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Original article

Assessment of biofilm formation by faecal strains of *Enterococcus hirae* from different species of animals

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Abstract

Enterococcus hirae belongs in the *Enterococcus faecium* group within the genus Enterococcus. This species occurs naturally in the environment, commensally in the alimentary tracts of animals, and pathologically for example in humans with urinary infections. Some strains of *E. hirae* possess virulence factors, including biofilm formation. Biofilm growth protects bacteria against host defences; biofilm can be a source of persistent infection. Testing bacterial strains for their ability to form biofilm might therefore facilitate their treatment or prevention. This study focuses on biofilm formation by *E. hirae* strains derived from various animals. This kind of testing has never been done before. A total of 64 identified *E. hirae* from laying hens, ducks, pheasants, ostriches, rabbits, horses and a goat were tested by means of three methods; using Congo red agar, the tube method and microtiter plate agar. The majority of strains were found to form biofilm. 62.5% of strains were biofilm-forming, four categorized as highly positive ($OD_{570} \geq 1$); most strains were low-grade biofilm positive ($0.1 \leq OD_{570} < 1$). Related to poultry, 55 *E. hirae* strains were tested and found to produce biofilm; 24 strains did not form biofilm, 31 strains were biofilm-forming; 27 strains showed low-grade biofilm formation, and four strains were highly biofilm-forming. Four strains from hens and ostriches reached the highest OD_{570} values, more than 0.500. Rabbit-derived *E. hirae* strains as well as strains isolated from horses and the goat were low-grade biofilm-forming. Microtiter plate assay proved to be the best tool for testing the *in vitro* biofilm formation capacity of *E. hirae* strains from different species of animals.

Key words: *Enterococcus hirae*, various animals, biofilm

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Introduction

The species *Enterococcus hirae* belongs to the *Enterococcus faecium* group within the genus Enterococcus based on 16S rRNA gene similarity analysis (Sistek et al. 2012). These Gram-positive, catalase-negative coccoid bacteria can be found in the natural environment and in the alimentary tracts of animals (Kondo et al. 1997, Devriese et al. 2002); they have also been isolated from human causing urinary infection in a patient with benign prostatic hyperplasia (Devriese et al. 2002, Bourafa et al. 2015). Moreover, this species has been found in diseased birds of all ages, causing for example septicaemia or focal necrosis of the brain in chicken (Morishita 2017). On the other hand, regarding healthy poultry, *E. hirae* has also been detected in ducks, farming laying hens, pheasants and ostriches (Kandričáková et al. 2015, Lauková et al. 2016); but also in rabbits, horses, goats or dogs (Kubašová et al. 2017). Some strains of the species *E. hirae* can possess genes for virulence factors (Kandričáková et al. 2015); they can be decarboxylase-positive (Lauková et al. 2016) or they can form biofilm (Bino-Glatzová 2017).

Biofilms are defined as consortia of microorganisms attached to biotic (intestinal wall) or abiotic surfaces (e.g. catheters and others; Sauer 2003). Biofilm formation is assumed to be a factor of pathogenicity, especially in pathogenic bacteria; in this case the biofilm protects the bacteria as a protective barrier against host defences and the action of antimicrobial agents; for this reason biofilm can be a source of persistent infection (Lee Wong 1998, Davies 2003). Both qualitative and quantitative methods are usually used in testing for biofilm formation in bacteria (Christensen et al. 1982, Freeman et al. 1989, Chaieb et al. 2007, Slížová et al. 2015). However, our aim was not to compare the three methods used regarding biofilm formation in *E. hirae* strains; our principal aim was to test the investigated *E. hirae* strains for their ability to form biofilm, especially when having a group of *E. hirae* strains isolated from different species of animals. This kind of testing has never been done before. This study focuses on testing biofilm formation in *E. hirae* strains because (as mentioned above) biofilm can make them potential agents threatening animal health.

Materials and Methods

Bacterial strains

Faeces from different species of animals (involving 405 animals altogether) were sampled; they included 215 poultry such as laying hens (8, *Gallus gallus domesticus*), ducks (7, *Anas platyrhynchos f. domestica*), pheasants (60, *Phasianus colchicus*), ostriches (140, *Struthio camelus*) as well as 150 broiler rabbits (*Oryctolagus cuniculus domesticus*-breed M91, Hyla or Hyplus lines), 39 horses (*Cabballus/Equs*, Norik breed, Slovak warm-blooded, Hucul breed, Polish warm-blooded, British blood-horse), and one goat (*Capra aegagrus hircus*). Faeces were sampled from private breeds, in aviaries and on farms. Samples were collected in the course of various experiments (from control animals). In the poultry mixed faecal samples were used; e.g. ten mixed samples from 60 pheasants (Kandričáková et al. 2015), 54 mixed faecal samples from 140 ostriches in three groups (Lauková et al. 2016), from laying hens (continually sampled), e.g. ten mixed samples from 20 hens (Lauková et al. 2015), six mixed faecal samples from 24 rabbits in each group (Lauková et al. 2012); and faeces were collected from individual horses (Lauková et al. 2018) and from the goat as well. Handling of the animals and sampling was approved by the breeders themselves as well as by the Slovak Veterinary and Food Administration. To isolate enterococci, faecal samples were treated using the standard microbiological dilution method (in Ringer solution, ratio 1:9, Merck, Germany). The appropriate dilutions were spread on M-Enterococcus agar (Difco, Michigan, USA, ISO 15214) and incubated at 37°C for 24-48 h. Representative colonies were picked up, controlled for their purity and submitted for identification. A total of 64 *E. hirae* strains were identified using the MALDI-TOF identification system, (Bruker Daltonics), PCR and phenotypic tests (commercial BBL Gram-positive Crystal kit, Becton and Dickinson, USA) previously reported by Kandričáková et al. (2015), Lauková et al. (2016) or Bino-Glatzová (2017). The identified *E. hirae* strains were stored with the Microbank™ system (Pro-Lab Diagnostic, USA) for further testing.

Biofilm production

To test for biofilm formation in our identified *E. hirae* strains, we decided to use two qualitative methods and one quantitative method. Growth of tested colonies on Congo red agar is the qualitative phenotypic method according to Freeman et al. (1989). The cultivation medium was composed of Brain-heart infusion (Difco, Michigan, USA, 37 g/l) enriched with sucrose (36 g/l), pure agar (30 g/l) and Congo red dye (0.8 g/l, Merck, Germany). The medium was autoclaved at 121°C for 15 minutes. Plates of the medium were inoculated with the tested strains and incubated at 37° for 24 hours. A positive result was indicated by black colonies with a dry crystalline consistency. Non-slime producers usually remained pink. The colour was also checked after 48 and 72 hours.

Table 1a. Biofilm formation by *Enterococcus hirae* strains from duck, hen and pheasant.

Strain	Congo24	Congo48	Congo72	Tube m.	Microtitre m.± SD
Kč1/b	-	-	-	0	0.586 ± 0.063
Kč2/b	-	-	-	1	0.210 ± 0.025
Kč4	-	-	-	0	0.189 ± 0.023
Kč5	-	-	-	0	0.157 ± 0.018
Kč5/a	-	-	-	0	0.141 ± 0.025
Kč6	-	-	-	0	0.460 ± 0.061
Kč7	-	-	-	0	0.350 ± 0.059
SLJ2/b	+	+	+	2	0.164 ± 0.033
SLH1/b	+	+	+	1	0.298 ± 0.047
SLH3/b	+	+	+	2	0.500 ± 0.060
SLJ1/a	+	+	+	3	0.697 ± 0.012
EH31	d	d	d	1	0.114 ± 0.005
EH32	d	d	d	1	0.176 ± 0.008
EH33	+	+	+	2	0.023 ± 0.008
EH41	+	+	+	2	0.035 ± 0.006
EH43	+	+	+	2	0.010 ± 0.009
EH51	d	d	d	2	0.010 ± 0.010
EH52	d	d	d	1	0.031 ± 0.009
EH53	d	d	d	0	0.036 ± 0.015
EHb41	-	-	-	0	0.039 ± 0.012
EHb52	-	-	-	0	0.060 ± 0.028

E. hirae from duck (Kč1/b-Kč7); *E. hirae* from laying hen (SLJ2/b-SLJ1/a); *E. hirae* from pheasant (EH31-EHb52); + means positive; d means dubious; - means negative; 0-negative; 1-slight biofilm formation; 2-medium biofilm formation; 3-strong biofilm formation; Biofilm formation was evaluated as highly positive (OD_{570}≥1), low-grade positive (0.1 ≤ OD_{570} <1), or negative (OD_{570} < 0.1).

The second qualitative method used in our testing was the modified tube method according to Christensen et al. (1982). Cultivation medium Trypticase soy broth (Difco, Michigan, USA) enriched with 0.25% glucose (2 ml) in plastic tubes was inoculated with one colony of overnight cultured strain on blood agar. After incubation at 37°C for 24 hours, the tubes were removed and washed using phosphate buffer (pH 7.4) and then dried. After drying, each tube was dyed with 0.1 % solution of crystal violet. Each tube was gently rotated to ensure uniform staining of any adherent material on the inner surface of the tube, and then the contents were gently decanted. The tubes were then placed upside-down to drain. A positive result was indicated by the presence of an adherent layer of stained material on the inner surface of the tubes, and then evaluated as 0, medium (1) and strong (2) slime (biofilm) formation.

Finally, the capacity for biofilm formation of the *E. hirae* strains was assessed with a quantitative method, a biofilm plate assay as previously described and evaluated by Chaieb et al. (2007) and Slížová et al. (2015). In brief, one colony of the tested strain grown on overnight at 37°C on Trypticase soy agar (Difco, Michigan, USA) was transferred into 5 ml of Ringer solution (pH 7.0, 0.75% w/v) to reach the McFarland standard 1 suspension that corresponded to 1 x 10^8 cfu/ml. A volume of 100 μl from that culture was then transferred into 10 ml of Trypticare soy broth (TSY). That standardized culture (200 μl) was inoculated in a well on a polystyrene microtiter plate (Greiner ELISA 12 Well Strips, 350 μl, flat bottom, Frickenhausen GmbH, Germany) and incubated for 24 h at 37°C. The biofilm formed in the well of the microtiter plate well was washed twice with 200 μl of deionized water and dried at 25°C for 30 min in an inverted position. The remaining attached bacteria were stained for 30 min at 25°C with 200 μl of 0.1 % (m/v) crystal violet in deionized

Table 1b. Biofilm formation by *Enterococcus hirae* strains from ostrich.

Strain	Congo24	Congo48	Congo72	Tube m.	Microtitre m.± SD
EH36	d	d	d	0	0.083 ± 0.012
EH111	+	+	+	2	0. 333 ± 0.031
EH131/Cl	-	-	-	0	0.097 ± 0.022
EH141/Cl	-	-	-	2	0.560 ± 0.054
EH141	+	+	+	1	0.309 ± 0.027
EH142	+	+	+	1	0.294 ± 0.032
EH151	+	+	+	0	0.059 ± 0.010
EH161/Cl	-	-	-	0	0.089 ± 0.02
EH210	+	+	+	1	0.164 ± 0.016
EH211	+	+	+	1	0.084 ± 0.013
EH221	+	+	+	0	0.079 ± 0.011
EH242/Cl	-	-	-	1	0.212 ± 0.033
EH272	+	+	+	1	0.270 ± 0.038
EH281	+	+	+	1	0.165 ± 0.017
EH282	+	+	+	1	0.202 ± 0.018
EH291	+	+	+	1	0.217 ± 0.037
EH371	-	-	-	1	0.240 ± 0.030
EH1101	-	-	-	0	0.053 ± 0.012
EH1102	-	-	-	0	0.168 ± 0.029
EH1131	+	+	+	1	0.127 ± 0.028
EH1132	+	+	+	1	0.245 ± 0.032
EH1151	-	-	-	0	0.227 ± 0.019
EH1152	-	-	-	0	0.112 ± 0.013
EH1161	-	-	-	0	0.085 ± 0.017
EH1181	-	-	-	1	0.079 ± 0.017
EH1182	-	-	-	0	0.074 ± 0.018
EH2131	-	-	-	0	0.043 ± 0.017

+ means positive; - means negative; 0-negative; 1-slight biofilm formation; 2-medium biofilm formation; 3-strong biofilm formation; Biofilm formation was evaluated as highly positive ($OD_{570} \geq 1$), low-grade positive ($0.1 \leq OD_{570} < 1$), or negative ($OD_{570} < 0.1$).

water. The dye solution was aspirated away and 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 and placed on a new microtiter plate for optical density (OD) determination 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. Moreover, a sterile culture medium was included in each analysis as negative control. *Streptococcus equi* subsp. *zooepidemicus* CCM 7316 was used as positive control in each method (kindly provided by Dr. Eva Styková, University of Veterinary Medicine and Pharmacy, Košice, Slovakia). Biofilm formation was then classified according to Chaieb et al. (2007) and Slížová et al. (2015) as highly positive ($OD_{570} \geq 1$), low-grade positive ($0.1 \leq OD_{570} < 1$) and negative ($OD_{570} < 0.1$).

Statistical evaluation

Data were analyzed using GraphPad Prism version 3.00 (Graph-Pad Software, San Diego, California,

Table 2. Biofilm formation by *Enterococcus hirae* strains from ostrich, rabbit, goat and horse.

Strain	Congo24	Congo48	Congo72	Tube m.	Microtitre m.± SD
EH2142	-	-	-	0	0.137 ± 0.014
EH2162	-	-	-	0	0.119 ± 0.015
EH2171	-	-	-	0	0.099 ± 0.019
EH2172	-	-	-	0	0.088 ± 0.010
EH2181	-	-	-	1	0.077 ± 0.017
EH3121	-	-	-	0	0.066 ± 0.011
EH3161	-	-	-	0	0.053 ± 0.010
Kr2/b	+	+	+	0	0.240 ± 0.035
Kr7a	+	+	+	0	0.344 ± 0.050
Kr8/a	+	+	+	1	0.388 ± 0.038
Kr9/b	+	+	+	0	0.273 ± 0.035
KAp.b2017a	+	+	+	1	0.204 ± 0.006
KAp.b2017	+	+	+	1	0.334 ± 0.054
K5od/1	-	-	-	0	0.352 ± 0.062
K7/1	+	+	+	2	0.303 ± 0.031
EH11Kz	+	+	+	2	0.193 ± 0.029

0-negative; 1-slight biofilm formation; 2-medium biofilm formation; 3-strong biofilm formation; *E. hirae* from ostrich (EH2142-EH3161); *E. hirae* from rabbit (Kr2/b-KAp.b2017); *E. hirae* from horse (K5od/1, K7/1); *E. hirae* from goat (EH11Kz); Biofilm formation was evaluated as low-grade positive ($0.1 \leq OD_{570} < 1$), or negative ($OD_{570} < 0.1$).

USA, www.graphpad.com), with one-way analysis of variance (ANOVA) and with Tukeys multiple comparison test.

Results

A total of 64 strains of *E. hirae* were tested for biofilm production. Regarding the growth of strains on Congo red agar, *E. hirae* strains from ducks were negative; using the tube method only *E. hirae* Kč2/b was biofilm-forming (grade 1 means medium biofilm formation, Table 1a). However, these strains formed biofilm in microtiter plate (Table 1a). In the case of *E. hirae* isolated from hens, they showed biofilm formation when tested with the qualitative as well as the quantitative methods (Table 1a). Evaluation by means of the tube method was equal in correlation with the quantitative assessment of biofilm production; that is, evaluation 1-3 and OD_{570} from 0.164 to 0.697. Regarding the qualitative method, *E. hirae* EH31 and EH32 from pheasants showed a dubious reaction (no clearly black colonies) on Congo red agar; the tube method confirmed medium- intensity biofilm formation by them (1), which was also confirmed by the quantitative microtiter plate method (0.114, 0.176, Table 1a). Strains EH52, EH53, EHb41and EHb52 did not show biofilm formation on Congo red agar; they were also negative using the quantitative microtiter plate method (in EH52 strain medium biofilm formation was measured using the tube method (1), (Table 1a). However, results for the strains EH33, EH41, EH43 and EH51 on Congo red agar and using the tube method showed low-grade biofilm formation; the quantitative method did not show biofilm formation in these strains (Table 1a). Some discrepancy was also found in strains EH371, EH1151, EH1152, EH2142, EH2162 isolated from ostriches; their reaction on Congo red agar was negative. Using the tube method negative or slight biofilm production was shown in these strains; however, the quantitative method evaluated these strains as low-grade biofilm-forming (Table 1a,b). On the other hand, EH221, EH151, EH211 showed biofilm formation on Congo red agar and slight or negative biofilm formation was revealed by the tube method; however, using the microtiter plate method, biofilm was not detected (Table 1a). In the case of EH141/Cl strain, no biofilm formation was demonstrated on Congo red agar, strong biofilm formation was detected using the tube method (2) and highly-positive biofilm formation was detected using the microtiter plate method (Table 2, OD_{570} 0.560±0.054). *E. hirae* isolated from the faeces of

broiler rabbits, goat-EH11Kz and horse (K7/1) showed biofilm formation on Congo red agar; this was also confirmed by the plate method (0.303±0.031, Table 2). Biofilm formation by *E. hirae* from horse K5od/1 was measured only using the quantitative method (0.352±0.062, Table 2).

As can be seen, the majority of strains (55) were isolated from poultry faeces (ducks, hens but also ostriches and pheasants). The rest of our *E. hirae* strains were isolated from the feaces of rabbits, horses and a goat. The majority of strains were found to be biofilm-forming. Forty strains (62.5%) were biofilm-producing. Four strains of *E. hirae* SLH3/b, SLJ1/a, both from hen faeces, Kč1/b from duck and EH141/Cl from ostrich, were categorized as highly positive ($OD_{570} \geq 1$). The rest of the strains were low-grade positive ($0.1 \leq OD_{570} < 1$). That is, four strains were highly positive and 36 strains were low-grade biofilm-forming. Biofilm negative results were recorded for 24 *E. hirae* strains (37.5 %) ($OD_{570} < 0.1$). Among 34 ostrichs-derived strains, 16 were found to produce biofilm (47.0 %, Table 1b, 2) and 11 strains did not form biofilm (47.1%). The OD_{570} values for those biofilm-forming strains ranged from 0.112 to 0.560. Concerning the *E. hirae* strains from pheasants, they mostly did not form biofilm (Table 1a); only two strains (EH31, EH32) out of the ten tested formed low-grade biofilm (Table 1a, OD_{570} 0.114, 0.176). Assessment of seven *E. hirae* strains from ducks revealed that they were biofilm-forming (Table 1a); one strain highly biofilm-forming and nine low-grade biofilm forming. *E. hirae* from hens (4) were biofilm-forming (Table 1a); the strain EH SLJ/1a even reached the highest value among all 64 tested strains (OD_{570} 0.697±0.112). In terms of the poultry, 55 *E. hirae* strains were found to produce biofilm; 24 strains did not form biofilm, 31 strains were biofilm-forming, four strains were highly biofilm positive and 27 were low-grade positive. Four strains reached the highest OD_{570} values; the already mentioned *E. hirae* SLJ1/a strain (OD_{570} 0.697±0.112), SLH3/b (0.500±0.060) from hens, EH141/Cl from ostrich (0.560 ±0.054) and Kč1/b from duck (0.586±0.063). *E. hirae* strains derived from rabbits as well as strains isolated from goat and horses were low-grade biofilm-forming (Table 2).

Discussion

Any type of microorganism, including spoilage or pathogenic, could form a biofilm. On the one hand, biofilm is supposed to be a virulence factor which can play a key role in many animal disorders (Parsek and Singh 2003); biofilms are the basis for persistent or chronic bacterial infection (Costerton et al. 1999). Ability to form biofilm has been studied predominantly in Gram-negative pathogens such as *Sallmonella* sp. (Seixas et al. 2014) or *E. coli* (Oliveira et al. 2014); as well as in Gram-positive *S. aureus* (Jian-Zhong He et al. 2014).

As previously mentioned, *E. hirae* can be detected in the gastrointestinal tract of animals generally, but particularly in diseased animals. Moreover, human infection caused by *E. hirae* strains is assumed to make up 1-3% of the *Enterococcus* spp. infections detected in clinical practice (Paosinho et al. 2016). For these reasons, testing the huge range of strains for their ability to form biofilm and after that their treatment (e.g. with enterocins, antimicrobial substances studied at our laboratory) can contribute to the knowledge on how to facilitate their treatment or prevention.The majority of *E. hirae* strains in our study produced biofilm; the microtiter plate assay used to assess biofilm formation proved to be the most confirmatory tool to assess the *in vitro* biofilm formation capacity of *E. hirae* strains from different sources; on the other hand, Jian-Zhong He et al. (2014) reported that the Congo red agar method used in testing for biofilm formation in *Staphylococcus aureus* showed that 80 out of 102 strains produced biofilm. Studying this property in a wide range of animal-derived *E. hirae* strains is therefore important and it can show a serious impact; e.g. as indicated the results of the microbial virulence factor study by Anderson et al. (2016). *E. hirae,* as a possible disease stimulating agent also possess virulence factors such as gelE, ccf, cylA, or they can produce toxic substances such as biogenic amines. In this study strains from pheasants and ostriches were found to produce biogenic amines (Lauková et al. 2017). It will be very useful then to know how to reduce/eliminate these strains; as mentioned above Lauková et al. (2016, 2017) reported sensitivity to enterocins (antimicrobial substances) in decarboxylase-positive enterococci from farm ostriches and from pheasants (tested in this study as well) which also possess virulence factor genes. Enterocins are antimicrobial substances of proteinaceous character with inhibition activity against more or less relative species (Franz et al. 2007). In our laboratory we have been involved in enterocin research for years (Lauková et al. 1993, Mareková et al. 2007). To reach our further aim, the inhibition effect of enterocins has been studied *in vitro* but also *in vivo*, e. g. in rabbits, hens or horses; enterocins were additionally used to treat enterococcal strains isolated from the faeces of the same range of animals (Lauková et al. 2008, Pogány Simonová a Lauková 2017). As mentioned above, enterocins inhibited enterococci possessing virulence factors, including *E. hirae* (Lauková et al. 2016,

2017). Obtaining information associated with the ability of *E. hirae* strains tested to form biofilm, in the future, our research will be more focused on continuous testing of sensitivity of biofilm-forming strains to bacteriocins as well as on the molecular basis of biofilm formation in *E. hirae*. The molecular basis of biofilm formation is well known, e.g. in *Staphylococcus epidermidis* (Barros et al. 2015). There the initial attachment phase includes the participation of proteins with adhesive properties (Fbe, AtlE) which bind to the host factors, fibrinogen and vitronectin respectively. In spite of the fact that the presence of genes associated with biofilm formation was not analyzed in this study, in our opinion it is a great contribution to research related to biofilm formation in enterococci, especially in those originating in food-producing animals particularly, since other studies are more focused on clinical isolates (Anderson et al. 2016).

In conclusion, the majority of *E. hirae* strains were found to form biofilm. 62.5% of strains were biofilm-producing, with four categorized as highly positive ($OD_{570} \geq 1$) and most strains were low-grade biofilm-forming ($0.1 \leq OD_{570} < 1$). The microtiter plate assay is proposed as the optimal way of confirming the *in vitro* biofilm formation capacity of *E. hirae* strains from different species of animals.

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