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Evolution and antimicrobial resistance of enterococci isolated from Pecorino and goat cheese manufactured on-farm in an area facing constraints as per EU Regulation 1305/2013 in Umbria, Italy.

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Abstract: The latest EU regulation on geographical indications (EU Regulation No. 1151/2012) has introduced a set of new tools for the protection and enhancement of food products in rural areas, under the group name of optional quality term (OQT). The Commission Delegated EU Regulation, No. 665/2014, regulated the conditions for the use of the optional quality term «mountain product» (MP), to support the implementation of a mountain value chain. This new tool is aimed at promoting local development, maintaining the economic activities in mountain areas and redistributing wealth, whilst, at the same time, promoting the territory. Pecorino and goat cheeses are typical Italian cheeses made usually with whole raw ewe's or raw goat's milk, without starter culture addition. In an attempt to characterize these productions, the aim of this study was to investigate the evolution of enterococci during the production and ripening of Pecorino cheese made in three different farms, located in Umbria, Italy in areas facing natural or other specific constraints as stipulated by Regulation 1305/2013 on support for rural development by the European Agricultural Fund for Rural Development (EAFRD). Enterococci are enteric organisms which are commonly isolated from ewe and goat's milk production in Umbria, Italy. Counts of enterococci in raw milk ranged from 1.75 for ovine milk to 3.62 for ewe milk and a marked reduction was observed after thermization especially in ovine milk. Out of 100 isolates, 69 were *E. faecium*, 23 *E. durans*, 8 *E. faecalis* and 2 *E. casseliflavus* and the distribution of species between farms and between samples showed a prevalence of *E. faecium* in ovine farms and *E. durans* in ewes farms, with an equal distribution between samples. High percentages of susceptible isolates were found for amoxicillin/clavulanic acid, ampicillin, chloramphenicol, sulphamethoxazole, sulphamethoxazole/trimethoprim, ticarcillin, vancomycin. A high prevalence of resistant strains (> 30%) was observed for amikacin, ciprofloxacin, ceftriaxone, kanamycin, tetracycline. A comparison of this results with those of previous works on similar dairy products revealed high levels of resistance to antimicrobials which needs to be addressed.

Keywords: Enterococcus, QPS, GRAS, safety, milk, cheese

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1. Introduction

One of the roles the EU covers is that of creating an equilibrium amongst the dimensions of governance, embedding, and marketing, in order to define policies and strategies to manage productive activities. One such activity is to define strategic choices for the promotion of European foodstuffs. In this context, the latest EU regulation on geographical indications (EU Regulation No. 1151/2012) has introduced a set of new tools for the protection and enhancement of food products in rural areas, under the group name of optional quality term (OQT). The Commission Delegated EU Regulation, No. 665/2014, regulated the conditions for the use of the optional quality term «*mountain product*» (MP), to support the implementation of a mountain value chain. This new tool aimed at promoting local development, maintaining the economic activities in mountain areas and redistributing wealth, whilst, at the same time, promoting the territory [1]. Umbria, Italy is a region with several areas facing natural or other specific constraints as stipulated by Regulation 1305/2013 on support for rural development by the European Agricultural Fund for Rural Development (EAFRD).

Pecorino and goat cheeses are typical cheeses produced in these areas, made usually with whole raw ewe's or raw goat's milk, without starter culture addition. Thus, only bacteria from milk contribute to ripening changes in the cheese [2]. Enterococci represent the predominant microbiota of these productions [2-7].

Enterococci are gram-positive bacteria and may fit within the general definition of lactic acid bacteria. With regard to safety and according to the Qualified Presumption of Safety (QPS) list from the European Food Safety Authority (EFSA) (https://www.efsa.europa.eu/en/topics/topic/qps), *Enterococcus* species are neither recommended for the QPS list [8] nor have GRAS (Generally Regarded As Safe) status [9], in spite of recent scientific knowledge allowing differentiation of commensal from pathogenic strains [10-12]. Modern classification techniques resulted, back in 1980s, in the transfer of some members of the genus Streptococcus, notably some of the Lancefield's group D streptococci, to the new genus Enterococcus [13]. Enterococci can be used as indicators of faecal contamination and have been implicated in outbreaks of foodborne illness. On the other hand, they have been ascribed a beneficial or detrimental role in foods [10-12]. In processed meats, enterococci may survive heat processing and cause spoilage, though in certain cheeses the growth of enterococci contributes to ripening and development of product flavour [2,10]. Some enterococci of food origin produce bacteriocins that exert anti-Listeria activity [14]. Enterococci are used as probiotics to improve the microbial balance of the intestine, or as a treatment for gastroenteritis in humans and animals [11,12,15,16]. On the other hand, enterococci have become recognized as serious nosocomial pathogens causing bacteraemia, endocarditis, urinary tract and other infections. This is in part explained by the resistance of some of these bacteria to most antibiotics that are currently in use [10]. Resistance is acquired by gene transfer systems, such as conjugative or nonconjugative plasmids or transposons. It appears that foods could be a source of vancomycin-resistant enterococci [11,17,18].

Regardless these considerations Enterococci are commonly found in milk and cheese [19] and there are two divergent opinions about the presence of enterococci in cheese. One is that enterococci should be considered more suitable than other groups commonly used as indicators of unhygienic procedures in food processing and handling (e.g. Enterobacteriaceae). This is related to their high heat resistance and salt tolerance [20]. The other opinion is that enterococci have a possible contribution to the ripening of cheese due to their lipolytic, proteolytic and caseinolytic activities [21]. Moreover it has been suggested that enterococci contribute to flavour due to the attitude of produce acetoin, diacetyl and acetaldehyde [21].

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In an attempt to characterize cheese production from a mountain area in Umbria, Italy, and define safety aspects, the aim of this work was to investigate the evolution of enterococci during the production and ripening of Pecorino cheese made in an area facing natural and specific constraints as per EU Regulation 1305/2013, with two different cheesemaking processes from sheep and ewe milk and characterize *Enterococcus* spp. isolates for antibiotic susceptibility all along the cheesemaking and ripening process.

2. Materials and Methods

2.1 Study area

The three farms are located in Umbria, Italy in an area that extends from latitude 42.977715°N to 43.227086°N and from longitude 12.141079°E to 12.310128°E (Figure 1). The area, partly hilly and mountainous and partly flat and fertile owing to the valley of the Tiber river, is separated from the central Apennines by the Tiber Valley ("Val Tiberina"). The thee farms are located in areas facing natural or other specific constraints as stipulated by Regulation 1305/2013 on support for rural development by the European Agricultural Fund for Rural Development (EAFRD). For the three farms, according to the cited Commission Delegated EU Regulation, No. 665/2014, most of the conditions for the use of the optional quality term «*mountain product*» (MP), to support the implementation of a mountain value chain, are present and might be the base for a regional development for rural areas.

2.2 General

The experiment was carried out on three farms in Umbria, farm A, has 500 Sardinian ewes, farm B has 200 Sardinian ewes, farm C has 80 Saanen goats. In farm A and B milk is collected with a milking machine and automatically filtered before the cheesemaking. In farm C goats are hand milked and the milk is filtered through a linen cloth.

Milk from each farm was collected, analysed and subsequently used to produce Pecorino or goat cheese. On each farm two different cheesemaking processes were carried out from raw milk and from heat-treated milk to which the autochthonous cultures were added as a starter. Bacterial strains used in the formulation were: *Lactococcus lactis* ssp. lactis, reference strain n. 340; *L. lactis* ssp. lactis, strain n. 16; *Lactobacillus casei* ssp. casei, strain n. 208. The morphological, biochemical and physiological characterization, the growth curves at several temperatures, including refrigeration conditions, the acidifying activity and their ability to improve palatability of cheeses have been reported by the authors in previous papers [15,22-26].

Each cheesemaking was replicated three times. According to this experimental procedure there were 18 manufacturing processes: 9 from raw milk and 9 from heat-treated milk.

2.3 Cheese manufacturing

Cheese was made from ewes and goats milk obtained from both evening and morning milking. The main steps of cheesemaking process were: milk coagulation in a tinned copper vat occurred at 37°C within about 30' by adding liquid calf rennet (Lima, Perugia, Italy, titre 1:10.000). The curd was cut into nut-sized granules (10 to 20 mm), then stirring for 5 minutes, heated at 42-43°. After a pause of 5 to 10 minutes the curd was put into frames (20 cm diameter by 8 cm high), pressed by hand for a few seconds, drained for 18 to 20 hours and salted in brine (20% NaCl w/v, at 12-15°C for 30 h). The cheeses were ripened in non-conditioned storage rooms at 12-15°C and 83-87% RH for 60 days (40 days for goat cheeses).

Heat treatment (where applied) included a heating step before the rennet addition. Raw milk was heated to 65-66 °C for 3-4 min in a double wall stainless steel vat. The thermic cycle 55-65-55°C (during heating and cooling) took about 14 min. After further cooling to 42 °C, the starter was added at 42°C at a final concentration of 2% (as a full-coagulated 24h culture in sterile milk)

2.4 Sampling

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The following samples were taken on each farm: raw milk, heat-treated milk (where applied), curd, 7 days cheese, 30 days cheese, full-ripened cheese. Triplicate samples were collected, transported to the laboratory in chilled containers and analysed on the same day. Sampling was made according to ISO 5538:2004 [27].

2.5 Bacterial counts

The following groups were evaluated:

- total viable count: pour plates of Plate Count Agar (Difco, Detroit, Mi, USA), were incubated at 30°C for 72 h; all colonies were counted;

- Enterobacteriaceae: pour plates of Violet Red Bile Agar (Difco) were incubated at 32°C for 48h; all pink to red colonies, irrespective of diameter or presence/absence of zone of precipitation were counted;

- enterococci: surface-inoculated plates of Barnes Agar (Biolife, Milano, Italy) were incubated at 44°C for 72 h; all pink, red or maroon colonies, irrespective of diameter, were counted.

2.6 Isolation and identification of enterococci

Two to five colonies from each sample were sub-cultured from Barnes medium into brain heart infusion broth (BHI, Difco) at 37°C for 24h and then tested for the following characteristics: cell morphology after Gram staining, presence of catalase, growth in bile-esculin-azide agar (Coccosel agar, BioMérieux, Marcy-l'Etoile, France) at 37°C, growth in 6.5% NaCl BHI agar (Difco) at 37°C, growth in the presence of 4% bile salts (Coccosel agar, Biolife, with added 4% bile salts) at 37°C, haemolysis type on tryptic soy agar (Biolife) to which 5% of ram blood was added, at 37°C.

Complementary biochemical tests were performed on colonies grown on blood agar using the API 20 STREP (BioMérieux). Computer program Apiweb (BioMérieux) was used for the results.

2.7 Antibiotics susceptibility

Enterococcus spp. isolates were tested for antimicrobial susceptibility against a panel of 12 antimicrobials by the disk diffusion method (Kirby Bauer Test) as described by the Clinical and Laboratory Standards Institute [28] The following antimicrobials were tested: amikacin 30 mg, amoxicillin/clavulanic acid 30 (20 + 10) mg, ampicillin 10 µg, ceftriaxone 30 µg, chloramphenicol 30 µg, ciprofloxacin 5 µg, kanamycin 30 µg, sulphamethoxazole 25 µg, sulphamethoxazole/trimethoprim 25 µg, tetracycline 30 µg, ticarcillin 75 µg, vancomycin 30 µg. This antimicrobial panel was selected to test the major groups of antimicrobials. Briefly, frozen isolates were thawed and cultured in BHI broth (Bio-Rad) at 35 to 37°C for 24 h. A portion of the culture broth was inoculated into 6 mL of 0.9% sterile physiological saline solution until a turbidity of 0.5 McFarland was reached (1.0 for vancomycin [29]). Using a sterile swab, the solution was spread on Muller-Hinton agar plates (Oxoid). Antimicrobial disks (Oxoid) were placed on Muller-Hinton agar plates which were incubated at 37°C for 18 to 24 h. At the end of incubation, the diameters of the growth inhibitory zones were measured, and these were interpreted using specific CLSI tables whereby the bacterium is classified as susceptible, intermediately susceptible or resistant [28].

3. Results and discussion

Results of the determination made on Pecorino and goat cheese during manufacture and ripening are given in tables 1-2-3-4-5-6.

Changes in total viable counts and Enterobacteriaceae.

The differences observed on the three farms comprised a range of variability which is common to on-farm cheesemaking (Table 1).

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Table 1. Evolution of microbiota during production and ripening of farm manufactured cheeses (log cfu ml^{-1} or g^{-1}, sd: standard deviation.

	Aerobic viable count		Enterobacteriaceae		*Enterococcus* spp.	
Farm A – raw milk	mean	sd	mean	sd	mean	sd
raw milk	5.9	0.34	3.93	0.42	2.74	2.81
curd	7.42	0.23	4.41	0.34	3.47	3.08
1 week	10.04	0.79	5.76	0.21	5.26	2.74
1 month	8.7	0.14	1.69	2.93	4.42	1.95
2 months	7.1	0.64	0.00	0.00	3.66	1.1
Farm A – heat treated milk						
raw milk	5.9	0.34	3.93	0.42	2.74	2.81
heat treated milk	3.06	0.25	0.00	0.00	0.33	0.58
curd	7.58	0.89	3.33	0.64	4.6	1.8
1 week	8.56	2.03	3.88	3.4	5.54	2.92
1 month	9.42	0.4	1.37	2.37	6.27	1.5
2 months	9.13	0.57	0.00	0.00	4.29	1.12
Farm B – raw milk						
raw milk	6.01	0.31	4.7	0.36	1.75	3.03
curd	7.19	0.66	5.34	1.49	4.72	0.59
1 week	9.13	0.38	5.3	0.45	5.74	1.1
1 month	8.19	0.44	1.78	0.88	5.33	1.01
2 months	7.44	0.85	0.00	0.00	3.66	1.1
Farm B – heat treated milk						
raw milk	6.01	0.31	4.7	0.36	1.75	3.03
heat treated milk	4.56	0.91	0.48	0.83	1.79	1.72
curd	8.15	0.46	4.61	1.35	5.92	0.86
1 week	10	0.44	5.86	0.49	6.27	0.33
1 month	8.01	0.42	3.15	1.32	4.67	0.75
2 months	8.34	0.22	0.00	0.00	5.14	0.36
Farm C – raw milk						
raw milk	7.54	0.36	3.63	0.85	3.62	1.11
curd	8.74	0.31	5.15	0.11	5.37	1.03
1 week	9.46	0.00	4.91	0.27	5.77	0.79
1 month	7.81	0.27	1.81	1.57	5.35	1.24
2 months	7.97	0.12	1.63	1.45	4.41	0.46
Farm C – heat treated milk						
raw milk	7.54	0.36	3.63	0.85	3.62	1.11
heat treated milk	4.91	0.09	0.72	0.72	2.47	0.04
curd	8.42	0.05	3.81	0.53	6.22	1.18
1 week	9.72	0.01	5.92	0.24	6.7	0.59
1 month	8.12	0.27	2.75	1.55	4.36	0.82
2 months	7.99	0.61	0.83	1.43	4.92	0.41

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The total mesophilic aerobes in raw milk ranged from log 5.9 log cfu ml^{-1} in farm A to log 7.54 log cfu ml^{-1} in farm C. At the early stage of production of cheeses made with raw milk, counts increased up to 1 week and then decreased. In the cheesemaking processes from heat-treated milk added with autochthonous starter cultures bacterial population in milk, after the treatment, was reduced to log 3.06 log cfu ml^{-1} in farm A, log 4.56 log cfu ml^{-1} in farm B and log 4.91 log cfu ml^{-1} in farm C. After the treatment counts showed the same evolution described for cheeses made with raw milk.

Enterobacteriaceae in raw milk ranged from 3.9 log cfu ml^{-1} in farm A to 3.9 log cfu ml^{-1} in farm B. In cheeses made with raw milk Enterobacteriaceae increased up to curd production and then decreased being not detectable or at low concentration at the end of the ripening. On farm B and C bacterial reduction was observed on 1-week cheese whilst on farm A an additional growth occurred in cheese during the first week of ripening, after which there was a decrease in population. Enterobacteriaceae were not detectable in full-ripened cheese made with raw ewe's milk whilst they were detected from full-ripened cheese made with raw goat's milk. In cheeses made with heat-treated milk with added autochthonous starter cultures, Enterobacteriaceae underwent great reduction in heat-treated milk being not detectable in farm A, whilst they were only partially reduced in farm B and C (4.22 log of reduction in farm B and 2.91 in farm C). Although heavy curd recontamination always occurred, no Enterobacteriaceae were detectable in full-ripened cheese made with heat-treated ewe's milk, whilst they were still detected in cheeses made with heat-treated goat's milk.

Samelis, *et al.* [30] studied the changes in microbial composition of raw milk induced by thermization and demonstrated that the treatment significantly reduced bacterial populations of raw milk. Although the 67°C treatment was much more effective against all bacterial groups than was the 60°C treatment, the latter had major inactivation effects against gram-negative bacteria but moderate effects against gram-positive bacteria. Reductions in populations of thermophilic LAB, including enterococci, in samples thermized at 60°C were not significant.

3.1 Behaviour of enterococci

Enterococci counts in milk were always lower than 4 log cfu ml^{-1}. The changes observed were similar to those observed previously [2]. At the early stage of production of cheeses made with raw milk, counts increased up to 1 week and then slightly decreased. In the cheesemaking processes from heat-treated milk with added autochthonous starter cultures no evident reduction of enterococci population was observed after the treatment. After the heat treatment counts showed the same behaviour described for cheeses made with raw milk.

Other authors have studied *Enterococcus* spp. populations in pecorino and other traditional cheeses. For instance Serio, et al. [7] found that the presence of enterococci in Pecorino Abruzzese ranged from log 4 cfu g^{-1} to log 4 cfu g^{-1} at the beginning of ripening and reached log 9 cfu g^{-1} in ripened cheeses. Similar results were described by Litopoulou-Tzanetaki and Tzanetakis [4] which described enterococci levels in traditional Greek cheeses as ranging from log 6 cfu g^{-1} to log 10 cfu g^{-1} for the different kind of products examined.

Identification

One-hundred strains of enterococci were identified. Forty-six from farm A, thirty-two from farm B and twenty-two from farm C. Sixty-nine were identified as *Enterococcus faecium*, twenty-two as *Enterococcus durans*, seven as *Enterococcus faecalis* and two as *Enterococcus casseliflavus* (Tables 2 and 3). *Enterococcus faecium* was found more frequently on farm A (80.4%) and on farm B (68.8%), whilst on farm C more than 95% of the isolates consisted of *Enterococcus faecium* and *Enterococcus durans* altogether. No strains of *Enterococcus faecalis* were found on Farm A, whilst *Enterococcus casseliflavus* was found only on farm B.

Recently, Russo, et al. [5] described the prevalence distribution of enterococci in Ragusano and Pecorino Siciliano: 35% of the isolate were ascribed to *E. durans*, 35% to *E.*

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faecalis, 28% to *E. faecium*. Overall data from previous studies are quite similar and the prevalence of *E. faecium*/*E. durans*, followed by *E. faecalis* is reported quite constantly [31-33].

Table 2. Distribution of *Enterococcus* spp. per sample.

Species	n.	milk	curd	7-day cheese	30-day cheese	full ripened cheese
E. faecium	69	8 (5)	4 (8)	10 (6)	4 (5)	10 (9)
E. durans	22	2 (-)	1 (4)	3 (4)	2 (-)	5 (1)
E. faecalis	8	- (-)	1 (-)	1 (-)	- (-)	5 (-)
E. casseliflavus	2	- (-)	- (-)	- (-)	- (-)	- (2)
Total	100	10 (5)	6 (12)	14 (10)	6 (5)	20 (12)

( )= cheesemaking processes from heat-treated milk.

Table 3. Distribution of *Enterococcus* spp. per farm.

Species	n.		Farm A		Farm B		Farm C	
	n.	%	n.	%	n.	%	n.	%
E. faecium	69	69	37	80.4	22	68.8	10	45.4
E. durans	22	22	9	19.6	2	6.2	11	50.0
E. faecalis	7	7	-	-	6	18.8	1	4.6
E. casseliflavus	2	2	-	-	2	6.2	-	-
Total	100	100	46	100	32	100	22	100

Bacterial cells under microscopic observation after Gram staining appeared Gram positive, in pairs or in short chains, ovoid elongated in direction of the chain. All strains were catalase negative. This result together with cells morphology confirmed the identification of the strains as belonging to the genus *Streptococcus*. Enterococci were identified as such on the basis of their growth in the presence of bile-esculin, 6.5% of NaCl solution, and 4% bile salts, and on the basis of the hydrolysis of the arginine. TTC reduction on Barnes medium differentiated *E. faecalis* from *E. faecium*. In fact, culture grown in the presence of triphenyl tetrazolium chloride (TTC) on Barnes medium were white or with a red centre and white border. The former was presumptively identified as *E. faecium* and the latter as *E. faecalis* prior to biochemical tests with API 20 Strep (BioMérieux). No strains were ß-haemolytic.

The principal physiological and biochemical characteristics of the isolates are given on Table 4.

Table 4. Principal physiological and biochemical characteristics of enterococci isolated from pecorino and goat cheese at various stages of ripening (figures are numbers of strains).

	E. faecium	*E. durans*	*E. faecalis*	*E. casseliflavus*
β-haemolysis	0	0	0	0
α-haemolysis	0	0	0	0
γ-haemolysis	69	22	7	2
growth in bile-esculin	69	22	7	2
growth in the presence of 6.5% NaCl	69	22	7	2
growth in the presence of 4% bile salts	69	22	7	2
growth in the presence of TTC	69	22	7	2
hydrolysis of arginine	69	22	7	2
hydrolysis of hippurate	34	16	7	2
acetoin production	69	22	7	2
fermentation of:				
mannitol	69	0	7	2
sorbitol	0	0	7	0
raffinose	0	0	0	2
inulin	0	0	0	0
L-arabinose	68	0	0	2
ribose	69	22	7	2
lactose	69	22	7	2
trehalose	69	22	7	2
starch	68	16	7	2
glycogen	0	0	0	0
Total number of strains	69	22	7	2

3.2 Antibiotics susceptibility

The antimicrobial susceptibility test data are shown in Table 5 and 6. High percentages (>80%) of susceptible strains were found for amoxicillin/clavulanic acid, ampicillin, chloramphenicol, sulphamethoxazole, sulphamethoxazole/trimethoprim, ticarcillin, vancomycin. A high prevalence of resistance strains (> 30%) was observed for amikacin, ciprofloxacin, ceftriaxone, kanamycin, tetracycline. It is important to note that 5% of the strains (one strain of *E. durans* and 4 strains of *E. faecium* all isolated from farm B) were resistant to vancomycin. In the last two decades, Enterococci have become major nosocomial pathogens. An increasing number of these infections are due to enterococci that are resistant to vancomycin. Accurate detection of vancomycin-resistant enterococci (VRE) is important so that appropriate therapy and infection control measures may be applied, including veterinary surveillance [34]. Russo, et al. [5] examined the susceptibility of *Enterococcus* spp. isolates to the most clinical antibiotics by microdilution method and found that 97% of the isolates, out of 110, exhibited a multidrug-resistant phenotype (resistance to at least three antimicrobials) with 14 strains, 13 E. *faecalis* and one *E. faecium*, resistant to 7 out of 9 antimicrobials tested. Furthermore, 19 strains (15 E. durans, 2 E. faecalis and 2 E. faecium) showed resistance to 5 antimicrobials. In particular, out of 15 E. durans, all were resistant to erythromycin, 14 to rifampicin and ampicillin and 7 to penicillin. Only three (2.7%) strains (*E. durans*, *E. faecalis*, and *E. faecium*) were susceptible to all tested antibiotics. These results highlighted the highest occurrence of resistance for rifampicin, erythromycin and ampicillin and the lowest for gentamicin. More recently Výrostková, *et al.* [35] reported that isolates of *Enterococcus* spp. showed high antibiotic resistance to vancomycin (84.62%; 44/52 isolates), teicoplanin (84.62%; 44/52 isolates), erythromycin (76.92%; 40/52 isolates), and rifampicin (76.92%; 40/52 isolates). Lower antibiotic resistance was detected against nitrofurantoin (46.15%; 24//52 isolates) and minocycline (38.46%; 20/52 isolates). Serio, et al. [7] analysed enterococci isolates from Pecorino

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Abruzzese and found a high incidence of antibiotic resistance, with a prevalence of erythromycin resistance especially for *E. faecium* (75.7%), followed by *E. faecalis* (48.3%), and *E. durans* (37.5%). Results for vancomycin by disk diffusion test have been criticized and in 2011 the CLSI M100-S19 document has recommended the disuse of vancomycin disks for staphylococci and informed that studies on the action of teicoplanin in disk-diffusion testing should be performed [36], however, for screening purposes, such as in this study, the disk diffusion test is still a viable choice, especially for enterococci [29,37].

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Table 5. Antimicrobial susceptibility data per *Enterococcus* spp.

	Total n=100			*E. durans* n=22			*E. faecium* n=69			*E. faecalis* n=7			*E. casseliflavus* n=2		
AK	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
total	31	21	48	7	5	10	21	15	33	3	1	3	0	0	2
%	31	21	48	31.8	22.7	45.5	30.4	21.7	47.8	42.9	14.3	42.9	0	0	100
AMC															
total	99	1	0	22	0	-	68	1	0	7	0	0	2	0	0
%	99	1	0	100	0	-	98.6	1.5	0	100	0	0	100	0	0
AMP															
total	98	0	2	22	0	0	67	0	2	7	0	0	2	0	0
%	98	0	2	100	0	0	97.1	0	2.9	100	0	0	100	0	0
C															
total	94	5	1	22	0	0	63	5	1	7	0	0	2	0	0
%	94	5	1	100	0	0	91.3	7.3	1.5	100	0	0	100	0	0
CIP															
total	23	47	30	8	9	5	15	29	25	0	7	0	0	2	0
%	23	47	30	36.4	40.1	22.7	21.7	42.0	36.2	0	100	0	0	100	0
CRO															
total	8	50	42	0	15	7	5	32	32	2	2	3	1	1	0
%	8	50	42	0	68.2	31.8	7.3	46.4	46.4	28.6	28.6	42.9	50	50	0
K															
total	0	26	74	0	5	17	0	21	48	0	0	7	0	0	2
%	0	26	74	0	22.7	77.3	0	30.4	69.6	0	0	100	0	0	100
RL															
total	100	0	0	22	0	0	69	0	0	7	0	0	2	0	0
%	100	0	0	100	0	0	100	0	0	100	0	0	100	0	0
SXT															
total	89	4	7	19	0	3	62	4	3	6	0	1	2	0	0
%	89	4	7	86.4	0	13.6	89.9	5.8	4.38	85.7	0	14.3	100	0	0
TE															
total	45	16	39	13	3	6	27	11	31	4	2	1	1	0	1
%	45	16	39	59.1	13.6	27.3	39.1	15.9	44.9	57.1	28.6	14.3	50	0	50
TIC															
total	91	7	2	19	3	0	65	2	2	6	1	0	1	1	0
%	91	7	2	86.4	13.6	0	94.2	2.9	2.9	85.7	14.3	0	50	50	0
VA															
total	82	13	5	19	2	1	57	8	4	6	1	0	0	2	0
%	82	13	5	86.4	9.1	4.5	82.6	11.6	5.8	85.7	14.3	0	0	100	0

S: susceptible, I: intermediate, R: resistant; AK: amikacin 30 μg, AMC: amoxicillin/clavulanic acid 30 (20 + 10) μg, AMP: ampicillin 10 μg, C: chloramphenicol 30 μg, CIP: ciprofloxacin 5 μg, C RO: ceftriaxone 30 μg, K: kanamycin 30 μg, RL: sulphamethoxazole 25 μg, SXT: sulphamethoxazole/trimethoprim 25 μg, TE: tetracycline 30 μg, TIC: ticarcillin 75 μg, VA: vancomycin 30 μg.

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Table 6. Antimicrobial susceptibility data per farm.

	total			Farm A n=46			Farm B n=32			Farm C n=22		
AK	S	I	R	S	I	R	S	I	R	S	I	R
total	31	21	48	17	9	20	9	3	20	5	9	8
%	31	21	48	37.0	19.6	43.5	28.1	9.4	62.5	22.7	40.9	36.4
AMC												
total	99	1	0	46	0	0	31	1	0	22	0	0
%	99	1	0	100	0	0	96.9	3.1	0	100	0	0
AMP												
total	98	0	2	46	0	0	30	0	2	22	0	0
%	98	0	2	100	0	0	93.8	0	6.3	100	0	0
C												
total	94	5	1	45	0	1	27	5	0	22	0	0
%	94	5	1	97.8	0	2.2	84.4	15.6	0	100	0	0
CIP												
total	23	47	30	17	15	14	2	18	12	4	14	4
%	23	47	30	37.1	32.6	30.4	6.3	56.3	37.5	18.2	63.6	18.2
CRO												
total	8	50	42	2	15	29	6	19	7	0	16	6
%	8	50	42	4.3	32.6	63.0	18.8	59.4	21.9	0	72.7	27.3
K												
total	0	26	74	0	16	30	0	7	25	0	3	19
%	0	26	74	0	34.8	65.2	0	21.9	78.1	0	13.6	86.4
RL												
total	0	0	100	0	0	46	0	0	32	0	0	22
%	0	0	100	0	0	100	0	0	100	0	0	100
SXT												
total	89	4	7	43	0	3	26	4	2	20	0	2
%	89	4	7	93.5	0	6.5	81.3	12.5	6.3	90.9	0	9.1
TE												
total	45	16	39	21	6	19	13	7	12	11	3	8
%	45	16	39	45.7	13.0	41.3	40.6	21.9	37.5	50	13.6	36.4
TIC												
total	91	7	2	46	0	0	27	3	2	18	4	0
%	91	7	2	100	0	0	84.4	9.4	6.3	81.8	18.2	0
VA												
total	82	13	5	43	3	0	18	9	5	21	1	0
%	82	13	5	93.5	6.52	0	56.3	28.1	15.6	95.5	4.5	0

S: susceptible, I: intermediate, R: resistant; AK: amikacin 30 μg, AMC: amoxicillin/clavulanic acid 30 (20 + 10) μg, AMP: ampicillin 10 μg, C: chloramphenicol 30 μg, CIP: ciprofloxacin 5 μg, C RO: ceftriaxone 30 μg, K: kanamycin 30 μg, RL: sulphamethoxazole 25 μg, SXT: sulphamethoxazole/trimethoprim 25 μg, TE: tetracycline 30 μg, TIC: ticarcillin 75 μg, VA: vancomycin 30 μg.

4. Conclusion

The results clearly supported the technological and hygienic reasons that cheese processors have for applying mild thermization rather than typical pasteurization treatments in raw ewe's and goat's milk intended for use in traditional hard cheese processing. In principle, empirically applied thermization treatments should contribute to the reduction

of the total microbial load of raw milk to levels assuring an optimal fermentation and increased safety of the resultant cheese while selecting for the beneficial part of the natural milk flora that maintains viability. Thermization of raw milk samples at 56°C for 30 s practically eliminated undesirable Enterobacteriaceae. In our previous study we demonstrated thermization of ewe's and goat's milk almost completely eliminated the coliforms and staphylococci in farm goat cheese, although these bacteria were present in high concentrations in raw milk. *S. aureus* strains (10% of total staphylococci) were isolated from raw milk cheese samples only [2,38].

Regarding the antimicrobial susceptibility, in Turkey a study by Sanlibaba and Senturk [39] in 215 traditional cheese samples identified 99.1% enterococcal isolates that were highly resistant to nalidixic acid (100%), kanamycin (98.6%), and rifampicin (78.4%), and were resistant to ampicillin, ciprofloxacin, erythromycin, tetracycline, penicillin G, chloramphenicol, gentamycin, and streptomycin [39]. On the other hand, according to Hollenbeck and Rice [40] the resistance profile of *Enterococcus* species according was as follows: erythromycin (49.2%); vancomycin (37.3%); and tetracycline (45.8%). Concurrently, the detected occurrence of antibiotic resistance genes in theses tested enterococci was: ermA 44.8%, vanA 63.6%, tetA 51.9%, tetM 55.6%, ermB 13.8%, and vanB 22.7%. This study may reveal that RTE food products may be reservoirs of detectable enterococci such as *E. casseliflavus*, *E. durans*, *E. hirae*, *E. gallinarum*.

Also Serio, et al. [7] found a high incidence of antibiotic resistance, with a prevalence of erythromycin resistance especially for E. *faecium* (75.7%), followed by *E. faecalis* (48.3%), and E. *durans* (37.5%). Food isolates resistant to this macrolide have been reported by other authors, such as Russo, et al. [5], but in lower percentages and with a higher incidence for E. faecalis than for E. faecium. The widespread erythromycin resistance could be related to the presence in enterococci of plasmids and transposons [41].

Our results, with an overall high level of resistance of *Enterococcus* spp. to erythromycin (48% of the strains), ciprofloxacin (30%), ceftriaxone (42%), kanamycin (74%) and tetracycline (39%), including a 5% of the strains resistant to vancomycin (and 13% intermediate) demonstrated that the resistance to antimicrobial of *Enterococcus* spp. is distributed over a wide variety of antibiotics groups. According to Werner, et al. [18] the genomic composition of enterococci, their robust nature, the frequent occurrence in many natural habitats and their flexibility to respond to varying environmental conditions make them a central hub (a «*drug resistance gene trafficker*») for resistance gene acquisition, conservation and dissemination, especially among related Gram-positive bacteria

In Pecorino and goat cheese the ripening processes are the result of the natural microbiological contamination of milk and of the characteristics cheesemaking technology. Therefore, not only lactic acid bacteria but also other bacterial groups, such as enterococci, must be considered. This study demonstrated that, regardless the cheesemaking technology, heavy curd recontamination occurs and that enterococci are always detected at high concentration at the end of ripening. A large proportion of isolates are also resistant to antibiotics and this pose the question whether microorganism belonging to *Enterococcus* spp. should be awarded in future the QPS or GRAS status.

The development of mountain farming and the promotion of mountain food production are a way to encourage sustainable development of mountain areas, which are generally considered places with specific geographic and climatic constraints. In order to promote strategies for mountain products, the European Union with the Regulation (EU) No 1151/2012 reserves the use of the term "Mountain Product" to food products produced and processed in mountain areas. This regulation was supplemented by the Delegated Act (EU) No 665/2014, which specifies the conditions of use of the optional quality term "Mountain Product". It is of the utmost importance to assess the interest in the application of the new mountain label and the perception of the food mountain products by the Umbria Region. However, we believe that the creation of a label to protect and certify mountain food could be the basis to improve the promotion of mountain quality food products and the sustainability of these areas

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