Phenotypic and genotypic characterization of *Enterococcus* spp. from yolk sac infections in broiler chicks with a focus on virulence factors

Dagmara Stępień-Pyśniak,[*,1] Tomasz Hauschild,[†] Marta Dec,[*] Agnieszka Marek,[*] Renata Urban-Chmiel,[*] and Urszula Kosikowska[‡]

*Department of Veterinary Prevention and Avian Diseases, Institute of Biological Bases of Animal Diseases, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, 20-612 Lublin, Poland; †Department of Microbiology and Biotechnology, Faculty of Biology, University of Bialystok, 15-245 Białystok, Poland; and ‡Department of Pharmaceutical Microbiology, Medical University in Lublin, 20-093 Lublin, Poland

ABSTRACT Bacterial infections of yolk sacs contribute to increased mortality of chicks, chronic infections during their rearing, or increased selection in the flock, which in turn leads to high economic losses in poultry production worldwide. The aim of this study was a phenotypic and genotypic characterization of enterococci isolated from yolk sac infections (**YSI**) of broiler chickens from Poland and the Netherlands. Biochemical, matrix-assisted laser desorption/ionization (**MALDI**)–time-of-flight (**TOF**) MS, and *rpoA* gene sequencing identification was performed. Moreover, phenotypic and genotypic characterization of virulence factors and analysis of the clonal relationship of isolates by MALDI-TOF MS and enterobacterial repetitive intergenic consensus—polymerase chain reaction (**ERIC-PCR**) were performed. The biochemical test identified 70 isolates as *Enterococcus faecalis* and 6 as *Enterococcus mundtii*. The results of MALDI-TOF MS were 100% concordant with those obtained by r*poA* gene sequencing, and all 76 isolates were identified as *E. faecalis*. Differences were noted in the β-glucuronidase, β-glucosidase, α-galactosidase, phosphatase, melibiose, lactose, and raffinose tests that is going about the results of biochemical identification. None of the isolates were beta-hemolytic on blood agar in aerobic conditions, but all but one were gelatinase positive. Among biofilm-forming isolates (30/76; 39.5%), as many as 66.7% (20/30) were Polish *E. faecalis* strains. Most of the isolates carried virulence genes, that is *gelE*, *ace*, *asa1*, *efaAfs*, *fsrA*, *fsrB*, *fsrC*, *cob*, *cpd*, and *ccf*, but none had the *hyl* gene. Some isolates harbored cyl operon genes. One Polish strain (ST16) had all of the tested *cyl* genes and the *esp* gene, considered clinically important, and showed the highest biofilm-forming ability. Nearly 50% of the isolates showed close genetic relatedness in ERIC typing. In contrast with MALDI-TOF MS cluster analysis, ERIC-PCR results did not show a relationship with the origin of the strains. Using MALDI-TOF MS, 7 peaks were found in Polish and Dutch isolates, which may type them as species-specific biomarkers in *E. faecalis* from YSI.

Key words: yolk sac infections, *E. faecalis* in chicks, *E. faecalis* virulence, MALDI-TOF MS, ERIC-PCR

INTRODUCTION

Enterococci are Gram-positive members of the gastrointestinal microbiota of animals and are also implicated in opportunistic infections. Among avian enterococci, *Enterococcus faecalis* is the most frequently isolated from clinical cases, including endocarditis in chickens, hepatic granulomas in turkeys, ascites in hens, pulmonary hypertension in broilers (Tankson et al., 2001), septicemia, yolk sac infection (**YSI**)/omphalitis (Olsen et al., 2012a), amyloid arthropathy, and concomitant systemic amyloidosis in brown layers and broiler breeders (Steentjes et al., 2002), arthritis in domestic ducks, and tracheitis in canaries (Devriese et al., 1990). *E. faecalis* is also significant bacterial pathogen at posthatching period (Stępień-Pyśniak et al., 2016) and even from hatching eggs or dead-in-shell embryos (Dolka et al., 2017b; Karunarathna et al., 2017).

Although the emergence of *Enterococcus*-associated infections has been reported in many countries, virulence factors associated with these infections are poorly understood, especially among *E. faecalis* from YSI/omphalitis. Routine laboratory diagnostics of these infections most often include a phenotypic assessment of the biochemical characteristics and drug resistance of isolated bacteria. However, these data are insufficient to determine the pathogenic potential of the bacteria and to compare their virulence. Similarly, only demonstrating of the presence of *E. faecalis* in the test material is insufficient to correctly determine the disease course and prognosis, as well as the epidemic potential of the bacteria. Knowledge of traits associated with the pathogenicity of isolated bacteria and determination of their virulence may help to reduce the occurrence and spread of diseases of *E. faecalis* etiology among farmed birds, as well as the potential spread of these microorganisms in the environment, and even among humans for example by using the high pathogenic isolates as the candidates for autogenous vaccines (Blanco et al., 2018).

Biofilms and a number of biochemical features can contribute to the virulence and antibiotic resistance of many bacteria, including enterococci. Although numerous genes involved in the pathogenicity of these bacteria have been identified, their distribution among *E. faecalis* from YSI has not been comprehensively studied, and their diagnostic ability to predict biofilm phenotypes is not fully established. Chickens colonized with enterococcal strains containing virulence genes are not only at risk of infection but are also a potential source for the dissemination of microorganisms to the environment and to people. This is very likely because of human participation in many aspects of poultry hatching and farming (e.g., manual segregation of chicks, individual vaccination, and monitoring of the flock during rearing–contact with bioaerosol) or the possibility of spread via the food chain (Lawniczek-Walczyk et al., 2013). In addition, opportunistic bacteria with increased pathogenic potential accompanied by the ability to form a biofilm may survive in the hatchery or poultry house environment because of ineffective disinfection, thus posing a threat in subsequent production cycles (Ali et al., 2013).

The virulence factors most frequently tested in human clinical isolates of enterococci include aggregation substance (*asa1*), collagen-binding protein (*ace*), enterococcal surface protein (*esp*), enterococcal endocarditis antigen for *E. faecalis* (*efaAfs*), gelatinase (*gelE*), hyaluronidase (*hyl*), cytolysin operons (*cylA*, *cylB*, *cylM*, and *cylL$_L$*), the quorum sensing *fsr* locus (*fsrA*, *fsrB*, and *fsrC*), and sex pheromones (*cpd*, *cob,* and *ccf*) (Eaton and Gasson, 2001; Vankerckhoven et al., 2004; Golińska et al., 2013).

Various techniques for identifying, typing, and comparing enterococci are still being sought or adapted, taking into account their cost and time requirements (Nowakiewicz et al., 2017, 2020; Blanco et al., 2018). One such technique is rep-PCR fingerprinting, which shows the manner in which repetitive sequences are distributed in bacterial genomes. Families of repetitive DNA sequences are dispersed within the genome of various bacterial species. One of the more thoroughly studied is the family of enterobacterial repetitive intergenic consensus (**ERIC**) sequences. The ERIC sequences 124–127 nucleotides in length usually occur between genes in polycistronic operons or in untranslated genome regions preceding or following open reading frames. The bases that form them exhibit intramolecular complementarity enabling the formation of secondary stem-loop structures in mRNA molecules, which can affect the arrangement of the bacterial genome structure. Genome organization can be influenced by the insertion, deletion, and/or dispersion of ERIC sequences. In addition, the distribution of repetitive sequences, including ERIC sequences, may indicate genomic similarity/relatedness among isolates within a species.

An alternative way to differentiate among similar species of the genus *Enterococcus* is phyloproteomic analysis of the isolates' spectral profiles by matrix-assisted laser desorption/ionization–time-of-flight (**MALDI-TOF**) mass spectrometry, involving laser ionization of the test sample using a matrix (MALDI) and analysis of the time of flight of the ions (**TOF**) (Giebel et al., 2008; Quintela–Baluja et al., 2013; Santos et al., 2015; Stępień-Pyśniak et al., 2017). Mass spectrometry is a universal analytical technique with applications in many fields, based on measurement of the ratio of the mass of a given ion to its electric charge (m/z). The main advantage of MALDI-TOF is that it enables direct detection of the composition of populations of high-molecular-weight molecules, such as mixtures of proteins. Thus, identification of bacteria by MALDI-TOF MS consists in analyzing the composition of cellular proteins, mainly those found in large quantities in the bacterial cell, such as ribosomal proteins. The final result of MALDI-TOF MS analysis is a spectrometric spectrum on which we can see signals from the masses of the ions formed and a nonionized molecule, which can also be used to create one's own database and compare strains with one another.

Therefore, the aim of this study was to identify and differentiate presumptive *E. faecalis* strains isolated from YSI of broiler chickens from Poland and the Netherlands using biochemical tests, MALDI-TOF MS, and *rpoA* gene sequencing. Moreover, a characterization of virulence factors and biofilm formation were carried out. In addition, the diversity of these isolates was analyed using MALDI-TOF MS and enterobacterial repetitive intergenic consensus—polymerase chain reaction (**ERIC-PCR**).

MATERIALS AND METHODS

Bacteriological Analysis

A collection of 35 Polish and 41 Dutch isolates of *Enterococcus* spp. from broiler chickens with YSI was examined. All isolates were stored at −80°C until analysis. The bacterial isolates were initially characterized

based on the colony morphology, catalase production, pyrrolidonyl arylamidase activity (PYRAtest; Erba Lachema, Brno, Czech Republic), and biochemical properties tested using STREPTOtest 24 (Erba Lachema) according to manufacturer's instruction.

Phenotypic Screening of Virulence

Hemolysin and gelatinase activity test and biofilm assays were performed based on a before described methods (Stępień-Pyśniak et al., 2019). Hemolysis was evaluated by plating the strains on Columbia Agar Base (OXOID, Hampshire, UK) supplemented with 5% defibrinated horse blood (Pro Animali Company, Wroclaw, Poland). The plates were incubated at 37°C for 24 h in aerobic conditions. A positive result was indicated by the formation of hemolytic (clear) zones around the colonies. Gelatinase production was detected by inoculating the *E. faecalis* isolates onto Trypticase Soy Agar (OXOID) containing 3% gelatine (Avantor Performance Materials, Gliwice, Poland). The appearance of a clear halo around the colonies after incubation at 37°C for 24 h in aerobic conditions followed by refrigeration at 4°C for 30 min was considered to be a positive indication of gelatinase production. *E. faecalis* ATCC 29212 was used as a positive control in both tests.

In brief, biofilm assays were conducted in 96-well flat-bottomed polystyrene microtiter plates with using 180 μL Brain Heart Infusion (**BHI**) supplemented with 2% glucose. Then 20 μL of bacteria suspension added to each of 4 wells per isolate. For the negative control, 200 μL of broth (BHI with glucose) was dispensed into 8 vertical wells per plate. After incubation for 24 h, the broth was carefully removed, and wells were washed with phosphate-buffered saline (pH 7.2). After staining of the adherent biofilm layer, the microplates were washed until the washings were free of the stain. The dye bound to the cells was resolubilized with 200 mL of 96% ethanol per well for 30 min without shaking. The optical density of the resolubilized crystal violet was then measured at 570 nm (OD_{570}) using a microplate reader (Bio-Rad, Hercules, CA, Model 680). Based on the bacterial biofilm OD, the isolates were classified into 4 categories: nonbiofilm producer, weak, moderate, or strong biofilm producer. The isolates were classified as follows: OD < ODc = nonbiofilm producer; ODc < OD < 2ODc = weak biofilm producer; 2ODc < OD < (4ODc) = moderate biofilm producer; and OD > 4ODc = strong biofilm producer.

Identification of Isolates by MALDI-TOF MS

The isolates were identified using MALDI-TOF MS (Bruker Daltonics, Bremen, Germany). The identification step was preceded by extraction of proteins with ethanol and formic acid as described by Stępień-Pyśniak et al. (2017). Automatic measurement of the spectrum and comparative analysis with reference spectra of bacteria were performed using an Ultraflextreme mass spectrometer and MALDI-Biotyper 3.0

software (Bruker Daltonics). The reliability of identification in the MALDI Biotyper system was expressed in points. The MALDI Biotyper output is a log (score) between 0 and 3.0, which is calculated by comparing the peak list from an unknown isolate with the reference main spectra in the database. According to the criteria proposed by the manufacturer, a log (score) below 1.700 does not allow for reliable identification; a log (score) between 1.700 and 1.999 enables identification to the genus level; a log (score) between 2.000 and 2.299 means highly probable identification at the genus level and probable identification at the species level; and a log (score) higher than 2.300 (2.300–3.000) indicates highly probable identification at the species level.

Dendrogram Construction for E. faecalis and MALDI-TOF MS Phyloproteomic Analysis

Based on cross-wise mass spectral tree matching, a dendrogram was created with similar main spectral profile (**MSP**) resulting in a high matching score value. Each MSP was matched against all MSP of the analyzed set. The list of score values was used to calculate normalized distance values between strains, resulting in a matrix of matching scores. The visualization of the relationship between the MSP was displayed in a dendrogram using MALDI Biotyper 3.0 software (Bruker Daltonik, Bremen, Germany) (Sauer et al., 2008).

Phyloproteomic analysis of the *E. faecalis* groups from Poland and the Netherlands was performed to characterize their proteomic relationships. For this purpose, mass spectra were collected using FlexControl software. Then, the spectra were baseline-corrected and noise-filtered using flexAnalysis 3.3. Data lists of m/z values with a signal-to-noise ratio higher than 3 were extracted from the mass spectral data. Then, all mass lists were analyzed and compared over a mass interval from 2,000 to 20,000 Da, as reproducibility in this range is good. All final strain-specific peak mass lists were then compared with one another to determine characteristic peak masses.

Identification of Isolates by RpoA Gene Sequencing

Genomic DNA was extracted using a commercial Genomic Mini kit (A&A Biotechnology, Gdynia, Poland). To improve nucleic acid extraction efficiency, lysozyme was used in the enzymatic lysis step.

Polymerase chain reaction (**PCR**) and *rpoA* gene sequencing were carried out as described previously (Naser et al., 2005). The primer pair rpoA-21-F (5′-ATGATYGARTTTGAAAAACC-3′) and rpoA-23-R (5′-ACHGTRTTRATDCCDGCRCG-3′) was used for amplification and sequencing of the gene. The *rpoA* gene sequences were compared with other bacterial *rpoA* sequences available in the GenBank database using BLAST software and aligned with sequences of the type

strains of the same *Enterococcus* species using Clustal X software (Thompson et al., 1997). Phylogenetic analysis was performed using MEGA7 software (Kumar et al., 2016). Distances were calculated according to the Kimura 2-parameter model (Kimura, 1980), and clustering was carried out by the neighbor-joining method using bootstrap values based on 500 replications.

Genotypic Analysis of Virulence

All isolates were screened for the presence of 16 virulence factors by PCR amplification with primers and conditions reported previously by Stępień-Pyśniak et al. (2019). The following determinants were tested: *esp* (enterococcal surface protein), *asa1* (aggregation substance), *ace* (collagen-binding protein), $efaA_{fs}$ (adhesin-like *E. faecalis* endocarditis antigen A), *cylA, cylB, cylM,* and *cylL* (cytolysin, i.e. a hemolytic exotoxin), *gelE* (gelatinase), *hyl* (hyaluronidase), *fsrA, fsrB,* and *fsrC* (quorum sensing), and *cpd, cob,* and *ccf* (sex pheromone).

Molecular Typing by Enterobacterial Repetitive Intergenic Consensus— Polymerase Chain Reaction

All 76 strains were genotyped by ERIC-PCR using the primers ERIC1 (5′-ATG TAA GCT CCT GGG GAT TCA C-3′) and ERIC2 (5′-AAG TAA GTG ACT GGG GTG AGC G-3′) as described previously (Versalovic et al., 1991). Reactions were carried out in a total volume of 25 µL containing 2.5 µL of 10x Taq reaction buffer, 2 µL of 25 mmol of $MgCl_2$, 2 µL of 25 mmol dNTPs, 2.5 U of Taq DNA polymerase (Qiagen, Hilden, Germany), 12.5 pmol of each primer, and 60 ng of template DNA. Amplification was performed with 5 min of initial denaturation at 95°C, 30 cycles consisting of denaturation at 94°C, for 30 s, annealing at 50°C for 1 min, and elongation at 72°C for 3 min, followed by a final elongation for 10 min at 72°C.

The ERIC-PCR profiles were examined by cluster analysis with NTSys ver. 2.02 software (Exeter Software Ltd, USA). The similarity distances between ERIC-PCR profiles were calculated using the Dice coefficient, and the dendrogram was based on the unweighted pair group method with arithmetic average.

RESULTS

Biochemical Characterization of Isolates Collection and Phenotypic Virulence Analysis

STREPTOtest 24 identified 70 isolates as *E. faecalis* at the 100% identification level (1 isolate), at 99.9% (67 isolates), at 97% (1 isolate), and at 86.48% (1 isolate). In addition, 6 isolates were identified as *Enterococcus mundtii* (1 isolate—94%; 4 isolate—88.7%, and 1 isolates—87%). The patterns of reactions of biochemical

tests were identical for 66 isolates of *E. faecalis*: N-acetyl-glucosaminidase, L-leucine-aminopeptidase, β-mannosidase, β-glucosidase, β-galactosidase, esculin, mannitol, sorbitol, ribose, lactose, arginine, growth in 6.5% NaCl, tagatose, maltose, and trehalose were positive; β-glucuronidase, α-galactosidase, phosphatase, inulin, melibiose, pullulan, α-methylglucosidase, raffinose, and sorbose were negative. In contrast with these 66 isolates, 1 isolate with 99.9% identification gave a negative reaction for lactose. The *E. faecalis* isolates with 100 and 97% identification were positive for phosphatase and raffinose, respectively, but *E. faecalis* with 86.48% identification was negative for β-glucosidase, in contrast with the phenotype of 66 isolates with a 99.9% identification level.

The patterns of the biochemical reactions for *E. mundtii* were similar as for the 66 *E. faecalis* isolates with a 99.9% identification level, but these isolates were additionally positive for α-galactosidase and melibiose in the case of *E. mundtii* with a 94% level of identification; melibiose for *E. mundtii* with 88.7% identification; and β-glucuronidase, α-galactosidase, melibiose, and raffinose for *E. mundtii* with 87% identification.

All isolates were positive for pyrrolidonyl arylamidase and negative for catalase production.

None of the isolates were beta-hemolytic in aerobic conditions. Gelatinase was produced by all but 1 isolate. Detailed data regarding the biochemical properties of the isolates are presented in Table 1.

The prevalence of *E. faecalis* with the ability to form a biofilm was 39.5% (30/76 strains), and the distribution characteristics of the biofilm phenotype were as follows: 5.3% of isolates were strong biofilm producers (1 Dutch and 3 Polish isolates), 26.3% were moderate biofilm producers (5 Dutch and 15 Polish isolates), and 7.9% were weak biofilm producers (4 Dutch and 2 Polish isolates). Among the biofilm-forming isolates, as many as 66.7% (20/30) were Polish *E. faecalis* strains. The biofilm strength among individual *E. faecalis* isolates is shown in Supplementary Table 1.

MALDI-TOF MS Species Identification

MALDI-TOF MS confirmed that 21/76 (27.6%) isolates belonged to the species *E. faecalis*, with a log (score) ranging from 2.301 to 2.828. The other 34 field strains (44.7%) attained a log (score) over 2.000 (2.009–2.292), which was sufficient to identify them as *E. faecalis*. The log (score) of 21 isolates (27.6%) was between 1.741 and 1.992 (Supplementary Table 1). Despite the log (score) < 2.000, we identified these strains as *E. faecalis* as well, because the best 3 matches obtained for them indicated this species. Moreover, analysis of the spectra corresponding to the protein and peptide ions of individual *E. faecalis* strains, ranging from 2,000 to 20,000 Da, reveals that both the position of the main peaks and their mass-to-charge ratios exhibit a high degree of similarity between strains.

Table 1. Biochemical properties of *Enterococcus* spp. isolates from yolk sac infections.

Code	Test	*E. faecalis* 99.9% n = 66 D = 35, P = 31[1]	*E. faecalis* 100% n = 1 D = 1	*E. faecalis* 99.9% n = 1 D = 1	*E. faecalis* 86.48% n = 1 D = 1	*E. faecalis* 97% n = 1 P = 1	*E. mundtii* 88.7% n = 4 D = 1, P = 3	*E. mundtii* 87% n = 1 P = 1	*E. mundtii* 94% n = 1 D = 1	*E. faecalis* 99.9% ATCC 29212
β-HEM	Beta-hemolytic activity	−	−	−	−	−	−	−	−	+
PYR	Pyrrolidonyl arylamidase	+	+	+	+	+	+	+	+	+
Catalase	Catalase production	−	−	−	−	−	−	−	−	−
GEL	Gelatinase activity	+[2]	+	+	+	+	+	+	+	+
NAG	N–acetyl–glucosaminidase	+	+	+	+	+	+	+	+	+
LAP	L–leucine–aminopeptidase	+	+	+	+	+	+	+	+	+
bMN	β-mannosidase	+	+	+	+	+	+	+	+	+
GLR	β-glucuronidase	−	−	−	−	−	−	**+**	−	−
bGL	β-glucosidase	+	+	+	**−**	+	+	+	+	+
bGA	β-galactosidase	+	+	+	+	+	+	+	+	**−**
aGA	α-galactosidase	−	−	−	−	−	−	**+**	**+**	−
PHS	Phosphatase	−	**+**	−	−	−	−	−	−	−
ESL	Esculin	+	+	+	+	+	+	+	+	+
INU	Inulin	−	−	−	−	−	−	−	−	−
MAN	Mannitol	+	+	+	+	+	+	+	+	+
SOR	Sorbitol	+	+	+	+	+	+	+	+	+
MLB	Melibiose	−	−	−	−	−	**+**	**+**	**+**	−
RIB	Ribose	+	+	+	+	+	+	+	+	+
LAC	Lactose	+	+	**−**	+	+	+	+	+	**−**
PUL	Pullulan	−	−	−	−	−	−	−	−	−
ARG	Arginine	+	+	+	+	+	+	+	+	+
SO6	Growth in 6.5% NaCl	+	+	+	+	+	+	+	+	+
AMG	α-methylglucosidase	−	−	−	−	−	−	−	−	- −
TGT	Tagatose	+	+	+	+	+	+	+	+	+
MLT	Maltose	+	+	+	+	+	+	+	+	+
RAF	Raffinose	−	−	−	−	**+**	−	**+**	−	−
TRE	Trehalose	+	+	+	+	+	+	+	+	+
SOE	Sorbose	−	−	−	−	−	−	−	−	−

(+) = positive reaction; (−) = negative reaction.
Boldface characters (+/−) indicate differences in biochemical properties compared to 66 isolates (in column 1).
[1]D—Dutch isolates; P—Polish isolates.
[2]One isolate negative for gelatinase activity; GEL–isolates positive for gelatinase activity.

Dendrogram analysis for the *E. faecalis* isolates indicates that Dutch and Polish *E. faecalis* strains have a similar protein profile (Figure 1). The bacterial isolates were classified into 2 main phylogenetic groups. One group (cluster 1) contained 36 isolates of *E. faecalis* from the Netherlands and the *E. faecalis* ATCC 29212 reference strain, whereas the second group (cluster 2) was formed mainly by 35 Polish strains as well as 4 Dutch strains (H41, H43, H17, and H32).

The dendrogram generated in BioTyper software shows the H42 strain as a single leaf between 2 main clades. Comparison of the custom MSP of H42 to the *E. faecalis* ATCC 29212 reference strain showed that these strains had 51 common peaks.

All of the spectra showed good resolution, with a variety of peaks and specific spectral profiles for each strain.

The strains in cluster 1 had 10 peaks in common, with masses at m/z 3,346 $\pm$ 2, **4,410 $\pm$ 2**, **4,426 $\pm$ 2**, 4,438 $\pm$ 1, **4,556 $\pm$ 2**, **4,762 $\pm$ 2**, **6,223 $\pm$ 2**, 6,668 $\pm$ 2, **9,107 $\pm$ 4**, and **9,521 $\pm$ 4**. In the second cluster, containing 39 strains, there were 31 common peaks, with masses of 2,204 $\pm$ 1, 2,212 $\pm$ 1, 3,039 $\pm$ 1, 3,336 $\pm$ 1, 3,429 $\pm$ 1, 3,666 $\pm$ 1, **4,410 $\pm$ 2**, **4,426 $\pm$ 2**, **4,557 $\pm$ 2**, **4,764 $\pm$ 2**, 5,104 $\pm$ 2, 5,558 $\pm$ 3, 6,078 $\pm$ 3, **6,225 $\pm$ 2**, 6,397 $\pm$ 3, 6,699 $\pm$ 4, 6,720 $\pm$ 2, 6,859 $\pm$ 3, 7,024 $\pm$ 3, 7,330 $\pm$ 3, 7,576 $\pm$ 2, 8,825 $\pm$ 3, 8,881 $\pm$ 3, **9,114 $\pm$ 3**, **9,528 $\pm$ 3**, 10,207 $\pm$ 3, 11,120 $\pm$ 4, 11,582 $\pm$ 4, 13,439 $\pm$ 4, 14,113 $\pm$ 4, and 15,148 $\pm$ 6.

Within the 2 main groups, there were 7 common masses (boldface peaks above) that may be characteristic for the analyzed *E. faecalis* isolates. The other peak masses, apart from the boldface peaks, were present only in a single source of origin, for example Dutch (H) or Polish (P) isolates (cluster 1 and 2, respectively), with some exceptions (H41, H43, H17, and H32).

Identification With Using RpoA Gene Sequencing

All 76 isolates were also identified to the species level by sequence analysis of a 716 bp fragment of the *rpoA* gene to confirm the identification of isolates by STREPTOtest 24 and MALDI-TOF MS. The *rpoA* sequences of the isolates showed the highest sequence similarity (99.9–100%) to *E. faecalis* type strain LMG7937 (Supplementary Table 1). The identification of isolates by *rpoA* gene sequencing was 100 and 92.1% concordant with the results obtained using the MALDI-TOF MS technique and biochemical identification, respectively.

Genotypic Virulence Analysis

The phenotypic and genotypic patterns of virulence factors in individual *E. faecalis* isolates are shown in Supplementary Table 1.

No *hyl* gene was detected in any of the isolates tested. The presence of the *gelE* gene associated with the *fsrABC* locus was detected in all 41 Dutch and 34 of 35 Polish *E. faecalis* isolates. The *ace* gene coding for collagen-binding protein was harbored in 33 (80.5%) Dutch and 30 (85.7%) Polish isolates. The *asa1* gene coding for aggregation substance was detected in
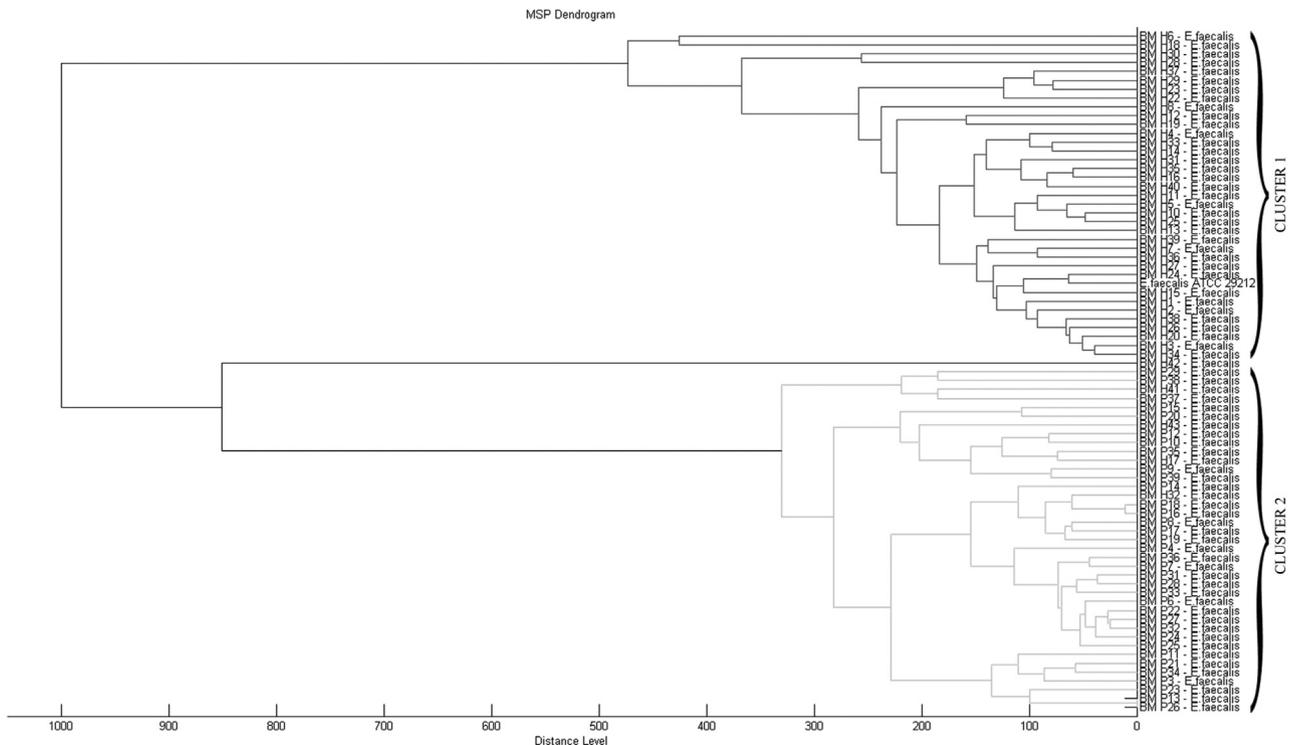
Figure 1. Dendrogram showing 76 *E. faecalis* isolates from yolk sac infections obtained by MALDI-TOF MS analysis of mass spectral profiles. *E. faecalis* ATCC29212 is the reference strain. Abbreviations: MALDI-TOF, matrix-assisted laser desorption/ionization–time-of-flight; MSP, main spectral profile.

Table 2. Prevalence of virulence genes in *E. faecalis* isolates [n(%)].

Isolates	Genes encoding virulence factors															
	gelE	*ace*	*asa1*	*efaAfs*	*cylA*	*cylB*	*cylM*	*cylL*	*esp*	*hyl*	*fsrA*	*fsrB*	*fsrC*	*cob*	*cpd*	*ccf*
Polish *E. faecalis* n = 35	34 (97.1)	30 (85.7)	21 (60)	35 (100)	3 (8.6)	3 (8.6)	3 (8.6)	3 (8.6)	1 (2.9)	0 (0)	34 (97.1)	34 (97.1)	34 (97.1)	31 (88.6)	35 (100)	35 (100)
Dutch *E. faecalis* n = 41	41 (100)	33 (80.5)	21 (51.2)	41 (100)	1 (2.4)	6 (14.6)	6 (14.6)	6 (14.6)	0 (0)	0 (0)	41 (100)	41 (100)	41 (100)	40 (97.6)	41 (100)	41 (100)
Total n = 76	75 (98.7)	63 (82.9)	42 (55.3)	76 (100)	4 (5.3)	9 (11.8)	9 (11.8)	9 (11.8)	1 (1.3)	0 (0)	75 (98.7)	75 (98.7)	75 (98.7)	71 (93.4)	76 (100)	76 (100)

51.2% (21/41) of Dutch *E. faecalis* isolates and 60% (21/35) of Polish isolates. The $efaA_{fs}$ gene coding for cell wall adhesin in enterococci was found in all analyzed *E. faecalis* strains. Three (8.6%) Polish and 1 (2.4%) Dutch *E. faecalis* strain had all investigated cytolysin determinants (*cylA*, *cylB*, *cylM*, and $cylL_L$). Additionally, 5 (12.2%) Dutch isolates carried 3 of these genes together (*cylB*, *cylM*, and $cylL_L$).

It is worth noting that the 1 Polish isolate with all the *cyl* genes tested also possessed the *esp* gene, but it did not carry the *gelE* or *fsrABC* gene.

Two of 3 sex pheromone determinants (*cpd* and *ccf*) were present in all tested *E. faecalis* isolates. However, the *cob* gene was not detected in 1 (2.4%) Dutch and 4 (11.4%) Polish isolates. Data pertaining to the prevalence of virulence determinants in *E. faecalis* isolates are presented in Table 2.

ERIC-PCR

Genotyping by ERIC-PCR demonstrated high genetic diversity among *E. faecalis* isolates (Figure 2). The ERIC1 and ERIC2 primers in *E. faecalis* generated 3–10 amplicons with molecular weights ranging from 550 to 13,000 bp and based on unweighted pair group method with arithmetic average analysis 21 distinct ERIC-PCR profiles were recognized within all 76 isolates. The ERIC-PCR profile D was predominant, characterizing 26 (34.2%) of all isolates, followed by profile C, which characterized isolates 9 (11.8%). Four ERIC-PCR profiles (B, G, K, and S) were represented by 2 isolates each and 3 profiles (F, E, and R) by 6 isolates each. Profile I grouped 4 isolates, whereas each of the 11 profiles (A, H, J, L, M, N, O, P, Q, T, and U) was detected in only 1 isolate.

DISCUSSION

Yolk sac infections contribute to increased mortality in chicks, chronic infections, or increased selection in the flock, which in turn leads to high economic losses in poultry production worldwide. Olsen et al. (2012a) observed a correlation between mortality caused by bacterial infections in chicks reared as future layers in the first week of life and subsequent mortality during rearing. They also noted that flocks of layers that were heterogeneous in terms of the size and development of chicks during the hatching period showed the same heterogeneity during the transfer of birds from the rearing room to the production hall.

According to available data, a few studies have investigated *E. faecalis* from YSI/omphalitis in meat turkeys and layer hens (Olsen et al., 2012a; Maasjot et al., 2019), but there is lack of such information on broiler chicks, which constitute the largest percentage of farmed poultry worldwide.

In our study, based on biochemical characteristics, 5 and 3 phenotypes were identified as *E. faecalis* and *E. mundtii*, respectively. Differences were noted in β-glucuronidase, β-glucosidase, α-galactosidase, phosphatase, melibiose, lactose, and raffinose tests, which was

ERIC profile (strains No.)
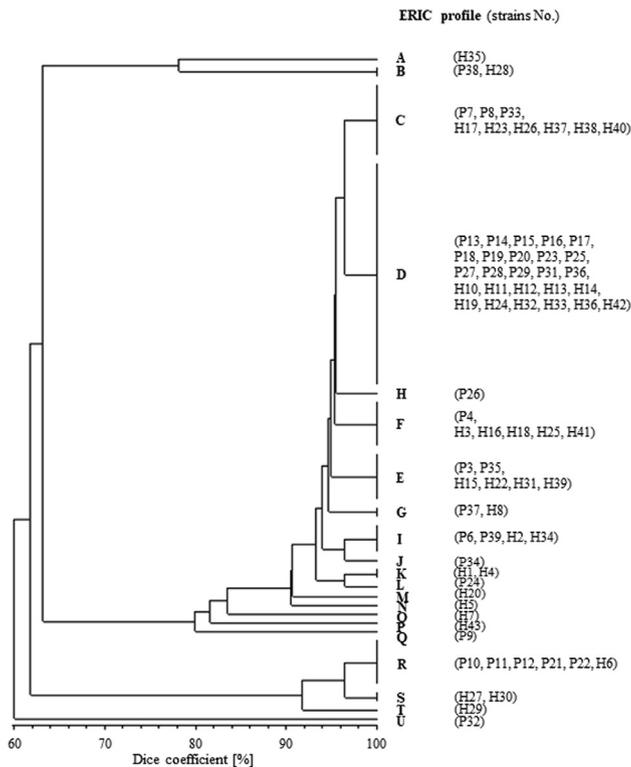
Figure 2. ERIC-based dendrogram showing genetic relatedness among *E. faecalis* isolates from yolk sac infections. Abbreviation: ERIC, enterobacterial repetitive intergenic consensus.

consistent with results for *E. faecalis* from pigeons reported by Dolka et al. (2020). In some cases, commercial tests are inaccurate for identification of veterinary isolates, especially from poultry. Their biochemical profiles usually differ slightly in phenotypic properties from the human isolates used to develop the database. For instance, *E. faecalis* ATCC 29212 control strains (originally isolated from the human urinary tract) were negative for β-galactosidase, in contrast to avian isolates. Manero and Blanch (1999) designed a key based on L-arabinose (−), arginine dihydrolase (+), mannitol (+), and ribose (+) for use in routine identification of clinical and environmental *E. faecalis* isolates. It has also been shown that common avian isolates can be separated by their differential ability to ferment mannitol, sorbitol, L-arabinose, sucrose, and raffinose (Christensen and Bisgaard, 2016). The present study showed that biochemical tests were able to correctly identify 92.1% of isolates, which is in agreement with Jin et al. (2011), who reported an identification rate of 92.3% for *Enterococcus* spp. Incorrect identification of the etiological agent of a disease may result in inappropriate treatment or prevention. For this reason, we used MALDI-TOF mass spectrometry to perform further diagnostics of isolates based on the protein profile specific to the bacteria, together with a verifying molecular method based on *rpo*A gene sequencing. The results obtained using both techniques indicated that all tested isolates from YSI in broiler chicks belonged to the species *E. faecalis*. Many studies indicate that MALDI-TOF mass spectrometry reliably identifies the species *E. faecalis* from

both farmed and free-living animals (Karunarathna et al., 2017; Nowakiewicz et al., 2017; Stępień-Pyśniak et al., 2017).

The MALDI-TOF mass spectrometry, in addition to identification of microorganisms, can be used for preliminary analysis and comparison of isolates, including differentiation of closely related enterococcal species, based on their protein profiles (Stępień-Pyśniak et al., 2017). Identification by MALDI-TOF MS is based on comparison of the mass spectrum of the test isolate (analysis of the distribution, number and intensity of peaks) with the spectra of reference strains in the database. Among the most intense mass peaks, Nowakiewicz et al. (2017) found 9 peaks occurring in all strains isolated from pigs in Poland, of which 9,522 m/z, 9,104 m/z, 6,669 m/z, 6,223 m/z, 6,077 m/z, and 4,428 m/z were also present in our isolates. In addition, only 5 peaks (4,411 m/z, 4,428 m/z, 4,556 m/z, 4,764 m/z and 6,223 m/z) among the common peaks found in all commensal *E. faecalis* isolates from pigs were simultaneously present in *E. faecalis* isolates from YSI from each of the 2 countries (the Netherlands and Poland). Therefore, they could be classified as potential species-specific markers for *E. faecalis*, regardless of the origin (pigs or chickens). Moreover, in our work, we showed the presence of specific peaks (apart from peaks that were in common) among *E. faecalis* isolates from each of the 2 countries that were not repeated in the group of isolates from the other country. Likewise, Giebel et al. (2008) showed a correlation between the presence of specific peaks and species of animals from which a given *Enterococcus* strain originated. Additionally, we noted 7 peaks simultaneously occurring in isolates from 2 sources of origin (from both countries), which can be designated as biomarkers for rapid identification of *E. faecalis* (species-specific biomarkers), especially from YSI in broiler chickens. In addition, based on the MSP dendrogram, we observed that this technique can be used to distinguish enterococci in terms of their place of origin, which is, in our opinion, important and practically useful information that can be used for preliminary inexpensive differentiation of strains in terms of their origin in an epidemiological investigation. Similarly, Quintela–Baluja et al. (2013) and Santos et al. (2015) suggest that MALDI-TOF MS can be useful for distinguishing strains from different sources, as the expression of specific biomarkers may be associated with the ecological niches of the strains or result from the selection of differentially expressed genes.

In contrast with MALDI-TOF MS cluster analysis, the ERIC-PCR results did not show a relationship to the origin of the strains. The ERIC-PCR result showed that 46.1% of the isolates were closely genetically related and belonged to types D and C. Most of the strains presented similar virulence patterns; however, the strains which possessed putative genes from the *cyl* operon, including 1 isolate with the *esp* gene, belonged to types D, F, C, and P. Similarly, in the work of Zalipour et al. (2019), the majority of human clinical *E. faecalis* isolates were clustered in the same genotypes, but in contrast

with our results, most isolates of these types were isolated from the same source. The ERIC-PCR cannot distinguish strains according to their geographic origin, likewise, as is not able to distinguish between virulent and avirulent avian strains as previously reported by Blanco et al. (2018).

In this study, all of the *E. faecalis* isolates were examined for the presence of the genes most often associated with clinical isolates (Fisher and Phillips, 2009; Olsen et al., 2012b). Previous research has shown that clinical isolates of the *Enterococcus* species, in comparison to food strains, possess a larger number of genes encoding virulence factors (Medeiros et al., 2014).

Many virulence determinants in *Enterococcus* spp. play an important role in the pathological process and in biofilm formation (Hashem et al., 2017). An important step in the development of infection and/or biofilm formation is the adhesion of bacterial cells to host tissues. In this study, the *asa1* gene coding for aggregating substance was detected in 55.3% of strains tested. Aggregating substance is encoded by genes located in sex pheromone-dependent plasmids. Two of the 3 determinants encoding sex pheromones (*cpd* and *ccf*) were present in all tested *E. faecalis* isolates. However, the *cob* gene was not detected in 6.6% of the strains. Similarly, Eaton and Gasson (2001) noted the presence of genes responsible for the production of sex pheromones in numerous strains of *E. faecalis*. The production of sex pheromones by *E. faecalis* may favor the acquisition of virulence as well as antibiotic resistance from other enterococci and lead to increased pathogenicity (Heaton et al., 1996). Both *efaAfs* and *ace* play a role in the pathogenesis of endocarditis (Singh et al., 1998, 2010). The *efaAfs* gene, coding for cell wall adhesin in enterococci (endocarditis antigen), was found in all analyzed strains of *E. faecalis*. In addition, in 82.9% of the isolates, we detected the *ace* gene, responsible for the production of the collagen adhesin protein Ace, which allows enterococci to bind to extracellular matrix proteins. The biofilm is thought to play an important role in the pathogenesis of various enterococcal infections (Mohamed and Huang, 2007). Growth of enterococci in the form of a biofilm increases bacterial resistance to antibiotics and decreases their susceptibility to phagocytosis and immune mechanisms (Distel et al., 2002). The ability to form a biofilm in BHI broth with 2% glucose was observed in 39.5% of isolates, with 5.3% of strains classified as strong biofilm producers, 26.3% as moderate, and 7.9% as having weak biofilm-forming ability. In contrast to our results, Woźniak-Biel et al. (2019) noted that turkey *E. faecalis* strains produced a strong biofilm after 24 h. However, they used LB broth (Lennox), and as previously observed, the type of medium affects the ability of the same bacterial species to form a biofilm (Stępień-Pyśniak et al., 2019).

The *hyl* gene encoding hyaluronidase was not detected in any of the isolates. This is in agreement with a previous studies reporting *E. faecalis* isolates from yolk sac or *Enterococcus cecorum* strains associated with infections in poultry without the *hyl* gene (Dolka et al., 2016, 2017a; Maasjot et al., 2019) The presence of the *gelE* gene encoding gelatinase, associated with the *fsrABC* locus, was detected in 98.7% of *E. faecalis* isolates. Studies on the FsrABC system have shown that it controls the development of *E. faecalis* biofilms through gelatinase synthesis (Nakayama et al., 2001; Hancock and Perego, 2004; Hashem et al., 2017). Phenotypic ability to produce gelatinase was shown in the same isolates that had the *gelE* gene. While infecting a macroorganism, bacteria can secrete substances that are toxic or destructive to host tissues. The appearance of an excess of proteolytic enzymes disturbs the natural balance between proteinases and their inhibitors in the host organism (Travis et al., 1995). This is particularly important in the infection process, as the host's proteinase inhibitors are unable to control bacterial enzyme activity, which can lead to degradation of proteins of the infected tissue (Travis et al., 1988).

All tested determinants of the cytolysin operon (*cylA*, *cylB*, *cylM*, and *cylL$_L$*) were found in 5.3% of strains. In addition, 6.7% of isolates contained 3 of these genes together (*cylB*, *cylM,* and *cylL$_L$*). The cylL, cylM, and cylB genes are relevant to the expression of component L (lysine), whereas cylA is necessary for the expression of component A (activator). None of the strains showed β-hemolytic activity on Columbia agar with the addition of 5% defibrinated sheep blood under aerobic conditions. Similarly, Dec et al. (2020) found no beta-hemolysis in strains with the *cylA* gene incubated under aerobic conditions, whereas they obtained beta-hemolysis in the same strains grown under microaerophilic conditions (7% CO_2). Phenotypic vs. genotype discrepancies have also been observed by Maasjot et al. (2019) in *E. faecalis* strains isolated from meat turkeys (including from cases of YSI); by Chajęcka-Wierzchowska et al. (2017) in isolates from food; and by Olsen et al. (2012b) in clinical and commensal enterococci from poultry.

This can be explained by downregulation of a gene that corresponds to a decrease in phenotypic expression or by the presence of silent genes, which may be activated by factors in vivo, for example factors resulting in infection (Eaton and Gasson, 2001). Environmental factors in in vitro conditions have also been observed to affect gene expression (Semedo et al., 2003; Cafini et al., 2015).

The low prevalence of the *esp* gene encoding enterococcal surface protein in isolates from broiler chicks with YSI was consistent with data obtained by Poeta et al. (2006) and Olsen et al. (2012b). It is worth emphasizing, however, that 1 strain in our study, belonging to the ST16 sequence type (data not shown), had all the *cyl* genes investigated and the *esp* gene, considered to be clinically relevant in medicine (Eaton and Gasson, 2001; Routsi et al., 2003), but did not have the *gelE* or *fsrABC* gene. Enterococcal surface protein is a surface protein involved in colonizing ability and immune evasion (Shankar et al., 2001). The presence of *esp* increases cell

hydrophobicity, adherence to abiotic surfaces, and biofilm formation in vitro (Toledo-Arana et al., 2001). This strain showed the highest biofilm capacity of all strains tested (OD value above the upper limit of detection by the spectrophotometer). Zheng et al. (2018) also noted that ST16 isolates displayed a higher biofilm-forming capacity than ST179 isolates from urinary tract infections. Moreover, Larsen et al. (2010) suggested that pigs were a source of high-level gentamicin-resistant ST16 *E. faecalis* in human infection. The same *E. faecalis* clones have been shown to be present in poultry, pigs, and humans, indicating that these bacteria can be transmitted between food animals and humans (Larsen et al., 2010; Olsen et al., 2012b; Poulsen et al., 2012).

CONCLUSION

The data demonstrate that most clinical *E. faecalis* strains from YSI in broiler chickens have the same biochemical properties irrespective of their source (country). Only a few strains show atypical biochemical characteristics. The results of MALDI-TOF mass spectrometry indicate that the technique can be used for preliminary comparison of *E. faecalis* isolates with respect to their origin and thus be a faster and less costly alternative to genotypic techniques such as ERIC-PCR. We noted 7 biomarkers which identified together may indicate *E. faecalis* from YSI in broiler chickens and could be used for rapid, routine laboratory diagnosis. Moreover, the results of the study indicate that broiler chicks with YSI can be a source of enterococci with virulent determinants for other animals, people working in hatcheries and on farms, or even consumers. Some *E. faecalis* strains have been shown to have a high biofilm-forming ability, which may help them survive in the environment and also promotes their pathogenicity. Veterinarians should also note that biofilm-capable *Enterococcus* spp. may increase resistance to antimicrobial agents, including antibiotics and disinfectants. This information can be used to take appropriate preventive measures, especially disinfection in poultry production facilities and monitoring of its effectiveness to limit the spread of pathogenic *E. faecalis* strains. The results of our research may also indicate the reason why treatment of enterococcal infections caused by strains characterized by increased virulence is ineffective despite the use of targeted therapy or explain why certain strains are persistently maintained in bird housing or hatcheries.

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DISCLOSURES

There are no known conflicts of interest associated with this publication.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.psj.2021.01.008.

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