ECRONICON OPEN ACCESS

EC MICROBIOLOGY
Research Article

Variability in Fecal Canine Staphylococci Identified by MALDI-TOF Mass Spectrometry, their Properties and Susceptibility to Bacteriocins

Andrea Lauková*, Eva Bino, Ivana Kubašová, Viola Strompfová and Monika Pogány Simonová

Institute of Animal Physiology, Centre of Biosciences of the Slovak Academy of Sciences, Košice, Slovakia

***Corresponding Author:** Andrea Lauková, Institute of Animal Physiology, Centre of Biosciences of the Slovak Academy of Sciences, Košice, Slovakia.

Received: March 02, 2021; **Published:** May 27, 2021

Abstract

Although staphylococci are a part of microbiota in gastrointestinal tract of animals, still not enough information exist regarding the properties of canine staphylococci. This study has been focused on canine fecal staphylococci, their susceptibility to antimicrobial substances of proteinaceous character-enterocins and lantibiotic gallidermin; their antibiotic phenotype and biofilm formation were also tested. Feces from 50 clients owned clinically healthy dogs of various breeds, sex and age (6 months to 7 years) were sampled in eastern Slovakia. The total counts of colonies enumerated on Mannitol salt agar reached 2.94 ± 0.71 CFU/g log10 on average. Species variability was noted in identified staphylococci; 11 different species were identified: *Staphylococcus arlettae*, *S. aureus*, *S. capitis*, *S. epidermidis*, *S. intermedius*, *S. pseudintermedius*, *S. vitulinus*, *S. equorum*, *S. hominis*, *S. haemolyticus* and *S. warneri*. Seven strains were hemolysis-negative (γ-hemolysis), 7 strains showed β-hemolysis and 7 strains were nuclease test positive. Five strains were low-grade biofilm - forming ($0.1 \leq A_{570} < 1$), the others were biofilm non-forming ($A_{570} < 0.1$). Staphylococci were mostly susceptible to enterocins with inhibition activity up to 25 600 AU/ml and gallidermin as well. Multiresistant strain *S. pseudintermedius* SPsi Beno2, low-grade biofilm-forming with β-hemolysis was susceptible to enterocins and gallidermin.

Keywords: *Dog; Staphylococci; Characteristic; Biofilm; Susceptibility; Bacteriocin*

Abbreviations

Ents: Enterocins; ATB: Antibiotics; S: Susceptible; R: Resistant; CoNS: coagulase-negative staphylococci; CoPS; Coagulase-Positive; A: Absorbance; Ents: Enterocins; AU/ml: Arbitrary Unit Per Milliliter; cfu/g: Colony Forming Unit Per Gram; SD: Standard Deviation

Introduction

Nowadays, the genus *Staphylococcus* includes 58 known species with 25 subspecies [1]. Most species validated are coagulase-negative staphylococci (CoNS). Bacteria in the genus *Staphylococcus* serve as resident members of the normal cutaneous and mucosal microbiota of both human and animals. They are also opportunistic pathogens commonly found in dogs with skin disorders [2]. Among mucous specimens from healthy dogs, the most frequently detected species were *Staphylococcus intermedius* and *S. xylosus* [3]. However, staphylococci are often involved in a wide variety of diseases in animals [4]; in diseased animals are the most detected agents coagulase-positive staphylococcal species *S. aureus* or *S. intermedius.* On the other side, staphylococci are a part of microbiota in gastrointestinal tract of animals, involving dogs. Individual strains belonging in different species can be resistant to antibiotics, they can form biofilm or they can possess virulence factor genes; these properties indicate their pathogenic character.

The most frequently examined virulence factors detected in CoNS are e.g. coagulase activity, nuclease activity, lecithinase, gelatinase and hyaluronidase activity as well as detection of genes encoding production of enterotoxin activity [5]. Moreover, biofilm as an aggregation of microorganism (attached to and growing on a surface) also belong among virulence factors [6]. Any type of microbiota, including those microbiota signed as spoilage and/or pathogenic, could form a biofilm and play a key role in many infections [7]. The other factor which can contribute to spoilage character of staphylococci is antibiotic resistance. In staphylococci, methicillin resistance has emerged as a significant interest [8]. In general, antibiotic resistant strains of staphylococci are problem in both human and animal clinical microbiology. Therefore, new ways how to solve this problem have been looked forward. To inhibit staphylococcal growth using bacteriocins has been previously reported by Lauková., *et al* [9]. Moreover, only limited information exists regarding the different properties of canine staphylococci, especially those species belonging in CoNS.

Enterocins are ribosomally synthesized antimicrobial proteinaceous substances (peptides), mostly thermo-stable which includes four classes: Class I, lantibiotic enterocins, Class II, enterocins, small, non-lantibiotic peptides, Class III, cyclic enterocins and Class IV, enterocins-large proteins [10,11]. Gallidermin is bacteriocin-lantibiotic containing unusual amino acid residues such as lanthionine, β-methyllanthionine or α, β-didehydroaminoacids firstly described in *Staphylococcus gallinarum* TU3928 [12]. Its mode of action integrates into the plasma membrane, forming pores and inhibiting cell membrane synthesis. Gallidermin is active predominantly against Gram-positive bacteria.

This study has been focused on fecal staphylococci isolated from various dogs, which were tested for their ability to form biofilm; their antibiotic profile, hemolysis, nuclease and enzymatic activities were tested as well as their susceptibility to antimicrobial substances of proteinaceous character-enterocins and lantibiotic gallidermin. Results obtained will contribute to basic microbiology regarding the CoNS and possible prevention of those antibiotic resistant and/or biofilm-forming strains using enterocins and gallidermin; moreover, it will be contribution in antimicrobial spectrum of used bacteriocins.

Materials and Methods

Sampling and strains identification: Feces from 50 clients owned clinically healthy dogs of various breeds (Rhodesian Ridgeback, Belgian shepherd dog, Labrador retriever, Czechoslovak wolfdog, Staffordshire bull terrier, Tibetian terrier, German shepherd, Slovakian Chuvach, Australian shepherd, Coarse-haired Styrian hound, cross-breed), different sex and age (6 months to 7 years) were sampled in eastern Slovakia region (Table 1). Fecal samples were treated as previously reported by Kubašová., *et al* [13]. They were homogenized in Ringer solution (Oxoid, Basingstoke, United Kingdom) by using a stomacher masticator (IUL, Barcelona, Spain), diluted according to the standard microbiological method (ISO-International Organization for Standardization, in ratio 1:9) and plated on selective Mannitol salt agar (MSA agar, Difco, Michigan, USA). Agar plates were cultivated at 37°C for 48 hrs. Bacterial richness was calculated as an average count of colonies grown in the highest dilution per sample and expressed in colony-forming units per gram of sample (log10 CFU/g ± SD). Nineteen randomly picked up colonies isolated from 50 fecal samples were streaked on MSA agar to check their purity.

Identification of presumed colonies was performed by using MALDI-TOF mass spectrometry (MS) based on protein "fingerprints" [14]. Moreover, phenotypization was performed using the BBL Gram-positive Crystal kit (Becton and Disckinson, Cockeysville, USA) as well as API STAPH kit (BioMerioux, L'Etoile, France). MALDI-TOF MS was performed using a Microflex MALDI-TOF mass spectrometer. A single colony from MSA was mixed with matrix (α-cyano-4-hydroxycinnamic acid and trifluoroacetic acid). The suspension was spotted onto a MALDI plate and ionized with a nitrogen laser (wavelength 337 nm, frequency 20 Hz). Taxonomic allocation was evaluated on the basis of highly probable species identification (value score 2.300-3.000), secure genus identification/probable species identification (2.000 - 2.299) and probable genus identification (1.700-1.999). Positive controls were those involved in the identification database (MALDI Biotyper 3.0, Bruker Daltonics, USA). Phenotypization using the BBL Crystal kit was peformed according to the manufacturer's instruction with the appropriate control strains and the following parameters such as saccharides fermentation, Voges-Proskauer test, nitrates, urease, esculin, etc. Similarly, API STAPH kit is based on different biochemical tests including previously mentioned. Fifteen strains (Table 1) were then stored with Microbank™ system (Pro-Lab Diagnostic, Richmond, Canada).

Hemolysis, nuclease activity and biofilm formation: To detect hemolysis, tested strains were stroke on Brain heart agar (BHA, Difco, USA) supplemented with 5% of defibrinated sheep blood. Plates were incubated at 37°C for 24 - 48 hrs in an incubator. The presence/absence of cleared zones around the colonies was interpreted as α, β-hemolysis or γ-hemolysis (meaning strains exhibited non, γ- hemolysis [15]).

For nuclease determination, each strain was inoculated onto the surface of DNase agar (Oxoid, USA) and incubated for 24 hrs at 37 ºC. After flooding and acidifying the agar with 1N hydrochloric acid (HCl), the DNA precipitated, and the agar became turbid with cleared zones around DNase-producing colonies. *Staphylococcus aureus* SA4 from a dog (Dr. Strompfová, isolated in our laboratory) was used as positive control.

Biofilm formation in strains was tested using biofilm plate assay [16,17]. One colony of the tested strain grown on Trypticase soy agar overnight at 37ºC (Difco, USA) was transferred into 5 ml of Ringer solution (pH 7.0, 0.75% w/v) to obtain suspension corresponding to 1 x 10^8 CFU/ml. A 100 µl aliquot from that dilution was transferred into 10 ml of Brian heart infusion/broth (BHI, Difco, USA), 200 µl of which was then inoculated into polystyrene microtiter plate wells (Greiner ELISA 12 Well Strips, 350 µl, flat bottom, Frickenhausen GmbH, Germany) and incubated for 24 hrs at 37ºC. The biofilm formed in the microtiter plate wells was washed twice with 200 µl of deionized water and dried at 25ºC for 40 min. The remaining attached bacteria were stained for 30 min at 25ºC with 200 µl of 0.1% (m/v) crystal violet in deionized water. The dye solution was aspirated away, and the wells were washed twice with 200 µl of deionized water. After the water removal was removed and the plate was dried for 30 min at 25ºC, the dye bound to the adherent biofilm was extracted with 200 µl of 95% ethanol and stirred. A 150 µl aliquot was transferred from each well into a new microplate well for absorbance- A at 570 nm using a Synergy TM4 Multi Mode Microplate reader (Biotek, USA). Each strain and condition was tested in two independent tests with 12 replicates. A sterile BHI was included in each analysis as a negative control. *Streptococcus equi* subsp. *zooepidemicus* CCM 7316 was used as positive control (kindly provided by Dr. Eva Styková, University of Veterinary Medicine and Pharmacy, Košice, Slovakia). Biofilm formation was classified as highly-positive ($A_{570} \geq 1$), low-grade positive ($0.1 \leq A_{570} < 1$) or negative ($A_{570} < 0.1$) according to Chaieb., *et al*. [16] and Slížová., *et al* [17].

Enzyme production: The commercial API-ZYM system (BioMérioux, Marcy l'Etoile, France) was used to test enzyme production. Evaluated enzymes involved in table 2 followed the manufacturer's recommendation; 1: alkaline phosphatase, 2: esterase (C4), 3: esterase lipase (C8), 4: lipase (C14), 5: leucine arylamidase, 6: valine arylamidase, 7: cystine arylamidase, 8: trypsin, 9: α-chymotrypsin, 10: acid phosphatase, 11: naftol-AS-BI-phosphohydrolase; 12: α-galactosidase, 13: β-galactosidase, 14: β-glucuronidase, 15: α-glucosidase, 16: β-glucosidase, 17: N-acetyl-β-glucosaminidase, 18: α-mannosidase, 19: α-fucosidase. Inocula (65 µl-microliter) of McFarland standard one suspensions were pipetted into each well of the kit. Enzymatic activities were evaluated after 4 hrs of incubation at 37°C and after the addition of Zym A and Zym B reagents. Color intensity values from 0 to 5 and their relevant value in nanomoles (nmol) were assigned for each reaction according to the color chart supplied with the kit.

Antibiotic resistance phenotype: Antibiotic phenotype in identified staphylococci (100 µl of an 18-h culture of each strain) was tested by the qualitative agar disc diffusion method on Brain heart blood agar (Difco, Clinical and Laboratory Standards Institute method [18]. Seventeen antibiotic discs (Oxoid, Basingstoke, United Kingdom) were applied: oxacillin (1 µg), clindamycin (2 µg), lincomycine (2 µg) novobiocin (5 µg), methicillin (10 µg), ampicillin (10 µg), neomycin (10 µg), tobramycin (10 µg), penicillin (10 IU), erythromycin (15 µg), azithromycin (15 µg), streptomycin (25 µg), chloramphenicol (30 µg), tetracycline (30 µg), vancomycin (30 µg), cefoxitin (30 µg), fosfomycin (50 µg), and gentamicin (120 µg). After incubation at 37ºC for 18 hrs, the strains were evaluated as resistant or susceptible according to the manufacturer's instruction; the inhibition zone was expressed in mm. Antimicrobial free agar plates were included as control for obligatory strain growth. The use of the antimicrobial agents and positive control strain was decided according to the manufacturer's guidance. Moreover, to differ resistant strains, their growth on ORSAB agar (Oxacillin resistance screening agar, Oxoid) was tested. Those colonies growing in blue are supposed to be resistant to oxacillin.

Susceptibility to enterocins and gallidermin: Eight partially purified enterocins (Ents) were used. They were prepared according to the protocols as follows (produced by the strains of different origin characterized in our laboratory): Ent A/P, Ent M, both produced by

environmental strains *Enterococcus faecium* EK13=CCM 7419, *E. faecium* AL41=CCM 8558 [19,20]; Ent 55, produced by *E. faecium* EF55 isolated from chicken [21], Ent EM41 produced by ostrichs strain *E. faecium* EM41, Ent 412 produced by *E. faecium* EF412 isolated from horses [22,23]. Ent 9296 produced by *E. faecium* EF9296 isolated from silage [24], Ent 4231 produced by *E. faecium* CCM4231 isolated from rumen content [25] and durancin ED26E/7produced by *E. durans* ED26E/7 isolated from ewes` lump cheese. *E. faecium* EK13=CCM 7419, *E. faecium* AL41=CCM 8558 are deponed in Czech Culture Collection of Microorganisms in Brno (CCM, Czech Republic). Inhibition activity of bacteriocins was tested by the agar spot test [26] against the principal indicator strain *E. avium* EA5 (inhibition activity reached up to 819 200 AU/ml). Briefly, Brain-heart infusion/broth supplemented with 1.5% agar (BHIA, Difco, USA) was used for the bottom layer. For the overlay, 0.7% BHIA enriched with 200 µl of an 18-hrs culture of indicator strain was used (Absorbance-A_{600} up to 0.800 nm). Dilution of enterocins (10 µl, in phosphate buffer, pH 6.5, ratio 1:1) was dropped on the surface of soft agar with each tested indicator (staphylococcal) strain. The plates were incubated at 37ºC for 18 hrs. Clear inhibition zones around dose of Ents were checked and the antimicrobial/inhibition activity was expressed in Arbitrary units per ml (AU/ml); this means the reciprocal of the highest two-fold dilution of Ent demonstrating complete growth inhibition of the indicator strain.

Gallidermin used was pure substance supplied by Enzo Life Sci. Corporation USA, (MW2069.4). Because of pure substance (evaluated from previous tests), it was used in concentration 0.5 mg/ml in a dose 2 µl. Identified staphylococci were tested for susceptibility to gallidermin also by using agar diffusion method according to De Vuyst., *et al* [26]. The antimicrobial activity of gallidermin was evaluated in arbitrary units per mililiter and also in minimum inhibition concentration-MIC in µg. All tests were performed twice. Positive control was the principal indicator strain *Enterococcus avium* EA5 (our isolate from piglets); its growth was inhibited by activity 25 600 AU/ml (MIC-0.0039 µg).

Results

The total counts of colonies detected on Mannitol salt agar was 2.94 ± 0.71 CFU/g log 10 on average. *S. arlettae* SArlI was identified with score value of 2.170; *S. aureus* Sa Darty had score of 2.164; *S. capitis* SciP8 had score of 1.800 (Table 1). *S. epidermidis* SeP5a and SeP9a reached score of 1.94 respectively 1.815; *S. equorum* Sq Muco was identified with score of 1.757; *S. haemolyticus* ShaeP5a reached score of 2.048; *S. haemolyticus* SHae Bado1 was allotted with score of 1.752; *S. hominis* SHo I/No5 had score of 1.75 (Table 1). *S. intermedius* SiBas possessed score of 2.007, *S. pseudintermedius* SpsBeno2 showed score of 2.096, *S. vitulinus* Sv Ayshe was with score of 1.989, *S. warneri* Sw Rocky2 had score of 1.839, *S. warneri* Sw Bado 1.752, and *S. warneri* Sw I/ŠtD as well were evaluated with score of 1.713 (Table 1). The most identified species (3 strains) was *S. warneri* (Table 1 and 2) following the species *S. haemolyticus* and *S. epidermidis* (2 strains). However, also the species *S. vitulinus* or *S. arlettae* were detected. Biochemical phenotypization of strains confirmed individual components reaction for those species evaluated [1] as well as for positive controls, e.g. *S. capitis* ATCC35661.

Strain	Canine Breed	Age	Sex	Ident. score
S. arlettae ArlI	Rhod. Ridgeb.	2 years	Male	2.170
S. aureus Sa	Austr. Sheph.	2.5 years	Male	2.164
S. capitis SciP8	Germ. Sheph.	15 months	Male	1.800
S. epidermidis SeP5a	Belg. Sheph.	2 years	Male	1.940
S. epidermidis SeP9a	Cross-breed	5 years	Male	1.815
S. equorum Sq Muco	Germ. Sheph.	5 years	Male	1.757
S. haemolyticus P5a	Belg. Sheph.	1 year	Male	2.048
S. haemolyticus Bado I	Labrador retr.	6 year	Male	1.752
S. hominis SHoI/No5	Germ. Sheph.	5 years	Female	1.750
S. *intermedius* Si Bas	Tibet. Ter.	6 months	Male	2.007
S. pseudinter. Beno2	Slov. Chuvach	1 year	Male	2.096
S. *vitulinus* Sv Ayshe	Czech. wolfdog	13 months	Female	1.989
S. warneri SwRocky 2	Staffsh. bullter.	3 years	Male	1.839
S. warneri SwBado	Labrador retr.	6 years	Male	1.752
S. warneri Sw I/Std	Coar-Styr.hound	6 months	Female	1.713

Table 1: *Canine staphylococcal strains, their source and identification score.*
SArlI-S. arlettae-Rhodesian Ridgeback; SA Darty-S. aureus-Australian shepherd; SciP8-S. capitis-German shepherd; SEP5a-Belgian shepherd; SEP9a-S. epidermidis-Cross-breed; I/StD-S. warneri-Coarse-haired Styrian hound; S. haemolyticus, S. warneri Bado-Labrador retriever; S. intermedius Bas-Tibetian terrier; S. pseudintermedius Beno 2-Slovakian Chuvach; S. warneri Rocky-Staffordshire bull terrier; S. vitulinus Ayshe-Czechoslovak wolfdog.

Seven strains were hemolysis-negative (γ-hemolysis), and 7 strains showed β-hemolysis phenotype. Seven strains also showed nuclease activity; among them both hemolytic strains of the species *S. haemolyticus.* (Table 2). Five strains were low- grade biofilm forming ($0.1 \leq A_{570} < 1$), the others did not show biofilm ability formation ($A_{570} < 0.1$; Table 2).

Strains	Hemolysis	Nuclease	Plate assay (± SD)
S. arlettae SArlI	γ	ng	0.005 (0.00)
S. aureus SA Darty	γ	+	nt
S. capitis Sci P8	β	+	0.101 (0.06)
S. epidermidis SEP5a	β	+	0.022 (0.02)
S. equorum Muco	γ	ng	0.071 (0.05)
S. haemolyticus ShaeP5a	β	+	0.111 (0.28)
S. haemolyticus ShaeBADo1	β	+	nt
S. hominis SHoI/No5	β	ng	0.102 (0.06)
S. intermedius Bas	β	+	0. 040 (0.01)
S. pseudintermedius Beno2	β	+	O. 123 (0.05)
S. vitulinus Sv Ayshe	γ	ng	0.103 (0.05)
S. warneri Sw Rocky2	γ	ng	0.089 (0.08)
S. warneri Sw BADO	γ	-	0.029 (0.09)
S. warneri Sw I/ŠtD	γ	-	0.079 (0.05)

Table 2: *Hemolysis, nuclease activity and biofilm formation testing in the identified staphylococci. SE 9a, not tested; β-hemolysis, γ-no hemolysis-negative, ng-negative result, +: nuclease activity was evaluated; -, negative reaction, no activity; nt-not tested; Biofilm formation tested using the plate assay.*

Among tested strains, only *S. pseudintermedius* Beno2 reached 40 nmol for alkaline phosphatase. Higher value (20 nmol) for this enzyme was also measured in *S. epidermidis* SeP5a and *S. warneri* Sw Bado. The value 10 nmol for alkaline phosphatase was measured in the strains *S. aureus* Sa Darty, *S. capitis* SciP8 and *S. arlettae* SArlI. In the rest of strains was measured minimal value (5 nmol) for this enzyme. Staphylococci also showed higher values for esterase (C4), esterase lipase (C8), acidic phosphatase, naftol-AS-BI-phosphohydrolase (range from 10 to 40 nmol). On the other hand, amounts of α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase or N-acetyl-β-glucosaminidase were very low (5 nmol) and enzyme β-glucuronidase (generally associated with cancer) reached 5 nmol in strains tested. Low values in enzymes were evaluated in *S. capitis* SciP8, *S. vitulinus* Ayshe and *S. warneri* Sw I/Št/D. The values of alkaline and acid phosphatase (40 nmol) were the highest in *S. pseudintermedius* SPsiBeno2.

Staphylococci were susceptible to vancomycin (Van), chloramphenicol-C, and gentamicin (CN). Coagulase-positive strain *S. aureus* Sa Darty was susceptible to ATBs used (Table 3). Multiresistant strains were noted among identified staphylococci- *S. pseudintermedius* SPsi Beno2 (Table 3) with resistance against 9 ATBs (out of 17); strains *S. warneri* SwRocky2 and Sw I/ŠtD were multiresistant against 5 and 7 ATBs (Table 3). *S. hominis* SHo I/No5 was resistant against 7 ATBs, *S. haemolyticus* SHae P5a showed resistant phenotype against 4 ATBs. *S. arlettae* and *S. capitis* SciP8 were resistant against 3 ATBs, *S. epidermidis* Se P5a and Sw Bado against 2 ATBs. Three strains were monoresistant - *S. equorum* Sq Muco (Te) and coagulase-positive strains species *S. intermedius* Si Bas (L) and *S vitulinus* Ayshe (S). Regarding the Met^R, *S. arlettae* SArlI, *S. hominis* SHo I/No5, Sw Rocky2 and Sw I/Št/D showed Met^R phenotype, SHo I/No5 and Sw I/Št/D were also penicillin resistant. Most strains were also susceptible to streptomycin (S), tetracycline (Te), phosphomycin, penicillin, ampicillin, azithromycin, erythromycin and neomycin. Resistance to lincomycine was the most detected. Their growth on ORSAB agar was in association with disc method results.

Strains	Ox/1	Da/2	L/2	Nv/5	Tb/10	Met/10	Amp/10	P/10	Azm/15	E/15	S/25	Te/30	Fos/50
SArlI	R	S/12	S/10	S/22	R	R	S/20	S/21	S/16	S/19	S/13	S/30	S/12
SA	S/22	S/25	S/16	S/24	S/21	S/24	S/21	S/21	S/22	S/16	S/16	S/28	S/27
SciP8	S/20	S/12	R	R	S/24	S/29	S/20	S/16	S/19	S/25	S/20	S/16	R
SeP5a	S/16	S/15	R	S/13	S/21	S/25	S/25	S/27	S/21	S/17	S/22	S/28	R
Sq	S/12	S/22	S/11	S/R/12	S/27	S/19	S/22	S/17	S/12	S/13	S/23	R	S/20
ShaeP5a	R	R	R	S/10	S/27	S/21	S/20	S/16	R	S/15	S/20	S/16	S/14
I/No	R	R	R	S/R/16	R	R	S/20	R	S/12	S/15	S/11	S/23	S/13
SiBas	S/14	S/19	R	S/16	S/23	S/21	S/12	S/13	S/20	S/23	S/15	S/17	S/12
Beno2	R	R	R	S/29	S/10	S/14	R	R	R	R	R	S/26	R
Ayshe	S/17	S/21	S/R17	S/14	S/25	S/21	S/22	S/21	S/17	S/21	R	S/25	S/11
Rocky2	R	R	R	S/21	R	R	S/18	S/20	S/14	S/15	S/11	S/27	S/13
Bado	S/18	R	R	S/20	S/25	S/20	S/14	S/15	S/11	S/13	S/12	S/28	S/12
I/ŠtD	R	R	R	S/16	R	R	S/14	R	S/16	S/15	S/11	S/26	R

Table 3: *Antibiotic phenotype of the identified staphylococci.*
R-resistant; S-susceptible; S/16-susceptible and 16 mm-size of the inhibition zone;
Abbreviations and antibiotics concentrations are involved in the part material and methods.

Staphylococci were mostly susceptible to Ents used (except Sv Ayshe which was resistant to Ents, but it was susceptible to duranc-in, inhibition activity 6 400 AU/ml). However, this strain was inhibited using gallidermin (12,800 AU/ml= MIC-0.0039 μg). The most susceptible to Ents were the strains *S. equorum* Sq Muco and *S. pseudintermedius* SPsi Beno2, which were inhibited by all Ents with activity 25,600 AU/ml as well as by gallidermin 12,800 AU/ml= MIC-0.0039 μg (Table 4). The other strains were inhibited by Ents with inhibition activity ranging from 400 up to 25,600 AU/ml and with gallidermin in activity 12,800 or 25,600 AU/ml corresponding with MIC 0.0039 and 0.0078 μg. The strains Sw Rocky 2 and Sw Bado as well as *S. hominis* SHo I/No5 were resistant to gallidermin.

Strain	EntM	EntA/P	Ent55	Ent4231	EM41	Ent9296	Ent412	ED26/7	Gall	G/MIC in μg
SArlI	3200	6400	3200	800	1600	25600	1600	1600	12800	0.0078
SeP5a	3200	12800	3200	800	1600	25600	3200	800	12800	0.0078
SciP8	nt	nt	nt	nt	nt	nt	nt	nt	12800	0.0078
Muco	25600	25600	25600	25600	25600	25600	25600	12800	25600	0.0039
ShaeP5a	nt	nt	nt	nt	nt	nt	nt	nt	12800	0.0078
I/No5	800	3200	3200	800	800	1600	3200	3200	R	R
Bas	6400	800	3200	400	800	12800	1600	800	3200	0.015
Beno2	25600	25600	25600	25600	25600	25600	25600	1600	25600	0.0039
Ayshe	ng	ng	ng	ng	ng	ng	ng	6400	12800	0.0078
Rocky2	6400	12800	3200	800	1600	25600	3200	1600	R	R
SwBado	1600	3200	3200	400	1600	25600	800	1600	R	R
I/ŠtD	6400	6400	6400	800	800	25600	6400	3200	12800	0.0078

Table 4: *Treatment of the identified staphylococci with enterocins and gallidermin expressed in AU/ml (Arbitrary unit per ml).*
AArlI-S. arlettae-Rhodesian Ridgeback; Sa Darty-S. aureus-Australian shepherd; SciP8-S. capitis-German shepherd;
SEP5a-Belgian shepherd; SEP9a-S. epidermidis-Cross-breed; I/StD-S. warneri-Coarse-haired Styrian hound; S. haemolyticus,
S. warneri Bado-Labrador retriever; S intermedius Bas-Tibetian terrier; S. pseudintermedius Beno 2-Slovakian Chuvach;
S. warneri Rocky-Staffordshire bull terrier; S. vitulinus Ayshe-Czechoslovak wolfdog; Sa Darty, SEP9a-not tested;
R-resistant, nt-not tested, ng-negative.

Discussion

The total count of staphylococci enumerated in canine feces were comparable with amount enumerated in the other animals [27]. However, their amount was lower than in feces of e.g. horses sampled in Slovakia [28]. In horses feces, total staphylococcal count reached 3.71 ± 0.96 CFU/g (log 10) on average.

Species variability was noted among staphylococci; 11 different species were identified - *S. arlettae*, *S. aureus*, *S. capitis*, *S. epidermidis*, *S. intermedius*, *S. pseudintermedius*, *S. vitulinus*, *S. equorum*, *S. hominis*, *S. haemolyticus*, and *S. warneri*. Based on 16S rRNA sequences [29], those 11 species have been involved in seven different clusters. Except coagulase-positive strains *S. aureus*, Sa Darty, *S. intermedius* SiBas, and *S. pseudintermedius* SPsiBeno2, the others were coagulase-negative. It is interesting that also *S. arlettae* species was identified in canine feces. Although using MALDI-TOF identification, only five strains were identified with score corresponding with secure genus identification/probable species identification and 10 strains were identified in range associated with probable genus identification, phenotypization of each one strain confirmed its taxonomical allocation by the reference strains [1]. *S. arlettae* was firstly isolated from the skin of poultry and goat, respectively [30] but it has never been isolated from canine feces. *S. intermedius* was described as opportunistic pathogen of dogs causing various infection [1]. Typical parameter for *S. pseudintermedius* is β-hemolysis. Hemolysis determination and nuclease activity represent virulence factors [31]; they are also identification marker for the species allocation. Seven strains with β-hemolysis were nucleaase-positive (except SHo I/No5), seven strains were hemolysis and nuclease-negative, except S. *aureus* Sa Darty which was nuclease-positive. Methicillin-resistant staphylococci pose a major public health treat. However, Vanderhaeghen., *et al*. [32] reported that in the population of 177 animals, only 2 were carrying *Met*^{R} gene. In tested strains phenotype resistance for Met was tested. Novobiocin is indicating diagnostic characteristic in staphylococci. Tested staphylococci are in association with this parameter reported in Bergey's Manual of Sytematic Bacteriology [1] for the appropriate species; although *S. hominis* Ho I/No5, *S. equorum* Sq Muco and *S. vitulinus* Sv Ayshe showed dubious reaction, zones were only up to 16 mm.

Alkaline phosphatase is enzyme playing role in metabolism within liver and skeleton. In human blood it works as an marker for hepatitis or osteomalacia diagnosis. Esterase, esterase lipase, acid phosphatase or naftol-AS-BI-phosphohydrolase represent hydrolyses. Results for enzymes were not depended on the staphylococcal species allocation; however, some not requested enzymes (high level) were measured in *S. epidermidis* SeP5a, *S. pseudintermedius* SPsiBeno2, and *S. hominis* SHo I/No5. In spite of that, enzymes production indicate that tested staphylococci are mostly not associated with the enzymes involving in diseases markers.

Staphylococci were susceptible to gallidermin (except *S. hominis* and two *S. warneri* strains). Gallidermin is lantibiotic bacteriocin predominantly inhibiting growth of Gram-positive bacteria, in which staphylococci belong. Anti-staphylococcal activity of gallidermin was also confirmed in previous study when biofilm-forming fecal staphylococci from roe deer and red deer were inhibited [9]. Even growth of multiresistant *S. pseudintermedius* SPsiBeno2 was inhibited by gallidermin. However, Met^{R} strains SHo I/No5, Sw Rocky2 and Sw Bado were resistant to gallidermin but they were susceptible to Ents. The growth of Met^{R} *S. arlettae* was inhibited with Ents. Growth of the strain *S. vitulinus* Ayshe was inhibited using gallidermin; however, it was resistant to Ents. There are only limited information regarding the strains resistance to Ents [11]. But it could be supposed that *S. vitulinus* can have this gene (here not tested). Also, resistance of *S. warneri* or *S. hominis* to gallidermin is surprising. Because gallidermin is known to have predominant inhibition activity against Gram-positive bacteria [33] in which formerly mentioned strains belong.

Conclusion

Species variability was noted in identified fecal staphylococci from 50 clients owned clinically healthy dogs of various breeds, sex and age sampled in eastern Slovakia. Eleven 11 different species were identified: *S. arlettae*, *S. aureus*, *S. capitis*, *S. epidermidis*, *S. intermedius*, *S. pseudintermedius*, *S. vitulinus*, *S. equorum*, *S. hominis*, *S. haemolyticus*, and *S. warneri.* Seven strains were hemolysis-negative (γ-hemolysis), 7 strains showed β-hemolysis and 7 strains were nuclease-positive. Five strains were low-grade biofilm- forming ($0.1 \leq A_{570} < 1$), the others were biofilm non-forming ($A_{570} < 0.1$). Staphylococci were mostly susceptible to Ents with activity up to 25 600 AU/ml and gallidermin

as well. Multiresistant strain *S. pseudintermedius* SPsi Beno2, low-grade biofilm-forming with β-hemolysis was susceptible to Ents and gallidermin.

Acknowledgements

The authors would like to thank Mrs. Margita Bodnárová for her skillful laboratory work and also dogs clients for their kindness to co-operate. Thanks also belongs Dr. Radomíra Nemcová from University of Veterinary Medicine and Pharmacy in Košice, Slovakia for her teaching in biofilm formation analysis. The results were achieved in the framework of the project Vega 2/0005/21 of the Slovak Scientific Agency.

Ethical Approval

Dog clients agreed with feces sampling.

Conflict of Interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Bibliography

1. Bergey's Manual of Systematic Bacteriology, Volume 3. The Firmicutes. ed. by De Vos, P., Garrity, G., Jones, P., Krieg NP., Ludwig, W., Rainey, F.A., Schleifer, K.H., Whitmann, W. New York (2009).
2. Jousson O., *et al*. "Genotypic versus phenotypic identification of staphylococcal species of canine origin with special reference to *Staphylococcus schleiferi* subsp. *Coagulans". Veterinary Microbiology* 123.1-3 (2007): 238-244.
3. Cox HU., *et al*. "Temporal study of staphylococcal species on healthy dogs". *American Journal of Veterinary Research* 49.6 (1988): 2039-2042.
4. Hoekstra KA and Paulton RJL. "Clinical prevalence and antimicrobial susceptibility of *Staphylococcus aureus* and *Staph*. intermedius in dogs". *Journal of Applied Microbiology* 93.3 (2002): 406-413.
5. Čuvalová Z., *et al*. "Virulence factors and antimicrobial resistance of coagulase-negative staphylococci isolated from drinking water". *Open Life Science* 10 (2015) 328-338.
6. Costerton JW and Stewart PS. "Battling biofilms". *Science American* 285.1 (2001): 74-81.
7. Parsek MR, Singh PK. "Bacterial biofilms: an emerging link to disease pathogenesis". *Annals and Review in Microbiology* 57 (2003): 677-701.
8. Sergelidis D., *et al*. "Isolation of methicillin-resistant *Staphylococcus* spp. from ready-to-eat fish products". *Letters in Applied Microbiology* 59.5 (2014): 500-506.
9. Lauková A., *et al*. "Characterisation of fecal staphylococci from roe deer (*Capreolus capreolus*) and red deer (*Cervus elaphus*) and their susceptibility to gallidermin*". Probiotic and Antimicrobial Proteins* 12.1 (2020): 302-310.
10. Franz CHMAP., *et al*. "Diversity of enterococcal bacteriocins and their grouping in a new classification scheme". *FEMS Microbiology Review* 31.3 (2007): 293-307.
11. Nes IF., *et al*. "Enterococcal bacteriocins and antimicrobial proteins that contribute to niche control". In Enterococci: from commensals to leading causes of drug resistant infection [Internet] eds. Gilmore MS, Clewel DB, Ike Y., *et al*. Boston: Massachusetts Eye and Ear Infirmary (2014): 21.

12. Pag U and Sahl HG. "Multiple activities in lantibiotics-models for the design of novel antibiotics". *Current Pharmaceutical Design* 8.9 (2002): 815-833.

13. Kubašová I., *et al*. "Safety assessment of commensal enterococci from dogs". *Folia Microbiologica* 62.6 (2017): 491-498.

14. Alatoom AA., *et al*. "Comparison of direct colony method versus extraction method for identification of Gram-positive cocci by use of Bruker Biotyper matrix-assisted laser desorption ionization-time of flight mass spectrometry". *Journal of Clinical Microbiology* 49.8 (2011): 2868-2873.

15. Semedo-Lemsaddek T., *et al*. "Virulence traits and antibiotic resistance among enterococci isolated from Eurasian otter (*Lutra lutra*) in Portugal". *Veterinary microbiology* 163.3-4 (2013): 378-382.

16. Chaieb K., *et al*. *"In vitro* effect of pH and ethanol on biofilm formation by clinical ica-positive *Staphylococcus epidermidis* strains". *Annals of Microbiology* 57 (2007): 431-437.

17. Slížová M*., et al.* "Analysis of biofilm formation by intestinal lactobacilli". *Canadian Journal of Microbiology* 61.6 (2015) 437-466.

18. Clinical Laboratory Standard Institute guideline, CLSI. Performance standards for antimicrobial susceptibility testing M100S, 26th Edition (2016).

19. Mareková M., *et al*. "Partial characterization of bacteriocin produced by environmental strain *Enterococcus faecium* EK13". *Journal of Applied Microbiology* 94.3 (2003): 523-530.

20. Mareková M., *et al*. "Isolation and characterization of a new bacteriocin, termed enterocin M, produced by environmental isolate *Enterococcus faecium* AL41". *Journal of Industrial Microbiology and Biotechnology* 34.8 (2007): 533-537.

21. Strompfová V and Lauková A. "*In vitro* study on bacteriocin of Enterococci associated with chickens". *Anaerobe* 13.5-6 (2007): 228-237.

22. Lauková A., *et al*. "Potential of enterococci isolated from horses". *Anaerobe* 14.4 (2008): 234-236.

23. Lauková A., *et al*. "*Enterococcus faecium* AL41, its Enterocin M and their beneficial use in rabbits husbandry". *Probiotics and Antimicrobial Proteins* 4.4 (2012): 243-249.

24. Marciňáková M., *et al*. "Occurrence of structural enterocin genes among silage enterococci". *Bulletin of Veterinary Institute in Pulawy* 49.4 (2005): 387-391.

25. Lauková A., *et al*. "Detection and antimicrobial spectrum of a bacteriocin-like substance produced by *Enterococcus faecium* CCM 4231". *Letters in Applied Microbiology* 16.5 (1993): 257-260.

26. De Vuyst L., *et al*. "Characterization of antagonistic activity of *Lactobacillus amylovorus* DCE471 and large-scale isolation of its bacteriocin amylovorin L471". *Systematic and Applied Microbiology* 19.1 (1996): 9-20.

27. Wats JL, Owens WE. "Prevalence of staphylococcal species in four dairy herds". *Research in Veterinary Science* 46.1 (1989): 1-4.

28. Bino E., *et al*. "Fecal coagulase-negative staphylococci from horses, their species variability, and biofilm formation". *Folia Microbiologica* 64.6 (2019): 719-726.

29. Schleifer KH and Bell JA. "Familly Staphylococcacae fam nov". In Bergey's Manual of Systematic Bacteriology, Volume 3. The Firmicutes, ed. by De Vos P, Garrity G, Jones P, Krieg NP, Ludwig W, Rainey FA, Schleifer KH, Whitmann W. New York (2009): 392-420.

30. Takashi T., *et al*. "Phylogenetic relationship of 38 taxa of the genus *Staphylococcus* based on 16S rRNA gene sequence analysis". *International Journal of Systematic Bacteriology* 49.2 (1999): 725-728.

31. Ebrahimi A., *et al*. "Some virulence factors of staphylococci isolated from wound and skin infections in Shahrekord, IT Iran". *Jundishapur Journal of Microbiology* 7.4 (2015): e9225.

32. Vanderhaeghen W., *et al*. "Screening for methicillin-resistant staphylococci in dogs admitted to a veterinary teaching hospital". *Research in Veterinary Science* 93.1 (2012): 133-136.

33. Kellner R., *et al*. "Gallidermin: a new lanthionine polypeptide antibiotic". *European Journal of Biochemistry* 177.1 (1988): 53-59.

Volume 17 Issue 6 Jane 2021