNase-positive colonies.
Isolated strains were also tested for resistance to novobiocin, which is one of the factors dividing CoNS into two groups. 0.1 ml of a 24-hour bacterial suspension of the tested strains of staphylococci in BHI broth, adjusted to the 0.5 McFarland turbidity standard was inoculated onto the surface of the Müller-Hinton agar (OXOID, Hampsire, UK). Subsequently, commercially produced standard disks with a novobiocin concentration of 30 μg.disk^{-1} were applied to the agar surface using a dispenser (OXOID, Hampsire, UK). No later than 15 minutes after disk application, the plates were placed in thermostats and incubated at 37°C for 24 hours. After 24 hours of incubation, the diameters of the inhibition zones (including the disc) in millimeters were measured using a calibrated measuring instrument. The results were evaluated according to the criteria set by the **Becton Dickinson & Comp (1996)**.

Matrix-assisted-laser-desorption-ionization-mass-spectrometry (MALDI-TOF-MS)

The extraction procedure using ethanol and formic acid was used to prepare the samples for MALDI-TOF-MS identification. Two full loops of bacterial cells containing 1 μl were resuspended in an test tube with 300 μl of distilled water. 900 μl of absolute ethanol was mixed into the test tube and the mixture was centrifuged (15 000 rpm/2 min). The supernatant was pipetted off 50 μl of 70% formic acid and 50 μl of acetonitrile were added to the pellet before analysis and the mixed mixture was centrifuged again (15 000 rpm/2 min).
Subsequently, 1.0 μl of supernatant was applied to a MALDI plate, which, after drying, was overlaid with 1.0 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA) in 50% acetonitrile and 2.5% trifluoroacetic acid **(Bruker Daltonics, 2008)**. The analysis of the results was performed in an Ultraflex III device. The obtained results were processed using Flex Analysis software, version 3.0 and evaluated using BioTyper software, version 1.1 (BRUKER DALTONICS, Massachusetts, USA).

Polymerase chain reaction (PCR)

For DNA isolation, *Staphylococcus* spp. were originally cultured on Petri dishes with Columbia blood agar (OXOID, Hampsire, UK). This was followed by reproduction in BHI liquid medium (OXOID, Hampsire, UK) at 37°C. Bacterial genomic DNA was extracted using the NucleoSpin Microbial DNA kit (MACHEREY-NAGEL GmbH & Co. KG, Duren, Germany).
Primers (Tab 1) synthesized in AMPLIA s.r.o (Bratislava, SR) were used for genus and species identification of individual staphylococcal species by PCR.

Table 1 Characteristics of the primers used in this study

***Staphylococcus* species**	**Target gene**	**Primer pair**	**Sequence (5′→3′)**	**annealing temp. (°C)**	**Amplicon size (bp)**	**GenBank accession no. (reference)**
Ubiquitous (*Staphylococcus*)	16S rDNA	16s 1 16s 2	CAGCTCGTGTCGTGAGATGT AATCATTTGTCCCACCTTCG	55	420	Y15856 **(Dunman *et al*., 2001)**
S. aureus	*S. aureus*-specific sequence	sau1 sau2	AATCTTTGTCGGTACACGATATTCTTCACG CGTAATGAGATTTCAGTAGATAATACAACA	55	107	AF033191 **(Martineau *et al*., 1996)**
S. epidermidis	*serp0107* gene	Serp0107F Serp0107R	TTGAGCTTGTCATTGGTTCG TGTAGAGGTTGCACGTCGAG	55	581	CP000029 **(Liu *et al*., 2006)**
S. capitis	*sodA* gene	ScapF ScapR	GCTAATTTAGATAGCGTACCTTCA CAGATCCAAAGCGTGCA	59	208	AJ343896 **(Iwase *et al*., 2007)**
S. haemolyticus	*sodA* gene	ShaeF ShaeR	GTTGAGGGAACAGAT CAGCTGTTTGAATATCTT	50	85	AJ343910 **(Iwase *et al*., 2007)**
S. warneri	*sodA* gene	SwarF SwarR	TGTAGCTAACTTAGATAGTGTTCCTTCT CCGCCACCGTTATTTCTT	60	63	AJ343932 **(Iwase *et al*., 2007)**

PCR reactions were performed in FIREPol® MasterMix (AMPLIA s.r.o, Bratislava, SR). In a total volume of 20 μl containing 5 ng/μl of template DNA and 10 pmol of each of the primers. This mixture was heated to 95°C for 5 minutes during the initial denaturation. 30 amplification cycles were performed (denaturation 95°C/30 s, annealing of each primer is indicated in Table 1/30 and extension 72°C/2 min) in thermocycler (TECHNE TC-512, London, UK) with final extension 7 minutes/72 °C.

Detection of Amplified DNA

A 5 μl portion of the amplified PCR product was analyzed on a 2% TBE agarose gel containing GelRed™ (Biotium, California, USA) at 120 V for 1 hour or until the desired resolution was achieved. Gels were monitored by UV transillumination (Mini Bis Pro®, DNR BIO- IMAGING SYSTEMS Ltd., Jerusalem, Israel) and photographed using a 35 mm camera. Ladders of 100 bp (AMPLIA s.ro, Bratislava, SK) were used as molecular size markers in all gels. PCR products were sequenced at the European Sequencing Center, GATC Biotech AG (Cologne, Germany). Subsequently, the obtained sequences were compared with the corresponding nucleotide sequences from the reference strains (accession no. GenBank in Tab 1). *S. aureus* CCM 4223, *S. haemolyticus* CCM 2737, *S. epidermidis* CCM 2124, *S. capitis* CCM 2734, *S. warneri* CCM 2730 (CZECH COLLECTION OF MICROORGANISMS, Brno, Czech Republic) served as positive controls in this study.

RESULTS

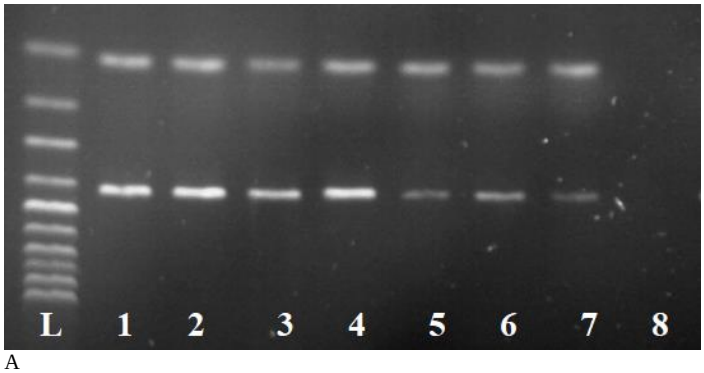
Our study confirmed the presence of *Staphylococcus* spp, from samples of fish meat, wild rabbit, wild pheasant, chickens and also bryndza cheese, by culture microbiological examination, on the basis of which 193 isolates were isolated. Specifically, in this study, 28 staphylococcal strains were isolated from alaska pollock samples, 21 staphylococcal strains isolated from Atlantic mackerel, 28 strains isolated from Atlantic herring, 29 staphylococcal strains from wild pheasants, 25 strains isolated from wild rabbit, 31 strains isolated from chicken and 31 staphylococal strains from bryndza cheese.
The obtained isolates were phenotypically and genotypically identified. When culturing individual isolates, their phenotypic features such as pigment formation, hemolysis, coagulase, deoxyribonuclease and susceptibility to the antibiotic novobiocin were evaluated. These features are characteristic of each type of CoNS. Staphylococcal plasma-coagulase activity was determined using a tube coagulase assay (TCA). Based on the TCA results, 170 (88%) isolates were included in the group of coagulase-negative staphylococci and 23 (12%) in the group of coagulase-positive strains (Tab 2). Of the total number of 23 coagulase-positive staphylococci, the formation of *α*-hemolysis was detected in 4 strains (17

%) and the formation of *β*-hemolysis in 19 strains (83%). Of the 170 coagulase-negative staphylococci, 54 isolates (32%) developed hemolysis.
Yellow pigment formation was confirmed in 23 coagulase-positive isolates (100%) and in 18 (11%) CoNS isolates. Nuclease production was also confirmed in isolates in 15 coagulase-positive staphylococcal (CoPS) isolates and in 6 CoNS isolates, where subsequent identification by MALDI-TOF-MS and PCR assay were nuclase-producing isolates assigned to *S. aureus* (15 isolates), *S. warneri* (4 zolates) and *S. epidermidis* (2 isolates).

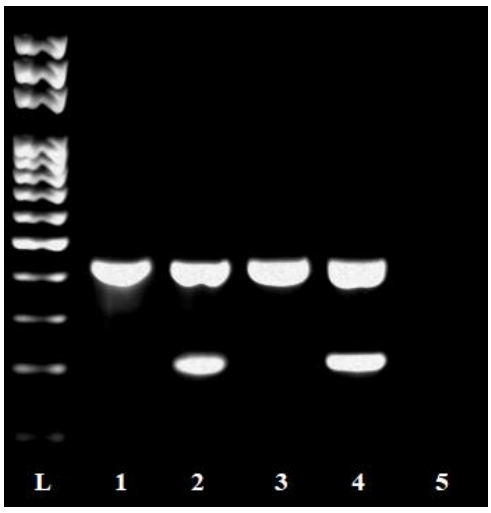
Table 2 Overview of phenotypic properties of individual species of staphylococcal isolates

Species	Hemolysis			Pigment	Nuclease
	α	*β*	without		
S. warneri	43	-	52	12	4
S. epidermidis	6	-	58	-	2
S. aureus	4	19	-	23	15
S. haemolyticus	5	-	3	6	-
S. capitis	-	-	3	-	-

The phenotypic features were originally classified by individual staphylococcal isolates into the CoPS and CoNS groups. Based on the results of the disk diffusion method, CoNS were classified into the group of novobiocin-sensitive staphylococci, where the sizes of the inhibition zones ranged from 25 mm to 32 mm. CoPS have been identified by phenotypic expression as the species of *S. aureus* that is most important for human pathogenicity.



A



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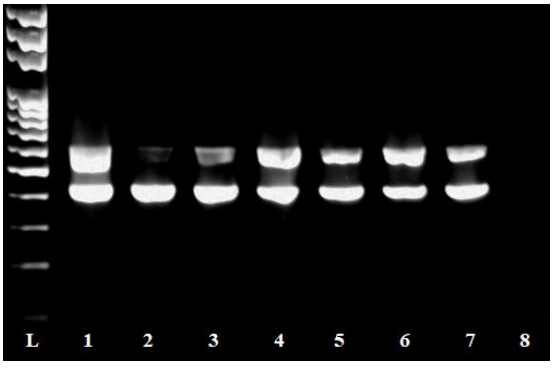

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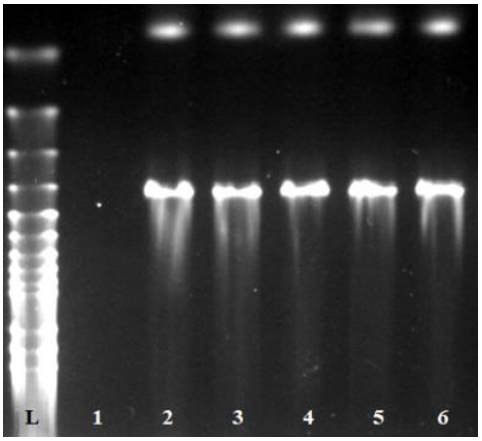

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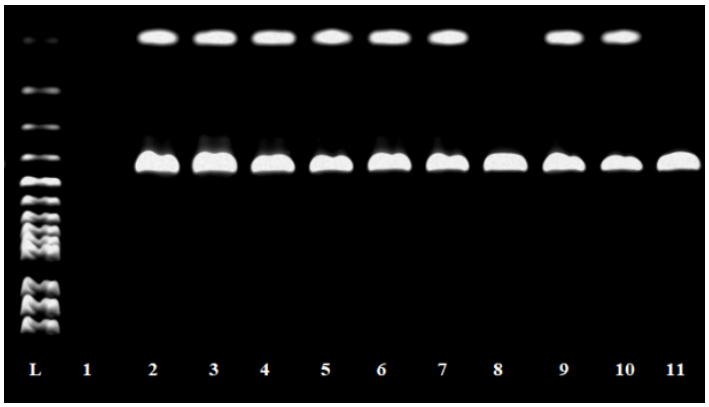

D

Figure 1 Identification of *S.aureus* (A), *S. capitis* (B), *S. epidermidis* (C), *S. haemolyticus* (D) and *S. warneri* (E) by PCR method.
Figure A: L – 100 bp ladder; Line 1 – reference strain *S. aureus* CCM 4223; Lines 2,3,4,5,6,7 – isolates of *S. aureus*. (107 bp – *S. aureus*-specific sequence; 420 bp – *16S rDNA*); Line 8 – negative control.
Figure B: L – 100 bp ladder; Lines 1,3 - unidentified isolates; Line 2 – isolates of *S. capitis* (208 bp - *S. capitis*-specific sequence; 420 bp – *16S rDNA*); Line 4 – reference strain *S. capitis* CCM 2734; Line 8 – negative control.
Figure C: L – 100 bp ladder; Lines 1,3,4,5,6 – isolates of *S. epidermidis* (587 bp - *S.epidermidis*-specific sequence; 420 bp – *16S rDNA*); Line 2 - unidentified isolates; Line 7 – reference strain *S. epidermidis* CCM 2124; Line 8 – negative control.
Figure D: L – 100 bp ladder; Line 1 – negative control; Line 2 – reference strain *S. haemolyticus* CCM 2737; Lines 3,4,5,6 – isolates of *S.haemolyticus* (63 bp - *S. haemolyticus*-specific sequence; 420 bp – *16S rDNA*).
Figure E: L – 100 bp ladder; Line 1 – negative control; Line 2 – reference strain *S. warneri* CCM 2730; Lines 3,4,5,6,7,9,10 – isolates of *S. warneri* (85 bp- *S. warneri*-specific sequence; 420 bp – *16S rDNA*); Lines 8,11 - unidentified isolates.

After evaluation of all observed phenotypic manifestations, the isolates were subjected to further species identification by PCR method. The *16S rDNA* gene, housekeeping gene, has been used in the past for species identification of staphylococci. However, the *16S rDNA* gene of *Staphylococcus epidermidis* is very similar to the gene in other CoNSs. Therefore, alternative target sequences were used, namely *S. aureus* specific sequences, *S. epidermidis* putative transcriptional regulator gene *serp0107* and the specific sequence of the *sodA* gene (Fig 1) was used to identify *S. warneri, S. haemolyticus, S. capitis*.
The phenotypic manifestations of the isolates correlated with species identification using the PCR method. Five species of staphylococci were identified by PCR and subsequently by MALDI-TOF-MS, namely: *S. aureus, S. epidermidis. S. capitis, S. haemolyticus, S. warneri*.
When comparing the results of identification by PCR and MALDI-TOF-MS, the identification at the genus level was identical in all cases (100%). However, discrepancies in the results were confirmed at the species level, where the congruence in the identification of isolates by PCR and MALDI-TOF-MS was only 80%. For the mentioned isolates, the score value ranged from 1.953 to 2.564 (Tab 3).

Table 3 Identification of staphylococcal isolates by MALDI-TOF-MS

MALDI-TOF-MS Identification	n	Score value
S. aureus	23	1.705-2.325
S. warneri	95	1.700-2.564
S. epidermidis	64	1.700-2.545
S.haemolyticus	8	1.874-2.420
S. capitis	3	1.721-2.501

The score value 2.000-2.300 indicated highly probable identification of the species, score value 1.7-1.999 indicated identification of the genus and probable identification of the species, and score value 1.699-0.000 not reliable identification **(Chen *et al*., 2014; Deng *et al*., 2014; Cheng *et al*., 2015)**.

Eight isolates were identified as *S. epidermidis* by MALDI-TOF-MS, but the presence of a specific sequence of *S. warneri* species (score valus 1.700-1.895) was confirmed in these isolates by PCR. The other 7 isolates confirmed the specific sequence for *S. haemolyticus* species, but MALDI-TOF-MS identified these isolates as *S. warneri* (score value 1.705-1.892).

In another 24 isolates identified by MALDI-TOF-MS as *S. aureus* (4 isolates), S. *epidermidis* (12 isolates), *S. warneri* (5 isolates), *S. haemolyticus* (1 isolate) and *S. capitis* (2 isolates) only *16S rDNA* sequence specific for the genus *Staphylococcus* spp. (Tab 3) was confirmed by PCR. In these cases, the score was less than 2 (1.700-1.874). However, the producer recommends using the score to determine species-level identification above 2.000.

When comparing the effectivity of the PCR method and MALDI-TOF-MS in genus and species identification, the presence of staphylococci in all tested food samples of animal origin was simultaneously confirmed.

Table 4 Number of species-identified staphylococci in individual types of food of animal origin

	methods	*S. aureus*	*S. epidermidis*	*S. warneri*	*S.haemolyticus*	*S. capitis*
Σ	MALDI-TOF-MS	23	64	95	8	3
	PCR	17	44	83	7	1
Alaska pollock	MALDI-TOF-MS	-	10	18	-	-
	PCR	-	8	12	-	-
Atlantic mackerel	MALDI-TOF-MS	-	5	16	-	-
	PCR	-	3	15	-	-
Atlantic herring	MALDI-TOF-MS	-	8	15	3	2
	PCR	-	5	13	3	-
Wild pheasant	MALDI-TOF-MS	-	11	16	2	-
	PCR	-	8	15	2	-
Wild rabbit	MALDI-TOF-MS	2	10	13	-	-
	PCR	2	6	12	-	-
Chicken	MALDI-TOF-MS	-	15	12	3	1
	PCR	-	11	12	2	1
Bryndza cheese	MALDI-TOF-MS	21	5	5	-	-
	PCR	15	3	4	-	-

As follows from Table 4, the presence of coagulase-positive species *S. aureus* was confirmed to a lesser extent in wild rabbit samples and to a greater extent in bryndza cheese samples. Coagulase-negative species *S. epidermidis* was most common in chicken thigh muscle samples, despite inconsistencies in the species identification of this species by both methods. *S. warneri* was also identified in all food samples, but the smallest number was detected in bryndza cheese samples. *S. haemolyticus* was confirmed only in small numbers in samples of herring, pheasant and chickens. The lowest number of identified isolates belonged to the species *S. capitis*. Its presence was confirmed by MALDI-TOF-MS in herring samples, but it was confirmed by PCR method only in a chicken thigh muscle sample (Tab 4).

DISCUSSION

The microflora of food is mainly influenced by hygiene in the food chain. Improper handling of food during the production process increases the risk of contamination of the final product by microbes, including multi-resistant and/or enterotoxigenic staphylococci **(Ferroni *et al*., 2010; Hammad *et al*., 2012)**. Therefore the detection of the presence of bacteria *Staphylococcus* spp. in food is very important. The presence of these bacteria is also confirmed by this study, where the presence of 170 isolates of coagulase-negative staphylococci and 23 isolates of coagulase-positive staphylococci was confirmed by microbiological examination of samples and tube coagulase test. Phenotypic features such as the formation of hemolysis and pigment, were further monitored in these isolates. Formation of coagulum and nuclease activity, which are important in distinguishing between pathogenic staphylococci and non-pathogenic resident flora, have also been studied **(Pfaller and Herwaldt, 1988)**. Nuclease production in CoNS was also confirmed by **Lambe *et al*. (1990)**, who reported that most strains of *S. epidermidis, S. warneri* and *S. haemolyticus* tested in their study produced nuclease. Similarly, **Cunha *et al*. (2006)** confirmed 117 nuclease activity in 9 *S. epidermidis* isolates and two *S. haemolyticus* isolates when 117 CoNS isolates were examined, but nuclease production was not confirmed in *S. warneri* isolates. In this study, nuclease production was confirmed in 15 CoPS isolates and 6 CoNS isolates.

The phenotypic features of staphylococcal isolates initially classified individual staphylococcal isolates, into the CoPS and CoNS groups. Based on the results of the disk diffusion method, CoNS were classified into the group of novobiocin-sensitive staphylococci. CoPS were identified by their phenotypic expression as the species of *S. aureus*, which is of the greatest importance for human pathogenicity. Recently, the importance of CoNS in terms of the development of serious infections, especially infections of nosocomial origin, has also increased. Strains causing nosocomial infections are more common than other CoNS strains which contain features considered as virulence factors (ability to form a biofilm, antimicrobial resistance). Accurate identification and typing of these strains is therefore important for clinical and epidemiological reasons **(Chomoucká, 2009)**.

Therefore, in this study, after initial identification by phenotypic expression of the isolates, more accurate identification was performed by MALDI-TOF-MS and PCR. The phenotypic manifestations of the isolates correlated with species identification by PCR. Five species of staphylococci were identified by PCR and subsequently by MALDI-TOF-MS, namely: S. *aureus, S. epidermidis. S. capitis, S. haemolyticus. S. warneri*. Until recently, the *16S rDNA* gene, a housekeeping gene**,** was used for PCR identification, which is generally targeted when species-specific detection by PCR is performed. This is also confirmed by **Sindhu *et al*. (2007)** and **Organji *et al*. (2018)**, who used sequence of *16S rDNA* gene as a target to identify staphylococcal isolates. However, the *16S rDNA* gene of *S. epidermidis* is very similar to the gene in other CoNSs. To solve this problem, alternative target sequences that show higher divergence than the *16S rDNA* gene have been used, namely the *S. aureus* specific sequence, the putative transcriptional regulator gene *serp0107* and the *sodA* gene **(Poyart *et al*., 2001)**.

When comparing the results of identification by PCR and MALDI-TOF-MS, the identification at the genus level was identical in all cases (100%). However, discrepancies in the results were confirmed at the species level, where the congruence in the identification of isolates by PCR and MALDI-TOF-MS was only 80%. For the remaining 20%, the identification between MALDI-TOF-MS and PCR did not match.

In the mentioned cases, the score was lower than 2.000 (1.700-1.874). Although the manufacturer recommends using scores to determine species-level identification above 2.000, lower cut-off scores have been used in the past to identify individual Gram-positive cocci species **(Deak *et al*., 2015)**. Also in the studies of **Dubois *et al*. (2012)**, **Kim *et al*. (2012)** and **Martiny *et al*. (2012)** they confirmed lower species identification accuracy by MALDI-TOF-MS for CoNS isolates.

Based on work on the application of MALDI-TOF-MS on microorganisms, it was confirmed that the choice of the correct matrix is a very important factor in the correct identification of microorganisms. Staphylococci are HCCA and ferulic acid, which allows the detection of high molecular weight proteins up to 70 kDa **(Keys *et al*., 2004; Madonna *et al*., 2000)**. However, in order to obtain quality spectra, it is necessary to first disrupt the Gram-positive bacterial cell and thus release its proteins **(Smole *et al*., 2002)**. When preparing their samples, it is

therefore necessary to first disrupt the thick peptidoglycan layer, which does not allow the ionization of proteins that are protected by the cell wall or are attached to it. One of the proven methods to disrupt the peptidoglycan layer is the use of lysozyme or lysostaphin. In addition to enzymatic methods for disrupting the cell wall, physical methods and chemical methods using various solvents, such as ethanol, are used, which we have also used in this study. The nature of the spectrum is also affected by the correct crystallization on the plate and the ionization capabilities of the analyte. The intensity (height) of the signal corresponds to the relative protein concentration at the laser impact site and depends on the degree of ionization **(Vaňousová, 2010)**. *Staphylococcus* spectra usually show 50-75 signals with a weight of 1.8 kDa **(Smole *et al*., 2002)**.

Identification of microorganisms is performed by comparing the obtained spectrum with a database of characteristic known spectra. A signal present in different spectra can be evaluated as a single protein if its size differs by a maximum of ±3 Da **(Smole *et al*., 2002)**. Spectrum analysis first searches for genus-specific signals common to all species. For example for the genus *Staphylococcus* it is the signal 9625 Da. Further, common intraspecific signals are searched. The identification and quantification of the signals of the examined spectrum is followed by the calculation of similarity with other mass spectra analyzed or obtained from the reference library **(Vaňousová, 2010)**. Other factors that may affect the accuracy of the identification of *Staphylococcus* isolates by MALDI-TOF-MS are the composition of the culture medium and the duration of the culture, as confirmed by the study by **Šedo *et al*. (2013)** on bacterial strains isolated from food, where a more accurate species distinction was achieved by changing the culture conditions.

This was similarly confirmed by **Prod'hom *et al*.(2010)**, who used blood culture to propagate staphylococci to identify bacteria by MALDI-TOF-MS, leading to the correct identification of 79% of the isolates tested at the species level. More specifically, all 25 cases of *S. aureus* bacteremia were identified, but scored only 1.7, indicating an identification that is only reliable at the genus level. Other groups have also confirmed the high efficiency of *S. aureus* identification in blood cultures at the genus level only using MALDI-TOF-MS **(Christner *et al*., 2010; Ferroni *et al*., 2010; Stevenson *et al*., 2010)**. **Clerc *et al*. (2014)** also identified *S. aureus* by MALDI-TOF-MS. In the study, 5.5% of *S. aureus* isolates were identified incorrectly. All showed scores <1.7; therefore, the species has not been correctly identified at the species level.

Manukumar and Umesha (2017) confirmed the presence of staphylococcal species in food samples. They identified by MALDI-TOF-MS, a species of *S. aureus* where 94% of the isolates tested had a score higher than 2.000, a species of *S. epidermidis* where a score higher than 2,000 showed 83.3 % of isolates and a species of *S. haemolyticus* where a score higher than 2.000 showed 100%. These results correlate with our study, where the smallest differences in identification were in the species *S. haemolyticus*. This staphylococcal species as well as *S. epidermidis* and *S. warneri* were confirmed by **Kačániová *et al*., 2019** by MALDI-TOF-MS in samples of Slovak cheese "Parenica". However, it also notes that while MALDI-TOF-MS has revolutionized speed and precision of microbial identification for clinical isolates, in contrast few performance studies have been published so far focusing on suitability for particularly industrial applications.

In addition to CoNS (**Tvarožková *et al*., 2020**), the species *S. aureus* (**Zigo *et al*. 2011**; **Tančin *et al*. 2017**; **Holko *et al*. 2018**) is often identified in milk and dairy products, similarly to our study where in bryndza its presence was confirmed by both methods.

Like the above-mentioned studies, **Pipová *et al*. (2012)**, **Chan *et al*. (2014)** and **Regecová *et al*. (2014)** also confirmed that MALDI-TOF-MS can be used as a complementary screening method in the identification of individual staphylococcal species. These claims are also supported by **Gekenidis et al. (2014)**, who proves that MALDI-TOF-MS has certain restrictions. First of all, unlike sequence databases such as GenBank, MALDI-TOF-MS databases are not publicly available. The low percentage of identification of some organisms can be improved by the addition of mass spectral records of under-represented species or strains. Due to the low score/percentage, retesting may be required. Growth on some media can also negatively affect scores/percentages. Identification of small or mucoid colonies can be a problem in the accurate identification of isolates by MALDI-TOF MS. Small colonies can be identified more quickly by gene sequencing than by MALDI-TOF-MS.

Low identification scores are also affected by laboratory errors that can occur when applying colonies to the diagnostic plate. Although MALDI-TOF-MS is generally reproducible, there are sources of variability, including mass spectrometry, matrix and solvent composition, methods of preparation, training and technologist competence, culture conditions (such as media, colony-age, temperature) and biological variability **(Patel, 2015)**.

CONCLUSION

The study confirmed the presence of staphylococci in food samples. A total of 193 isolates were tested, which were classified as *S. aureus, S. epidermidis, S. capitis, S. haemolyticus, S. warneri* on the basis of phenotypic expression and identification by MALDI-TOF-MS and PCR. In the identification of MALDI-TOF-MS and PCR method, there were discrepancies in the identification of staphylococcal species in 20% of the tested isolates. Therefore, we can conclude that MALDI-TOF mass spectrometry is a suitable and quick method for the identification of food isolates of staphylococci at the genus level.

However, further studies are needed to standardize culture procedures, sample preparation of bacterial cultures, as well as procedures for the handling of prepared samples before testing by MALDI-TOF-MS, which affect the accuracy and resulting success scores for food staphylococcal isolates, especially at the species level. Our results point to MALDI-TOF-MS as a rapid method for the identification of staphylococci, which, however, requires verification by PCR.

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