

Supplementary Information

Biofilm formation capacity and presence of virulence factors among commensal *Enterococcus* spp. from wild birds

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Origin ^a	Isolates	Strain number	Phenotype				Hydrophobicity (%)	Genotypic patterns of virulence factors
			Gelatinase	Beta-haemolysis	Biofilm in TSB +1% glucose ^b	Biofilm in BHI +2% glucose ^b		
White-tailed Eagle	<i>E. faecalis</i>	2B	+	-	II	IV	62.5	<i>ebpA, ebpB, ebpC, srt, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
White-tailed Eagle	<i>E. faecalis</i>	3	+	-	IV	IV	100	<i>ebpA, ebpB, ebpC, pil,srt, ace, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
Eurasian Tawny Owl	<i>E. faecalis</i>	8	+	-	II	IV	100	<i>ebpA, ebpB, ebpC, srt, ace, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
Little Bittern	<i>E. faecalis</i>	11	+	-	II	IV	50	<i>ebpA, ebpB, ebpC, pil,srt, ace, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
Hoopoe	<i>E. faecalis</i>	12	+	-	II	IV	70	<i>ebpA, ebpB, ebpC, pil,srt, ace, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
Mallard	<i>E. faecalis</i>	20	+	-	I	I	0	<i>ebpA, ebpB, ebpC, pil,srt, ace, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
Mallard	<i>E. faecalis</i>	25	+	-	II	IV	100	<i>ebpA, ebpB, ebpC, srt, ace, agg, asa1, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
Mallard	<i>E. faecalis</i>	26	+	-	IV	I	0	<i>ebpA, ebpB, ebpC, srt, ace, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
Common Blackbird	<i>E. faecalis</i>	28	+	-	III	I	0	<i>ebpA, ebpB, pil, srt, ace, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
Eurasian Jay	<i>E. faecalis</i>	30	+	-	III	III	44.4	<i>ebpB, ebpC, ace, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
Short-eared Owl	<i>E. faecalis</i>	31	+	-	I	III	100	<i>ebpA, ebpB, ebpC, srt, ace, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>

Eurasian Tawny Owl	<i>E. faecalis</i>	32	+	-	III	I	0	<i>ebpB, pil, srt, ace, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
Mallard	<i>E. faecalis</i>	33	+	-	I	II	33.3	<i>ebpA, ebpB, pil, srt, ace, asaI, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
Little Bittern	<i>E. faecalis</i>	35	+	-	I	III	50	<i>ebpA, ebpB, ebpC, srt, ace, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
Eurasian Sparrow Hawk	<i>E. faecalis</i>	36	+	-	I	III	57.1	<i>ebpA, ebpB, ebpC, srt, ace, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
Common Buzzard	<i>E. faecalis</i>	42	+	-	II	III	100	<i>ebpC, srt, ace, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
Little Owl	<i>E. faecalis</i>	44	+	-	II	III	100	<i>ebpB, srt, ace, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
White-tailed Eagle	<i>E. faecalis</i>	46	+	-	I	IV	55.6	<i>ebpA, ebpB, ebpC, srt, ace, agg, asaI, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
Mallard	<i>E. faecalis</i>	48A	+	-	I	III	100	<i>ebpC, srt, ace, agg, asaI, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
Mallard	<i>E. faecalis</i>	50	+	-	I	III	66.7	<i>srt, ace, agg, asaI, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
Lesser Spotted Woodpecker	<i>E. faecalis</i>	53	+	-	III	III	50	<i>ebpA, ebpB, ebpC, srt, ace, agg, asaI, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
European Green Woodpecker	<i>E. faecalis</i>	54	+	-	IV	III	42.9	<i>ebpA, ebpC, srt, ace, agg, asaI, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
Eurasian Tawny Owl	<i>E. faecalis</i>	56	+	-	III	III	57.1	<i>ebpA, ebpB, ebpC, srt, ace, agg, asaI, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
Great Spotted Woodpecker	<i>E. faecalis</i>	58	+	-	III	III	42.9	<i>ebpB, ebpC, srt, ace, agg, asaI, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>

Grey Heron	<i>E. faecalis</i>	61	+	-	II	II	100	<i>ebpA, ebpB, srt, ace, agg, asa1, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
Western Marsh Harrier	<i>E. faecalis</i>	62	+	-	I	II	33.3	<i>ebpB, ebpC, srt, ace, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
Grey Heron	<i>E. faecalis</i>	64A	+	-	II	III	100	<i>ebpA, ebpB, srt, ace, gelE, sprE, efaAfs, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
Great Egret	<i>E. faecium</i>	1	-	-	I	I	14.3	<i>efaAfm, ccf</i>
White-tailed Eagle	<i>E. faecium</i>	2A	-	-	I	III	25	<i>efaAfm, ccf</i>
Mute Swan	<i>E. faecium</i>	5	-	-	I	III	10	<i>efaAfm, ccf</i>
Common Raven	<i>E. faecium</i>	10	-	-	I	III	37.5	<i>efaAfm, ccf</i>
Eurasian Golden Oriole	<i>E. faecium</i>	13	-	-	I	III	0	<i>efaAfm, ccf</i>
Eurasian Sparrow Hawk	<i>E. faecium</i>	14	-	-	I	IV	0	<i>efaAfm, ccf</i>
Common Buzzard	<i>E. faecium</i>	15	-	-	I	IV	44.4	<i>efaAfm, ccf</i>
Common Tern	<i>E. faecium</i>	16	-	-	I	IV	0	<i>ebpC, srt, ace, gelE, sprE, efaAfm, fsrA, fsrB, fsrC, cpd, ccf</i>
Mallard	<i>E. faecium</i>	17	-	-	II	IV	66.7	<i>efaAfm, ccf</i>
Mallard	<i>E. faecium</i>	19A	-	-	I	III	0	<i>efaAfm</i>
Mallard	<i>E. faecium</i>	19B	-	-	I	III	0	<i>efaAfm, ccf</i>
Mallard	<i>E. faecium</i>	22	-	-	I	III	0	<i>efaAfm, ccf</i>
Fieldfare	<i>E. faecium</i>	29	-	-	IV	II	0	<i>efaAfm, ccf</i>
Mallard	<i>E. faecium</i>	34	-	-	I	I	0	<i>ebpA, ebpC, srt, ace, gelE, sprE, efaAfm, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
Mallard	<i>E. faecium</i>	38A	-	-	II	II	12.5	<i>ebpA, ebpC, srt, ace, gelE, sprE, efaAfm, fsrA, fsrB, fsrC, cpd, ccf</i>
Mallard	<i>E. faecium</i>	39A	+	-	II	III	0	<i>ebpA, srt, ace, gelE, sprE, efaAfm, fsrA, fsrB, fsrC, cpd, ccf</i>

Short-eared Owl	<i>E. faecium</i>	57	-	-	I	I	0	<i>ebpA, ebpC, srt, ace, agg, asaI, gelE, sprE, efaAfm, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
European Green Woodpecker	<i>E. faecium</i>	60	-	-	I	II	10	<i>ebpC, srt, asaI, efaAfm, ccf</i>
White Stork	<i>E. hirae</i>	4	-	-	II	IV	22.2	<i>ebpC, ccf</i>
Mallard	<i>E. hirae</i>	23	-	-	I	III	0	-
Mallard	<i>E. hirae</i>	41	-	-	II	II	0	<i>srt, gelE, sprE, fsrA, fsrC, cpd, ccf</i>
Barn Owl	<i>E. hirae</i>	43	-	-	II	III	28.6	<i>ebpC, srt, asaI, gelE, sprE, fsrA, fsrC, cpd, cob, ccf</i>
Long-eared Owl	<i>E. hirae</i>	45	+	-	I	II	37.5	<i>ebpA, ebpC, pil, srt, ace, gelE, sprE, fsrA, fsrB, fsrC, cpd, cob, ccf</i>
Mallard	<i>E. durans</i>	24	-	-	I	III	22.2	<i>ccf</i>
Mallard	<i>E. durans</i>	37	-	-	I	II	42.9	<i>ccf</i>
Mallard	<i>E. casseliflavus</i>	49	-	-	I	IV	62.5	<i>ebpC, ccf</i>
Mallard	<i>E. casseliflavus</i>	52	-	-	I	III	42.9	<i>ebpC, ccf</i>

Table S1. Phenotypic and genotypic properties in *Enterococcus* strains isolated from wild birds.

^a Western Marsh Harrier (*Circus aeruginosus*), White-tailed Eagle (*Haliaeetus albicilla*), Eurasian Sparrow Hawk (*Accipiter nisus*), Common Buzzard (*Buteo buteo*), Lesser Spotted Woodpecker (*Dendrocopos minor*), Great Spotted Woodpecker (*Dendrocopos major*), European Green Woodpecker (*Picus viridis*), Mute Swan (*Cygnus olor*), Mallard (*Anas platyrhynchos*), Barn Owl (*Tyto alba*), Little Owl (*Athene noctua*), Eurasian Tawny Owl (*Strix aluco*), Long-eared Owl (*Asio otus*), Short-eared Owl (*Asio flammeus*), Eurasian Golden Oriole (*Oriolus oriolus*), Little Bittern (*Ixobrychus minutus*), Grey Heron (*Ardea cinerea*), Great Egret (*Ardea alba*), Hoopoe (*Upupa epos*), Fieldfare (*Turdus pilaris*),

Common Blackbird (*Turdus merula*), Eurasian Jay (*Garrulus glandarius*), Common Raven (*Corvus corax*), Common Tern (*Sterna hirundo*),
White Stork (*Ciconia ciconia*)

^b Category of biofilm formation: I- no biofilm; II- weak biofilm; III- moderate biofilm; IV- strong biofilm

Genes	Encoded virulence factor	Primer sequence 5' – 3'	Annealing temperature	Amplicon length (bp)	Reference
<i>ebpA</i>	endocarditis and biofilm-associated pili (fibre tip pilin)	F: CCATTGTCAGAAGCAAGAATG R: GAGTGAAAGTTCCTCCTCTAG	54	613	1
<i>ebpB</i>	endocarditis and biofilm-associated pili (cell wall-anchored pilin)	F: CATTAGCAGAGGCATCGCAA R: CAAGTGGTGGTAAGTCATAGG	54	504	1
<i>ebpC</i>	endocarditis and biofilm-associated pili (major shaft pilin)	F: CTGCTACGAATATGGTGGTG R: GGTGTTTGATTGTTTGCTTC	54	487	1
<i>pil</i>	pili	F: GAAGAAACCAAGCACCTAC R: CTACCTAAGAAAAGAAACGCG	54	620	1
<i>srt</i>	pilus-associated sortase	F: GTATCCTTTTGTTAGCGATGC R: TGTCTCGAACTAATAACCGA	54	612	1
<i>ace</i>	collagen-binding protein	F: AAAGTAGAATTAGATCCACAC R: TCTATCACATTCGGTTGCG	48	320	2
<i>agg</i>	aggregation substance	F: CACGTAATTCTTGCCCACCA R: CAAGCATTATTGGCAGCGTT	55	520	3
<i>asa1</i>	aggregation substance	F: GCACGCTATTACGAACTATGA R: TAAGAAAGAACATCACCACGA	56	375	4
<i>esp</i>	enterococcal surface protein	F: AGATTCATCTTTGATTCTTGG R: AATTGATTCTTTAGCATCTGG	56	510	4
<i>efaA_{fs}</i>	<i>E. faecalis</i> specific endocarditis antigen	F: GACAGACCCTCACGAATA R: AGTTCATCATGCTGTAGTA	51	705	5

<i>efaA_{fm}</i>	<i>E.faecium</i> specific surface antigen	F: AACAGATCCGCATGAATA R: CATTTCATCATCTGATAGTA	48	735	5
<i>gelE</i>	gelatinase	F: ACCCCGTATCATTGGTTT R: ACGCATTGCTTTTCCATC	48	419	6
<i>sprE</i>	serine protease	F: TTGAGCTCCGTTCTGCCGAAAGTCATTC R: TTGGTACCGATTGGGGAACCAGATTGACC	55	591	6
<i>hyl</i>	hyaluronidase	F: ACAGAAGAGCTGCAGGAAATG R: GACTGACGTCCAAGTTTCCAA	56	276	4
<i>fsrA</i>	response regulator	F: ATGAGTGAACAAATGGCTATTTA R: CTAAGTAAGAAATAGTGCCTTGA	49	740	6
<i>fsrB</i>	signalling peptide	F: GGGAGCTCTGGACAAAGTATTATCTAACCG R: TTGGTACCCACACCATCACTGACTTTTGC	63	566	6
<i>fsrC</i>	histidine kinase	F: ATGATTTTGTCTGTTATTAGCTACT R: CATCGTTAACAACTTTTTACTG	49	1343	6
<i>cylA</i>	cytolysin activator	F: ACTCGGGGATTGATAGGC R: GCTGCTAAAGCTGCGCTT	56	688	4
<i>cylB</i>	transport of cytolysin	F: ATTCCTACCTATGTTCTGTTA R: AATAAACTCTTCTTTTCCAAC	51	843	5
<i>cylM</i>	cytolysin synthetase	F: CTGATGGAAAGAAGATAGTAT R: TGAGTTGGTCTGATTACATTT	53	742	5

<i>cylL</i>	cytolysin precursor	F: GCTTCACCTCACTAAGTTTTATAG R: GATGGAGGGTAAGAATTATGG	53	253	7
<i>cpd</i>	sex pheromone	F: TGGTGGGTTATTTTTCAATTC R: TACGGCTCTGGCTTACTA	48	782	5
<i>cob</i>	sex pheromone	F: AACATTCAGCAAACAAAGC R: TTGTCATAAAGAGTGGTCAT	51	1405	5
<i>ccf</i>	sex pheromone	F: GGGAATTGAGTAGTGAAGAAG R: AGCCGCTAAAATCGGTAAAAT	52	543	5

Table S2. Target genes and primers used in this study.

1. Hashem, Y.A., Amin, H.M., Essam, T.M. & Aziz, R.K. Biofilm formation in enterococci: genotype-phenotype correlations and inhibition by vancomycin. *Sci Rep.* **7**, 5733 (2017).
2. Duprè, I., Zanetti, S., Schito, A.M., Fadda, G. & Sechi, L.A. Incidence of virulence determinants in clinical *Enterococcus faecium* and *Enterococcus faecalis* isolates collected in Sardinia (Italy). *J Med Microbiol.* **52**, 491-498 (2003).
3. Seputiene, V., Bogdaite, A., Ruzauskas, M. & Suziedeliene, E. Antibiotic resistance genes and virulence factors in *Enterococcus faecium* and *Enterococcus faecalis* from diseased farm animals: pigs, cattle and poultry. *Pol J Vet Sci.* **15**, 431-438 (2012).
4. Vankerckhoven, V., et al. Development of a multiplex PCR for the detection of *asa1*, *gelE*, *cylA*, *esp*, and *hyl* genes in enterococci and survey for virulence determinants among European hospital isolates of *Enterococcus faecium*. *J Clin Microbiol.* **42**, 4473-4479 (2004).

5. Eaton, T.J. & Gasson, M.J. Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. *Appl Environ Microbiol.* **67**, 1628-1635 (2001).
 6. Lopes Mde, F., Simões, A.P., Tenreiro, R., Marques, J.J. & Crespo, M.T. Activity and expression of a virulence factor, gelatinase, in dairy enterococci. *Int J Food Microbiol.* **112**, 208-214 (2006).
 7. Semedo, T., et al. Comparative study using type strains and clinical and food isolates to examine hemolytic activity and occurrence of the cyl operon in enterococci. *J Clin Microbiol.* **41**, 2569-76 (2003).
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