Article

Enterococcus faecium Isolates Present in Human Breast Milk Might Be Carriers of Multi-Antibiotic Resistance Genes

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Abstract: Using *Enterococcus faecium* strains as probiotics raises several controversies related to their antibiotic resistance (AR). In the current study, we examined isolates of *E. faecium* obtained from human breast milk. Catalase-negative and γ-haemolytic isolates were identified by analyzing the sequences of 16S rRNA gene and their phenotypic resistance to antibiotics was investigated. We examined the expression of genes that were found on plasmids. The majority of isolates tested were resistant to erythromycin (96%), followed by trimethoprim (67%), tetracycline (57%), and gentamicin (55%). Ninety-seven percent of *E. faecium* isolates were resistant to at least two antibiotics. We detected the presence of the following genes on plasmids: *ErmB* (erythromycin), *dfrA17* (trimethoprim), *tetO*, *tetK* (tetracycline), *Aph(3′)-IIIa* (neomycin), and *marA* (rifampicin). *TetO* was not expressed in all cases, *dfrA14* was not expressed in CDCP1449, while *tetK* was only expressed in CDCP1128 and CDCP1331 isolates. In the majority of isolates, AR genes were located on chromosomes since they were not detected on plasmids. Our study shows that due to the spread of AR, human milk could be one of the first sources of the bacteria resistant to antimicrobials to infants.

Keywords: human breast milk; probiotics; antibiotic resistance; *Enterococcus faecium*; multi-drug resistance; gene expression

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1. Introduction

According to the European Food Safety Authority (EFSA), all bacterial strains used as food or animal feed additives must be tested against the resistance to relevant human or veterinary antimicrobial agents. Such examination ought to be conducted in accordance with international standards such as ISO 10932:2010 [1]. There are dozens of antibiotics mentioned in the paper published by EFSA [2] that need to be considered: ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline, chloramphenicol, and, in specific cases, tylosine, apramycin, nalidixic acid, sulfonamide, and trimethoprim. To distinguish strains resistant from susceptible ones, the cutoff values of antimicrobial concentrations were established. Strains which growth is inhibited at minimum inhibitory concentration (MIC) or below it are considered susceptible. MIC values are strictly defined for particular groups, genera, or species of microorganisms [2]. Strains that are considered resistant can be further investigated with molecular biology techniques to determine the nature of antimicrobial resistance. It is necessary to distinguish between strains that show acquired or intrinsic resistance. Strains that demonstrate intrinsic antibiotic resistance are generally considered as safe for human or animal consumption, whereas strains with acquired resistance must not be added to food or animal feed [2].

Even though probiotic strains are mostly known among *Lactobacillus* and *Bifidobacterium* genera, there are some representatives of the *Enterococcus* genus that have already received probiotic status, e.g., *E. faecium* SF68 [3]. There are more *Enterococcus* strains that are being assessed for their potential use as probiotics for human and animals. Some of

them have been shown to be safe for use in various fish species [4,5] or piglets at different growth stages [6,7]. Moreover, others may reduce carryover infections with chlamydia in sows and piglets [8]. It has also been demonstrated that *E. faecium* C2 strain isolated from human breast milk could be a potential novel probiotic [9]. Therefore, the search for putative probiotics among that species in human breast milk seems justified.

On the other hand, it has been demonstrated that *E. faecium* might carry antibiotic resistance genes on plasmids. Moreover, the presence of those genes might be correlated with the presence of some virulence genes such as *hyl*, which encodes hyaluronodisase [10]. It has also been shown that antibiotic resistance could be transferred through breast milk [11]. Considering the above, it seems justified to search for antibiotic resistance genes on plasmids.

Originally, the project in which we obtained all tested isolates was focused on searching putative probiotics in human milk microbiota, but since antibiotic resistance was shown to be very common among tested bacteria, we decided to observe that phenomenon more closely. In the current study, we selected various *E. faecium* isolates that could be considered as potential probiotics, tested their susceptibility to antibiotics, determined the presence of resistance genes on plasmids, and then verified if those genes were truly responsible for antibiotic resistance by determining their expression.

2. Results

2.1. Identification of Bacterial Isolates

We originally collected 2000 isolates (20 from each donor) and 310 among them were classified as catalase negative and gamma-haemolytic. For those isolates, we carried out antibiotic susceptibility tests and Sanger sequencing of 16S rRNA gene. We established that 51 of them were *Enterococcus faecium* (Table 1). We also found many isolates that were *Enterococcus faecalis* (73 isolates) and few isolates that were identified as *Enterococcus casseliflavus* (1), *Enterococcus durans* (5), and *Enterococcus hirae* (2). The other 130 isolates were identified only to the genus level (data not shown). Since the study was focusing on the potential probiotics among *E. faecium* species, we continued further analysis using only isolates that were identified as such.

Based on the phylogenetic analysis, it could be stated that the vast majority of isolates created a common cluster (Figure 1). Among them, isolates CDCP523 and CDCP497 or isolates CDCP531 and CDCP976 formed subclusters, with the similarity of 71% and 79%, respectively. Another cluster was formed by isolates CDCP968 and CDCP1512 with the similarity of 74%.

2.2. Assessment of Antibiotic Resistance

In the next research step, we tested all 51 isolates against the resistance to antibiotics using ISO 10932:2010 norm. The majority of tested isolates were resistant to erythromycin (96%), followed by trimethoprim (67%), tetracycline (57%), and gentamicin (55%) (Table 1). There were three antibiotics that demonstrated particularly high efficacy and isolates were susceptible to them: linezolid (98%), then ampicillin (94%), and chloramphenicol (92%). It must be highlighted that cutoff values were not established for linezolid or many other antibiotics (neomycin, quinupristin/dalfopristin, trimethoprim, ciprofloxacin, and rifampicin), thus we decided to consider tested isolates as resistant to those antibiotics only when bacterial growth was noted in the whole tested concentration range, including trimethoprim.

There were no isolates susceptible to all tested antibiotics. Only three isolates (5.7%) were resistant to one antibiotic and it was erythromycin (Table 1). Eleven isolates (20.8%) were resistant to two antibiotics, 5 isolates to three (9.4%), 11 isolates to four (20.8%), 6 isolates to five (11.3%), 4 isolates to six (7.6%), 3 isolates to seven (5.7%), 1 isolate to eight (1.9%), 5 isolates to nine (9.4%), and 1 isolate to twelve (1.9%).

Table 1. Determination of phenotypic antibiotic resistance of *Enterococcus faecium* isolates obtained from human breastmilk.

Isolate Number	Accession Number	MIC Values (µg/mL)															
		Gent	**Kan**	**Strep**	**Tet**	**Ery**	**Clin**	**Chlor**	**Amp**	**Neo**	**Van**	**Q/D**	**Lin**	**Trim**	**Cip**	**Rif**	**Tyl**
CDCP13	MT814617	>	>	>	>	>	>	>	0.5 S	64	2 S	1	2	>	>	32	2 S
CDCP18	MT883426	>	>	>	>	>	>	>	1 S	128	2 S	1	2	>	>	64	1 S
CDCP29	MT814618	>	>	>	>	8 R	>	64 R	8 R	>	2 S	>	2	>	128	>	1 S
CDCP37	MT814619	>	>	>	>	>	>	64 R	16 R	>	4 S	>	>	>	>	>	2 S
CDCP74	MT882797	64 R	512 S	256 R	>	8 R	>	8 S	0.5 S	>	2 S	>	2	32	128	32	2 S
CDCP195	MT882798	128 R	256 S	256 R	>	>	>	8 S	0.5 S	>	2 S	>	2	16	>	32	2 S
CDCP238	MT814620	128 R	512 S	>	>	8 R	4 S	8 S	0.5 S	>	2 S	8	2	>	128	32	2 S
CDCP252	MT882787	128 R	256 S	>	>	>	>	8 S	1 S	>	2 S	8	2	64	64	>	1 S
CDCP351	MT814240	256 R	512 S	>	64 R	>	4 S	8 S	1 S	>	2 S	2	2	2	128	32	1 S
CDCP477	MT882695	64 R	8 S	64 S	8 R	>	0.125 S	8 S	1 S	256	2 S	1	2	>	128	32	2 S
CDCP495	MT883429	32 S	256 S	64 S	8 R	>	0.25 S	4 S	0.5 S	64	1 S	1	2	>	>	32	1 S
CDCP497	MT882696	64 R	8 S	128 S	0.5 S	>	0.125 S	16 S	0.S5	64	1 S	1	2	4	128	32	2 S
CDCP521	MT882697	32 S	4 S	32 S	4 S	>	0.125 S	0.5 S	0.5	64	1 S	1	2	4	128	32	2 S
CDCP522	MT882698	64 R	1024 S	64 S	4 S	8 R	0.125 S	16 S	1	64	1 S	1	2	16	128	32	2 S
CDCP523	MT882699	64 R	8 S	64 S	4 S	8 R	0.125 S	4 S	0.5	64	1 S	1	2	64	128	64	2 S
CDCP531	MT882700	16 S	8 S	64 S	4 S	>	0.125 S	4 S	0.5	64	1 S	1	2	2	128	32	2 S
CDCP533	MT882701	32 S	256 S	128 S	4 S	>	0.125 S	8 S	1	32	8 R	>	4	128	128	32	2 S
CDCP579	MT883425	64 R	256 S	128 S	4 S	4 S	0.25 S	4 S	1	64	1 S	0.5	2	>	128	32	2 S
CDCP749	MT882799	32 S	128 S	64 S	2 S	8 R	0.125 S	2 S	1	32	0.5 S	0.5	2	4	128	32	1 S
CDCP750	MT882800	32 S	128 S	64 S	2 S	8 R	0.25 S	4 S	1	64	0.5 S	0.5	2	>	128	16	2 S
CDCP753	MT882801	64 R	512 S	64 S	2 S	>	0.5 S	8 S	1	64	0.5 S	0.5	2	>	128	32	1 S
CDCP787	MT882802	32 S	128 S	32 S	2 S	>	0.25 S	4 S	2	64	0.5 S	0.5	2	>	128	32	2 S
CDCP791	MT882803	64 R	128 S	64 S	16 R	>	0.125 S	4 S	1	32	0.5 S	0.5	2	>	>	32	1 S
CDCP795	MT882804	32 S	512 S	64 S	16 R	8 R	0.125 S	4 S	1	64	0.5 S	0.5	2	>	>	32	1 S

Table 1. *Cont.*

Isolate Number	Accession Number	MIC Values (μg/mL)															
		Gent	**Kan**	**Strep**	**Tet**	**Ery**	**Clin**	**Chlor**	**Amp**	**Neo**	**Van**	**Q/D**	**Lin**	**Trim**	**Cip**	**Rif**	**Tyl**
CDCP825	MT882805	8 S	32 S	32 S	8 R	4 S	0.25 S	16 S	>	64	16 R	0.5	2	>	16	0.125	1 S
CDCP850	MT882788	64 R	256 S	64 S	4 S	>	0.25 S	8 S	1	64	1 S	1	4	>	128	32	1 S
CDCP868	MT882789	64 R	256 S	64 S	4 S	>	0.25 S	8 S	1	64	1 S	2	4	>	>	32	1 S
CDCP941	MT882790	32 S	256 S	128 S	4 S	>	0.25 S	4 S	0.5	256	1 S	1	2	>	128	32	1 S
CDCP942	MT882791	32 S	256 S	64 S	4 S	>	0.25 S	4 S	0.5	128	1 S	1	2	>	128	16	2 S
CDCP950	MT882792	32 S	128 S	256 R	4 S	>	0.5 S	16 S	0.5	64	1 S	0.5	2	>	128	16	1 S
CDCP968	MT882793	16 S	128 S	32 S	2 S	>	0.25 S	4 S	0.5	>	1 S	1	2	16	128	16	2 S
CDCP976	MT882794	32 S	256 S	128 S	2 S	>	0.25 S	4 S	0.5	64	1 S	0.5	2	>	128	32	2 S
CDCP979	MT882795	32 S	256 S	64 S	2 S	>	0.25 S	4 S	0.5	64	0.5 S	0.25	1	>	128	32	2 S
CDCP1121	MT814241	32 S	128 S	64 S	16 R	>	0.25 S	4 S	1	32	>	1	2	>	128	32	2 S
CDCP1123	MT882796	128 R	1024 S	256 R	4 S	>	1 S	16 S	2	256	1 S	2	4	>	>	32	2 S
CDCP1124	MT814242	256 R	>	>	32 R	>	8 R	16 S	0.5	>	2 S	4	2	64	>	8	>
CDCP1129	MT883427	32 S	64 S	64 S	>	>	0.5 S	4 S	1	32	>	1	2	16	>	16	>
CDCP1205	MT814243	32 S	128 S	64 S	32 R	>	0.25 S	8 S	1	32	1 S	1	2	>	>	32	1 S
CDCP1228	MT814244	64 R	256 S	128 S	>	>	0.25 S	8 S	1	32	>	1	2	>	>	64	1 S
CDCP1248	MT814245	32 S	512 S	64 S	64 R	>	0.5 S	4 S	1	32	>	1	1	>	>	64	4 S
CDCP1249	MT814246	32 S	128 S	128 S	16 R	>	0.25 S	4 S	1	64	1 S	1	2	>	128	32	2 S
CDCP1270	MT814247	32 S	128 S	128 S	64 R	>	0.125 S	4 S	1	64	>	1	1	64	128	32	2 S
CDCP1331	MT814248	64 R	1024 S	128 S	32 R	>	0.5 S	8 S	1	128	>	2	4	>	>	>	>
CDCP1359	MT814249	32 S	1024 S	128 S	32 R	>	0.25 S	4 S	1	64	2 S	1	2	>	128	64	>
CDCP1380	MT814250	256 R	1024 S	256 R	64 R	>	2 S	4 S	1	256	4 S	8	16	>	128	64	>
CDCP1446	MT814251	32 S	128 S	128 S	>	>	0.5 S	8 S	1	64	>	1	2	>	>	16	>
CDCP1447	MT814621	32 S	128 S	64 S	64 R	>	0.5 S	4 S	1	64	>	1	2	>	>	16	2 S
CDCP1449	MT814252	64 R	128 S	64 S	64 R	>	0.5 S	4 S	1	64	>	1	2	64	>	16	1 S
CDCP1512	MT883428	64 R	128 S	64 S	>	>	0.125 S	4 S	1	64	>	1	2	>	>	16	>

Table 1. *Cont.*

Isolate Number	Accession Number	MIC Values (μg/mL)															
		Gent	**Kan**	**Strep**	**Tet**	**Ery**	**Clin**	**Chlor**	**Amp**	**Neo**	**Van**	**Q/D**	**Lin**	**Trim**	**Cip**	**Rif**	**Tyl**
CDCP1532	MT814253	128 R	512 S	>	>	>	>	16 S	1	256	1 S	>	4	4	128	8	2 S
CDCP1692	MT814254	128 R	512 S	>	2 S	>	1 S	8 S	1	>	1 S	1	2	>	128	64	2 S
Cut-off values		32	1024	128	4	4	4	16	2	ns	4	ns	ns	ns	ns	ns	4
Percentage of resistance strains		54.9	11.8	31.4	56.9	96.1	19.6	7.8	5.9	19.6	21.6	11.8	2.0	66.7	37.3	7.8	13.7

Abbreviations: Gent: Gentamicin, Kan: Kanamycin, Strep: Streptomycin, Tet: Tetracycline, Ery: Erythromycin, Clin: Clindamycin, Chlor: Chloramphenicol, Amp: Ampicillin, Neo: Neomycin, Van: Vancomycin, Q/D: Quinupristin/Dalfopristin, Lin: Linezolid, Trim: Trimethoprim, Cip: Ciprofloxacin, Rif: Rifampicin, Tyl: Tylosine; ns–cut-off value was not specified for particular antibiotic; ">"–resistant within the whole concentration range, S–isolate is susceptible to a particular antibiotic, R–isolate is resistant to a particular antibiotic.

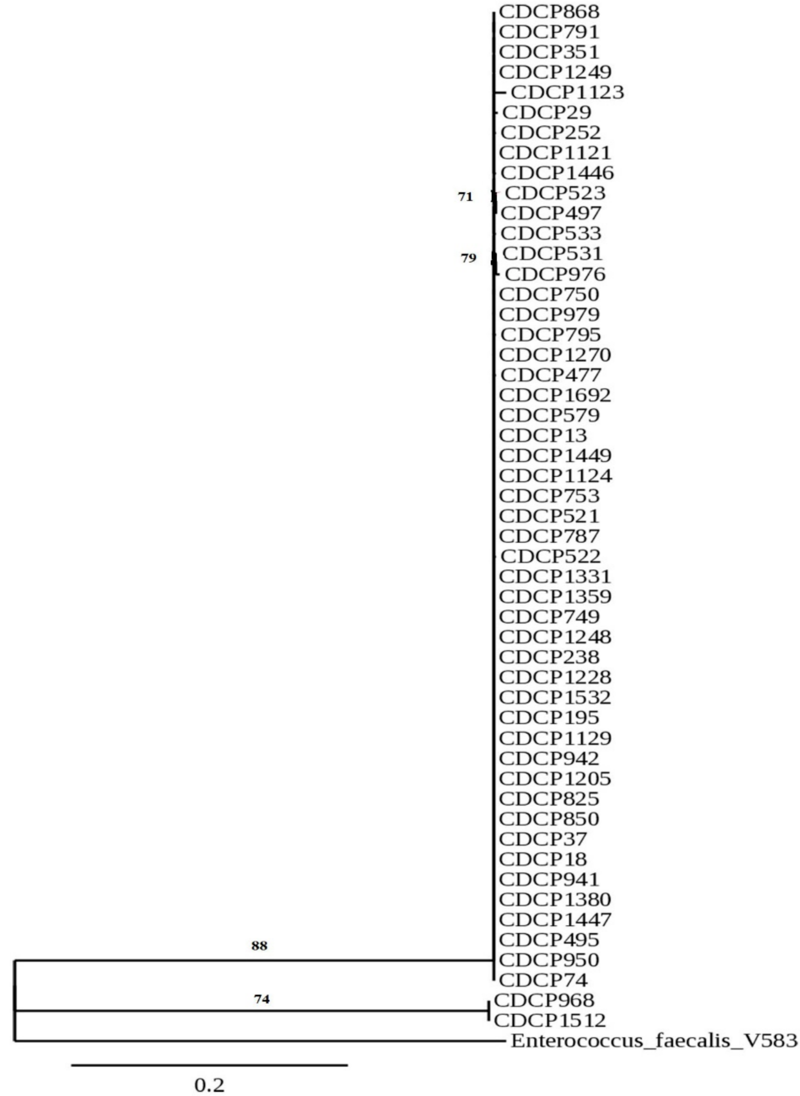

Figure 1. Phylogenetic tree of *Enterococcus faecium* isolates originated from human breast milk based on their 16S rRNA gene sequences. The sequence of *Enterococcus faecalis* V583 was used as an outgroup.

We also tested two other methods for the assessment of antibiotic resistance, such as disk diffusion method and E-test, but those were proven inaccurate because tested strains did not demonstrate even growth on solid media used for that analysis (brain heart infusion agar, data not shown).

2.3. Verifying the Presence of Transferable Genes

After examining phenotypes of antibiotic resistance for each isolate, we verified if those genes were located on plasmids. We found out that only one contained *ant(6)* gene, which encodes the resistance to streptomycin (Table 2). In the case of tetracycline, we detected three genes: *tetM* (2 isolates), *tetO* (7 isolates), and *tetS* (1 isolate). Ten isolates had *ErmB* gene, which provided the resistance to erythromycin (Table 2). *Aph(3′)-IIIa* gene encoding the resistance to neomycin was detected in four isolates while *marA* (rifampicin) was present in one isolate. The most frequently occurring gene was *dfrA14*. It encodes the resistance to trimethoprim, and it was found in 19 isolates (Table 2).

Table 2. Isolates of *Enterococcus faecium* obtained from human breast milk in which antibiotic resistance genes were found on plasmids.

Antibiotic	**Gent**		**Kan**	**Strep**							**Tet**					**Ery**		**Clin**		**Chlor**		**Amp**		**Van**		**Tyl**		**Neo**	**Q/D**	**Trim**	**Lin**	**Cip**		**Rif**
Isolate Number	**aac(6′)-aph(2″)**	**aph3′-IIIa**	**aph2′-IIIa**	**ant(2″)**	**ant(6)**	**ant(2)**	**ant(2″)-Ia**	**aadA**	**strA**	**strB**	**tetM**	**tetK**	**tetL**	**tetO**	**tetS**	**ErmB**	**ErmB1**	**lunA**	**lunB**	**catA4**	**catII**	**mecA**	**BlaZ**	**ErmG**	**vanH**	**ErmA**	**ErmT**	**Aph(3′)-IIIa**	**ermB**	**dfrA14**	**clcD**	**gyrA**	**parC|**	**marA**
CDCP238	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
CDCP351	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
CDCP477	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDCP495	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDCP521	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDCP532	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDCP531	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDCP533	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDCP750	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
CDCP753	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
CDCP787	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
CDCP791	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
CDCP795	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
CDCP825	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
CDCP850	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
CDCP868	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
CDCP941	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
CDCP950	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
CDCP979	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
CDCP1208	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDCP1228	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
CDCP1248	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
CDCP1249	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 2. *Cont.*

Antibiotic	Gent	Kan			Strep						Tet					Ery		Clin		Chlor		Amp		Van		Tyl		Neo	Q/D	Trim	Lin	Cip		Rif
Isolate Number	**aac(6′)-aph(2″)**	**aph3′-IIIa**	**aph2′-IIIa**	**ant(2″)**	**ant(6)**	**ant(2)**	**ant(2″)-Ia**	**aadA**	**strA**	**strB**	**tetM**	**tetK**	**tetL**	**tetO**	**tetS**	**ErmB**	**ErmB1**	**lunA**	**lunB**	**catA4**	**catII**	**mecA**	**BlaZ**	**ErmG**	**vanH**	**ErmA**	**ErmT**	**Aph(3′)-IIIa**	**ermB**	**dfrA14**	**clcD**	**gyrA**	**parC**	**marA**
CDCP1331	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
CDCP1359	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
CDCP1380	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
CDCP1446	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
CDCP1447	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDCP1449	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
CDCP1512	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CDCP1532	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-
CDCP1692	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-

Abbreviations: Gent: Gentamicin, Kan: Kanamycin, Strep: Streptomycin, Tet: Tetracycline, Ery: Erythromycin, Clin: Clindamycin, Chlor: Chloramphenicol, Amp: Ampicillin, Van: Vancomycin, Tyl: Tylosine; Neo: Neomycin, Q/D: Quinupristin/Dalfopristin, Lin: Linezolid, Trim: Trimethoprim, Cip: Ciprofloxacin, Rif: Rifampicin; "+"—tested gene was detected; "-"—tested gene was not detected.

Isolate CDCP238 had four tested genes encoding antibiotic resistance: *ant(6)*, *tetM*, *tetO*, and *aph(3′)-IIIa*; while CDCP351 had *tetO*, *ErmB*, and *aph(3′)-IIIa* (Tables 2 and 3). There were few isolates for which we detected two genes: CDCP753 and CDCP787–*ErmB* and *dfrA14*; CDCP1228 and CDCP1449–*tetO* and *dfrA14*; CDCP1532 and CDCP1692–*aph(3′)-IIIa* and *dfrA14*; CDCP1331–*tetO* and *marA*. There were also other isolates that contained only one of considered genes: CDCP1205, CDCP1249, CDCP1447–*tetK*; CDCP477, CDCP495, CDCP521, CDCP523, CDCP531, CDCP533–*ErmB*; CDCP749, CDCP795, CDCP825, CDCP850, CDCP868, CDCP941, CDCP950, CDCP979, CDCP1248, CDCP1359, CDCP1380, CDCP1446–*dfrA14* (Tables 2 and 3).

Table 3. Summary of the expression of antibiotic resistance genes located on plasmids of *Enterococcus faecium* isolates originating from human breast milk.

Antibiotic (Gene)	Isolate No.	ΔCt for the Target	ΔCt for the Reference	Relative Ratio
Tetracycline (*tetK*)	CDCP351	0	0	0
	CDCP1228	6.74×10^{-4}	4.52×10^{-9}	1.49×10^{5}
	CDCP1331	2.03×10^{-7}	1.11×10^{-9}	1.82×10^{2}
	CDCP1449	0	0	0
Erythromycin (*ErmB*)	CDCP351	1.14×10^{-2}	1.48×10^{-7}	7.69×10^{4}
	CDCP753	9.72×10^{-3}	2.06×10^{-7}	4.72×10^{4}
	CDCP787	1.68×10^{-3}	3.02×10^{-7}	5.58×10^{3}
Neomycin (*Aph(3′)-IIIa*)	CDCP351	3.69×10^{-2}	1.27×10^{-4}	2.90×10^{2}
	CDCP1532	1.72×10^{-2}	4.48×10^{-4}	3.83×10^{1}
	CDCP1692	1.88	3.14×10^{-4}	5.98×10^{3}
Rifampicin (*marA*)	CDCP1331	1.65×10^{-2}	3.92×10^{-6}	4.21×10^{3}
Trimethoprim (*dfrA14*)	CDCP753	5.08×10^{-3}	3.94×10^{-7}	1.29×10^{4}
	CDCP787	3.77×10^{-3}	1.35×10^{-7}	2.80×10^{4}
	CDCP1228	2.14×10^{-3}	7.91×10^{-7}	2.70×10^{3}
	CDCP1449	5.07×10^{-3}	0	0
	CDCP1532	4.74×10^{-2}	9.92×10^{-5}	4.78×10^{2}
	CDCP1692	1.79×10^{-2}	4.30×10^{-4}	4.17×10^{1}

We also noted that if analyzed isolates originated from a common donor, they carried the same AMR genes: CDCP521, 531 and 533–*ErmB1*, CDCP750 and 753–*dfrA14* gene; CDCP 787, 791 and 795–*dfrA14* gene; and CDCP1446 and 1449–*dfrA14* gene as well.

2.4. Gene Expression Analysis

For gene expression analysis, we selected isolates that carried at least two antibiotic resistance genes on plasmids (Table 2). Isolates CDCP238 and CDCP1512 were not included in those experiments because when we took them out of stock, they did not grow in any culture media that were used for their cultivation before banking. That left us with the following isolates: CDCP351 CDCP753, CDCP787, CDCP1228, CDCP1331, and CDCP 1449. There was no gene expression of the *TetO* gene, so we did not include those results. Data collected in that experiment for other genes (Table 3) showed very high relative ratios but with some exceptions: *tetK* was not expressed in CDCP351 and CDCP1449, while *dfrA14* was not expressed in CDCP1449. It could also be stated that the most significant gene expression occurred in the following cases: *tetK* in CDCP1228 (1.49 10^{5}), *ErmB* in CDCP351 (7.69×10^{4}), and CDCP753 (4.72×10^{4}) or *dfrA14* in CDCP753 (1.29×10^{4}) and CDCP787 (2.80×10^{4}).

3. Discussion

3.1. Identification of Bacterial Isolates

It is suspected that bacterial isolates in breastmilk are transferred from intestines with blood [12]. The majority of those microorganisms are nonculturable; however, Fernández and Rodríguez (2020) stated that if proper conditions are provided, most of the microorganisms might be cultured using traditional microbiology techniques. In our research, we applied three different media and culture conditions to support the isolation of the greatest diversity of potent probiotic microorganisms. Originally, we aimed at isolating putative probiotics from human breast milk among the representatives of *Lactobacillus, Bifidobacterium*, and *Streptococcus* genera; therefore, the media selected in the research were dedicated to lactic acid bacteria (LAB). The majority of isolates collected grew on MRS supplemented with L-cysteine. In general, the majority of probiotic isolates are microaerophilic [13], so it is not surprising that isolates in the current study demonstrated best growth at 5% of CO_2 rather than under strictly anaerobic or aerobic conditions.

The representatives of *Streptococcus* and *Staphylococcus* genera occur most frequently in human breastmilk [12], but the presence of the *Enterococcus* genus is confirmed as well [14]. The majority of staphylococci are catalase-positive [15] while streptococci cause haemolysis [16], so the representatives of those genera were not considered for further investigation. Since various species of the *Enterococcus* genus are able to produce lactic acid and have similar metabolism to LAB [17], it is not surprising that they grew on the media dedicated for that group of bacteria. We did not obtain any *Bifidobacterium* isolates, and there were only 20 strains of *Lactobacillus*. It is not surprising since those genera constitute no more than 5% of milk microbiota [14]. We did not include the results obtained for those isolates because their application could be patented in the future after further studies.

Enterococcus faecalis is able to cause beta-haemolysis and those isolates were not considered for further analysis. Since in the current study we only considered catalase-negative and gamma-haemolytic *Enterococcus faecium*, the number of isolates considered was reduced to 51.

Phylogenetic analysis revealed a close relationship between analyzed isolates and the main reason for that observation could be that all donors came from the same region (50 km radius). Since Kielce is a relatively small city (less than 200,000 citizens), there is a chance that some of them had the same general practice doctors and obtained the same treatment for various diseases, especially regarding skin infections or medical care during pregnancy. Another possible route of acquiring strains is through food, especially meat. However, the questionnaire that was given to donors in the current study was insufficient to provide this type of information.

3.2. Identification of Antibiotic Resistance

Based on our results, it seems that almost all of the isolates tested were susceptible to linezolid (Table 1). The same findings were reported by Golob et al. (2019, Slovenia) [18], and Chakraborty et al. (2015, Northern India) [19] or in the metadata analysis study [20]. The resistance to linezolid among *E. faecium* isolates occurs quite sporadically in general [21]. In Poland, this antibiotic is recommended for use in lung infections caused by Gram positive bacteria, complicated skin infections, infections of soft tissues, or in the cases when infections are caused by vancomycin-resistant enterococci [22]. Enterococcal isolates identified in various hospitals in Poland seemed to be susceptible to linezolid [23], so it could also be related to the occurrence of lineages that are characteristic for the Polish population.

In our study, the isolates tested were also susceptible to ampicillin (Table 1), which contrasts with the results of metadata analysis from 2019 [20] showing that the prevalence of the resistance to that antibiotic is the greatest among *E. faecium* isolates collected from blood in Europe. On the other hand, Miller et al. (2014) [24] demonstrated that ampicillin is the most effective beta-lactam against enterococci. The reason for those differences could be that the application of ampicillin in different countries could vary so the exposure of women to that antimicrobial would vary as well. In Poland, ampicillin is mostly prescribed

to patients who suffer from enterococcal infections [22] and, apparently, those occur more frequently as infections acquired in hospitals [23].

The vast majority of isolates considered in the current study were susceptible to chloramphenicol, which is also confirmed in studies considering *E. faecium* obtained from food products [25,26]; however, Hollenbeck and Rice (2012) stated that the resistance to that antibiotic is very common among enterococci. It is possible that isolates in our study were susceptible to chloramphenicol because that antibiotic is not present on the list of recommended antimicrobials in Poland [22]; it is rarely prescribed to patients.

Donors who were involved in our study were most likely not exposed to the antibiotics noted above; as a result, their microbiota did not have a chance to develop the resistance to that antimicrobial.

When isolates of *E. faecium* collected from various parts of human body in Slovenia were tested, they demonstrated the resistance to erythromycin, ampicillin and ciprofloxacin [18]. In our case, the vast majority of strains demonstrated the resistance to erythromycin as well (Table 1). This phenomenon could occur because that antibiotic is used in skin infections and considering the age of women that were donors of breast milk (25–35 years), they could have been exposed to that antibiotic since it is commonly used for acne treatment in Poland [27], but is also allowed for the treatment of some infections during pregnancy [22]. Therefore, microbiota of donors in the current study could have a chance to develop the resistance to that antibiotic.

More than half of the isolates tested in our study demonstrated the resistance to trimethoprim. Metadata analysis from Iran indicated that it occurred in 81% of the analyzed isolates [28]; however, the authors did not consider potential differences in methodologies used for assessing such phenomenon. Trimethoprim is prescribed to patients with urinary tract infections [22], which are quite common—up to 20% of infections treated in primary practice [29], so there are strong chances that donors involved in the current project were exposed to that antibiotic at some point of their life or even during pregnancy.

Our results showed that more than half of the isolates considered were resistant to tetracycline (Table 1). The same phenomenon was reported in other studies taking place in various parts of the world [20,28]. In Poland, that antibiotic is prescribed mostly in the cases of lower respiratory tract infections, inflammation of lesser pelvis, Lyme disease, or other zoonotic diseases [22]. The questionnaire given to donors along with the voluntary consent did not include questions regarding those diseases, but it is very unlikely that the majority of them could have suffered from at least one of them before donating a breast milk sample. Therefore, it is more probable that the resistance of the isolates present in human breast milk was acquired through a different route. One of the possibilities is that tetracycline-resistant strains were ingested with meat, especially that this antibiotic is still used as a growth promoter or in veterinary practices [30].

The metadata analysis [20] demonstrated that the second antibiotic that is ineffective against *E. faecium* is gentamicin. The resistance to that antimicrobial was also confirmed in studies involving patients suffering from nosocomial infections in Eastern India [19] or among *E. faecium* isolates from rectal swabs [10]. Our findings confirm the resistance to that antibiotic as well (Table 1). Considering such a wide geographical spread of the resistance to gentamicin, it could be speculated that *E. faecium* is able to develop intrinsic resistance.

In general, it can be stated that only one isolate met the criteria of EFSA and that was CDCP579. It could be considered as a putative probiotic strain, but then further analysis would be needed: whole genome sequence data analysis, assessment of strain cytotoxicity, survival in gastrointestinal tract, assessment of antimicrobial properties of the strain, etc.

3.3. Verifying the Presence of Transferable Genes

Despite the fact that almost all tested isolates were resistant to erythromycin, *ErmB* gene was detected only on plasmids of 10 of them (Table 2) and the expression of that gene was confirmed in the case of three (CDCP351, CDCP753, and CDCP787). In the case of other isolates, genes encoding the resistance to that antibiotic were either present

on a chromosome or there were other genes present on plasmids that were responsible for that feature. Garrido et al. (2014) [31] stated that numerous genes could be involved in that phenomenon and many of them could be intrinsic. *ErmB* gene was detected in isolates of *E. faecium* obtained from diseased farm animals [32], but the authors were using genomic DNA so it is impossible to determine whether that gene was located on plasmids or chromosomes.

There is still relatively little known about the molecular background of the resistance of *E. faecium* to trimethoprim. Our study suggested that it could be *dfrA14* gene located on plasmids while the study carried out with *E. faecalis* revealed that *dfrF* gene was involved in that process and it could be an acquired gene but located on a chromosome [33]. Our results confirmed that *dfrA14* could also be involved in the resistance to that antibiotic because that gene was expressed in five isolates carrying multiple AMR genes.

In the case of tetracycline, it was already shown that genes encoding the resistance to that antibiotic that could be acquired by enterococci are *tetK, tetL, tetO, tetS*, and *tetM* [21]. Our study indicates that *tetO* gene occurred most frequently on plasmids but only among very few strains. We excluded the expression of that gene in tested isolates (Table 3). Since *tetO, tetS*, and *tetM* genes could also be present on chromosomes [24], it is possible in the case of isolates obtained in the current study that the resistance to that antibiotic was intrinsic, especially that resistance genes were present on plasmids of only nine strains (Table 2). On the other hand, the expression of *tetK* gene was confirmed in the case of two isolates. It was previously shown in a study carried out in Scandinavia and published in 2018 that breast milk could be the source of tetracycline resistance genes or transposases that could contribute to the relocation of those genes [11].

As for the gentamicin resistance, none of the tested isolates had *aac(6′)-aph(2″)* gene on plasmids. It is possible that this gene was present on chromosomes of those isolates since that gene was responsible for intrinsic resistance of enterococci in previous studies [24].

The presence of neomycin resistance gene in *Enterococcus faecium* has been previously confirmed [34]. In our study, we demonstrated the expression of aph(3′)-IIIa gene in the case of isolates in which that gene was discovered on plasmids. On the other hand, it seems that our study is the first to report the presence of *marA* gene on the plasmid of *E. faecium*.

It is safe to say that molecular mechanisms of the resistance to vancomycin are best known in the *Enterococcus* genus [35] and some of them, namely the clusters *vanA, vanB, vanG, vanM,* and *vanT* are transferable. Curiously, in the current study, only 21% of tested isolates were resistant to vancomycin; however, none contained tested resistance genes on plasmids (*vanHa* and *ErmG*, Table 2). We decided to choose those genes because after careful analysis of CARD and the search in BLAST we discovered that those two genes could be frequently distributed among *Enterococcus* spp.

It is possible then that the isolates tested held other genes that determined their resistance to vancomycin or that they developed intrinsic resistance to that antibiotic that has already been reported [21]. Another explanation is that acquired resistance genes could be located on the chromosome. The same conclusion applies to all the other antibiotics tested, in which case genes encoding resistance were not detected in plasmid DNA of the tested isolates.

We also noticed that if isolates originated from the same donor, then they carried the same AMR genes on plasmids. This suggests that either those isolates acquired those genes in the same environment and were then transferred to human body—the transfer of those genes between bacterial cells in human body is very easy and happens spontaneously—or that the human microbiome acquired the resistance in contact with the antibiotic that was taken incorrectly (the treatment interrupted before it was complete).

4. Materials and Methods

4.1. Chemicals

All dry culture media and their components or supplements used for their preparation were purchased from Biomaxima (Lublin, Poland), unless otherwise stated. L-cysteine

hydrochloride, L-tryptophan, biotin, thiamine hydrochloride, adenine, guanine, xanthine, uracil, glucose, cyanocobalamin, lactose monohydrate, sucrose, soluble starch, gelatine, sodium chloride, disodium hydrogen phosphate, sodium acetate, zinc sulfate heptahydrate, manganese(II) chloride tetrahydrate, magnesium glycerophosphate monohydrate, calcium D-gluconate monohydrate, cobalt(II) sulfate monohydrate, copper(II) sulfate pentahydrate, and Gram staining kit were purchased from Pol-Aura (Dywity near Olsztyn, Poland). Menadione, pyridoxine, pantothenate, nicotinamide, and ascorbic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). Peptone was purchased from A&A Biotechnology (Gdynia, Poland). Seventeen antibiotics were obtained for susceptibility testing: gentamycin, neomycin sulfate, tetracycline, chloramphenicol, vancomycin hydrochloride, tylosine phosphate (Pol-Aura, Dywity near Olsztyn, Poland), kanamycin sulfate, streptomycin sulfate salt, erythromycin, clindamycin hydrochloride, ampicillin, linezolid, trimethoprim, ciprofloxacin, rifampicin (Sigma Aldrich, St. Louis, MO, USA), and quinupristin–dalfopristin mesylate complex (Santa Cruz Biotechnology Inc., Dallas, TX, USA). All kits for the extraction of nucleic acids, purification of PCR products, and cDNA synthesis were purchased from A&A Biotechnology (Gdynia, Poland): Genomic Mini AX Bacteria+ Spin for the extraction of genomic DNA; Plasmid Mini AX and mutanolisine solution for the extraction of plasmid DNA; EPPiC for the purification of products obtained after PCR; Total RNA Mini for the extraction of RNA; TranScriba Kit for cDNA synthesis. Luna Universal qPCR Master Mix (New England Biotechnology, Ipswich, MA, USA) was used for the determination of gene expression. Primers were synthesized and purified by Future Synthesis sp. z o.o. (Poznań, Poland). Agarose, 10X TAE buffer, SimplySafe dye, Perfect Plus Molecular Weight Quantitative Ladder, Perfect Plus 50–500 bp DNA Ladder, and Color Taq PCR Master Mix (2x) were purchased from EURx sp. z o.o. (Gdańsk, Poland).

4.2. Isolation of Bacterial Isolates

Breastmilk samples from healthy women up to 4 days after giving birth were collected from July to October 2019 in Szpital Kielecki Św. Aleksandra (Kielce, Poland). Samples were collected with the breast pump Madela Swing (Madela Polska sp. z o.o., Warszawa, Poland) to 30 mL sterile capped containers after cleaning the breast with water. Samples were then transferred immediately to the laboratory, diluted with peptone (1 g/L) saline water (8.5 g/L), and plated on MRS agar supplemented with L-cysteine (0.3 g/L, MRS-Cys), bifidobacterium medium (BM), and M17 medium with lactose. Plates were incubated for 72 h under following conditions: MRS-Cys at 37 °C (5% of CO_2), BM at 37 °C under anaerobic conditions in a Bactron 300 anaerobic chamber (Sheldon Manufacturing Inc., Cornelius, OR, USA), and M17 at 45 °C under aerobic conditions. After incubation we selected 10 random colonies from MRS-Cys, 5 from BM, 5 from M17, and we streaked them on plates with fresh medium. We then carried out catalase testing and Gram staining. We deposited pure cultures in Microbank vials (Biomaxima, Lublin, Poland) and stored them at −80 °C until further analysis.

4.3. Assessment of Haemolytic Properties and Catalase Activity Testing

Collected isolates were tested against their haemolytic properties. Prior to experiments, isolates were incubated overnight in fresh liquid medium. We then transferred bacterial cultures to plates with Colombia agar (g/L): casein peptone 10, beef extract 5, brain heart infusion 3, yeast extract 5, soluble starch 1, sodium chloride 5, and agar 13 supplemented with 5% of defibrinated sheep blood. Plates were incubated for 48 h at 37 °C and 5% CO_2. When we inoculated isolates on Colombia agar, we tested in parallel their catalase activity by transferring a drop of overnight culture and a drop of 3% hydrogen peroxide solution on the glass slide. Only isolates that demonstrated gamma haemolysis (no halo or clearance zone around colonies) and were catalase negative were considered for further experiments.

4.4. Antibiotic Susceptibility Testing

Resistance to antibiotics was examined according to ISO 10932:2010 [1]. Firstly, we prepared sterile solutions of each antibiotic at concentrations required in the norm and 50 µL was transferred into a certain well of a 96-well sterile plate. In columns for negative control, we pipetted 50 µL of sterile water. Plates were stored at −20 °C until the day of analysis but no longer than one month. Twenty-four hours prior to experiments, we inoculated Elliker broth with bacterial culture and incubated at 37 °C and 5% CO_2. We then adjusted the optical density of the suspensions to 1 MF (DEN-1B densitometer, Biosan Medical-Biological Research and Technologies, Riga, Latvia) and diluted it 500 fold with IST broth. The bacterial suspension obtained was added (50 µL) to wells containing antibiotic solutions and to the column for positive control. In the column designated for negative control, sterile IST broth (50 µL) was added. Lids were placed on the top of plates and the plates were incubated at 37 °C and 5% of CO_2 for 48 h. Antibiotic concentration at which there was at least 80% visual growth reduction in comparison to positive control was indicated as MIC.

4.5. Extraction of Genomic DNA, Sequencing and Identification

For the extraction of genomic DNA, we collected 1 mL of overnight culture of bacterial isolate, centrifuged it at 9800× *g* (Gusto high-speed mini centrifuge, Heathrow Scientific, Vernon Hills, IL, USA), discarded the supernatant, added 1 mL of sterile water, vortexed (V3, Elmi Skyline, Riga, Latvia), and repeated centrifugation. We then discarded the supernatant and carried out the extraction of genomic DNA with Genomic Mini AX Bacteria+ Spin kit per manufacturer's instruction with slight modification—we extended the time of each incubation with enzymes by 5 min. We then measured DNA concentration and purity using a NanoDrop™ One/OneC Microvolume UV–Vis Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). DNA samples were stored at −20 °C for further analysis. PCR for 16S rRNA gene was carried out in a LightCycler 96 thermal cycler (Roche Polska, Warszawa, Poland). Reactions were carried out in 50 µL volume: 25 µL of Color Taq PCR Master Mix (2x); 1 µL of 10 µM solution of each primer—27F 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1495R 5′-CTACGGCTACCTTGTTACGA-3′ [36]; 5 µL of 25 mM $MgCl_2$; 5 µL of DNA solution containing 30 ng of DNA; and 13 µL of nuclease-free water. Time/temperature profile was as follows: initial denaturation 94 °C/5 min; 30 cycles: denaturation 94 °C/30 s, annealing 58 °C/30 s, elongation 72 °C/2 min; final elongation 72 °C/7 min; and cooling at 37 °C [36]. Reaction products were visualized on 1.5% (*w*/*v*) agarose gel in TAE containing 5 µL of SimplySafe dye per 100 mL of gel against Perfect Plus Molecular Weight Quantitative Ladder. Electrophoresis was carried out for 60 min/100 V in Wide Mini-Sub Cell GT Cell (Bio-Rad Polska sp. z o.o., Warszawa, Poland) and gels were visualized in Gel Doc EZ System (Bio-Rad Polska sp. z o.o., Warszawa, Poland). After PCR, 10 µL of product obtained was combined with 2 µL of EPPiC and purification was carried out according to the manufacturer's instructions (37 °C/15 min and 70 °C/15 min). We then adjusted the concentration of DNA to 50 ng/µL and added a primer (5 µM solution) and sent samples for Sanger sequencing to Macrogene Europe (Amsterdam, Netherlands). The sequences obtained were trimmed in Chromas (version 2.6.6; http://www.technelysium.com.au/chromas.html, accessed on 22 February 2022) to remove ambiguous sequences and subjected to analysis in BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome, accessed on 22 February 2022) for identification. Only results including identity above 98% and e-value lower than 1×10^{-6} were considered. After that process, we submitted sequences for assigning accession numbers. Additionally, we carried out the alignment of all collected sequences to exclude the possibility that the isolates obtained could be the same strains. We used the phylogeny.fr platform for that purpose. For the rest of the analysis and experiments, we selected isolates that were identified to the species level. Isolates that belonged to other species were not considered in the current study.

4.6. Phylogenetic Analysis

Phylogenetic relationships between tested isolates were examined on the phylogeny.fr platform [37] with the settings as described by Wajda et al. (2019) [38]. If support values were below 70%, then branches were dropped. We selected *Enterococcus faecalis* V583 (accession no. AE016830.1) as an outgroup.

4.7. Extraction of Plasmid DNA and Detection of Antibiotic Resistance Genes with PCR

The selection of reference genes was carried out in The Comprehensive Antibiotic Resistance Database (CARD, https://card.mcmaster.ca/, accessed on 22 February 2022) based on the distribution of those among species that could come into contact with *E. faecium* in the intestine [39] or on skin [40]. The extraction of plasmid DNA was carried out with Plasmid Mini AX according to manufacturer's instructions; however, 5 µL mutanolisin (10 U/µL) was added in the first extraction step. PCR was carried out in a 20 µL volume: 10 µL of Color Taq PCR Master Mix (2x); 0,4 µL of each primer (10 µM) solution (Table 4); volume of 25 mM MgCl2 solution adjusted to the particular primer pair (Table 4); 1 µL of DNA solution containing 2 ng of plasmid DNA; and adjusted to 20 µL with nuclease free water. Time/temperature profile was as follows: initial denaturation 94 °C/5 min; 35 cycles: denaturation 94 °C/30 s, annealing temperature established for particular primer pair /30 s, elongation 72 °C/2 min; final elongation 72 °C/7 min; and cooling at 37 °C. PCR products were visualized as above; however, for products smaller than 500 bp, Perfect Plus 50–500 bp DNA Ladder and 2% agarose gel were used.

4.8. Determination of Gene Expression

We determined gene expression only for those isolates that held genes encoding resistance to at least two antibiotics. We prepared bacterial biomass as for antibiotic susceptibility testing. We then combined 0.5 mL of resulting suspension with 0.5 mL of the antibiotic solution that the isolate tested was resistant to and obtained final antibiotic concentration one fold lower than the MIC value determined previously. We used sterile water for control samples instead of the antibiotic solution. After incubation (37 °C/48 h), we isolated total RNA using Total RNA Mini and synthesized cDNA with the TranScriba Kit, and used it together with the Luna Universal qPCR Master Mix to verify if detected genes were involved in antibiotic resistance. The reaction mixture (20 µL) contained 10 µL of Luna Universal qPCR Master Mix, 0.5 µL of each primer (10 µM solution, Table 4), 2 µL cDNA (30 ng), and 7 µL of nuclease free water (NFW). In the case of the negative control of reverse transcription, we used NFW instead of TranScriba. For positive controls, we used primers designed for the 16S rRNA region. The thermal/time profile used in those reactions was: 95 °C/60 s, 95 °C/15 s, 40 cycles at 95 °C for 15 s and 60 °C for 30 s. Reactions were carried out in triplicate using a LightCycler 96 thermal cycler (Roche Polska, Warszawa, Poland).

Relative ratios were automatically generated by the software according to Equation (1):

$$\text{Relative ratio} = E_{\text{target}}^{\Delta\text{Ct target (control}-\text{sample)}} / E_{\text{reference}}^{\Delta\text{Ct reference (control}-\text{sample)}}, \quad (1)$$

where E is reaction efficiency obtained for the target and reference gene, respectively, (E = 2); ΔCt is a value of the difference of Ct values calculated for the target gene and a reference gene.

Table 4. PCR and real-time PCR conditions for the detection of antibiotic resistance genes in *Enterococcus faecium* isolates obtained from human breast milk.

Antibiotic	Gene Name	Primer Sequences (5′-3′)	Primer Name	Tm of Primers (°C)	Annealing Temperature (°C)	Final Mg^{2+} Concentration (mM) for the Regular PCR	Product Size (bp)	Reference
Kanamicin	*aph3′ IIIa*	GCCGATGTGGATTGCGAAAA	Aph3F	59.83	55	4	292	[41]
		GCTTGATCCCCAGTAAGTCA	Aph3R	56.92				
	Aph(2″)-IIIa	TCGCTTGGTGAGGGCTTTAG	Aph2F	60.04	55	4	402	Current study
		CTGATCCTCCACAGCTTCCG	Aph2R	60.18				
	ant(2″)-I	CAGATGAGCGAAATCTGCCG	Ant2F	59.42	54	4	226	Current study
		CAAGCAGGTTCGCAGTCAAG	Ant2R	59.76				
Tetracyclin	*tetM*	CTTGTTCGAGTTCCAATGC	tetMF	54.74	55	4	401	[42]
		GGTGAACATCATAGACACGC	tetMR	56.62				
	tetK	TTAGGTGAAGGGTTAGGTCC	tetKF	55.84	56	4	697	[42]
		GCAAACTCATTCCAGAAGCA	tetKR	56.62				
		CGATAGGAACAGCAGTATATGGAA	tetK2F *	59.76	60 *	3	164 *	Current study
		AGATCCTACTCCTTGTACTAACCT	tetK2R *	59.13				
	tet(L)	CATTTGGTCTTATTGGATCG	tetLF	52.04	52	4	456	[42]
		ATTACACTTCCGATTTCGG	tetLR	52.77				
	tetO	GCATTCTGGCTCACGTTGAC	tetOF	59.83	56	4	985	Current study
		TGCGGCAACAGTATTTCGTTC	tetOR	59.8				
		ATTAACTTAGGCATTCTGGCTCA	tetO2F *	59.76	60 *	3	176 *	Current study
		GATGTCACTGCTGTCTGGAT	tetO2R *	59.13				
	tetS	GATAAGGCAGAGCCTGGTGAG	tetSF	60.2	55	4	414	Current study
		AGCCCAGAAAGGATTTGGAGG	tetSR	59.99				

Table 4. *Cont.*

Antibiotic	Gene Name	Primer Sequences (5′-3′)	Primer Name	Tm of Primers (°C)	Annealing Temperature (°C)	Final Mg^{2+} Concentration (mM) for the Regular PCR	Product Size (bp)	Reference
Chloramphenikol	*Cat(A4)*	CAATGCACCTTTAGCCAGACCG	catA4F	62.08	60	4	310	Current study
Chloramphenikol	*Cat(A4)*	AGGCTAGATCGTCGCCGTATTG	catA4R	62.27	60	4	310	Current study
Chloramphenikol	*Cat(II)*	TTCTCTGCACTGTCCTGCCG	catIIF	62.44	60	4	311	Current study
Chloramphenikol	*Cat(II)*	AACCGTGCTGCATGAAAGCC	catIIR	62.15	60	4	311	Current study
Gentamicin	*aac(6′)-aph(2″)*	CCTCGTGTAATTCATGTTCTGGC	GentF	59.11	58	4	675	[41]
Gentamicin	*aac(6′)-aph(2″)*	ACAGAGCCTTGGGAAGATGAA	GentR	57.75	58	4	675	[41]
Erythromicin	*erm(B)-1*	GCATTTAACGACGAAACTGGC	ermB1F	58.76	55	4	247	Current study
Erythromicin	*erm(B)-1*	ATAGATGTCAGACGCACGGC	ermB1R	60.25	55	4	247	Current study
Erythromicin	*erm(B)-1*	ACTACTTAGGATGATGTCGTGGAA	EryF *	60.93	60	5	188	Current study
Erythromicin	*erm(B)-1*	CCCTGAACAATTGGTGGCATA	EryR *	60.40	60	5	188	Current study
Klindamicin	*lnu(A)*	TTGGTTAGATGGTGGCTGGG	lnuAF	59.67	60	4	253	Current study
Klindamicin	*lnu(A)*	ACCTTCTGGGTTTGCTTGGG	lnuAR	60.47	60	4	253	Current study
Klindamicin	*lnu(B)*	TGACGTAGCTCCGTACTTGATG	lnuBF	59.9	57	4	166	Current study
Klindamicin	*lnu(B)*	AAGCATAGCCTTCGTATCAGG	lnuBR	57.61	57	4	166	Current study
Ampicillin	*mecA*	CAGGTACTGCTATCCACCCTC	mecAF	59.31	55	4	770	Current study
Ampicillin	*mecA*	TTCTGCAGTACCGGATTTGCC	mecAR	60.95	55	4	770	Current study
Ampicillin	*Bla(Z)*	AACAGTTCACATGCCAAAGAG	BlaZF	57.00	57	4	485	Current study
Ampicillin	*Bla(Z)*	AAAGTCTTGCCGAAAGCAGC	BlaZR	59.69	57	4	485	Current study
Cyprofloksacin	*gyrA*	TTCCATTCGGATACGCGGAG	gyrAF	59.97	60	2.75	432	Current study
Cyprofloksacin	*gyrA*	CCACGCAAAATATGAGCCCG	gyrAR	59.97	60	2.75	432	Current study
Cyprofloksacin	*parC*	CCCTTGAACATGAACGTCCT	parCF	57.81	60	4	177	Current study
Cyprofloksacin	*parC*	GAGATAGGCGATCAGCAAGC	parCR	58.57	60	4	177	Current study

Table 4. *Cont.*

Antibiotic	Gene Name	Primer Sequences (5′-3′)	Primer Name	Tm of Primers (°C)	Annealing Temperature (°C)	Final Mg^{2+} Concentration (mM) for the Regular PCR	Product Size (bp)	Reference
Streptomicin	*ant(6)*	ACTGGCTTAATCAATTTGGG	Ant6F	53.79	59	1.5	597	Current study
		GCCTTTCCGCCACCTCACCG	Ant6R	56.11				
	Ant(2)	ACACAACGCAGGTCACATTG	Ant2F	59.34	56	4	421	Current study
		ACTGGTGGTACTTCATCGGC	Ant2R	59.75				
	Ant(2″)-Ia	CAGATGAGCGAAATCTGCCG	Ant2IaF	59.42	56	4	226	Current study
		CAAGCAGGTTCGCAGTCAAG	Ant2IaR	59.76				
	aadA	AGGTAGTTGGCGTCATCGAG	aadAF	59.54	55	4	724	Current study
		TCGCCTTTCACGTAGTGGAC	aadAR	60.04				
	Str(A)	CTTGGTGATAACGGCAATTC	straF	55.06	55	4	549	[41]
		CCAATCGCAGATAGAAGGC	straR	55.87				
	Str(B)	ATCGTCAAGGGATTGAAACC	strbF	55.76	56	4	509	[41]
		GGATCGTAGAACATATTGGC	strbR	53.68				
	ant(6)	AGGGACATAGTTCCGACTGAT	StrepF *	60.93	60 *	3	198 *	Current study
		AACCTTCCACGACATCATCC	StrepR *	60.40				
Vankomicin	*EmrG*	TGAAATAGGTGCAGGGAAAGG	EmrGF	59.09	58	4	330	Current study
		AGCAATGCTAGTGATCTGTTTG	EmrGR	58.19				
	vanHa	TCGGAATCCAACGCCAAATC	vanHaF	60.05	60	4	428	Current study
		CTTCGGCTGCGACTATAAGC	vanHaR	59.98				
Tylosine	*ErmT*	GGGAAAGGTCATTTCTCGTTTG	ErmTF	58.99	57	4	252	Current study
		ACTTTCTGTAGCTGTGCTTTC	ErmTR	57.62				
	ErmA	TCGTTGAGAAGGGATTTGCG	ErmAF	59.7	59	1.5	273	Current study
		TCAAAGCCTGTCGGAATTGG	ErmAR	59.62				

Table 4. *Cont.*

Antibiotic	Gene Name	Primer Sequences (5′-3′)	Primer Name	Tm of Primers (°C)	Annealing Temperature (°C)	Final Mg^{2+} Concentration (mM) for the Regular PCR	Product Size (bp)	Reference
Dalfopristin	*ermB*	GGCATTTAACGACGAAACTGGC	ermBDF	60.98	60	4	322	Current study
		TGAGTGTGCAAGAGCAACCC	ermBDR	60.82				
Trimethoprim	*dfrA14*	TGGTTGCGGTCCAGACATAC	dfrA14F *	60.04	60 *	2.75	261	Current study
		ATTTCTCCGCCACCAGACAC	dfrA14R *	60.32				
Linesolid	*clcD*	TGCGTTGTTTGCTTTAAGTCCG	CfrBF	60.54	60	4	490	Current study
		ACCGCAAGCAGCGTCTATATC	CfrBR	60.6				
Rifampicin	*marA*	ACAACCTGGAATCGCCACTG	marAF *	60.61	60 *	5	270 *	Current study
		TCATCCGGTATTTATGCGGCG	marAR *	60.94				
Neomycin	*Aph(3′)-IIIa*	AAGATACGGAAGGAATGTCTCC	NeoF	57.33	57	4	600	Current study
		TGTCATACCACTTGTCCGCC	NeoR	60.04				
		CATCAGGCTCTTTCACTCCAT	NeoF *	59.57	60 *	3	200 *	Current study
		CAAGTTCCTCTTCGGGCTT	NeoR *	59.18				
Positive control	16S rRNA	CCTGCAATCCGAACTGAGA	16SEF *	58.96	60 *	3	105 *	Current study
		CCTTATGACCTGGGCTACAC	16SER *	59.20				

* Marked primers were used for the analysis of gene expression, while in the case of trimethoprim and rifampicin, primer pairs were used for both regular and real-time PCR.

5. Conclusions

Many bacterial specimens isolated from human breast milk, especially the representatives of the *Enterococcus* genus, are resistant to multiple antibiotics. The investigation of the molecular background of that phenomenon could be very complex and time consuming because sometimes acquired resistance genes can be present on chromosomes due to the presence of transposons. It also seems justified to sequence plasmid DNA for each isolate considered as a putative probiotic because that information could help verify the presence of transferable antibiotic genes. We demonstrated that some of the genes found on plasmids are expressed, especially *dfrA14* that encodes the resistance to trimethoprim. However, it is possible to find potential candidates for probiotics in human breast milk because one of the tested isolates (CDCP539) did not show any antibiotic resistance.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Bioethics Committee of Swietokrzyski Medical Board (protocol code 11/2019-VII approved on 07.02.2019).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Informed consent has been obtained from the patient(s) to publish all papers based on collected data.

Data Availability Statement: Since the project was performed by a private company, data were not published in any publicly available databases.

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