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Biofilm-forming ability and virulence factor detection in *Enterococcus hirae* strains of canine origin

Eva Bino, Ivana Kubašová, Viola Strompfová and Andrea Lauková*

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

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ABSTRACT

Bacteria, including enterococci, occur and exist in biofilm which is more favourable for them than existence in a planktonic form. In this study, 28 faecal canine strains of the species *E. hirae* were assessed to have virulence factor genes when studying their antibiotic status (susceptibility and/or resistance) but also their biofilm-forming ability, to assess their pathogenic potential. Biofilm-forming ability was associated with the presence of the intercellular adhesion gene cluster (*ica*) mediating the production of the polysaccharide intercellular adhesion. The *bap* gene (for biofilm-associated protein) encodes a protein associated with the cell wall. Only *E. hirae* CH+P/2a possessed the *ica* gene (for biofilm production) but the strains did not possess the *bap* gene. However, regarding the biofilm-forming ability testing using a quantitative method, 16 out of the 28 *E. hirae* showed low-grade positive biofilm-forming ability. Twelve strains did not form a biofilm. In two strains (EH/CH10b, EH/CH+P/4a), the adhesin gene *efaAfm* was found, and also *esp* and *gelE* genes (for surface protein and gelatinase). Gentamicin was the most effective antibiotic against canine *E. hirae* strains (100% susceptibility). On the other hand, the highest resistance (57.1%) was recorded in the case of ampicillin. Following assessment of *E. hirae* in this study, it was found that they did not show pathogenic potential.

Key words: biofilm; enterococci*;* faeces; dog

Introduction

Enterococci are Gram-positive cocci from the phylum Firmicutes. The species *Enterococcus hirae* belongs to the *Enterococcus faecium* group of the genus *Enterococcus* based on the 16S rRNA gene similarity analysis (ŠVEC and FRANZ, 2014). Representatives of this species can be found in the natural environment and in the alimentary tract of animals and also humans **(**ŠVEC and FRANZ, 2014; BOURAFA et al., 2015). *E. hirae* strains can possess virulence factor genes (KANDRIČÁKOVÁ et al., 2015). These can predict their pathogenic character (FISHER and PHILLIPS, 2009). The species strains *E. hirae* can be for instance decarboxylase-positive (PLEVA et al., 2012) and/or show biofilm-forming ability (BINO et al., 2018). According to SAUER (2003),

*Corresponding author:
Andrea Lauková, Centre of Biosciences of the Slovak Academy of Sciences, Institute of Animal Physiology, Šoltésovej 4-6, 040 01 Košice, Slovakia, phone: +421557922964; fax: +42155787842; e-mail mail: laukova@saske.sk

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biofilm is defined as a consortium of microbiota attached to a biotic (the intestinal wall) or abiotic surfaces (e.g. catheters and similar). Biofilm growth protects the bacteria against host defences and the action of antimicrobial agents (SAUER, 2003). Therefore, biofilm can be a source of persistent infection (DAVIES, 2003). Many species strains grow in a biofilm environment, including the strains of the species *E. hirae* (SAHIN, 2019). This is more beneficial for the bacteria than the planktonic form (SAHIN, 2019). ANDERSON et al. (2016) reported that microtiter plate assay proved to be a suitable tool for testing biofilm formation. SOARES and COLOMBO (2014) even found a significant association of the virulence factor genes *esp, gelE* detection with the ability to form biofilm (virulence factor), especially in the clinical strains of *E. faecalis*. The composition of the microbial biofilm in the digestive system varies depending on the site of attachment. Identification of some genes involved in biofilm formation is needed to understand the molecular basis of strain variations, and the pathogenic mechanisms involved in bacterial infection which differ in some aspects. The microbiota of the animal intestine is strongly influenced by the composition of the diet administered and by the quality of the digestive processes in the higher parts of the digestive tract (KERN et al., 2018). Insufficient digestion and protein breakdown in the stomach and small intestine can lead to the overgrowth of clostridia at the expense of lactobacilli and the other symbiotic microbiota (KERN et al., 2018). The secreted virulence factors of enterococcal species have a function in pathogenesis (FISHER and PHILLIPS, 2009). Bacterial pathogenicity can lead to diseases occurrence in the host, stimulated by virulence factor genes (gelatinase, aggregation substance, surface protein, adhesions, cytolysin, IS element, hyaluronidase, sex pheromones), by biofilm-forming ability, and/or antibiotic resistance. The c*ytolysin* gene is detected in high numbers in clinical strains (SEMEDO et al., 2003). Sex pheromones, cpd, cob, ccf, are small peptides which facilitate the conjugative transfer of plasmids between cells. Extracellular surface protein (esp) is a cell-wall-associated protein that occurs in enterococcal species. Esp and aggregation substance are factors associated with supporting the adhesion and colonization of pathogenic bacteria (KUBAŠOVÁ et al., 2017). Hyaluronidase acts on hyaluronic acid. It is a degradative enzyme associated with tissue damage (KAYAOGLU and ORSTAVIK, 2004). The main role of gelatinase in enterococcal pathogenesis lies in providing nutrients to the bacteria by degrading host tissue. It also has a function in the biofilm-forming ability of individual strains (FISHER and PHILLIPS, 2009). Although IS 16 is a marker specific for the species *E. faecium*/*E. faecalis*, it can also occur in *E. hirae* strains. Adhesins help to adhere host cells to mucosa (KUBAŠOVÁ et al., 2017). Therefore, the aim of this study was to assess the biofilm-forming ability of selected *E. hirae* strains, as well as other virulence factors such as: antibiotic resistance, gelatinase, enterococcal surface protein, *E. faecium* adhesin, cytolysin A, hyaluronidase, aggregating substance, cell wall adhesins, sex pheromones, and element *IS16*. These parameters may be responsible for pathogenicity of some *E. hirae* strains as potential disease agents.

Materials and methods

The target of E. hirae strains and virulence factor gene analysis. Twenty-eight *E. hirae* strains were involved in this study. They were selected out of 160 faecal isolates from 105 clinically healthy dogs in the eastern Slovakia region during the years 2014 and 2015, as previously described by KUBAŠOVÁ et al. (2017). The faeces were sampled from individual dogs of different breeds, sexes and ages. Detailed identification and characterization of the strains was previously reported by KUBAŠOVÁ et al. (2017). They were identified using the MALDI-TOF BioTyper™ identification system (Bruker Daltonics, USA) on the basis of bacterial protein analysis (KUBAŠOVÁ et al., 2017). The *E. hirae* species strains were evaluated using scores ranging from 2,300 to 3,000 corresponding with highly probable species identification. They were also associated with the scores for the reference *E. hirae* strain in the MALDI -TOF database.

The following virulence factor genes were tested: *gelE* (gelatinase), *esp* (enterococcal surface protein), *efaAfm* (*E. faecium* adhesin), *cylA* (cytolysin A), *hylEfm* (hyaluronidase), *agg* (aggregating substance), *efaAfs* (cell wall adhesins), *ccf, cob, cpd* (sex pheromones) and element *IS16* (IS 16). The PCR method was applied, as previously reported by KUBAŠOVÁ et al. (2017). DNA was extracted using the rapid alkaline lysis method (BAELE et al., 2001). Individual primers were then used and a C1000™ thermocycler (BioRad Laboratories, Hercules, USA). The PCR products were separated by electrophoresis on an agarose gel (1.2% w/v Sigma-Aldrich, Saint Louis, USA) containing 1 µl/ml ethidium bromide (Sigma-Aldrich) using 0.5 x TAE buffer (Merck, Darmstadt, Germany), as previously reported by KUBAŠOVÁ et al. (2017). PCR fragments were visualized under UV light. The PCR was carried out in a volume of 25 µl with a mixture consisting of 1x reaction buffer, 0.2 mmol/l deoxynucleoside triphosphate, 3 mmol MgC_{l2}, 1 µmol/l of each primer, 1 U of Taq DNA polymerase, and 1.5 µl DNA template (KUBAŠOVÁ et al., 2017). The cycling conditions were as follows (except for IS16 and hyl Efm): an initial step of 95°C for 3 min, 35 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C, followed by an additional step at 72°C for 5 min. The conditions for amplification of IS*16* were as follows: 94°C for 4 min, 30 cycles of 30 s at 94°C, 30 s at 50°C, and 30 s at 72°C, followed by 72°C 4 min (KUBAŠOVÁ et al., 2017). For the *hylEfm* gene, the following conditions were provided: 94°C for 4 min; 94°C for 30 s, 50°C for 30 s, 72°C for 30 s (30 cycles), and 72°C for 4 min. The strains *E. faecalis* P36 and *E. faecium* F10 (kindly provided by Dr. Teresa Semedo-Lemsaddek, University of Lisbon, Portugal) and *E. faecium* UW 9086 (provided by Dr. I. Klare, of the Robert Koch Institute in Germany) were used as positive controls in gene detection. *E. faecium* UW 9086 was the control for hyaluronidase gene testing, F10 for the *IS 16* gene and the P36 strain for the remaining gene testing. The oligonucleotides used in this study to amplify the virulence factor genes in canine enterococci were also previously reported by KUBAŠOVÁ et al. (2017) with the procedures according to EATON and GASSON (2001), KLARE et al. (2003), WERNER et al. (2011) and SEMEDO-LEMSADDEK et al. (2013).

Antimicrobial profile testing using antibiotic discs. The antimicrobial profile was tested using the disc diffusion method on Mueller-Hinton agar (Oxoid) according to CLSI (2016). The antibiotic discs used (12) were supplied by Oxoid. Antibiotics from different antibiotic groups were used: penicillin (10 µg) and ampicillin (10 µg) from β-lactams; the aminoglycosides group was represented by gentamicin (120 µg); the macrolide antibiotic erythromycin (15 µg) was also involved in testing, as well as the broad-spectrum antibiotics - chloramphenicol (30 µg) and tetracycline (30 µg). Fluroquinolon ciprofloxacin (5 µg) and quinupristin-dalfopristin (15 µg) were also analysed. Peptidoglycan teicoplanin (30 µg) and ansamycin antibiotic rifampicin (30 µg) were also involved in the testing. The vancomycin antibiotic glycopeptide (30 µg) was also involved in the testing. Moreover, trimethoprim (5µg) was involved in the test. Agar plates with broth cultures (100 µl) of the tested strains with the antibiotic discs applied were incubated at 37°C overnight. After incubation, the inhibitory zones were measured and expressed in mm. The results were interpreted as resistant and/or susceptible strains on the basis of the Clinical and Laboratory Standards Institute breakpoint table (CLSI, 2016). *Enterococcus faecalis* ATCC 29212 was used as the positive control.

Biofilm formation assay. The biofilm-forming ability of *E. hirae* strains was tested using the quantitative method in microtiter plates (CHAIEB et al., 2007; SLÍŽOVÁ et al., 2015). The tested strain (10 µl, grown on Trypticase-Soy-Yeast Infusion agar - TSYA, Becton and Dickinson, USA) was transferred into Ringer solution (5 ml, pH 7.0, 0.75% w/v, Merck, Darmstadt, Germany) to reach the McFarland standard 0.5 suspension. This corresponds to 1 x 10^8 CFU/ml. Then a volume of 100 µl was transferred into 10 ml TSY broth. The standardized culture (200 µl) was inoculated into a polystyrene microtiter plate well (Greiner ELISA 12 Well Strips, 350 µl, flat bottom, Frickenhausen GmbH, Germany) and incubated

for 24 h at 37°C. The biofilm that formed in the well of the microtiter was washed twice with 200 µl of deionized water, and dried at laboratory temperature for 30 min. The remaining attached bacteria were stained for 30 min at 25°C with 200 µl of 0.1 % (m/v) crystal violet in deionized water. The dye solution was aspirated away. The wells were washed twice with 200 µl of deionized water. After water removal and drying for 30 min at 25°C, 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 to a new microtiter plate for absorbance (A) determination at 570 nm, using a Synergy TM4 Multi Mode Microplate reader (Biotek USA). Each strain and condition were tested in two independent tests with 12 replicates. Moreover, a sterile medium was included in each analysis as the negative control. *Streptococcus equi* subsp. *zooepidemicus* CCM 7316 was used as the positive control in each analysis (kindly provided by Dr. Eva Styková, University of Veterinary Medicine and Pharmacy, Košice, Slovakia). Biofilm formation by *E. hirae* was then classified according to CHAIEB et al. (2007) and SLÍŽOVÁ et al. (2015) as high-grade positive (A570 ≥1), low-grade positive (0.1 ≤ A570 < 0.1) and negative (A570 < 0.1).

Detection of the ica and bap genes responsible for the biofilm-forming ability of E. hirae strains. *E. hirae* strains were cultured on M-Enterococcus agar for 24 h at 37°C. One colony was picked up from each pure *E. hirae* strain and subsequently mixed in 20 µl of sterile injection water. After mixing, the supernatant was used as a DNA template for PCR analysis. The genes *ica* and *bap* are found most often in biofilm-forming bacteria. Therefore, the presence of *ica* and *bap* genes was tested in this study (Table 1) as follows: initial denaturation at 94°C for 2 min, with 37 cycles at 95°C for 1 min, annealing at 42°C for 1 min, and elongation at 72°C for 10 min. The size of the PCR products was analysed in a 1.2% agarose gel (Sigma, Germany) using 1 × TAE buffer (Merck, Germany) on a Techgene KRD thermocycler (Techne, UK). The results were visualized with UV light. The strain *E. hirae* KČ1/b was used as a positive control for the *ica* gene. To confirm the *bap* gene, the strain *E. hirae* 211 was used as a positive control.

Table 1. The primers for *ica* and *bap* genes related with biofilm production

Gene	Primers	Sequencing 5´-3´
ica	Ica4F	TGGGATACTGAYATGATTAC
	Ica4r	CCTCTGTCTGGGCTTGACCATG
bap	Sasp-6m	CCCTATATCGAAGGTGTAGAATTGCAC
	Sasp-7c	GCTGTTGAAGTTAATACTGTACCTGC

The primers for virulence factor gene detection which are not in Table 1 have already been published previously by KUBAŠOVÁ et al. (2017)

Results

Virulence factor gene detection, antibiotic profile, biofilm-forming ability. E. hirae strains were absent from the genes *efaAfs, cylA, agg, cob, cpd, IS16,* and *hylEfm* (Table 2). The genes *gelE* and *efaAfm* were detected in the strains EH/CH+P4a and EH/CH10b. In addition, the *esp* and *ccf* genes were also detected in the strain *E. hirae* EH/CH+P4a (Table 2).

Table 2. Virulence factor gene analysis and biofilm-forming ability in canine strains *Enterococcus hirae*

Enterococcus hirae	Absorbance reached in microtiter plate assay	*gelE*	efa_{Afm}	*esp*	*ccf*
EH/e/Doz/b	0.031 ± 0.016	-	-	-	-
EH/G01	0.167 ± 0.011	-	-	-	-
EH/G/49/a	0.125 ± 0.016	-	-	-	-
EH/CH/6	0.021 ± 0.021	-	-	-	-
EH/CH/10b	0.055 ± 0.015	+	+	-	-
EH/CH/4a	0.119 ± 0.09	-	-	-	-
EH/CH+P/10a	0.237 ± 0.025	-	-	-	-
EH/CH+P/2a	0.140 ± 0.018	-	-	-	-
EH/CH+P/4a	0.209 ± 0.019	+	+	+	+
EH Badoo	0.029 ± 0.025	-	-	-	-
EH Olina	0.051 ± 0.016	-	-	-	-
EH/CH+P/2b	0.219 ± 0.035	-	-	-	-
EH/CH/8	0.027 ± 0.024	-	-	-	-
EH Darty/a	0.002 ± 0.017	-	-	-	-
EH Begy/b	0.019 ± 0.019	-	-	-	-
EH Chaster/b	0.006 ± 0.029	-	-	-	-
EH/L/a/žltá	0.272 ± 0.016	-	-	-	-
EH/CH/7	0.168 ± 0.027	-	-	-	-
EH/Ro1	0.294 ± 0.024	-	-	-	-
EH/AD2	0.100 ± 0.025	-	-	-	-
EH/P7a	0.177 ± 0.035	-	-	-	-
EH/P13	0.002 ± 0.017	-	-	-	-
EH/l/Kyy	0.035 ± 0.023	-	-	-	-
EH/l/Doz/a	0.232 ± 0.018	-	-	-	-
EH/fifi/a	0.229 ± 0.019	-	-	-	-
EH/Army/b	0.280 ± 0.028	-	-	-	-
EH/AL1 b	0.208 ± 0.019	-	-	-	-
EH/A1-KO1	0.004 ± 0.031	-	-	-	-

± SD - standard deviation, Biofilm formation was classified as high-grade positive biofilm- forming (A570 ≥1), low-grade positive biofilm-forming strains (0.1 ≤ A570 < 1.0) or no biofilm-forming strains (A570 < 0.1). In summary, virulence factor gene presence was indicated as + (positive) or absence – (negative).

Table 3. Antibiotic susceptibility/resistance of canine faecal strains *E. hirae*

Strain	AMP	CIP	CHLF	ERY	GEN	P	RFM	QNS	TTR	TEI	TMP	VAN
EH/e/Doz/b	S (20)	S (20)	S (16)	S (19)	S (13)	S (20)	R	R	R	R	S (26)	S (16)
EH/G01	R	S (14)	S (15)	S (19)	S (12)	R	R	R	S (17)	R	R	S (15)
EH/G/49/a	R	R	S (13)	R	S (13)	S (16)	S (17)	R	S (15)	R	R	S (17)
EH/CH/6	R	S (20)	S (18)	S (20)	S (13)	S (19)	S (21)	R	S (15)	R	R	S (15)
EH/CH/10b	R	S (21)	S (18)	S (22)	S (15)	S (18)	S (21)	S (19)	S (17)	S (17)	S (28)	S (19)
EH/CH/4a	R	S (22)	S (19)	S (22)	S (15)	S (16)	S (25)	S (16)	R	S (16)	S (22)	S (17
EH/CH+P/10a	R	S (22)	S (19)	S (20)	S (14)	S (18)	S (19)	R	S (19)	R	S (25)	S (20)
EH/CH+P/2a	R	S (26)	S (20)	S (21)	S (16)	S (20)	S (27)	S (16)	S (18)	S (19)	S (30)	S (21)
EH/CH+P/4a	R	S (22)	S (20)	S (23)	S (17)	S (17)	S (21)	S (16)	S (18)	S (16)	S (30)	S (20)
EH Badoo	R	S (20)	S (16)	S (18)	S (12)	S (19)	S (18)	R	S (20)	R	S (26)	R
EH Olina	R	S (18)	S (18)	S (14)	S (13)	R	S (18)	R	R	S (20)	R	S (15)
EH/CH+P/2b	R	S (24)	S (20)	S (20)	S (15)	S (21)	S (21)	R	S (20)	S (17)	S (27)	S (22)
EH/CH/8	S (22)	S (20)	S (18)	S (20)	S (15)	S (20)	S (25)	R	S (18)	S (17)	S (25)	S (16)
EH Darty/a	S (29)	S (25)	S (20)	S (22)	S (17)	S (23)	S (30)	S (18)	R	S (20)	S (35)	S (23)
EH Begy/b	S (28)	S (24)	S (24)	S (24)	S (18)	S (17)	S (25)	S (17)	R	S (16)	S (32)	S (22)
EH Chaster/b	S (28)	S (24)	S (22)	S (20)	S (17)	S (16)	S (25)	S (17)	R	R	S (33)	S (21)
EH/L/a/žltá	S (18)	S (20)	S (17)	S (17)	S (14)	R	S (19)	S (16)	S (20)	R	S (25)	S (16)
EH/CH/7	R	S (22)	S (20)	S (22)	S (15)	S (15)	S (20)	R	S (17)	R	S (30)	S (19)
EH/Ro1	S (22)	S (26)	S (21)	S (20)	S (15)	S (19)	S (23)	S (16)	S (21)	S (18)	S (35)	S (19)
EH/AD2	R	S (25)	S (18)	S (19)	S (15)	S (19)	S (27)	S (16)	S (20)	S (17)	S (31)	S (19)
EH/P7a	R	S (19)	S (14)	S (15)	S (14)	S (22)	S (20)	R	R	R	S (22)	S (19)
EH/P13	S (22)	S (20)	S (20)	S (20)	S (15)	S (23)	S (25)	R	R	S (16)	R	S (16)
EH/l/Kyy	R	S (21)	S (17)	S (19)	S (14)	S (20)	S (20)	S (16)	S (17)	R	S (25)	S (17)
EH/l/Doz/a	R	S (20)	S (18)	S (20)	S (15)	S (19)	S (22)	R	S (16)	R	S (27)	S (20)
EH/fifi/a	S (22)	S (20)	S (22)	R	S (15)	S (17)	R	R	S (22)	R	S (21)	S (16)
EH/Army/b	S (30)	R	R	S (22)	S (20)	S (25)	S (22)	S (16)	R	S (20)	S (37)	S (22)
EH/AL1 b	S (22)	R	S (20)	S (20)	S (15)	S (16)	S (23)	R	R	S (16)	S (30)	S (19)
EH/Al-KO1	S (26)	S (22)	S (20)	S (21)	S (17)	S (31)	S (21)	S (16)	S (23)	S (19)	S (30)	S (20)

AMP - ampicillin 10 µg, CIP - ciprofloxacin 5 µg, CHC - chloramphenicol 30 µg, E - erythromycin 15 µg, GN - gentamicin 120 µg, P - penicillin 10IU, RFM - rifampicin 30 µg, QNS - quinopristin 15 µg, TC - tetracycline 30 µg, TEI - teicoplanin 30 µg, TMP - trimetoprim 5 µg, VAN - vancomycin 30 µg, R - resistant; S - susceptible (inhibitory zone size in mm); *E. hirae* strains were susceptible to gentamicin (inhibitory zones ranging from 12 mm to 20 mm). Susceptibility to ampicillin was represented by the inhibitory zones in a range from 18 to 30 mm. Susceptibility to ciprofloxacin was represented by the inhibitory zones ranging from 14 to 26 mm. Susceptibility to chloramphenicol was represented by the inhibitory zones from 13 mm to 24 mm. Susceptibility to erythromycin was represented by the inhibitory zones ranging from 14 to 24 mm. Susceptibility to penicillin was represented by the inhibitory zones from 15 to 31 mm. Susceptibility to rifampicin was represented by the inhibitory zones from 17 to 30 mm. Susceptibility to quinopristin was represented by the inhibitory zones from 16 to 19 mm, to teicoplanin 16-20mm, to tetracycline 15-23 mm, to vancomycin 16-23 mm and to trimethoprime 21-37 mm.

Regarding antibiotic resistance and/or susceptibility, aminoglycoside gentamicin (Gn) was 100% effective against canine *E. hirae* strains. This means the tested strains were susceptible to gentamicin (Table 3). The majority of *E. hirae* strains (57. 2%) were resistant to ampicillin. *E. hirae* strains (53.6%) were resistant to quinupristin-dalfopristin. 46.4% strains were resistant to teicoplanin (Table 3). Resistance to tetracycline was found in 35.7% of the *E. hirae* tested. In contrast, 89.3% *E. hirae* strains were susceptible to penicillin and rifampicin; this means that 10.7% of strains were resistant to penicillin and rifampicin. A similar percentage of resistance was found for ciprofloxacin. Susceptibility (96.4% and 92.8%) to broad-inhibitory spectrum antibiotics, such as chloramphenicol and tetracycline, was detected. The strain EH/G01 was resistant to seven antibiotics from different groups, meaning it is multidrug resistant strain. The strain EH/G/49/a was resistant to six antibiotics. The strain EH/Olina was found with resistance to five antibiotics (Table 3). *E. hirae* EHRo1 and EH/Al-K01 were susceptible to the tested antibiotics. In total, antibiotic resistance was recorded in 57.14% of canine *E. hirae* strains. Two *E. hirae* strains, EH/G/49a and EH/Fifi/a were resistant to macrolide erythromycin. There was only one strain *E. hirae* resistant to broad-inhibitory spectrum antimicrobial chloramphenicol (EH/Army/b) and glycopeptide vancomycin (EH Badoo). The EH/e/Doz/b strain was resistant to four antibiotics and had no virulence factor genes. This strain also did not form a biofilm**.** Multidrug resistant, biofilm-forming strains EHG01 and EH/G/49/a had no virulence factor genes. However, the *E. hirae* strain EH/CH/6 with no virulence factor genes was resistant to four antibiotics and did not form a biofilm. In contrast the ampicillin resistant strain *E. hirae* EH/CH/10b did not form a biofilm; however, it possesses g*elE* and *efaAfm* genes. The bi-resistant strain EH/CH/4a did not possess virulence factor genes but it had biofilm formation ability. The biofilm-forming strain EH/CH+P/10a had no virulence factor and was resistant to three antibiotics. The mono-resistant, biofilm-forming strain EH/CH+P/2a did not possess virulence factor genes (Table 2, 3). The biofilm-forming strain EH/CH+P/4a possessed four virulence factor genes. However, it was only resistant to ampicillin. The other non-biofilm-forming strains, with various degrees of antibiotic resistance, did not possess virulence factor genes (Table 2, 3). EH/Ro1, with susceptibility to all tested antibiotics, was biofilm-forming and had no virulence factor genes. On the other side, the antibiotics susceptible strain EH/AL-KOI did not form a biofilm and it was lacking virulence factor genes.

Nine out of 28 *E. hirae* strains were evaluated as high-grade-positive (biofilm-forming, A_{570} 0.208–0.294). Seven strains were low-grade biofilm-forming (A_{570}: 0.1–0.177). *E. hirae* EHRo1 showed the highest value (0.294) regarding biofilm-forming ability, and the lowest value was measured in the strain EH/AD2 (0.1) (Table 3). Twelve strains (12) were non- biofilm-forming (Table 3). Only *E. hirae* CH+P/2a possessed the *ica* gene. *E. hirae* strains did not possess the *bap* gene.

Discussion

Enterococci, lactic acid bacteria comprising both pathogenic and commensal strains, are ubiquitous in the environment, even as gut symbionts (HANCHI et al., 2018). The microtiter plate assay is used for the rapid assessment of biofilm production for a large number of bacterial strains. In this study, the biofilm assay was shown to be useful to obtain new information regarding the canine *E. hirae* strains. In our study, 28 canine *E. hirae* species strains were analysed. However, the most commonly occurring species among enterococci in healthy dogs was reported to be the species *Enterococcus faecalis* (JACKSON et al., 2009). KUBAŠOVÁ et al. (2017) also detected other species in canine faeces, such as *Enterococcus faecium, E. faecalis, E. casseliflavus,* and *E. mundtii.* DEVRIESE et al. (1991, 1992) reported that the *E. hirae* species strains were also isolated from dogs and cats, but also from poultry intestines, with their basic characteristic.

Pathogenic bacteria are essentially able to adhere to, grow on, and invade the host. Bacteria need to survive the host's defense mechanisms, compete with other bacteria, and cause pathological changes (SAUER, 2003). The presence of virulence

factors does not necessarily mean that isolated strains cause disease (SAHIN, 2019), but they may have pathogenic potential since virulence factors have been found to contribute to the severity of infections (SAHIN, 2019). The decline in the emergence of new antibiotics on the market over the past two decades is worrying, particularly in view of the rise in bacterial resistance against many currently used antibiotics (TEWARI et al., 2018). Given antibiotic resistance and biofilm formation in such strains, enterococcal infection can easily occur in animals (TEWARI et al., 2018). In our study, 16 out of 28 *E. hirae* strains formed a biofilm, but only one strain possessed the *ica* gene. In this study, *E. hirae* did not possess the *bap* gene. In general, biofilm has an immense negative impact on the world's economy and pose serious problems to industry, public health and medicine, due to increased rates of genetic exchange, altered biodegradability, increased resistance to antibiotics/antimicrobials and chemical biocides, and increased production of secondary metabolites (DI LODOVICO et al., 2017). Although, many antibiotics are effective against planktonic cells, fewer are active against biofilms (SHANKS et al., 2012). In our study, biofilm formation was monitored in order to evaluate it as one of virulence factors related to the pathogenicity potential of the *E. hirae* strains. DI ROSA et al. (2006) reported an association between the presence of the *esp* gene and biofilm formation in *E. faecium* species strains isolated from infection, compared to other sources. This may suggest a synergy between these factors. However, e.g. in other canine enterococcal species detected, such as *E. casseliflavus* and *E. mundtii,* no virulence factor genes appeared (KUBAŠOVÁ et al., 2017). The same author has also reported on the majority of multi-resistant canine enterococci (KUBAŠOVÁ et al., 2017).

KANDRIČÁKOVÁ et al. (2015) tested enterococci (*E. hirae*) from pheasants, and they found resistance to ampicillin, which is in contrast to results presented for canine *E. hirae.* The majority of *E. hirae* from our study were susceptible to vancomycin, except EH Badoo. Currently, genomic characterization of *E. hirae* from beef cattle feedlots and associated environmental continua has been reported by SANI-U ZEHRA et al. (2022). They also showed that *E. hirae* from bovine faeces has susceptibility to vancomycin and resistance to ampicillin. Van-resistance in enterococci, especially among clinical strains, has tended to increase, therefore the susceptibility to vancomycin in our *E. hirae* is an excellent property. From the beneficial point of view, the *E. hirae* species strains could be a source of bioactive compounds – bacteriocins - which may be effective against other bacteria, as previously reported by LAUKOVÁ et al. (2019) in the case of *E. hirae* from ducks or CAVICCHIOLI et al. (2019) in the case of the *E. hirae* ST57ACC strain from artisanal cheese. This indicates the need for testing canine *E. hirae* for bacteriocin activity, especially when *E. faecium* canine strains demonstrated this property (STROMPFOVÁ et al., 2019). Therefore, future studies of canine *E. hirae* strains could be directed towards testing their beneficial properties.

Conclusions

Sixteen (16) out of 28 canine faecal *E. hirae* strains were found to form a biofilm. However, only two out of 16 strains possessed the virulence factor genes tested. Gentamicin was found to be the most effective antibiotic against *E. hirae* strains. The majority of strains (57.2%) were resistant to ampicillin. From the results achieved regarding virulence factors (pathogenicity parameters), the *E. hirae* strains tested in this study did not show pathogenic potential. Additional studies are in progress to confirm this aspect.

Ethics satement

Samples from live animals were taken by responsible persons involved in the project. They were analysed for diagnostic purposes and safety, and approved by the relevant Ethics Committee.

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Author contributions

E. B. Methodology, Investigation, Data Curation, Writing; I.K. Methodology; V. S. Resources; A.L. Conceptualization, Project Administration, Data Curation, Writing Editing. All authors agreed with the final version of the manuscript and its submission.

Declaration of competing interest

The authors declare no conflict of interest.

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Some parameters have been already reported in our previous article: Kubašová et al. (2017), Safety assessment of commensal enterococci from dogs in Folia Microbiologica, 62, 491-498.

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BINO, E., I. KUBAŠOVÁ, V. STROMPFOVÁ, A. LAUKOVÁ: Sposobnost stvaranja biofilma i čimbenici virulencije kod sojeva bakterije *Enterococcus hirae* podrijetlom od pasa. Vet. arhiv 94, 375-386, 2024.

SAŽETAK

Bakterije, uključujući enterokoke, pojavljuju se i opstaju u biofilmu koji je znatno povoljnije okruženje za njih u odnosu na ono u planktonu. U ovom je istraživanju 28 sojeva *E. hirae*, izoliranih iz psećeg izmeta, analizirano kako bi se procijenio patogeni potencijal s obzirom na čimbenike i gene virulencije, antibiotički status (osjetljivost odnosno rezistenciju) i sposobnost stvaranja biofilma. Sposobnost stvaranja biofilma povezana je s prisutnošću klastera gena međustanične adhezije (*ica*) te s posredovanjem u proizvodnji polisaharidnog međustaničnog adhezina. Gen *bap* (gen za protein povezan s biofilmom) kodira protein povezan sa staničnom stijenkom. Samo je soj CH+P/2a bakterije *E. hirae* posjedovao gen *ica* (za proizvodnju biofilma), no sojevi nisu pronađeni u genu *bap*. Što se tiče sposobnosti stvaranja biofilma kvantitativnom metodom, 16 od 28 sojeva bakterije *E. hirae* pokazalo je nizak stupanj sposobnosti stvaranja biofilma. Biofilm nije formiralo 12 sojeva. U dva soja (EH/CH10b i EH/CH+P/4a) nađen je gen za adhezin *efaAfm*, također gen *esp* i geni *gelE* (za površinski protein i gelatinazu). Gentamicin je bio najučinkovitji antibiotik protiv sojeva *E. hirae* podrijetlom od pasa (osjetljivost 100%). Najveća je rezistencija pak zabilježena za ampicilin (57,1%). Rezultati ovog istraživanja nisu pokazali patogeni potencijal bakterije *E. hirae*.

Ključne riječi: biofilm; enterokoki; izmet; pas