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Original article

Influence of pH and temperature on the growth of *Enterococcus faecium* and *Enterococcus faecalis*

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Abstract – A total of 70 enterococcal strains isolated from traditional Italian raw milk cheeses were identified by API 20 Strep and Biolog GP. All the identifications were confirmed by species-specific PCR. Four species of genus *Enterococcus* were found: *E. faecalis, E. faecium, E. durans* and *E. hirae*. *E. faecalis* and *E. faecium* were the dominant enterococcal species isolated in artisanal cheeses. According to the most representative species and the origin of isolation, 21 strains (11 *E. faecalis* and 10 *E. faecium*) were selected for the conductimetric analysis. The influence of pH (5.0; 5.5; 6.0 and 6.5) and temperature (25 °C and 37 °C) on the metabolism and the development of enterococci was evaluated by determination of Generation Time (GT), Detection Time (DT) and the maximum conductance value (ΔμS) of the curve during a period of 48 hours. Conductance trials showed that both species were able to adapt to adverse cultural conditions (low values of pH and temperature) during cheese-making and ripening. *E. faecalis* appeared to be less sensitive on the whole. In the worst conditions (pH 5.0, 25 °C), after an adaptation phase (about 10 h), these microorganisms showed GT values almost double those at 37 °C. The same behavior can occur in cheese, where enterococci can reach numbers of up to 10^5–10^8 cfu·g^{-1} during ripening. This could explain the fact that enterococci (in particular, *E. faecalis* and *E. faecium*) represent the typical and important microflora in raw milk cheese.

***E. faecalis* / *E. faecium* / conductimetric analysis / pH / temperature / growth**

Résumé – Influence du pH et de la température sur la croissance d'*Enterococcus faecalis* et *Enterococcus faecium*. Un total de 70 souches d'entérocoques, isolées de certains fromages italiens traditionnels, a été identifié à l'aide des galeries API 20 Strep et Biolog GP. Toutes les identifications ont été confirmées par PCR espèce-spécifique. Quatre espèces de ce genre ont été isolées : *E. faecalis*, *E. faecium*, *E. durans* et *E. hirae*. *E. faecalis* et *E. faecium* étaient les espèces dominantes isolées des fromages artisanaux. Un total de 21 souches (11 *E. faecalis* et 10 *E. faecium*) a été sélectionné pour les analyses de conductimétrie selon les espèces les plus représentées et selon l'origine de l'isolement. L'influence du pH (5,0 ; 5,5 ; 6,0 et 6,5) et de la température (25 °C et 37 °C) sur le métabolisme et sur le développement des entérocoques a été évaluée par détermination du temps de génération (GT), du temps de détection (DT) et de la valeur conductimétrique maximale (ΔμS) de la courbe au cours d'une période de 48 h. Les mesures de conductance ont démontré que les deux espèces ont la capacité de s'adapter aux conditions de culture défavorables (valeurs faibles du pH

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et de la température) aussi bien pendant la fabrication du fromage que l'affinage. Les deux espèces ont un comportement différent : *E. faecalis* s'est avéré être moins sensible. Dans les plus mauvaises conditions (pH 5,0–25 °C), après la phase d'adaptation (environ 10 h), ces micro-organismes montrent une valeur du temps de génération deux fois plus élevée par rapport à celle relevée à 37 °C. Le même phénomène peut se produire dans le fromage où, pendant la maturation, les entérocoques peuvent atteindre 10^5–10^8 ufc·g^{-1}. Ceci pourrait expliquer le fait que les entérocoques (en particulier *E. faecalis* et *E. faecium)* représentent la flore typique dominante dans les fromages au lait cru.

***Enterococcus faecalis* / *Enterococcus faecium* / conductimétrie / pH / température / croissance**

1. INTRODUCTION

Enterococci consist of ubiquitous microorganisms commonly found in dairy products and other foods. The ability to grow at 10 °C and 45 °C, to initiate growth in 6.5% NaCl broth at pH 9.6 and to survive at 60 °C for 30 min, are commonly used traits to segregate enterococci from other streptococci [11, 26].

Enterococci are widely distributed in nature. They are found in such diverse habitats as the gastrointestinal tract, oral cavity and upper genital tract of man and other mammals, and also in birds, reptiles, insects, plants, soil and water [28].

The significance of their presence in food, particularly in dairy products, has been a source of discussion for a long time. Their possible use as fecal contamination indicators and their contribution to the development of flavor are controversial topics.

The ubiquitous nature of enterococci and their ability to survive in unfavorable conditions almost guarantee the presence of this group in many types of foods, particularly in dairy products.

Enterococci are the predominant microflora in a large number of typical Mediterranean cheeses with the European Protected Designation of Origin. They produce acetaldehyde and diacetyl, and consequently they contribute to the organoleptic characteristics of cheese and are considered to be an important part of the microflora [2, 24, 39].

Some enterococci of food origin also produce bacteriocins that exert activity against *Listeria* and *Salmonella* [3, 23, 27].

Moreover, enterococci are used as probiotics to improve the microbial equilibrium of the intestine, or as treatments for gastroenteritis in humans and animals. However, recent studies have pointed out that *E. faecium* and *E. faecalis* might be a potential recipient of vancomycin resistance genes, and consequently, the FAO/WHO have recommended that *E. faecium* should not be considered as probiotics for human use. Furthermore, enterococci have become recognized as serious nosocomial pathogens causing bacteraemia, endocarditis and infections of the urinary tract [14].

This is in part explained by the resistance of some of these bacteria to most of the antibiotics that are currently in use. Resistance is acquired by gene transfer systems, such as conjugative or nonconjugative plasmids or transposons. The virulence of enterococci is not well understood, but adhesins, haemolysin, hyaluronidase, aggregation substance and gelatinase are potential virulence factors. It also appears that food could be a source of vancomycin-resistant enterococci [14, 16, 25].

Impedimetric methods have been developed to analyze a range of foods and pharmaceuticals. These methods are based on the principle that as bacteria grow and metabolize, uncharged or weakly charged substrates are transformed into charged end products. The accumulation of these products leads to increased conductance of the medium and capacitance at the electrode-medium interface [29].

Measurement of the rate of growth permits investigation into the effects of inhibitors, sensitivity to some parameters such as pH, temperature and salt concentration, in a much shorter time than conventional techniques.

Impedance measurement, defined as the resistance to flow of an alternating current through a conducting material, can be used to monitor the growth of LAB [10] and to

Table I. Microbiological counts of enterococci in different Italian raw milk cheeses and identification of 70 isolated strains.

	Ripening (d)	*Enterococcus* sp. (cfu·g^{-1})	*E. faecalis*	*E. faecium*	*E. durans*	*E. hirae*
Scimudin	20	10^6–10^7	8 (3*)	3 (3*)		
Goat cheese	20	10^5–10^7	2 (1*)		1	3
Formai de Mut	45	10^5–10^7	4		1	
Caciocavallo Ragusano	60	10^5–10^8	1	1 (1*)	1	
Fontina	60	10^6–10^8	2	2		
Semuda	60	10^6–10^7		1		
Valtellina Casera	70	10^5–10^7	8 (4*)	1 (1*)		
Bitto	70	10^5–10^7		5 (3*)		
Hard cheese	70	10^6–10^7	13 (3*)	7 (2*)	5	1

* Strains selected for the conductimetric analysis.

determine the shelf life and bacteriological quality of raw materials of different food products [6, 15, 29, 30, 32].

Conductance is considered a valid method for evaluating the growth and acidity of LAB in milk [4, 22, 31, 36].

Changes in the conductance of the medium, which is the reciprocal of resistance, have been successfully employed for the detection of several microbial species, including *E. faecalis* and *E. faecium* [5, 20].

The aim of this study was to characterize and investigate the effects of different cultural conditions (pH and temperature), present in cheese during different cheese-making processes and different ripening periods, on the metabolism and growth rates of enterococci, using conductance measurements that allowed us to accurately evaluate the growth of these species (Tab. I).

2. MATERIALS AND METHODS

2.1. Enumeration, isolation and identification of enterococci

Different Italian cheeses produced in northern Italy from raw milk (Valtellina Casera, Bitto, Scimudin, pasta filata cheese, Formai de Mut, goat cheese, Fontina, Semuda and hard cheese) were analyzed in order to verify the presence of enterococci.

For the enumeration, a 5-g sample of each kind of cheese was placed in 45 mL of dipotassium hydrogenphosphate solution [21] and homogenized with a Stomacher for approximately 1 min.

A selective medium for detection and isolation of enterococci (Kanamycin Aesculin Azide agar, Sharlau Microbiology, Spain) was used. After 24 h of incubation at 37 °C, typical colonies of presumptive enterococci (surrounded by a black halo) were randomly picked from the plates. After purification, the ability to initiate growth in 6.5% NaCl broth and in broth at pH 9.6 at 10 °C and 45 °C, and survival at 60 °C for 30 min were verified. The enterococci were cultured in MRS broth (Sharlau Microbiology, Spain) with incubation at 37 °C for 16 h. Identification was performed by using API 20 STREP galleries (bioMérieux, Marcy l'Étoile, France) and Biolog GP MicroPlate (Biolog, USA) according to the instructions of the manufacturer. Since *E. faecium* and *E. faecalis* are the dominant enterococcal species, their identification was also performed by separate PCR reactions using either primer pair E

(with sequence: 5' ATCAAGTACAGTTAGTCTT 3' and 5' ACGATTCAAAGCTAACTG 3') or F (5' CGAAGGCTTCTTAGAGA 3' and 5' CATCGTGTAAGCTAACTTC 3') which were specific for amplification of two intragenic fragments of 941 and 550 bp from the D-alanyl:D-alanyl ligases of *E. faecalis* and *E. faecium*, respectively [13].

E. faecalis ATCC 19433^T and *E. faecium* ATCC 19434^T were used as positive controls.

Amplification reactions were carried out in a total volume of 25 µL containing 250 µmol·L^{-1} of each dNTPs, 2.5 µL of 10 × Taq reaction buffer, 1.5 µmol·L^{-1} of each of the two primers, 2.5 mmol·L^{-1} of $MgCl_2$, 0.5 U of AmpliTaq DNA polymerase (PE Biosystem, PE Italia, Monza, Italy) and between 2.5 and 10 µL of template of DNA extracted by the Instagene matrix (Bio-Rad laboratories, Milano, Italy) according to the manufacturer's instructions. DNA amplifications were performed in a Perkin Elmer thermal cycler (model 2400 PE Biosystem, PE Italia, Monza, Italy). The cycling program consisted of an initial denaturation step at 94 °C for 120 s and then 35 cycles of 94 °C for 60 s, 54 °C for 30 s and 72 °C for 70 s. The final elongation was performed at 72 °C for 10 min. PCR products were analyzed by electrophoresis through 1.0% agarose gels at 70 V for 3 h in 1 × Tris-acetate EDTA (TAE) buffer (1 × TAE: 40 mmol·L^{-1} Tris acetate, 1 mmol·L^{-1} EDTA, pH 8.0).

2.2. Conductimetric methods

Conductance trials were carried out using the Malthus-AT System (Malthus Instruments Ltd., Crawley, UK). The Malthus-AT System consists of two individual water-bath incubators, each of which contains up to 128 cells simultaneously over a temperature range 15–47 °C and 2-mL conductance electrodes.

Cells containing inoculated media were placed in two Malthus water-baths set at 25 and 37 °C. Changes in conductance of the medium were recorded automatically at selected intervals (6 min) for 48 h.

The results are given in ΔµS.

2.2.1. Preparation of the inoculum

Enterococci were grown overnight in MRS broth, then dilutions of broth culture (1:10, 1:50, 1:100) in reconstituted non-fat dry milk containing Bromo Cresol Purple (0.025 g·L^{-1}) were made and incubated at 37 °C.

The highest dilution showing clotted milk and yellow color was selected as the inoculum for conductance trials.

2.2.2. Milk inoculation

The pH of the reconstituted non-fat dry milk was adjusted with 1N HCl to 5.0, 5.5, 6.0 and 6.5 after sterilization.

Milk was inoculated into triple Malthus cells at a rate of 1% (v/v) using the inoculum prepared as in Section 2.2.1. Six decimal dilutions for each pH and each temperature tested were made.

All the cells were incubated simultaneously in two water-baths at 25 and 37 °C for 48 h.

2.3. Experimental design

Conductimetric analyses were performed on 11 *E. faecalis* and 10 *E. faecium* strains.

The influence of four different levels of pH (5.0, 5.5, 6.0 and 6.5) and two levels of temperature (25 °C and 37 °C) on the metabolism and the development of enterococci was evaluated by the determination of the Generation Time (GT), the maximum conductimetric values of the curve during a period of 48 h (ΔµS max) and the Detection Time (DT). Overnight cultures were diluted and then inoculated into triple Malthus cells by preparing 6 decimal dilutions for each pH and temperature combination tested. The cells were incubated simultaneously in water-bath incubators at 25 and 37 °C for 48 h.

2.3.1. Growth parameters

The Detection Time (DT), the maximum conductimetric value (ΔµS max), and the Generation Time (GT) were determined for each strain subjected to each combination of tested parameters (Fig. 1).

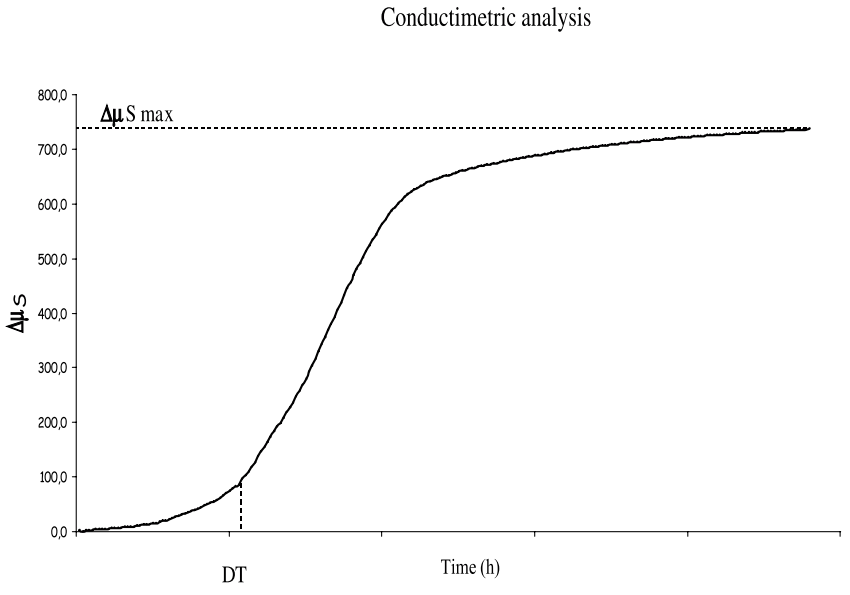

Figure 1. Example of conductimetric curve of *Enterococcus* sp.

The Generation Time was determined according to Firstenberg-Eden and Eden [16] by recording the delay in DT for 6 serial dilutions of the initial samples.

The maximum conductimetric value of the curve was used to estimate the enterococcal metabolic activity during a period of 48 h.

The DT is the time elapsed between the start of a test and the detection of an accelerating impedance signal by the instrument.

The DT is a function of numerous parameters, including initial concentration of microorganisms, lag phase, generation time and metabolic activity [16, 32].

The Malthus computer program automatically records the DT; however, in some cases it is necessary to correct values by directly reading them from the curves.

2.4. Statistical treatment of the data

The data were subjected to general linear model (GLM) analysis of the Statistical Analysis System [35]. All means shown in this paper are least square means (LSM). Statistical significance was accepted if the probability value was less than or equal to 0.05.

3. RESULTS AND DISCUSSION

3.1. Isolation and identification

Enterococci were recovered from all the fully ripened cheeses at levels ranging from 10^5 to 10^8 cfu·g^{-1}, thus confirming them to be an important part of the microflora of many Italian cheeses, especially for those produced from raw milk (Tab. I).

A total of 70 isolates were identified as belonging to *E. faecalis, E. faecium, E. durans* and *E. hirae*. The identifications performed by API 20 Strep, Biolog GP and species-specific PCR were always in agreement, except for *E. faecium*: for this species Biolog identification differed from API and species-specific PCR. *E. durans* and *E. hirae* were identified by phenotypical methods.

Among the isolated strains, *E. faecalis* and *E. faecium* were the dominating species present, as reported by many authors for different kinds of cheese [19, 34].

According to the most representative species and the origin of isolation (Tab. I), 21 strains (11 *E. faecalis* and 10 *E. faecium*) were selected for the conductimetric analysis.

3.2. Influence of pH and temperature on enterococcal growth

3.2.1. Generation Time (GT)

GT mean values related to the 21 tested strains of enterococci were highly correlated to both pH and, in particular, temperature ($P < 0.001$): GT values increased when pH decreased, and they ranged from 0.85 to 1.21 h ($P < 0.001$) at 25 °C while they varied from 0.44 to 0.67 h at 37 °C ($P < 0.001$).

At the same pH, when the temperature decreased the growth of all the enterococcal strains was greatly slowed down; in fact, GT values were almost double those at 25 °C than at 37 °C (Fig. 2, Tab. II).

In fact, at 37 °C a GT average emerges, which is about half that at 25 °C in the same conditions of acidity (Fig. 2, Tab. II).

Both species showed similar behavior at optimal temperature (37 °C), but *E. faecium* was more sensitive than *E. faecalis* at 25 °C and with increasing acidity: the maximum value of GT for this species was recorded at 25 °C and pH 5.0 (1.50 h), while in the same conditions *E. faecalis* duplicated in 0.93 h.

The development of *E. faecium* was influenced by temperature in the same way as by pH ($P < 0.001$); in fact, the GT values were bigger at 25 °C than at 37 °C, and differed in a remarkable way with increases in medium acidity. In addition, this species was particularly sensitive at pH 5.0; in fact, at both temperatures, at this pH there was a considerable increase in GT (Tab. II).

For *E. faecalis* at 25 °C there were no particular differences regarding the GT when acidity varies, while at 37 °C we had a bigger increase in this parameter with a decrease in the pH.

Thus, it seems that the development of the strains belonging to the species *E. faecalis* is above all influenced by variations in temperature ($P < 0.001$), rather than by pH, which is significant only at 37 °C ($P < 0.002$) (Tab. II).

3.2.2. Metabolic activity (ΔμS max)

Although grown in acid media, the same strains have high ΔμS max values; consequently, we can assume that the max values of ΔμS are an index of the metabolic activity of strains in acid media as well.

Observing the mean of ΔμS max values of the 21 tested strains, we can deduce that the metabolic activity of enterococci is also influenced by pH and temperature ($P < 0.0001$) (Fig. 3); in fact, the maximum values of ΔμS for both temperatures decreased with increasing acidity in a similar way (Tab. II), and at 37 °C they were always higher than at 25 °C.

Conductimetric trials pointed out that the metabolic curves took a longer time to reach the ΔμS max at 25 °C than at 37 °C; moreover, the count of the cfu·mL^{-1} carried out at the plateau of the curves for each strain at different pH indicated that the varying ΔμS max values are not due to decreased microbial development. We can suppose that the different activity is not bound to a minor microbial development nor to a different reaction of the medium at different pH, but rather to minor metabolic activity at decreasing pH, even if the strains show the same number of cells.

Metabolic activity of *E. faecalis* was less influenced than *E. faecium* by temperature: at 25 °C the ΔμS max was 335 vs. 301 ($P < 0.05$), while at 37 °C it was 394 vs. 424 (P = NS) (Fig. 3).

Otherwise *E. faecium* and *E. faecalis* showed a similar behavior for pH: when the acidity of the medium decreased, ΔμS max increased for both species without significant differences.

Considering all the different conditions of temperature and pH, *E. faecium* showed a wider range of metabolic activity (from 188 ΔμS at 25 °C and pH 5.0 to 530 ΔμS at 37 °C and pH 6.5) than *E. faecalis* (from

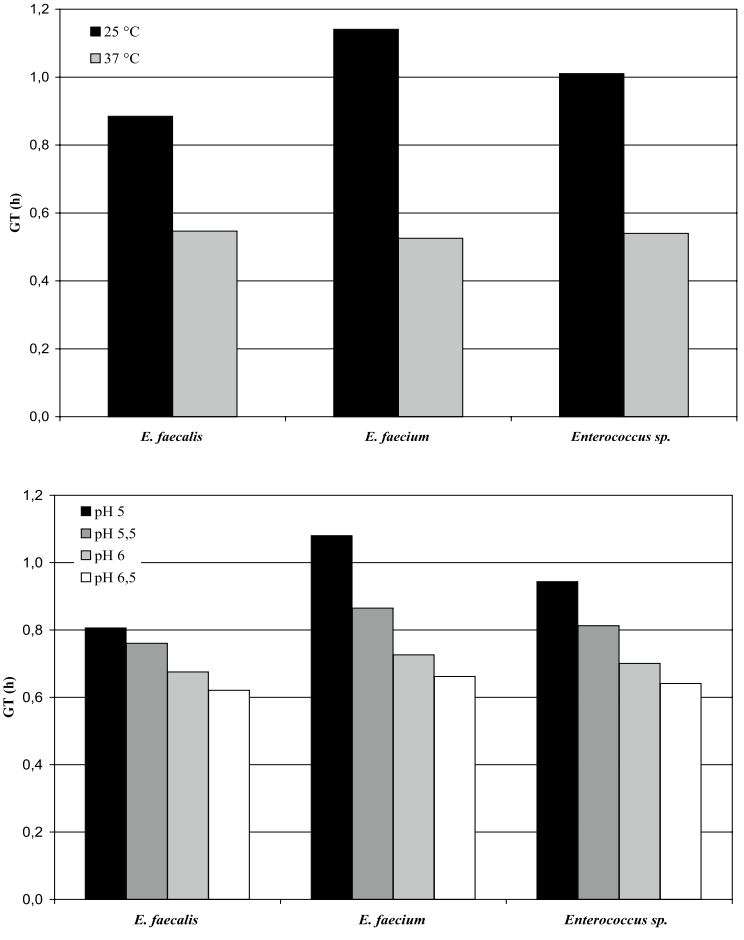

Figure 2. GT mean values (h) of *E. faecalis* (11 strains), *E. faecium* (10 strains) and *Enterococcus* sp. (21 strains) at different temperatures and pH.

235 ΔμS at 25 °C and pH 5.0 to 484 ΔμS at 37 °C and pH 6.5) (Tab. II).

3.2.3. Detection Time (DT)

Even the DT of cells, the expression of time necessary for the adaptation to different culture conditions was very correlated with species, pH and temperature. DT values were higher when temperature decreased and acidity increased (Fig. 4).

The ratio between DT values related to ideal conditions (pH 6.5, 37 °C) and the worst ones (pH 5.0, 25 °C) is almost 1:10 (Tab. II).

Also in this case *E. faecium* was sensitive to medium acidity and temperature; in fact, a shift in the DT for growth was detected with respect to the control curve (37 °C and pH 6.5). At 37 °C, it adapted better, with values ranging from 1.3 h to 4.6 h; at 25 °C from 3.5 h to 9.9 h.

For *E. faecalis* DT values at 37 °C were not significantly different when acidity increased to 5.5 but at pH 5.0 they doubled with respect to other pH levels.

Table II. Least square means and significance of temperature and pH on Generation Time (GT), Metabolic Activity (DT) of *Enterococcus* sp. (21 strains), *E. faecalis* (11 strains) and *E. faecium* (10 strains).

		T (° C)	pH							
			5.0	5.5	6.0	6.5	SE	5.0 vs. 5.5	5.0 vs. 6.0	5.0 vs. 6.
GT (h)	*Enterococcus* sp.	25	1.21	1.05	0.94	0.85	0.1	NS	**	***
		37	0.67	0.58	0.46	0.44	0.1	NS	**	***
	E. faecalis	25	0.93	0.91	0.87	0.83	0.1	NS	NS	NS
		37	0.69	0.61	0.48	0.41	0.1	NS	NS	*
	E. faecium	25	1.50	1.18	1.02	0.86	0.1	***	***	***
		37	0.66	0.55	0.44	0.46	0.1	NS	**	*
MAX (ΔμS)	*Enterococcus* sp.	25	211	311	345	406	18	***	***	***
		37	293	379	457	507	17	***	***	***
	E. faecalis	25	235	345	326	434	29	**	*	***
		37	280	354	459	484	27	*	***	***
	E. faecium	25	188	278	363	377	22	**	***	***
		37	306	405	455	530	22	**	***	***
DT (h)	*Enterococcus* sp.	25	10.0	5.6	4.2	3.1	0.4	***	***	***
		37	4.5	2.1	1.7	1.1	0.4	***	***	***
	E. faecalis	25	10.1	5.4	4.2	2.8	0.7	***	***	***
		37	4.5	1.7	1.9	1.0	0.6	**	**	***
	E. faecium	25	9.9	5.8	4.1	3.5	0.5	***	***	***
		37	4.6	2.5	1.6	1.3	0.5	**	***	***

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS: not significant; SE: standard error.

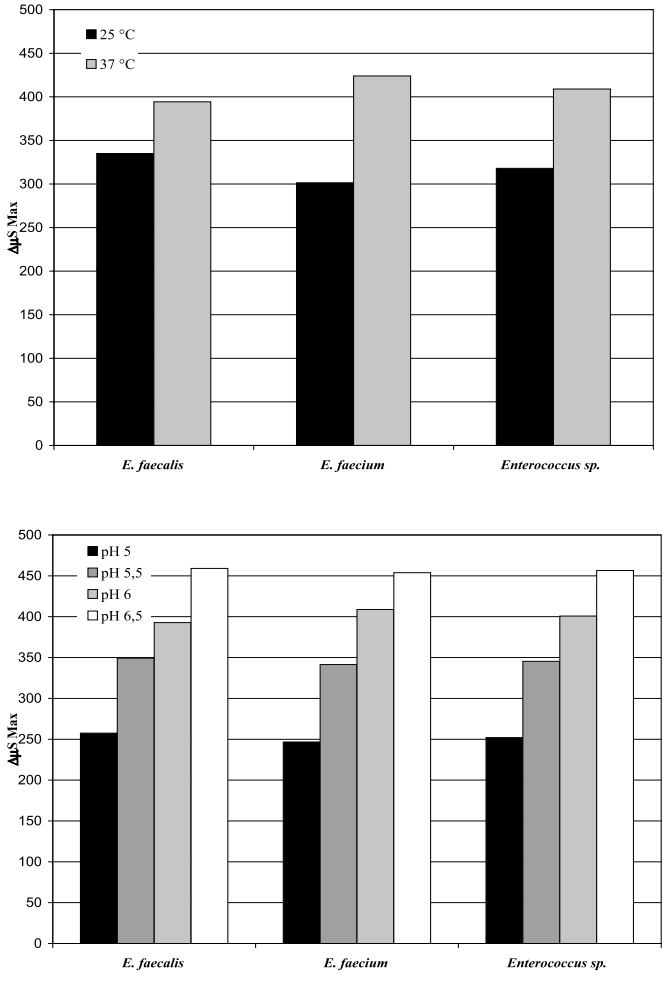

Figure 3. Metabolic activity mean values (ΔμS max) of *E. faecalis* (11 strains), *E. faecium* (10 strains) and *Enterococcus* sp. (21 strains) at different temperatures and pH.

At 25 °C they were higher and strictly dependent on the pH (from 2.8 h to 10.1 h). Both species were influenced in particular at pH 5.0 (Tab. II, Fig. 4).

4. CONCLUSION

Enterococci occur and grow in a variety of cheeses, especially in artisanal cheeses produced from raw milk in southern Europe (Italy, Spain, Portugal and Greece) [1, 2, 7, 17, 18, 33, 37, 38]. In our research we found, as remarked by other authors, that levels of enterococci in milks and cheese curds range from 10^2 to 10^4 cfu·mL^{-1} or g^{-1}, whereas in the fully ripened cheeses (from 20 to 70 days) they reach a level from 10^5 to 10^8 cfu·g^{-1} (numbers vary with cheese type and production season).

The dominance or persistence of enterococci in some cheeses during ripening can

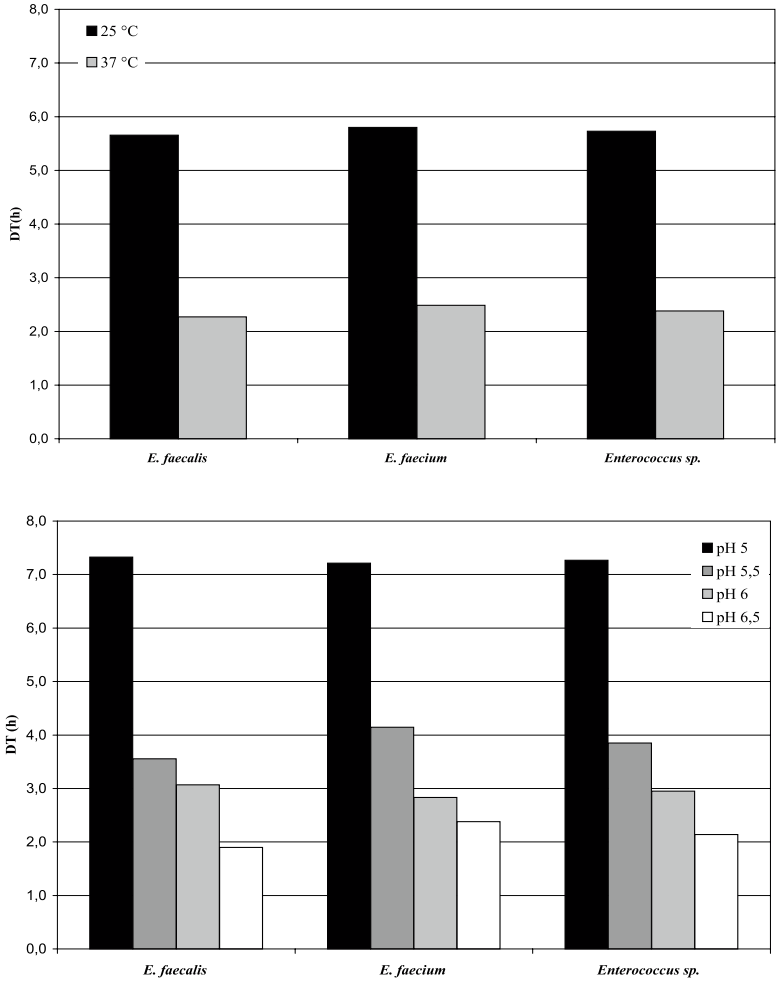

Figure 4. DT mean values (h) of *E. faecalis* (11 strains), *E. faecium* (10 strains) and *Enterococcus* sp. (21 strains) at different temperatures and pH.

be attributed to their wide range of growth temperatures and their high tolerance to heat, salt and acidity [1, 12, 18, 34]. For this reason the enterococci, in particular *E. faecalis* and *E. faecium*, usually represent an important part of the bacterial flora of ripened cheeses [1, 2, 7, 17, 18, 33, 37, 38].

Conductance analysis allowed the evaluation of the influence of pH and temperature on the growth and metabolic activity of enterococci in milk and to measure their ability to adapt to conditions present during cheese-making and ripening (without antagonists). In particular, it was possible to evaluate that speed of duplication and adaptation to low temperatures and acidity for *E. faecium* was slower than *E. faecalis*. All this information can justify the different behavior of these species in different cheeses, as reported by many authors.

Enterococci produce typical flavor components such as acetaldehyde, acetoin and

diacetyl [7, 17, 34]. Some enterococci of dairy origin have also been reported to produce bacteriocins (enterocins) inhibitory against pathogenic bacteria (e.g. *Listeria monocytogenes* and *Salmonella* sp.) [3, 23, 27]. The technological application of enterocins, which have been shown to be produced during food manufacture, led to proposing enterococci as adjunct or protective cultures in cheeses [8]. Moreover, *E. faecium* strain SF68 finds application in probiotic preparations for humans [9].

However, the fact that enterococci have recently assumed importance as nosocomial pathogens is not neglected. Enterococci are low grade pathogens, but their intrinsic resistance to many antibiotics and their acquisition of resistance to the few antibiotics available for treatment in clinical therapy, such as the glycopeptides, have led to difficulties and searching for new drugs and therapeutic options [17, 19].

Clearly, there is no consensus on the acceptance of their presence in foodstuffs and their role as primary non starter lactic acid bacteria (NSLAB) is still a question mark.

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