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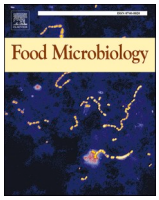

Biodiversity and antibiotic resistance profile provide new evidence for a different origin of enterococci in bovine raw milk and feces.

Stefano Morandi [a,*], Tiziana Silvetti [a], Vincenzo Lopreiato [b], Fiorenzo Piccioli-Cappelli [c], Erminio Trevisi [c], Milena Brasca [a]

[a] Institute of Sciences of Food Production (ISPA), Italian National Research Council, Milan, Italy
[b] Department of Veterinary Sciences, University of Messina, Viale Palatucci 13, Messina, 98168, Italy
[c] Department of Animal Sciences, Food and Nutrition (DIANA), Alimentari e Ambientali, Università Cattolica Del Sacro Cuore, Piacenza, 29122, Italy

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ABSTRACT

Enterococci are widely distributed in dairy sector. They are commensals of the gastrointestinal tract of animals, thus, via fecal contamination, could reach raw milk and dairy products. The aims of this study were: 1) to investigate the enterococcal diversity in cow feces and milk samples and 2) to evaluate the antibiotic resistance (AR) of dairy-related enterococci and their ability to transfer resistance genes.

E. faecalis (59.9%), *E. faecium* (18.6%) and *E. lactis* (12.4%) were prevalent in milk, while *E. faecium* (84.2%) and *E. hirae* (15.0%) were dominant in bovine feces. RAPD-PCR highlighted a high number of *Enterococcus* biotypes (45 from milk and 37 from feces) and none of the milk strains exhibited genetic profiles similar to those of feces biotypes.

A high percentage of enterococci isolated from milk (71%) were identified as multidrug resistant and resistance against streptomycin and tetracycline were widespread among milk strains while enterococci from feces were commonly resistant to linezolid and quinupristin/dalfopristin. Only *E. faecalis* strains were able to transfer horizontally the *tetM* gene to *Lb. delbrueckii* subsp. *lactis*. Our results indicated that *Enterococcus* biotypes from milk and bovine feces belong to different community and the ability of these microorganisms to transfer AR genes is strain-dependent.

1. Introduction

Enterococcus genus represents a controversial group of lactic acid bacteria commonly present in dairy products. Different studies focused on the microbiota of traditional Mediterranean cheeses highlighted as these bacteria are involved in fermentation and play an important role in cheese ripening (Morandi et al., 2005). Their metabolic activity and the resulting compounds impact the cheese flavor, texture, and rheological properties, thus contributing to the development of cheese typical sensorial properties (Quigley et al., 2013). In addition, these bacteria produce antimicrobial substances that have a broad-spectrum activity against foodborne pathogens, including *Listeria monocytogenes* and *Staphylococcus aureus* (Franz et al., 2007), and some strains show probiotic properties and health-promoting capabilities (Dapkevicius et al., 2021). Recently, it has been proven that some *Enterococcus* strains can lower total and low-density lipoprotein cholesterol levels and produce high level of folates in cheeses (Albano et al., 2018, 2020).

On the other hands, these bacteria are considered as human opportunistic pathogens since are frequently involved in hospital-acquired infections due to the presence of virulence factors that mediate adhesion to cells and biofilm synthesis in the process of host tissues colonization (Chajęcka-Wierzchowska et al., 2017). Resistance to antibiotics is a natural occurrence that characterizes the evolution of bacteria, but the application of these molecules in the veterinary field has accelerated this phenomenon by promoting the development of microorganisms capable of surviving in unfavorable environments. Particularly involved in the resistance phenomena are the bacterial species that make up the ESKAPE group (*Enterococcus* spp., *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and Enterobacteriaceae) (Pendleton et al., 2013). Enterococci are intrinsically resistant to several antimicrobial agents, and they can easily acquire and transfer further drug resistances across genetic mobile elements such as transposons and plasmids resulting in the risk of dissemination of determinants for antibiotic resistance (AR) and/or

* Corresponding author.
E-mail address: stefano.morandi@ispa.cnr.it (S. Morandi).

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virulence. Here, then, this microbial genus constitutes an effective indicator of the spread of antibiotic resistance of cross-cutting interest encompassing food, environmental and clinical microbiology (Mathur and Singh, 2005).

Several *E. faecalis* and *E. faecium* clinical isolates causing bacteremia, endocarditis, urinary and pelvic infections were resistant to many commonly used antibiotics, including ampicillin and vancomycin. Additionally, resistance to the last-resort antibiotics, such as daptomycin and linezolid, is also emerging among enterococcal strains (Zarzecka et al., 2022a). Novel antibacterial agents (teixobactin, lipopeptides derived from nisin, dimeric vancomycin analogues and tarocins) were discovered and developed and their targets and mode of action represent a new strategy for the treatment of *E. faecalis* and *E. faecium* infections (van Harten et al., 2017).

Due to the uncertainty on the safety, this genus does not possess the Generally Regarded as Safe (GRAS) status in the USA and the Qualified Presumption of Safety (QPS) in the European Union (EU). The lack of a recognized safety status has hampered the employment of these microorganisms as starter/adjunct cultures or probiotics, despite their potential benefits (Dapkevicius et al., 2021).

Given the interest in the use of these microorganisms for their healthy traits, many studies compared safe strains and those cause of human infections and is now recognized that in *Enterococcus faecium* population are present two different clades (clade A and B) that differ in ampicillin and vancomycin susceptibility and from the absence in three virulence factors and markers associated with hospital strains (IS*16*, *esp* and *hyl*-like gene) (EFSA, 2012; Daza et al., 2021).

Although numerous studies are focused on dairy-related enterococci, the origin of these bacteria in milk remains a matter of debate. Their presence is generally attributed to fecal contamination or poor hygienic conditions during milking, but data on this topic are limited and controversial (Kagkli et al., 2007; Quigley et al., 2013; Oikonomou et al., 2020).

Thus, the main purpose of the study was to provide a better understanding on the origin of dairy related enterococci to contribute to their safety evaluation. To this aim, genotypic diversity of enterococci isolated from individual cow milk and bovine fecal samples was investigated along with virulence traits and AR pattern. Lastly, we verified the strains' ability to transfer resistance genes.

2. Materials and methods

The research was conducted in accordance with Italian laws on animal experimentation and ethics (Italian Health Ministry Authorization N 444/2019-PR in agreement with D. Lgs. n. 26, March 04, 2014).

2.1. Experimental design and samples collections

The study was conducted in the CERZOO research and experimental center (San Bonico, Piacenza, Italy), and enrolled thirty-nine clinically healthy Italian Holstein dairy cows. During the experimental period (from May 2019 to April 2020) the cows were milked twice daily and were fed *ad libitum* with a total mixed ration, formulated in accordance with the protein and energy requirements (NRC, 2001; INRA, 1989).

Milk samples were collected from the morning milking at 65, 57 and 50 days before calving, the day of calving (colostrum) and at 3 and 35 days in lactating (234 samples in total). The first streams of foremilk were manually discarded and after the teat cleaning about 10 mL of raw milk was collected aseptically from each quarter, into the same sterile vial. Fecal samples were collected directly from the rectum of each individual animal at the same time points of milk samples and placed in 50 mL sterile container. All samples were frozen and kept at - 20 °C until analysis (Masters et al., 2015). Before the analysis the samples were thawed in a refrigerator.

2.2. Enterococci enumeration and isolation

One milliliter of raw milk samples was serially diluted in quarter-strength Ringer's solution (Scharlau Microbiology, Barcelona, Spain) and inoculated in Kanamycin Aesculin Azide (KAA) agar (Biolife Italiana, Milan, Italy). Fecal samples (10 g) were homogenized in 90 mL of a 2% (w/v) sterile Buffered Peptone water (Biolife Italiana) for 2 min in a Stomacher BagMixer (Interscience, St. Nom, France). Samples were serially diluted in quarter-strength Ringer's solution (Scharlau Microbiology) and plated in KAA agar (Biolife Italiana). All plates were incubated in aerobic conditions at 37 °C for-48 h and white or grey colonies surrounded by a black halo were counted as enterococci. Typical *Enterococcus* colonies from samples of three different time points (-65, +5 and + 35 days), were picked and sub-cultured in de Man Rogosa and Sharpe (MRS) broth (Biolife Italiana) at 37 °C. The purity of the isolates was checked by streaking repeatedly on Homofermentative Heterofermentative Differential (HHD) agar (Biolife Italiana). After purification, the isolates were examined for cell morphology and catalase activity and successively stored at - 20 °C in Litmus milk (Biolife Italiana).

2.3. Molecular identification and typing

Genomic DNA of the selected isolates was extracted from overnight cultures by the Microlysis kit (Aurogene, Rome, Italy) following the manufacturer's instructions. Yield and purity of DNA were evaluated using the Infinite F200 PRO microplate reader (Tecan, Mannedorf, Switzerland).

Enterococcal strains were identified by 16S rRNA sequencing as previously described by Silvetti et al. (2017) (Table S1). Amplification products were sent to Macrogen Europe (Amsterdam, the Netherlands) for sequencing and sequences were analyzed with Basic Local Alignment Search Tool (BLAST) software (National Center for Biotechnology Information, MD, USA). Species names were assigned whenever the degree of homology was higher than 98%. Enteroccocci identification was further confirmed by Randomly Amplified Polymorphic DNA-PCR (RAPD-PCR) analysis as previously described by Quednau et al. (1998) and Ong et al. (2014).

RAPD-PCR method was also applied to explore the biodiversity and genetic relatedness within the strains isolated. RAPD-PCR analysis was performed with 3 primers (M13, D11344 and D8635) (Table S1) as described by Morandi et al. (2015). Resulting fingerprints were compared with the BioNumeric 5.0 software package (Applied Maths, Sint-Martens-Latem, Belgium), using the UPGMA (unweighted pair group method with arithmetic averages) cluster analysis. Strains with a similarity coefficient equal to or higher than 90% were considered belonging to the same biotype (Morandi et al., 2019).

2.4. Antibiotic susceptibility testing and minimal inhibitory concentrations (MIC) determination

The susceptibility of *Enterococcus* biotypes to 12 antimicrobial agents selected on basis of the European Food Safety Authority scientific report on the AR in enterococci isolates from food-producing animals and food (EFSA, 2019). The antibiotics used in this study were ampicillin (AMP), chloramphenicol (C), daptomycin (DAP), erythromycin (E), gentamicin (CN), linezolid (LNZ), quinupristin/dalfopristin (QD) (only for non-*E. faecalis*), streptomycin (ST), teicoplanin (TEC), tetracycline (TE), tigecycline (TGC) and vancomycin (VA) was determined by applying the MIC test strips (Liofilchem, Roseto degli Abruzzi, Italy) on Mueller-Hinton (MH) agar plate (Biolife Italiana) according to the manufacturer's instructions. Briefly, saline suspensions with concentration of $\sim 10^8$ cells/mL were produced from overnight cultures. A sterile cotton swab was dipped in this cell suspension and used to inoculate the MH agar plates by swabbing in three directions. After drying the surface, the gradient strips were placed on the agar plates and

incubated aerobically. The MIC, defined by the intersection of the growth ellipse margin with the gradient strip, was recorded after 18 h incubation at 37 °C. Enterococcal biotypes were classified as susceptible, intermediate or resistant according to criteria from the Clinical and Laboratory Standards Document M100 (31st edition) (CLSI, 2021) and European Society of Clinical Microbiology and Infectious Diseases (Version 12.0) (EUCAST, 2021) only for tigecycline. All determinations were performed in duplicate.

2.5. Determination of AR and virulence factors genes

The presence of genes responsible for tetracycline, erythromycin, quinupristin/dalfopristin and vancomycin resistance was investigated for all strains using specific PCR assays. Multiplex PCR was employed to explore the presence of tetracycline resistant genes encoding efflux proteins (*tetK* and *tetL*) and ribosomal protection proteins (*tetM* and *tetS*) (Ng et al., 2001). The presence of the transposon integrase gene (*int* gene) of the Tn*916/*Tn*1545* family responsible for TE resistance transmission was determined according to Doherty et al. (2000). Erythromycin resistance gene (*ermB*) was revealed using specific primers and conditions reported by Jensen et al. (1999), while *vatD* and *vatM* genes, connected with the quinupristin/dalfopristin resistance, were analyzed as described by Soltani et al. (2000) (Table S1). The *vanA* and *vanB* genes were detected according to protocol proposed by Morandi et al. (2015).

In addition, one strain representative for each enterococcal biotype was analyzed for the presence of the virulence genes involved in the expression of aggregation substances (*asa1*), enterococcal surface protein (*esp*), gelatinase (*gelE*), glycosyl hydrolase (*hly*), cytolysin (*cylA*) and adhesion of collagen (*ace*). Multiplex-PCR was employed to detect the presence of *asa1*, *esp*, *gelE*, *hyl* and *cylA* (Vankerckhoven et al., 2004) while *ace* gene monitored as described by Ben Omar et al. (2004) (Table S1). All PCR reactions were performed in an Eppendorf Mastercycler Nexus (Eppendorf AG, Hamburg, Germany), using the AccuPrime Taq DNA polymerase (Thermo Fisher Scientific, Walthman, MA, USA) for Multiplex-PCRs and PCR Master Mix (2X) (Thermo Fisher Scientific) for single PCRs. The amplified PCR products were visualized by gel electrophoresis in a 3% agarose gel (GellyPhor, Euroclone, Milan, Italy), stained with Atlas Clear Sight DNA Stain (Bioatlas, Tartu, Estonia). Molecular size markers TrackIt 1 Kb Plus DNA Ladder (Thermo Fisher Scientific) were included in each agarose gel. All determinations were performed in duplicate.

Six *E. faecalis* strains were used as positive control to confirm the correct performance of a PCR reaction: SV6 (*tetK*, *ermB*, *asa1*, *gelE*); VC45 (*int*); VC124 (*ace*); VC148 (*cylA*); VS368 (*tetL*, *tetM*) and VS526 (*tetS*, *esp*) (Silvetti et al., 2019).

2.6. Conjugal transfer of tetracycline resistance tetM gene

In vitro conjugation experiments were carried out to investigate the ability of tetracycline resistant biotypes (containing the *tetM* and *int* genes) to transfer their resistance to other lactic acid bacteria species. Transfer was investigated using the filter mating technique as previously described by Toomey et al. (2010). The four biotypes belonging to different *Enterococcus* species that showed high tetracycline MIC values and that harboured simultaneously *tetM* and *int* genes were selected as donors. *Lactobacillus delbrueckii* subsp. *lactis* VC107, susceptible to tetracycline and free from *tet* genes, was considered the recipient strain (Morandi et al., 2015).

Donor strains were cultured in MRS broth (Biolife Italiana) supplemented with tetracycline (10 μg/mL) (Thermo Fisher Scientific). Recipient *Lb. delbrueckii* subsp. *lactis* VC107 was grown without antibiotic in MRS broth. All strains were incubated aerobically overnight at 37 °C. From the resulting cultures, 1 mL was added to 5 mL of fresh MRS broth (without antibiotic) and incubated for 4 h at 37 °C until the mid-exponential phase of growth was obtained. Then 1 mL of donor culture was added to the 1 mL recipient culture and the mixture was filtered through a sterile cellulose membrane filter with a pore diameter of 0.45 μm (Sartorius GmbH, Gottingen, Germany). After the donor and recipient were filtered, the filters were rinsed with 10 mL sterilized peptone physiological saline (PPS) solution (0.85% w/v NaCl and 0.1% w/v neutralized bacteriological peptone (Biolife Italiana)) to ensure cells attached tightly to the membrane.

Filters were aseptically placed, cell side up, on HHD agar plates and incubated anaerobically (AnaeroGen, Thermo Fisher Scientific) overnight at 37 °C. Following incubation, filters were placed in 2 mL PPS and a further 1 mL PPS was used to wash each plate and once again the washings were placed in a sterile tube along with the original mating filter. Filters were vortex-mixed to dislodge all cells and serial ten-fold dilutions were plated onto media selective or differential for donors (KAA agar), recipients (HHD agar) and transconjugants (HHD agar with the addition of 10 μg/mL of Tetracycline). Plates were incubated anaerobically at 37 °C for 48 h. All matings were repeated in duplicate. The transfer frequency was expressed as the ratio of the number of transconjugants to the number of recipients (Zarzecka et al., 2022b).

2.7. Confirmation of transconjugant identity

Presumptive transconjugants taken from selective agar plates were grown in MRS broth with tetracycline (10 μg/mL) and incubated at 37 °C overnight. From the cultures obtained, genomic DNA was extracted using the Microlysis kit (Aurogene) and the presence of *tetM* and *int* genes was determined using primers and conditions as described above. Moreover, these isolates were typed to distinguish them from mutants by RAPD-PCR analysis with primer M13, D11344 and D8635 (Rizzotti et al., 2009). Mutants and the stability of the transferred resistance were also assessed by culturing twice the transconjugant strains in MRS broth without antibiotics and then verifying the maintenance of tetracycline resistance (Toomey et al., 2010) and detecting the presence of *tetM* gene and Tn916-1545 transposons sequences as indicated above.

2.8. Statistical analysis

All data related to microbiological assays are presented as means ± standard deviation (SD). Significant differences ($P < 0.001$) among the data were calculated by one-way ANOVA using Minitab ver. 14.13 (Minitab Inc.).

3. Results

3.1. Enterococci enumeration, identification and typing

The total counts of enterococci in the raw milk and feces samples were reported in Table 1. *Enterococcus* content in milk from individual cows was quite stable ranging from 2.0 ± 1.4 $\log_{10}$ CFU/mL at calving time to 3.1 ± 1.8 $\log_{10}$ CFU/mL on the 35th day in milk. The lowest content was observed at the calving time point where the mean values of enterococci load differed significantly as compared to the other time points ($P < 0.05$). In feces samples, the enterococcal level was around 3.0 $\log_{10}$ CFU/g and remained constant for the entire experimental period ($P > 0.001$) (Table 1).

A total of 310 presumptive *Enterococcus* spp. isolates were recovered at the three different time points from raw milk (n. 177) and bovine feces (n. 133) samples (Table 1). The sequences of partial 16S rRNA gene from all the strains showed high degrees of similarity to those of the GenBank reference strains (between 99.8 and 100%) and thus were assigned to a specific species. Later, the *Enterococcus* species was confer clustering the RAPD-PCR patterns of the new isolates with the RAPD fingerprint profiles of other enterococcal strains (more than 500) contained in our database.

Clear differences in enterococcal community composition were observed between the milk and feces samples. Raw milk was dominated

Table 1
Enterococcus spp. content and number of strains recovered during the experimental period.

Sampling	Raw milk			Feces		
	n. samples	log_{10} CFU/mL	n. strains	n. samples	log_{10} CFU/mL	n. strains
−65 days	39	2.8 ± 1.5 [a]	51	39	3.2 ± 1.5 [a]	51
−57 days	39	3.1 ± 1.8 [a]		39	2.8 ± 1.5 [a]	
−50 days	39	2.4 ± 1.5 [a]		39	3.1 ± 1.3 [a]	
Calving	39	2.0 ± 1.4 [b]		39	3.0 ± 1.4 [a]	
5 days	39	2.6 ± 1.4 [a]	58	39	2.9 ± 1.6 [a]	37
35 days	39	3.1 ± 1.4 [a]	68	39	3.1 ± 1.2 [a]	45
total	234		177	234		133

Means with different lowercase letters in the same column are significantly different ($P < 0.05$).

by *E. faecalis* (59.9%), *E. faecium* (18.6%) and *E. lactis* (12.4%), followed by low percentages of *E. casseliflavus* (2.8%), *E. malodoratus* (2.3%), *E. durans* (1.1%), *E. gallinarum* (1.1%), *E. hirae* (1.1%) and *E. gilvus* (0.6%). Differently, cow feces were mainly composed by *E. faecium* (84.2%) *and E. hirae* (15.0%), and only one isolates was identified as *E. faecalis* (0.8%) (Fig. 1, Table 2).

RAPD–PCR fingerprinting was performed to establish the number of genotypically different biotypes among the *Enterococcus* isolates. A total of 82 biotypes were recovered (45 from milk and 37 from cow feces) and none of the milk biotypes clustered with the cow feces ones (Table 2; Fig. S1).

Cluster analysis showed that the three main species detected in milk (*E. faecalis*, *E. faecium* and *E. lactis*) were grouped in 14, 14 and 8 biotypes with a similarity level of 25.0%, 54.5% and 59.8% respectively, while 31 and 5 biotypes were recovered within the *E. faecium* and *E. hirae* strains from feces (similarity level of 32.9% and 52.7%). The higher biodiversity was found in the *E. faecalis* species. It is interesting to notice that some strains belonging to *E. faecalis* (FC4), *E. faecium* (FU11, FU26, FU27, FU37 and FU39) and *E. hirae* (HR5) were isolated in all the three time points considered only in milk or only in feces (Table 2). It is worth noting that the most widespread *E. faecalis* biotypes FC4 and FC5 were detected in the milk from 19 cows and never in feces and similarly *E. faecium* FU26 and FU27 were isolated from the feces of 11 different cows.

3.2. Virulence factors

Enterococci biotypes considered in this study were also characterized for the presence of six virulence factors genes. Virulence traits encoding for enterococcal surface protein (*esp*), gelatinase (*gelE*) and aggregation substances (*asa1*) were detected only in *E. faecalis* and *E. hirae* isolated from milk samples (Table 3). All the fourteen *E. faecalis* biotypes (100%) recovered from milk were positive for *esp*, 13 (92.8%) for *gelE* and only one harbored the *asa1* gene. No virulence traits were detected among the fecal biotypes.

3.3. Antibiotic susceptibility and AR resistance genes

The susceptibility of all *Enterococcus* biotypes to 12 different antimicrobial agents (EFSA, 2019) was assessed by MIC test strip method. The first evidence is an important difference between strains isolated from milk and strains isolated from feces. All the strains screened were susceptible to ampicillin, daptomycin, gentamicin, teicoplanin and vancomycin (Table 4).

Among the milk biotypes, considered as a whole, the highest percentages of resistance occurred in response to streptomycin (86.7%), tetracycline (73.3%), erythtomycin (46.6%), tigecycline (33.3%) and quinupristin/dalfopristin (29.0%). The percentage of resistant strains to the remaining antibiotics (linezolid and chloramphenicol) was 13.3 and 2.9% (Table 4).

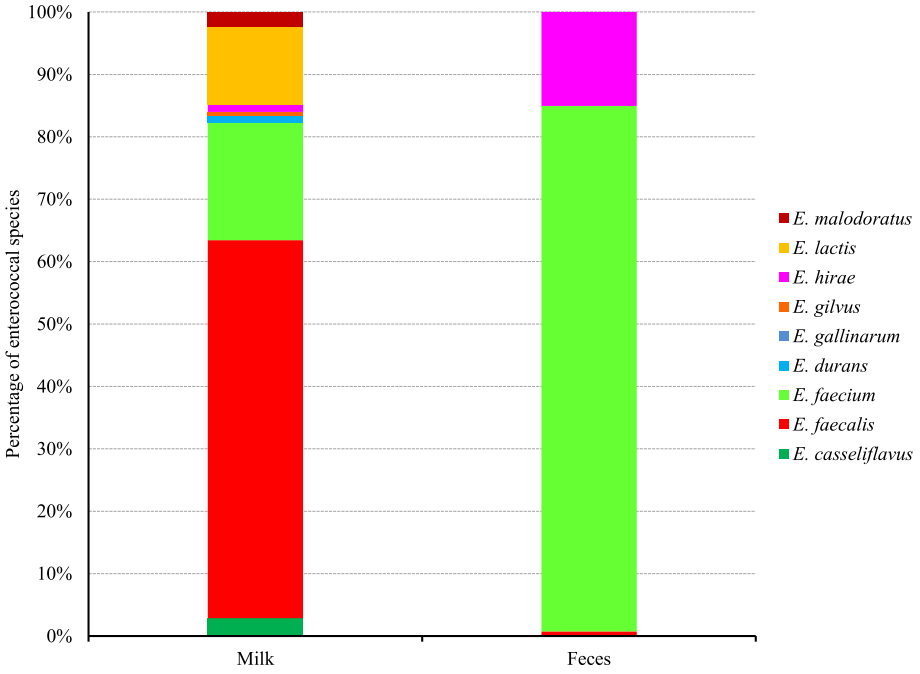

Fig. 1. Distribution of the *Enterococcus* species isolates in raw milk and bovine feces samples.

Table 2
Occurrence of *Enterococcus* biotypes obtained by RAPD-PCR in raw milk and feces samples. In bold are reported the biotypes detected in all the time points analyzed.

Sources	Species	n. isolates	Similarity level	Biotypes (n. of strains)				
Raw milk	*E. casseliflavus*	5	90.3%	CS1 (5)				
	E. durans	2	86.2%	DR1 (1)	DR2 (1)			
	E. faecalis	106	25.0%	FC1 (2)	FC2 (3)	FC3 (3)	**FC4** (38)	FC5 (19)
				FC6 (6)	FC7 (5)	FC8 (11)	FC9 (6)	FC10 (4)
				FC11 (4)	FC12 (1)	FC13 (3)	FC14 (1)	
	E. faecium	33	54.5%	FU1 (1)	FU2 (1)	FU3 (5)	FU4 (4)	FU5 (2)
				FU6 (1)	FU7 (2)	FU8 (4)	FU9 (4)	FU10 (1)
				FU11 (4)	FU12 (1)	FU13 (2)	FU14 (1)	
	E. gallinarum	2	84.5%	GA1 (1)	GA2 (1)			
	E. gilvus	1	–	GL1 (1)				
	E. hirae	2	27.7%	HR1 (2)				
	E. lactis	22	59.8%	LC1 (6)	LC2 (1)	LC3 (8)	LC4 (1)	LC5 (3)
				LC6 (1)	LC7 (1)	LC8 (1)		
	E. malodoratus	4	77.1%	ML1 (1)	ML2 (3)			
Cow feces	*E. faecalis*	1	–	FC15 (1)				
	E. faecium	112	32.9%	FU15 (2)	FU16 (3)	FU17 (10)	FU18 (1)	FU19 (1)
				FU20 (1)	FU21 (1)	FU22 (1)	FU23 (3)	FU24 (6)
				FU25 (7)	**FU26** (16)	**FU27** (16)	FU28 (2)	FU29 (2)
				FU30 (3)	FU31 (5)	FU32 (1)	FU33 (1)	FU34 (1)
				FU35 (1)	FU36 (1)	**FU37** (5)	FU38 (1)	**FU39** (9)
				FU40 (2)	FU41 (1)	FU42 (2)	FU43 (1)	FU44 (1)
				FU45 (5)				
	E. hirae	20	52.7%	HR2 (3)	HR3 (1)	HR4 (5)	**HR5** (10)	HR6 (1)
	Total	310						

Table 3
Virulence factors genes in *Enterococcus* biotypes isolated from raw milk and feces.

Sources	Species	n. biotypes	Genes	N.of biotypes (%)
Raw milk	*E. casseliflavus*	1	–	–
	E. durans	2	–	–
	E. faecalis	14	*esp-gelE*	13 (92.8)
			esp-asa1	1 (7.2)
	E. faecium	14	–	–
	E. gallinarum	2	–	–
	E. gilvus	1	–	–
	E. hirae	1	*esp-gelE*	1 (100)
	E. lactis	8	–	–
	E. malodoratus	2	–	–
Cow feces	*E. faecalis*	1	–	–
	E. faecium	31	–	–
	E. hirae	5	–	–

None of the biotypes tested harboured the *hyl*, *cylA* and *ace* genes.
- trait not detected.

Substantially different AR profiles were observed among the *Enterococcus* isolated from cow feces where linezolid (45.9%), quinupristin/dalfopristin (36.1%), tigecycline (32.4%) and erythromycin (29.7%) were the most prevalent AR phenotypes detected. Low-level resistances to chloramphenicol (16.2%), streptomycin (8.1%) and tetracycline (8.1%) were observed in strains isolated from feces (Table 4).

In milk, a high proportion of *E. faecalis* (14/14), *E. faecium* (13/14) and *E. lactis* (8/8) biotypes were found to be high-level streptomycin-resistant (HLSR) showing MIC values higher than 1024 μg/mL, while only 5 out of 31 *E. faecium* isolated from feces showed resistance to streptomycin. Thirteen *E. faecalis* biotypes (92.8%) isolated from milk were simultaneously resistant to tetracycline and tigecycline, while *E. faecium* and *E. lactis* showed high percentages of tetracycline (85.7% and 87.5%) and erythromycin (71.4% and 100%) resistant strains (Table 5). Resistance to tetracycline was conferred by the *tetM* and *tetL* genes; *tetM* gene was detected in all *E. faecalis* resistant biotypes while *E. faecium* and *E. lactis* showed the presence of *tetL* and *tetM* in combination. The Tn*916*/Tn*1545* family transposons (*int* gene) were detected in *E. faecalis* (6 biotypes) and the only *E. malodoratus* biotype that harboured the *tetM* gene. Erythromycin resistance was always associated with the presence of the *ermB* gene (Table 6).

Quinupristin/dalfopristin-resistant biotypes were noted within the *E. durans*, *E. faecium* and *E. malodoratus* species and were conferred by *vatD* gene (Tables 5 and 6). A high percentage of *E. faecalis* biotypes (92.8%) showed phenotypic resistance to tigecycline.

Resistance to three or more antimicrobials is generally defined as multidrug resistance (MDR). A high percentage of milk biotypes (71.1%) showed MDR and about the 33.3% these were simultaneously resistant to three different antibiotics. The most common resistance phenotypes detected were S-TE-TGC, E-QD-ST-TE and E-ST-TE. A lower MDR percentage was observed in fecal enterococci (24.3%) and 5 strains (13.5%) showed resistance to three antibiotics (Fig. 2).

Lizenolid-resistant biotypes were noted also within the *E. hirae* species (80%). Interesting to notice that the six *vatD* positive strains showed the higher quinupristin/dalfopristin MIC values (≥4 μg/mL) (Tables 5 and 6). Among the enterococcal strains no *vanA* or *vanB* genes were identified.

Eleven out of 31 (35,5 %) and 10 out of 14 (71,4) *E. faecium* strains respectively from fecal and milk biotypes showed resistance to erythromycin that was always associated to the *ermB* gene. Moreover, as described for milk isolates, also in the two *E. faecium* from feces the tetracycline resistance was linked to *tetL-tetM* genes. The *int* gene was detected in *E. hirae* HR2 that harboured the *tetM* gene (Table 6).

3.4. Tetracycline resistance genes transfer

To examine the *tetM* transfer from the *Enterococcus* biotypes to the recipient strain *Lb. delbrueckii* subsp. *lactis* VC107, filter matings technique was applied. *E. faecalis* FC1 and FC5, *E. malodoratus* ML2 and *E. hirae* HR2 were selected as donors since they were the only strains showing tetracycline resistance and harbouring the *tetM* and *int* genes. Only the *E. faecalis* biotypes were able to successfully transfer their resistance determinant *tetM* to the recipient strain, and the frequency from donors to *Lb. delbrueckii* subsp. *lactis* ranged from 10^3 to 10^6 transconjugants per recipient (Table 7).

Molecular analysis of the phenotypically tetracycline-resistant transconjugants showed that all these strains harbored *tetM* gene, while Tn*916-1545* transposon was found only in transconjugants from *E. faecalis* FC5 donor biotype (Table 7). RAPD-PCR analysis excluded the

Table 4
Antibiotic resistance profile of *Enterococcus* biotypes isolated from raw milk and feces.

Antibiotic	Resistance breakpoint	References	Sources (n. biotypes)	MIC (μg/mL) min	MIC (μg/mL) max	Susceptible (n.)	Intermediate (n.)	Resistant (n.)	Resistant (%)
Ampicillin	≥16 μg/mL	CLSI (2021)	Raw milk (45)	0.19	2	41	4	–	0
			Feces (37)	0.094	3	37	–	–	0
Chloramphenicol	≥32 μg/mL	CLSI (2021)	Raw milk (45)	1.5	48	4	40	1	2.9
			Feces (37)	2	>256	6	25	6	16.2
Daptomycin	≥8 μg/mL	CLSI (2021)	Raw milk (45)	0.125	2	45	–	–	0
			Feces (37)	0.19	4	36	1	–	0
Erythromycin	≥8 μg/mL	CLSI (2021)	Raw milk (45)	0.125	>256	6	18	21	46.6
			Feces (37)	0.38	>256	8	18	11	29.7
Gentamicin	≥500 μg/mL	CLSI (2021)	Raw milk (45)	6	48	45	–	–	0
			Feces (37)	6	256	37	–	–	0
Linezolid	≥8 μg/mL	CLSI (2021)	Raw milk (45)	0.5	12	9	30	6	13.3
			Feces (37)	0.5	16	6	14	17	45.9
Quinupristin/ Dalfopristin	≥4 μg/mL	CLSI (2021)	Raw milk (31)	0.75	6	3	19	9	29.0
			Feces (36)	0.38	12	4	19	13	36.1
Streptomycin	≥1024 μg/mL	CLSI (2021)	Raw milk (45)	96	>1024	6	–	39	86.7
			Feces (37)	8	>1024	32	1	5	8.1
Teicoplanin	≥32 μg/mL	CLSI (2021)	Raw milk (45)	1	3	45	–	–	0
			Feces (37)	0.5	3	37	–	–	0
Tetracycline	≥16 μg/mL	CLSI (2021)	Raw milk (45)	0.38	24	6	6	33	73.3
			Feces (37)	0.064	24	28	6	3	8.1
Tigecycline	>0.25 μg/mL	Eucast (2021)	Raw milk (45)	0.064	>256	30	–	15	33.3
			Feces (37)	0.047	3	25	–	12	32.4
Vancomycin	≥32 μg/mL	CLSI (2021)	Raw milk (45)	0.25	3	47	–	–	0
			Feces (37)	0.064	1.5	37	–	–	0

Quinupristin/dafopristin resistance was not evaluated against the *E. faecalis* strains, since these bacteria are intrinsically resistant to this antibiotic.

Table 5
Antibiotic resistance in enterococci species recovered in raw milk and feces. In bracket the percentage of resistant biotypes.

Species	n. biotypes	C	E	LNZ	QD	ST	TE	TGC
Raw milk								
E. casseliflavus	1	S	S	S	S	S	S	S
E. durans	2	S	2 (100)	S	1 (50)	2 (100)	S	S
E. faecalis	14	S	1 (7.1)	3 (21.4)	(*)	14 (100)	13 (92.8)	13 (92.8)
E. faecium	14	S	10 (71.4)	1 (7.1)	6 (42.8)	13 (92.8)	12 (85.7)	S
E. gallinarum	2	S	S	S	S	S	S	S
E. gilvus	1	S	S	1 (100)	S	S	S	1 (100)
E. hirae	1	1 (100)	1 (100)	1 (100)	S	S	S	S
E. lactis	8	S	8 (100)	S	S	8 (100)	7 (87.5)	S
E. malodoratus	2	S	S	S	2 (100)	2 (100)	1 (50)	S
Feces								
E. faecalis	1	S	S	1 (100)	(*)	S	S	S
E. faecium	31	4 (12.9)	11 (35.4)	12 (38.7)	11 (35.4)	5 (16.1)	2 (6.4)	10 (32.2)
E. hirae	5	2 (40)	S	4 (80)	2 (40)	S	1 (20)	2 (40)

C: Chloramphenicol; E: Erythtomycin; LNZ: Linezolid; QD: Quinupristin/Dalfopristin; ST: Streptomycin; TE: Tetracycline; TGC: Tigecycline.
S: susceptible; (*): Intrinsic resistance.

presence of mutants within the donors and transconjugants isolates (data not shown). Transconjugants were grown in MRS broth without tetracycline and tested for the presence of *tetM* to confirm that these isolates were true transconjugants and not reverted mutants. The *tetM* gene was stably maintained in all transconjugants in the absence of selective pressure.

4. Discussion

Enterococci represent a significant share of the culturable microbiota of milk and cow feces (Kagkli et al., 2007; Quigley et al., 2013).

Although by the necessity of organization the milk samples were stored frozen, and it is known a possible effect of freezing that can reduce the bacterial load up to 1 to 2 logs with greater reduction occurring in Gram negative bacteria (Hubáčková and Ryšánek, 2007; Alrabadi 2015), our results clearly showed that the lactation stage may influence the *Enterococcus* content in milk, in fact, we observed an increase of their content from the colostrum to the mature milk (35 days). The lowest load of enterococci in milk samples at the calving time could be related with the strong antimicrobial activity of bovine colostrum that could hamper the growth of Gram-positive and Gram-negative bacteria (Bagwe-Parab et al., 2020; Costa et al., 2023). To date, no data are available on the content of these microorganisms with regard to cow lactation stage, but similar results were obtained by Khodayar-Pardo et al. (2014) in breast milk.

Differences in enterococcal community structure were observed between the milk and feces samples. Similar results were reported by McAuley et al. (2015) and Gelsomino et al. (2001) that evaluated the impact of seasonality and cheese-making environment on growth of enterococci in raw milk and cow feces. In contrast, Zaheer et al. (2020) found that *E. hirae* was the most frequently enterococcal species isolated in bovine feces.

RAPD-PCR analysis allowed us to detect a high number of *Enterococcus* biotypes and none of the strains present in raw milk exhibited

Table 6
Antibiotic resistance genes in *Enterococcus* biotypes isolated from raw milk and feces.

Antibiotic	Sources	Species	AR biotypes	Genes	N.of biotypes (%)
Erythromycin	Raw milk	*E. durans*	2	*ermB*	2 (100)
		E. faecalis	1	–	–
		E. faecium	10	*ermB*	10 (100)
		E. hirae	1	*ermB*	1 (100)
		E. lactis	8	*ermB*	8 (100)
	Feces	*E. faecium*	11	*ermB*	11 (100)
Quinupristin/ Dalfopristin	Raw milk	*E. durans*	1	*vatD*	1 (100)
		E. faecalis	14	(*)	(*)
		E. faecium	6	*vatD*	5 (83.3)
		E. malodoratus	2	*vatD*	2 (100)
	Feces	*E. faecalis*	1	(*)	(*)
		E. faecium	11	*vatD*	4 (36.4)
		E. hirae	2	*vatD*	2 (100)
Tetracycline	Raw milk	*E. faecalis*	13	*tetM*	7 (53.8)
				tetM-int	6 (46.2)
		E. faecium	12	*tetL-tetM*	11 (91.7)
		E. lactis	7	*tetL-tetM*	7 (100)
		E. malodoratus	1	*tetM-int*	1 (100)
	Feces	*E. faecium*	2	*tetL-tetM*	1 (50)
		E. hirae	1	*tetM-int*	1 (100)

None of the biotypes tested harboured the *tetK*, *tetS* and *vatM* genes.
(*): has not been analyzed due to intrinsic resistance.
-: trait not detected.

genetic profiles similar to those from cow feces. These results suggest that the bovine feces are not a source of enterococci for raw milk, confirming the few data available in the literature (Gelsomino et al., 2001; Kagkli et al., 2007). Gelsomino et al. (2002) and Ortigosa et al. (2008) supposed that these organisms may enter milk by the milking equipment (teat cups) and the presence of genes encoding for biofilm formation (*esp*, *asa1* and *gelE*) detected in *E. facalis* biotypes could support this hypothesis (Chajęcka-Wierzchowska et al., 2017). The study of different biotypes in the milk and feces of individual animals revealed the persistence of specific matrix-dependent strains over time, nevertheless the question regarding the genesis of the *Enterococcus* community in raw milk remains largely unanswered.

As suggested by EFSA, all enterococcal biotypes were characterized for their susceptibility to twelve different antibiotics. It is remarkable to underline that in our research no vancomycin-resistant enterococci (VRE) were detected. The low spread of VRE in dairy sector was supported by the observations of previous studies (Morandi et al., 2006; Kagkli et al., 2007; Silvetti et al., 2019). Vancomycin was introduced in

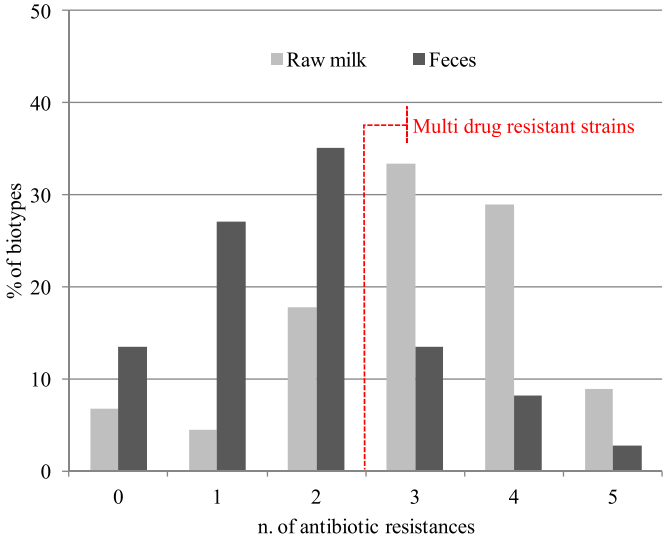

Fig. 2. Multidrug resistant biotypes (%) isolated from raw milk and feces samples.

the 1956 as a possible treatment of infection caused by multidrug-resistant *Enterococcus* strains. This antibiotic resulted to be active against *E. faecalis* and *E. faecium* until the 1986 when VRE strains were isolated in England (García-Solache and Rice, 2019). The rise of VRE in the EU led to the sanction of a glycopeptide (avoparcin) used as growth promoter in animal production in 1997. In the post-ban years, the prevalence of VRE in animal-based foods has been steadily declining (Casewell et al., 2003; Ortigosa et al., 2008). Nevertheless, low percentages of VRE were recently detected in dairy products from Poland and Portugal (Chajęcka-Wierzchowska et al., 2020; Rocha et al., 2022).

Markly different AR profiles were observed among the *Enterococcus* biotypes isolated from milk and feces samples. A high percentage of enterococci isolated from individual milk were identified as HLSR. HLSR detection is of particular importance since aminoglycosides are used in combination with β-lactams against enterococcal infections. Resistance to clinically achievable concentrations of aminoglycosides has been attributed to the poor penetration of these agents through the enterococcal cell envelope related to the presence of the *ant-6* gene or to ribosomal mutations (García-Solache and Rice, 2019).

As reported by many authors, tetracycline and erythromycin resistances are extensively widespread in the dairy environment (Flórez et al., 2021). Their diffusion was attributed to the overexploitation of these agents as growth promoters in veterinary practices (Teuber, 2001). In our study tetracycline and erythromycin resistant strains harboured *tetM*, *tetL* and *ermB* genes as previously reported (Jamet et al., 2012; Chajęcka-Wierzchowska et al., 2020). It is interesting to note that only one *E. faecalis* biotype was susceptible to tigecycline. This antibiotic is a next-generation tetracycline and represents the final line of defense against severe infections by VRE and multidrug-resistant pathogens. As described by Fiedler et al. (2016), this AR may be associated with an overexpression of *tetL* and *tetM* genes.

A high percentage of MDR was found among the milk biotypes. Multiple ARs may be a consequence of the pressure exerted by intramammary antibiotic administration that leads to a selection/development of AR bacteria within the milk microbiota (Oikonomou et al., 2020).

A different AR pattern was observed among the *Enterococcus* biotypes from bovine feces. The most presence of linezolid-resistant biotypes is concerning but not unexpected. This antibiotic was introduced in the 2000 years and is usually used in the treatment of important infections caused by multidrug-resistant Gram-positive bacteria. Linezolid

Table 7
Conjugal transfer frequencies obtained with the plate mating protocol in enterococci biotypes from raw milk and feces samples (data expressed in CFU/mL).

Mating biotypes	MIC	Donor	Recipient	Transconjugant	Transfert Rate	TET genes	
	(μg/mL)	(D)	(R)	(TC)	(TC/R)	D	TC
E. faecalis							
FC1	16	6.40×10^{8}	4.90×10^{8}	2.47×10^{3}	5.04×10^{-6}	*tetM-int*	*tetM*
FC5	24	1.47×10^{9}	4.90×10^{8}	2.30×10^{5}	4.69×10^{-3}	*tetM-int*	*tetM-int*
E. malodoratus							
ML2	16	2.50×10^{8}	1.32×10^{9}	–	–	*tetM-int*	-
E. hirae							
HR2	16	1.10×10^{7}	2.28×10^{9}	–	–	*tetM-int*	-

resistance in enterococci can be attributable to mutations in the rRNA genes or by the acquisition of the *cfr* or *cfrB* genes (García-Solache and Rice, 2019). As described by Zarzecka et al. (2022a), the number of linezolid-resistant enterococci is increasing over time and our results confirmed this growing trend.

In vitro filter conjugation trials showed that both *E. faecalis* biotypes were able to transfer horizontally the tetracycline resistance gene *tetM*. Transfer frequencies ranged from 10^3 to 10^6 transconjugants per recipient suggesting that this ability is a strain-dependent feature. As previously observed by Rizzotti et al. (2009), the *tetM* gene is frequently transferred in mating experiments from *Enterococcus* strains to other bacteria and this ability can be explained by the presence of a Tn*916-1545* transposon. On the contrary, under the conditions of the present study, no transfer of *tetM* from *E. malodoratus* and *E. hirae* was seen. The failure in *tetM* transfer observed in strains that harboured the *int* gene could be related to nucleotide changes and mutations in Tn*916* sequence (Sirichoat et al., 2020) or to the presence of non-conjugative plasmids in their genetic material (Zarzecka et al., 2022b). Our results suggests that the ability or the failure of the AR genes transfer is a strain-dependent feature, and the conjugation system of the resistant bacteria may depend on many environmental factors. Despite that, the ability of the enterococci isolated from milk samples to transfer the *tet* genes represents an undesirable phenomenon since they contribute to the spread of AR within the dairy environment.

5. Conclusion

This study allows to gain better knowledge of the raw milk and bovine feces enterococcal communities. Our results indicated that milk and feces enterococci belong to different niches, and therefore, cow feces do not represent a source and a potential AR enterococci reservoir for the milk microbiota. The presence of the same strain in milk from different animals, together with the presence of genes encoding for proteins that promote biofilm development, supports the hypothesis that farm surfaces i.e., the milking equipment may be a primary source of enterococci for milk. The high percentage of the MDR detected among the milk biotypes and their ability to transfer the AR genes through dairy products should not be neglected. It is remarkable to underline the absence of VRE from milk and feces samples that confirms the low dissemination of vancomycin resistant strains in European dairy environment, on the other hand the presence of one strain resistant to tigecycline demands attention. Based on our knowledge, the present study represents the first investigation carried out in Italy regarding the relationship among the origin, biotype and AR of enterococci isolated from milk and bovine feces.

CRediT authorship contribution statement

Stefano Morandi: Formal analysis, Investigation, Validation, Writing – original draft. **Tiziana Silvetti:** Investigation, Writing – review & editing. **Vincenzo Lopreiato:** Conceptualization, Writing – review & editing. **Fiorenzo Piccioli-Cappelli:** Conceptualization, Writing – review & editing. **Erminio Trevisi:** Conceptualization, Supervision, Writing – review & editing. **Milena Brasca:** Conceptualization, Funding acquisition, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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