Ready-to-eat dairy products as a source of multidrug-resistant *Enterococcus* strains: Phenotypic and genotypic characteristics

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ABSTRACT

The enterococci are ubiquitous bacteria able to colonize the human and animal gastrointestinal tracts and fresh and fermented food products. Their highly plastic genome allows *Enterococcus* spp. to gain resistance to multiple antibiotics, making infections with these organisms difficult to treat. Food-borne enterococci could be carriers of antibiotic resistance determinants. The goal of this work was to study the characteristics of *Enterococcus* spp. in fermented milk products from Poland and their antibiotic resistance gene profiles. A total of 189 strains were isolated from 182 dairy products out of 320 samples tested. The predominant species were *Enterococcus faecium* (53.4%) and *Enterococcus faecalis* (34.4%). Isolates were resistant to streptomycin (29.1%), erythromycin (14.3%), tetracycline (11.6%), rifampicin (8.7%), and tigecycline (8.1%). We also detected 2 vancomycin-resistant and 3 linezolid-resistant strains; however, no *vanA* or *vanB* genes were identified. A total of 57 high-level aminoglycoside resistance strains (30.2%) were identified, most of which have the *ant(6')-Ia* gene, followed by the *aac(6')-Ie-aph(2')-Ia* and *aph(3'')-IIIa* genes. Resistance to tetracycline was most often conferred by *tetM* and *tetL* genes. Macrolide resistance was most frequently encoded by *ermB* and *ermA* genes. Conjugative mobile genetic element (transposon Tn916–Tn1545) was identified in 15.3% of the strains, including 96.3% of strains harboring the *tetM* gene. This study found that enterococci are widely present in retail ready-to-eat dairy products in Poland. Many isolated strains are antibiotic resistant and carry transferable resistance genes, which represent a potential source of transmission of multidrug-resistant bacteria to humans.

Key words: *Enterococcus*, antibiotic resistance, antimicrobial agents, food, dairy, resistance genes

INTRODUCTION

Enterococci are ubiquitous bacteria that colonize different niches. They are commensals of the gastrointestinal tract of animals, thus, via fecal contamination, able to reach raw milk and meat (Ben Braïek and Smaoui, 2019). The ubiquitous nature of *Enterococcus* spp. in ready-to-eat food can be attributed to their resistance to food processing and storage operations as well as to their remarkable adaptive capacity. These microorganisms frequently constitute the residual microflora of foods. During cheese production, milk pasteurization factors play the key role in microbial selection. According to estimates, more than 50% of *Enterococcus* spp. populations survive high-temperature short-time pasteurization (72°C/15 s), and some populations are unharmed by higher processing temperatures (85°C/16 s; Ziarno, 2006). Bacteria of the genus *Enterococcus* are ubiquitous in cottage and rennet-coagulated cheeses made of both raw and pasteurized milk (Giraffa et al., 1997; Andrighetto et al., 2001; Gelsomino et al., 2001; Citak et al., 2004; Schirru et al., 2012). Enterococci are characterized by higher levels of proteolytic and lipolytic activity than other lactic acid bacteria, and they are also capable of breaking down citrates into acetaldehyde, acetoin, and diacetyl, which play an important role in the ripening of selected cheese types (Manolopoulou et al., 2003). Enterococci enhance the sensory attributes of cheese, and they are included in starter cultures for the production of various cheeses, particularly Italian, Argentine, and Greek cheeses (Giraffa, 2003). The abundance of enterococci in cheese is determined by the *Enterococcus* spp. counts in milk, the purity of the starter culture, and the hygiene standards in the processing plant (Ziarno, 2006). The presence of enterococci in milk can result from direct contamination with animal feces or indirect contamination with water or impurities from milking equipment (Bulajić et al., 2015). *Enterococcus* spp. counts in the fresh curd of rennet-coagulated cheese range from 10³ to 10⁶ cfu/g, and numbers fluctuate in the cheese-making process, depending on bacterial resistance to pH and salt (Pluta et al., 2004). Enterococci are most abundant in cheese

Received August 3, 2019.

Accepted January 16, 2020.

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made of unpasteurized milk, in particular in artisan cheeses produced in southern Europe. In these types of products, enterococci are regarded as non-starter lactic acid bacteria (Giraffa, 2003). Raw-milk cheese is not produced industrially in Poland. The presence of *Enterococcus* spp. in cheese made of pasteurized milk is attributed to their thermal resistance or recontamination after pasteurization (Giraffa, 2002).

Enterococci can also be pathogenic to humans. The antibiotic resistance of bacteria in the *Enterococcus* genus has been increasing steadily in recent years (Asadollahi et al., 2018). The most virulent and multidrug-resistant strains are still isolated from hospitals, but multidrug-resistant enterococci are increasingly being identified in foods (Kim et al., 2018, 2019). Antibiotic-resistant enterococci have also been isolated from ready-to-eat foods that are not heat-processed before consumption, giving grounds for concern (Chajęcka-Wierzchowska et al., 2016a). Meat and meat products are the most common sources of multidrug-resistant enterococci, but such strains have also been found in dairy products (Silvetti et al., 2019). Enterococci consumed with cheese could establish themselves in the intestine, even if their number in the cheese is small (Gelsomino et al., 2003). Enterococci have developed numerous adaptive strategies, and they can temporarily or permanently colonize the gastrointestinal tract, increasing the risk of bacterial gene transfer to the gut microbiota. In vivo transfer of the *vanA* resistance gene from an *Enterococcus faecium* isolate of animal origin to an *E. faecium* isolate of human origin in the intestines of human volunteers has been reported (Lester et al., 2006). This suggests that high counts of food-borne enterococci capable of transmitting resistance genes could serve as a reservoir for resistance transfer to other enterococcal populations, with effects on human health. Food-associated enterococci can survive gastric passage and multiply, leading to sustained intestinal carriage (Sørensen et al., 2001). In view of all of this, the aim of our study was to evaluate the antibiotic resistance and the genetic determinants in *Enterococcus* spp. strains isolated from ready-to-eat retail dairy foods purchased in Poland.

MATERIALS AND METHODS

Sampling

In this study, samples were selected according to several categories: soft rennet cheese, semi-hard rennet cheese, hard rennet cheese, sour milk cheese, and other dairy products (condensed milk, powdered milk, sour cream, butter). The number of samples depended on the

store assortment. The samples came from 11 supermarkets, markets, and local stores in the city of Olsztyn. In total, we investigated 320 ready-to-eat dairy samples, including 75 soft rennet cheeses, 46 semi-hard rennet cheeses, 91 hard rennet cheeses, 88 sour milk cheeses (unripened, aged), and 20 other dairy products. The samples were brought to the laboratory in cold chain and taken into analysis for the presence of *Enterococcus* spp. on the same day.

Isolation and Presumptive Identification of *Enterococcus* spp.

From each dairy food, 25 g of sample was blended for 2 min in a stomacher (BagMixer 400P, Interscience, Saint-Nom-la-Bretèche, France) with 225 mL of buffered peptone water and incubated overnight at 37°C. After that, a loopful was streaked on Slanetz and Bartley agar (Merck, Darmstadt, Germany). We selected 1 or 2 colonies per sample with typical enterococcal morphology. Because none of our samples had more than 2 morphologically different colonies, 1 or 2 were taken for further analysis. Presumptive identification of isolates was carried out as previously described (Chajęcka-Wierzchowska et al., 2016a, 2016b); briefly, Gram staining, catalase and oxidase production, growth at 10°C and 45°C, growth in the presence of 6.5% NaCl, growth at pH 9.6, and growth and esculin hydrolysis on bile-esculin agar (Merck). The enterococcal isolates were deposited in the laboratory culture collection in the Department of Industrial and Food Microbiology, University of Warmia and Mazury (Olsztyn, Poland) and were stored in a Microbank at -80°C (Biocorp, Warsaw, Poland).

PCR Identification of *Enterococcus* spp. Strains

Species identification was performed using PCR primers and conditions previously described for *Enterococcus* species *faecium*, *faecalis*, *casseliflavus*, *gallinarum*, *durans*, and *hirae* (Dutka-Malen et al., 1995; Deasy et al., 2000; Kariyama et al., 2000; Knijff et al., 2001). For DNA extraction we used methodology described in our previous papers (Chajęcka-Wierzchowska et al., 2014, 2016a, 2016b). Bacterial strains were streaked onto brain heart infusion agar (Merck) and incubated overnight at 37°C. The colonies were resuspended in a Tris-EDTA buffer (Sigma Aldrich, Poznan, Poland) and were lysed by lysozyme (0.6 mg/mL; A&A Biotechnology, Gdynia, Poland). The total genomic DNA of isolated and reference strains was extracted using the Genomic Mini DNA Purification Kit (A&A Biotechnology) according to the manufacturer's instructions. All PCR reactions

were analyzed by electrophoresis through a 1.5% high-resolution agarose gel (Promega, Madison, WI) in $1 \times$ TBE buffer, pH 8.3. The sizes of the amplification products were estimated by comparison with a 100-bp increment molecular size ladder (Fermentas, Thermo Fisher Scientific, Warsaw, Poland). Gels were stained with ethidium bromide (Sigma Aldrich) and viewed under UV light using a G-BOX F3 system (Syngene, Bangalore, India) and Gene Tools software (Syngene). Each profile was visually compared with those obtained from the enterococcal reference strains: *E. faecalis* ATCC 29212, *E. faecium* ATCC 19434, *E. casseliflavus* ATCC 49605, *E. gallinarum* ATCC 700425, *E. durans* ATCC 49479, and *E. hirae* ATCC 8043.

Antibiotic Susceptibility Testing

Susceptibility patterns were determined via the agar disc diffusion method on Mueller-Hinton agar (Merck) according to Clinical Laboratory Standards Institute guidelines (CLSI, 2017), as previously described (Chajęcka-Wierzchowska et al., 2016a,b). Eighteen antibiotics were used: ampicillin 10 µg, penicillin G 10 IU, gentamicin 120 µg, streptomycin 300 µg, vancomycin 30 µg, teicoplanin 30 µg, norfloxacin 10 µg, levofloxacin 5 µg, ciprofloxacin 5 µg, tetracycline 30 µg, tigecycline 15 µg, rifampicin 5 µg, erythromycin 15 µg, nitrofurantoin 300 µg, linezolid 30 µg, fosfomycin 200 µg, chloramphenicol 30 µg, and quinupristin-dalfopristin 15 µg (for *E. faecium*). Cartridges with commercially prepared paper discs containing the appropriate antibiotic dosage were purchased from Oxoid (Basingstoke, UK). An overnight culture of enterococcal isolates was streaked on the surface of the Mueller-Hinton agar. Antibiotic discs were then placed on the plates and incubated at 37°C. Zone diameters were recorded after a 24-h incubation period. Strains were classified as resistant or susceptible according to criteria from CLSI (2017) and EUCAST (2019) for tigecycline. *Staphylococcus aureus* ATCC 25923 was used as quality control. In vancomycin-resistant strains, MIC was defined. We used commercial Etest (bioMérieux, Craponne, France) according to the manufacturer's instructions.

Analysis of the Molecular Mechanisms of Antibiotic Resistance

All isolates were tested for the presence of the tetracycline resistance genes encoding efflux proteins (*tetK* and *tetL*) and genes encoding ribosomal protection proteins (*tetM*). The tetracycline efflux gene *tetK* amplification was performed according to Gevers et al. (2003) and *tetW* according to protocol described by Aminov et al. (2001). The tetracycline-resistant *tetM*

and *tetL* genes and the macrolide-resistant *ermB* gene were detected via multiplex PCR (triplex) using the specific primers and conditions reported by Rizzotti et al. (2009). The presence of conjugative transposons of the Tn916 and Tn1545 families was determined using primers targeting the integrase *int* gene, according to Macovei and Zurek (2006). The *ermA*, *ermC*, *mefA* and *E*, and *mrsC* genes were detected according to Sutcliffe et al. (1996). Multiplex PCR was employed to detect the following 4 genes encoding aminoglycoside-modifying enzymes: *aac(6')-Ie-aph(2')-Ia*, *aph(3')-IIIa*, *ant(4')-Ia*, and *ant(6')-Ia*. These genes were monitored according to protocols provided by Vakulenko et al. (2003) and Kobayashi et al. (2001). The *vanA*, *vanB*, *vanC1*, and *vanC2* and *C3* genes were detected according to protocol proposed by Dutka-Malen et al. (1995).

RESULTS

Of 320 samples of dairy products, 182 were contaminated with enterococci, and 189 strains were isolated. The predominant species were *E. faecium* (53.4%) and *E. faecalis* (34.4%). The analyzed products were characterized by low counts of *E. gallinarum* (6.3%) and *E. casseliflavus* (2.5%), whereas *E. durans* and *E. hirae* were not detected (Table 1).

Among the analyzed *Enterococcus* strains, the highest percentages of resistance occurred in response to streptomycin (29.1%), erythromycin (14.3%), tetracycline (11.6%), rifampicin (8.7%), and tigecycline (8.1%). The percentage of strains resistant to the remaining antibiotics was determined to be 3.2 to 1.1%, and none were resistant to ampicillin, teicoplanin, or ciprofloxacin. Two *E. faecalis* strains were resistant to vancomycin, a drug of last resort against serious gram-positive bacterial infections (MIC > 256 mg/mL); however, no *vanA* or *vanB* genes were identified. Genes encoding resistance to vancomycin were noted only in *E. casseliflavus* and *E. gallinarum*, and these were associated with natural bacterial resistance to vancomycin encoded by *vanC1* and *vanC2* or *C3* genes. We identified 26 (13.75%) multidrug-resistant strains and 23 different

Table 1. Occurrence of *Enterococcus* species in dairy food samples (n = 320)

Species	Number (%) of isolates	% of positive samples
<i>Enterococcus faecalis</i>	65 (34.4)	20.3
<i>Enterococcus faecium</i>	101 (53.4)	31.6
<i>Enterococcus gallinarum</i>	12 (6.3)	3.8
<i>Enterococcus casseliflavus</i>	5 (2.5)	1.6
Other <i>Enterococcus</i> spp.	6 (3.2)	1.9
Total	189	59.2

Table 2. Number (%) of antibiotic-resistant *Enterococcus* strains isolated from dairy food samples¹

Antimicrobial agent	Strain					Total n = 189
	<i>Enterococcus faecalis</i> n = 65	<i>Enterococcus faecium</i> n = 101	<i>Enterococcus gallinarum</i> n = 12	<i>Enterococcus casseliflavus</i> n = 5	Other <i>Enterococcus</i> spp. n = 6	
AMP	0	0	0	0	0	0
P	2 (3.1)	0	0	0	0	2 (1.1)
CN	0	3 (3.0)	0	0	0	3 (1.6)
S	24 (36.9)	30 (27.9)	0	0	0	55 (29.1)
VA	2 (3.1)	0	0	0	0	2 (1.1)
TEC	0	0	0	0	0	0
NOR	0	4 (4.0)	0	0	0	4 (2.1)
LEV	2 (3.1)	0	0	0	0	2 (1.1)
CIP	0	0	0	0	0	0
TE	11 (16.9)	11 (10.9)	0	0	0	22 (11.6)
TGC	9 (13.8)	3 (3.0)	2 (16.7)	1 (28.0)	1 (16.7)	15 (8.1)
RD	5 (7.7)	7 (6.9)	3 (25.0)	1 (28.0)	2 (33.3)	16 (8.7)
F	5 (7.7)	1 (1.0)	0	0	0	6 (3.2)
LZD	3 (4.6)	0	0	0	0	3 (1.6)
FOT	2 (3.1)	3 (3.0)	0	1 (28.0)	0	6 (3.2)
C	4 (6.2)	0	0	0	0	4 (2.1)
QD	nc [†]	3 (3.0)	0	1 (28.0)	0	4 (2.1)
E	12 (18.5)	15 (14.9)	0	0	0	27 (14.3)

[†]*Enterococcus faecalis* strains are intrinsically resistant to quinopristin and dalbopristin. AMP = ampicillin, P = penicillin G, CN = gentamicin, S = streptomycin, VA = vancomycin, TEC = teicoplanin, NOR = norfloxacin, LEV = levofloxacin, CIP = ciprofloxacin, TE = tetracycline, TGC = tigecycline, RD = rifampicin, F = nitrofurantoin, LZD = linezolid, FOT = fosfomycin, C = chloramphenicol, QD = quinupristin-dalbopristin, E = erythromycin. nc = not checked.

multidrug resistance phenotypes, of which S, TE, TGC, and E were the most common.

Three *E. faecalis* isolates were resistant to linezolid, an antibiotic that was approved for use in 2000. Multidrug resistance phenotypes were observed for both *E. faecalis* and *E. faecium*, as well as for other enterococci species isolated in this study (Tables 2 and 3).

Our study revealed a high percentage of enterococci with high-level aminoglycoside resistance (**HLAR**; Table 4). A total of 57 HLAR strains (30.2%) were identified in the studied isolates (n = 189). The HLAR phenotype was noted only in *E. faecium* (n = 33; 15.9%) and *E. faecalis* isolates (n = 24; 12.7%). The high-level gentamycin resistance (**HLGR**) and HLSR phenotype was identified only in 3 *E. faecium* strains. The most prevalent phenotype in *E. faecium* and *E. faecalis* strains was HLSR. In the isolated bacterial strains, resistance to streptomycin was most frequently associated with the presence of the *ant(6')-Ia* gene. Resistance to the remaining aminoglycosides was encoded by only 2 genes: *aac(6')-Ie-aph(2'')-Ia* and *aph(3'')-IIIa*. Isolates characterized by HLGR most often harbored the *aac(6')-Ie-aph(2'')-Ia* gene, localized on transposon Tn5281. Isolates with HLSR were characterized by greater genetic variation than isolates with HLGR. Streptomycin-resistant strains most often harbored the *ant(6')-Ia* gene, which could be located on a transposon (Clark et al., 1999), and which was detected individually or in combination with the *aph(3'')-IIIa* gene encoding

low-level kanamycin resistance or with the *aac(6')-Ie-aph(2'')-Ia* gene encoding HLGR (Table 5).

Resistance to tetracyclines was most often conferred by the *tetM* and *tetL* genes. The *tetK* gene was identified in only 2 *E. faecalis* isolates and the *tetW* gene in only 1 isolate of *Enterococcus* spp. The Tn916/Tn1545 family transposons were detected in 26 out of 27 strains (96.3%) harboring the *tetM* gene. The group of strains with phenotypic resistance to tetracyclines was characterized by greater species diversity than the erythromycin-resistant isolates (Tables 6 and 7). In *E. faecalis* strains, resistance to tetracyclines was associated with presence of the *tetM* gene (Table 4). In *E. faecium* strains, tetracycline resistance was associated with the presence of either a single *tetM* gene or a combination of the *tetM* and *tetL* genes (Table 6). The presence of the *tetL* gene or a combination of the *tetM* and *tetL* genes was also observed in other enterococcus strains (*E. casseliflavus*, *E. gallinarum*, and other *Enterococcus* spp.) The presence of the *tetK* gene was usually accompanied by the amplification of *tetM* gene. In the group of 2 *E. gallinarum* strains characterized by phenotypic resistance to tetracyclines, the *tetM* and *tetL* genotype was observed (Table 6).

Macrolide resistance was associated with the presence of the *ermB* and *ermA* genes, only in individual strains. Conjugative mobile genetic elements were identified in 15.3% of the strains, including in all strains harboring the *tetM* gene (Table 7). Regardless of species, eryth-

Table 3. Antibiotic resistance genes isolated from dairy products (% in parentheses)

Gene	Strain					Total n = 189
	<i>Enterococcus faecalis</i> n = 65	<i>Enterococcus faecium</i> n = 101	<i>Enterococcus gallinarum</i> n = 12	<i>Enterococcus casseliflavus</i> n = 5	Other <i>Enterococcus</i> spp. n = 6	
Aminoglycosides						
<i>aac(6′)-Ie-aph(2′′)-Ia</i>	7 (10.8)	14 (13.9)	0	0	0	21 (11.1)
<i>aph(2′′)-Ib</i>	0	0	0	0	0	0
<i>aph(2′′)-Ic</i>	0	0	0	0	0	0
<i>aph(2′′)-Id</i>	0	0	0	0	0	0
<i>aph(3′′)-IIIa</i>	12 (18.5)	19 (18.8)	0	0	0	32 (16.4)
<i>ant(4′)-Ia</i>	2 (3.1)	0	0	0	0	2 (1.1)
<i>ant(6′)-Ia</i>	19 (29.2)	26 (25.7)	0	0	0	45 (23.8)
Tetracyclines						
<i>tetM</i>	15 (23.1)	10 (9.9)	2 (16.7)	0	0	27 (14.3)
<i>tetL</i>	8 (12.3)	7 (6.9)	2 (16.7)	1 (20.0)	1 (16.7)	19 (10.1)
<i>tetK</i>	2 (3.1)	0	0	0	0	2 (1.1)
<i>tetO</i>	0	0	0	0	0	0
<i>tetW</i>	0	0	0	0	1 (16.7)	1 (0.5)
Macrolides						
<i>ermA</i>	4 (6.2)	6 (5.9)	0	0	0	10 (5.5)
<i>ermB</i>	12 (18.5)	12 (11.9)	0	0	0	24 (12.7)
<i>ermC</i>	0	1 (1.0)	0	0	0	1 (0.5)
<i>msrC</i>	2 (3.1)	1 (1.0)	0	0	0	3 (1.6)
<i>mefA/E</i>	0	2 (2.0)	0	0	0	2 (1.1)
Glikopeptides						
<i>vanA</i>	0	0	0	0	0	0
<i>vanB</i>	0	0	0	0	0	0
<i>vanC1</i>	0	0	12 (100)	0	0	12 (6.3)
<i>vanC2/C3</i>	0	0	0	5 (100)	0	5 (2.6)
Transposon						
<i>int</i> (Tn916–Tn1545)	15 (23.1)	12 (11.9)	2 (0.0)	0	0	29 (15.3)

Table 4. Phenotypes occurring in strains that are resistant to high concentrations of aminoglycosides¹

Species	Number (%) of strains			
	HLAR	HLGR	HLSR	HLGR + HLSR
<i>Enterococcus faecalis</i>	24	0	24 (100)	0
<i>Enterococcus faecium</i>	33	0	30 (90.9)	3 (9.1)
Total	57	0	54	3

¹HLAR = high-level aminoglycoside resistance; HLGR = high-level gentamycin resistance; HLSR = high-level streptomycin resistance.

Table 5. Gene profiles encoding aminoglycosides (AME) enzymes in strains resistant to high concentrations of streptomycin [high-level streptomycin resistance (HLSR) phenotype]

Species	Number of HLSR strains	Genotype	Number (%) of strains
<i>Enterococcus faecalis</i>	24	<i>ant(6')-Ia</i>	10 (42.0)
		<i>aph(3'')-IIIa + ant(6')-Ia</i>	7 (29.0)
		<i>aac(6')-Ie-aph(2'')-Ia + aph(3'')-IIIa</i>	3 (13.0)
		<i>aac(6')-Ie-aph(2'')-Ia + ant(6')-Ia</i>	2 (8.0)
		<i>aph(3'')-IIIa + ant(4')-Ia</i>	2 (8.0)
<i>Enterococcus faecium</i>	30	<i>aph(3'')-IIIa + ant(6')-Ia</i>	15 (50.0)
		<i>ant(6')-Ia</i>	6 (20.0)
		<i>aac(6')-Ie-aph(2'')-Ia + ant(6')-Ia</i>	5 (17.0)
		<i>aac(6')-Ie-aph(2'')-Ia + aph(3'')-IIIa</i>	4 (13.0)

Table 6. Genotypic diversity of tetracycline-resistant *Enterococcus* strains (TETR)

Species	Number of TETR strains	Antibiotic resistance genes	Number (%) of strains
<i>Enterococcus faecalis</i>	24	<i>tetM</i>	11 (45.8)
		<i>tetM</i> + <i>tetL</i>	6 (25.0)
		<i>tetL</i>	3 (12.5)
		<i>tetM</i> + <i>tetK</i>	2 (8.3)
		None	2 (8.3)
<i>Enterococcus faecium</i>	14	<i>tetM</i>	5 (17.7)
		<i>tetM</i> + <i>tetL</i>	5 (17.7)
		<i>tetL</i>	2 (7.7)
		None	2 (7.7)
<i>Enterococcus gallinarum</i>	2	<i>tetM</i> + <i>tetL</i>	2 (100)
<i>Enterococcus casseliflavus</i>	1	<i>tetL</i>	1 (100)
Other <i>Enterococcus</i> spp.	2	<i>tetL</i>	1 (50)
		<i>tetW</i>	1 (50)

romycin resistance was associated with the presence of the constitutive *ermB* gene. A high percentage of strains also harbored either a combination of the *ermA* and *ermB* genes or only the *ermA* gene (Table 7). The presence of *mefA* or *mefE* and *msrC* genes was far less frequently detected. In the group of genes encoding N-6 methyltransferase, only *ermB* and *ermA* were identified separately. The amplification of the *ermC* gene was always accompanied by the amplification of the *ermB* gene. Genes encoding efflux pumps (*mefA* or *mefE*; *msrC*) were always accompanied by the *ermB* or *ermA* genes, or both (Table 7).

DISCUSSION

More than half of the analyzed ready-to-eat dairy products contained bacteria of the genus *Enterococcus*, and *E. faecium* was the predominant species. It was previously reported that *E. faecium* (52.6%), *E. durans* (17.7%), *E. hirae* (16.4%), and *E. faecalis* (12.8%) were also most prevalent in Serbian cheeses (Bulajić et al., 2015). The proportions of *E. faecium*, *E. durans*, and *E. faecalis* in Slovak bryndza cheese were determined at 57, 22, and 16%, respectively (Belicová et al., 2007). Jamet et al. (2012) evaluated 126 samples of soft, semi-hard, and hard traditional French cheeses and observed a high prevalence of *E. faecalis* isolates (81%) relative to *E. faecium* (9.5%) and *E. durans* isolates (7.7%). Similar results were reported by Oguntuyinbo and

Okueso (2013) in a study of 30 samples of traditional fermented dairy products (wara and nunu) made from unpasteurized milk in Nigeria. The high levels of contamination of samples by enterococci may be caused by the fact that these bacteria are resistant to pasteurization temperature and show resistance to different substrates and conditions of development (low and high temperature, extreme pH, salinity, and more) and to reproduce in these environments (Biendo et al., 2010). The presence of enterococci in cheese from thermalized or pasteurised milk indicates that these bacteria are not eliminated as a result of heat treatment or recontamination (Jamet et al., 2012). Besides these factors, enterococcal contamination of the milk used to produce cheese can result from bacteria on the teat surface, in manure, or in the water used on the farm, or bacteria that cannot be cleaned from the farm workers or from the milking machines and storage tanks (Gelsomino et al., 2001; Gökmen et al., 2017).

Multidrug resistance is a growing public health concern, mainly due to the possible failure of therapeutic treatment for enterococcal infections, particularly in immunocompromised individuals, which may develop into severe urinary tract infection, endocarditis, or bacteremia (Kayser, 2003). Our research has shown that enterococci isolated from ready-to-eat dairy products can be resistant to antibiotics commonly used to treat human enterococcal infections. Most of the analyzed isolates from dairy products were resistant

Table 7. Genotypic diversity of erythromycin-resistant *Enterococcus* strains (ER)

Species	Number (%) of strains						
	ER	<i>ermA</i>	<i>ermB</i>	<i>ermA</i> + <i>ermB</i>	<i>ermB</i> + <i>ermC</i>	<i>ermB</i> + <i>mefA/E</i>	<i>ermA</i> + <i>ermB</i> + <i>msrC</i>
<i>Enterococcus faecalis</i>	12	0	8 (66.7)	2 (16.7)	0	0	2 (16.7)
<i>Enterococcus faecium</i>	15	3 (20.0)	6 (40.0)	2 (13.3)	1 (6.7)	2 (13.3)	1 (6.7)
Total	27	3 (11.1)	14 (51.9)	4 (14.8)	1 (3.7)	2 (7.4)	3 (11.1)

to aminoglycosides. The HLSR and HLGR phenotype is of particular importance because aminoglycosides used in combination with β -lactams have synergism against enterococcal infections (Bartash and Nori, 2017). Nearly half of the evaluated strains were highly resistant to streptomycin. The HLSR phenotype was most frequently associated with the Ant (6') enzyme with nucleotidyltransferase activity, but isolates of *E. faecalis* and *E. faecium* were also found to harbor the *ant(4'')-Ia* gene, which is frequently identified in resistant *Staphylococcus aureus* strains (Yadegar et al., 2009; Duran et al., 2012). Our results indicated a higher percentage of strains with the HLSR phenotype than those recently noted in other countries. In a study by Kürekci et al. (2016), only 5.8% (8 out of 139) of enterococci isolated from cheeses in Turkey were HLSR. A few more streptomycin-resistant strains (22%) were found by Gomes et al. (2008) in cheese and raw meat in Brazil. In agreement with our findings, Teuber et al. (1999) detected the HLAR phenotype in 80% of *Enterococcus* spp. isolated from cheese, indicating that HLSR has wide local variation.

Enterococci are increasingly resistant to antibiotics (Asadollahi et al., 2018). Hospital infections caused by enterococci resistant to vancomycin and linezolid, drugs of last resort in the treatment of *Enterococcus* infections, are particularly concerning. These bacteria pose serious therapeutic problems. Vancomycin is used in the treatment of infections caused by multidrug-resistant enterococci. In the present study, 2 of the isolated *E. faecalis* strains were resistant to this antibiotic; the high MIC values of these strains ($>259 \mu\text{g/mL}$ of vancomycin) are cause for concern. Genotypic analyses did not reveal the presence of the *vanA* or *vanB* genes; therefore, we hypothesized that vancomycin resistance could be encoded by other genes that we did not analyze in this study, such as *vanD*, *vanE*, or *vanG*. Similar observations were made by Gomes et al. (2008) in 3 *E. faecium* strains isolated from cheese. Resistance to vancomycin was also noted in *E. casseliflavus* isolates, but genotypic analyses revealed only the presence of the *vanC2* or *vanC3* genes, which encode natural resistance associated with low constitutive resistance to this antibiotic. These findings were confirmed by MIC values, not exceeding $32 \mu\text{g/mL}$ of vancomycin. The presence of vancomycin-resistant enterococci has been associated with avoparcin, a glycopeptide antibiotic with a similar structure, which has been added to animal feed. Historically, vancomycin-resistant isolates have been identified in the non-hospital environment in countries where avoparcin was approved for use as a growth promoter (Lauderdale et al., 2007). In Europe, the prevalence of vancomycin-resistant isolates from animal-based food products has been decreasing steadily ever since avo-

parcin was banned from animal production (Casewell et al., 2003). In the United States, where avoparcin was never used in farming, vancomycin-resistant enterococci have rarely been isolated in food animals (Donabedian et al., 2010).

The reported presence of linezolid-resistant enterococci (**LRE**; MIC $8 \mu\text{g/mL}$) is concerning but not unexpected (Krull et al., 2016). Linezolid was the first oxazolidinone antimicrobial in medical practice. It is used in the treatment of infections caused by multidrug-resistant strains, including methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Staphylococcus aureus* and enterococci (Flamm et al., 2013; Mendes et al., 2016). Linezolid is often used in the treatment of multidrug-resistant and extensively drug-resistant pneumonia as well as of acute bacterial skin and skin structure infections (Anger et al., 2010). Linezolid was approved for use in the year 2000, and the first *Enterococcus* strain resistant to this antimicrobial was isolated only 1 year later, which illustrates the ability of these bacteria to acquire resistance (Mutnick et al., 2003). The first LRE in Poland was isolated in September 2008 from a hospital patient, and from 2012 the number of LRE per year has been generally increasing (Gawryszewska et al., 2017). In enterococci, resistance to linezolid was initially associated with a mutation in the central loop of domain V of the 23S rRNA gene (Bourgeois-Nicolaos et al., 2007; Ntokou et al., 2012) or a mutation in the ribosomal proteins L3 and L4 (Locke et al., 2009). Another mechanism for linezolid resistance involves the acquisition of the *cfr* (chloramphenicol-florfenicol resistance; Kaminska et al., 2010) or *cfr* (B) genes (Deshpande et al., 2015) and the resistance can be transferred. In 2015, another gene, *optrA*, which can also be transferred between bacterial strains, was detected in linezolid-resistant isolates of *E. faecalis* and *E. faecium* from China (Wang et al., 2015). The *optrA* gene was also identified in isolates from Italy, Ireland, and Malaysia (Brenciani et al., 2016; Mendes et al., 2016). In Poland, research into the underlying mechanism and molecular epidemiology of LRE isolates was conducted only on clinical strains from the collection of the National Reference Center for Susceptibility Testing of the National Medicines Institute in Warsaw (Gawryszewska, et al., 2017) and demonstrated that in Polish isolates, linezolid resistance was most frequently caused by mutation G2576T in domain V of the 23S rRNA and mutation T511G in *rplD*. The *OptrA* transporter was detected in 2 epidemiologically unrelated non-high-risk enterococcal clonal complexes of *E. faecalis* strains belonging to ST116 (Gawryszewska et al., 2017). Further research is required to characterize the analyzed bacterial isolates from dairy products.

It should be noted that resistance to linezolid was also observed in *E. casseliflavus* isolates. Linezolid-resistant enterococci were first identified in Poland in 2008 in the hospital setting (Gawryszewska et al., 2017). The presence of LRE in raw foods has not been reported in Poland to date. In our study, LRE were identified for the first time in ready-to-eat dairy products.

Many hypotheses exist about the possible ways of spreading antibiotic-resistant strains. Some studies have shown that the same resistance gene has been found in bacteria isolated from both food and patient samples (Eaton and Gasson, 2001; Donabedian et al., 2003). Our previous studies have demonstrated that enterococci isolated from ready-to-eat food can transfer resistance genes to *E. faecalis* JH2-2 strain (Chajęcka-Wierzchowska et al., 2019). It was found that more than 70 percent of tested strains were able to conjugally transfer at least 1 of the antibiotic resistance genes. We confirm the possibility of the horizontal transfer of genes encoding resistance to aminoglycosides [*aac*(6')-Ie-aph(2'')-Ia, *ant*(6')-Ia], tetracyclines (*tetM*, *tetL*, *tetO*), and macrolides (*ermA*, *ermB*, *msrC*, *mefAB*). The *int* gene was transferred simultaneously with the *tetM* gene, which indicated that a transposon of the Tn916 or Tn1545 also participated in the conjugation process. Studies by other authors have shown that the resistance plasmid pAMβ1 has been transferred between *E. faecalis*, *E. faecium*, and *Lactobacillus reuteri* in the gastrointestinal tract of mice (Morelli et al., 1988). The same plasmid was also transferred between *Lactobacillus curvatus* strains during the fermentation of sausages (Marcinek et al., 1998). These observations support the idea that resistant enterococci can contaminate food, enter the human gastrointestinal tract, colonize people, and transmit resistance genes to commensal bacteria present in the human gastrointestinal tract (Giraffa et al., 1997). Growing antibiotic resistance of enterococci and its spread in food suggests a situation of risk for the community.

CONCLUSIONS

Enterococcus spp. strains are common microflora of dairy products. Enterococci are a reservoir of genes encoding multidrug resistance that could be transferred to other pathogenic and non-pathogenic microorganisms. Very little is known about the antibiotic resistance of food-borne enterococci other than *E. faecalis* and *E. faecium*. The results indicate that *E. casseliflavus* and *E. gallinarum* can be a source of genes encoding resistance to tetracyclines in dairy products. The isolation of *Enterococcus* spp. strains resistant to vancomycin and linezolid, drugs of last resort in the treatment of enterococcal infections, are particularly concerning. *En-*

terococcus spp. isolated from food products, including ready-to-eat foods, are becoming resistant to multiple antibiotics, highlighting the need to monitor the prevalence and occurrence of multidrug-resistant bacteria to guarantee food safety.

ACKNOWLEDGMENTS

This research was supported by the National Science Centre, Kraków, Poland, project number 2018/29/B/NZ9/00645. The publication was written as a result of the authors' (Wioleta Chajęcka-Wierzchowska and Anna Zadernowska) internship in the Department of Hygiene of Food of Animal Origin, National Veterinary Research Institute (Pulawy, Poland), co-financed by the European Union under the European Social Fund (Operational Program Knowledge Education Development), carried out in the project Development Program at the University of Warmia and Mazury in Olsztyn, Poland (POWR.03.05. 00-00-Z310/17). The authors have not stated any conflicts of interest.

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