

Inclusion of Enterococcus Faecalis and Enterococcus Faecium to UF White Cheese

H. Rasouli Pirouzian¹, J. Hesari^{1*}, S. Farajnia², M. Moghaddam³, S. Ghiassifar¹ and M. Manafi⁴

Abstract—Lighvan cheese is basically made from sheep milk in the area of Sahand mountainside which is located in the North West of Iran. The main objective of this study was to investigate the effect of enterococci isolated from traditional Lighvan cheese on the quality of Iranian UF white during ripening. The experimental design was split plot based on randomized complete blocks, main plots were four types of starters and subplots were different ripening durations. Addition of Enterococcus spp. did not significantly ($P < 0.01$) affect the pH and gross composition of cheeses. In the cheeses produced with Ent. faecalis and Ent. faecium strains, lipolysis rates were higher and flavor were improved. Moreover, proteolysis assay by measuring percentage of soluble nitrogen at pH 4.6 and urea polyacrylamide gel electrophoresis indicated the increase in proteolysis rate in the cheese containing Ent. faecalis and Ent. faecium strains compared to the control cheeses. Furthermore, the highest percentage of non-protein nitrogen was observed in the cheese containing Ent. faecium. In conclusion, the results showed the positive effect of the Ent. faecalis and Ent. faecium on secondary proteolysis, lipolysis and sensorial characteristics development of UF white cheeses.

Keywords—Enterococcus faecalis, Enterococcus faecium, Lighvan cheese, Lipolysis, Proteolysis, UF cheese

I. INTRODUCTION

NOWADAYS, researchers are examining the potential of native microorganisms obtained from raw milks. Lighvan cheese is the most popular traditional cheese made from raw sheep's milk in West Azerbaijan province of Iran. This variety of cheese is characterised by unique hardness (semi-hard), saltiness and spiciness. Milk coagulation is usually carried out at 23-25 °C for 120 min using rennet without deliberate addition of a starter culture. It has been indicated that wild types of strains produce better aroma than industrial starters. Occasionally combination of these microorganisms is preferred [4]. Enterococci have important implication in the dairy industry. They play an important role in the development of sensory characteristics during ripening of many cheeses probably through proteolysis, lipolysis and citrate breakdown, hence contributing to their typical taste and flavor [8]. Because of their role in ripening, flavor development and bacteriocin production in cheese, it has been suggested that enterococci with

desirable technological and metabolic traits could be included in starter cultures of various cheeses [8]. The aim of the present work was to evaluate the effect of Ent. faecalis and Ent. faecium strains when used as an adjunct starter on the proteolytic, lipolytic activity and sensory characteristics of Iranian UF white cheese.

II. MATERIAL AND METHODS

2.1 Isolation of enterococci

Four samples of cheese were made in four different cheese producers in Lighvan region. Strains of enterococci in these samples were isolated by standard microbiology methods and selective medium of Kanamycin Esculin Azide Agar (KAA) [30], then identified by biochemical methods. To identify species of enterococci, sugar fermentation tests were performed using six sugar types including Arabinose (L), Raffinose (D), Lactose (D), Sorbitol (D), Sorbose (D) and Melibiose (D) [19]. Thereby catalase and curd formation tests [10] were done on isolated strains. Finally, the dominant strains were recognized as Ent. Faecalis and Ent. Faecium. Both enterococci strains had not haemolytic properties when were tested on sheep or human blood, and exhibited no resistance against vancomycin and penicillin.

2.2 Chemical composition

Cheeses were analysed for moisture by the oven drying method at 102 ± 2 °C [15], salt by a potentiometric method [9], fat by Gerber method [20], and total protein by the macro-Kjeldahl method [14]. The pH of the cheese was measured by direct insertion of an electrode (PHC3031-9, Radiometer Analytical, Copenhagen, Denmark) into cheese [20]. All analysis were repeated three times and results were reported as means \pm standard deviations.

2.3 Proteolysis

The nitrogen fractions of the cheese samples, including pH 4.6-soluble nitrogen as a percentage of total nitrogen (pH 4.6-SN/TN) and soluble nitrogen in 12% trichloroacetic acid as non-protein nitrogen (NPN) were obtained by a slight modification of the procedure of Kuchroo and Fox [17] as described by Sousa and McSweeney [29]. Urea-polyacrylamide gel electrophoresis (PAGE) of the pH 4.6-insoluble fractions of the cheeses was performed using a vertical slab gel unit (Akhtariyan- Tehran- Iran) according to the method of Andrews [1] as modified by Shalabi and Fox (28). The gels were stained directly with Coomassie Brilliant Blue G250, as described by Blakesley and Boezi [3].

2.4 Lipolysis

The levels of free fatty acids (FFA) was estimated using the method of Nunez [24]. Results were expressed as milliequivalents FFA in 100 g cheeses.

2.5 Sensory evaluation

Sensory evaluation was performed at days 1, 15, 30, 45 and 60 of ripening by a sixteen-member non-professional tasting panel familiar with UF and Lighvan cheeses. Sensory evaluation were assayed on a scale of 1 to 5 (1: low value; 5: high value) [16].

¹- Department of Food Science and Technology, Faculty of Agriculture, University of Tabriz, Tabriz, Iran

²- Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

³- Department of Agronomy and Plant Breeding- Faculty of Agriculture- University of Tabriz, Tabriz, Iran.

⁴- Department of Food Science and Technology, Faculty of Agriculture, Islamic Azad University, Branch of Khoy, Khoy, Iran

* Corresponding author, Email: jhesari@tabrizu.ac.ir

2.6 Statistical analysis

The experimental design was split plot based on randomized complete blocks with four replications. The main factor included four experimental cheeses (made with *Ent. faecalis* strains, made with *Ent. faecium* strains, made with *Ent. faecalis* and *Ent. faecium* strains and control cheese) and the subplots were the days of ripening. After analysis of variance, means were compared by the 1-way ANOVA method. Statistical analyses were carried out using SPSS Version 9 for Windows 2003 (SPSS Inc., Chicago, IL, USA).

III. RESULTS AND DISCUSSION

3.1 Composition

The compositions of 1-d old experimental Iranian UF white brined cheeses are shown in Table 1. There were no significant ($P < 0.05$) differences among the gross compositions of cheeses. These results showed that changes the starter did not have significant effect on the gross composition of cheeses as reported by other workers for various cheeses [13].

TABLE I COMPOSITION OF 1-D-OLD EXPERIMENTAL ULTRAFILTERED (UF) IRANIAN WHITE CHEESES. RESULTS ARE PRESENTED AS AVERAGE OF DATA FROM THREE INDEPENDENT REPLICATED TRIALS \pm STANDARD DEVIATIONS

Cheeses	NaC 1 (%)	Moisture (%)	fat (%)	Protein (%)
<i>Ent.</i>	3.80	62.39	15.5	13.55 \pm
<i>Faecalis</i>	± 0.12	± 0.8	± 0.39	0.32
<i>Ent.</i>	3.75	63.37 $\pm 0.$	15.5	14.03 \pm
<i>Faecium</i>	± 0.14	36	± 0.41	0.51
<i>Ent.</i> <i>faecalis</i> +	3.72	62.50 $\pm 0.$	15.5	13.95 \pm
<i>Ent.</i> <i>Faecium</i>	± 0.11	23	± 0.42	0.38
Control	3.66	62.59 $\pm 0.$	15.5	14.30 \pm
	± 0.13	25	± 0.40	0.25
F value	NS	NS	NS	NS

NS: not significant ($P < 0.05$).

3.2 pH

The levels of pH of UF Iranian white cheeses during ripening are shown in Fig 1. In the cheeses made with *Ent. faecalis* and *Ent. faecium* strains, pH levels were lowest at day 60, while the cheeses made with *Ent. faecium* and control cheese had the higher pH. In the cheese samples inoculated with *Ent. faecium* strains, the pH levels were slightly higher than the cheeses made with *Ent. faecalis* strains.

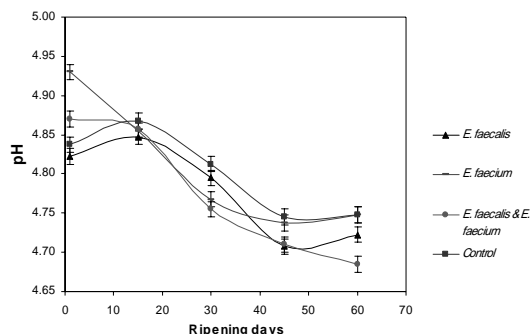


Fig. 1. pH changes of UF Iranian white cheese made without (control cheese) or with added *Enterococcus* strains during ripening. The values reported are the mean pH of all samples.

The pH decreased rapidly during ripening, due to the rapid growth of the starter cultures and *Enterococcus*. A rapid decrease in pH during the initial steps of cheese preparation is of crucial importance in cheesemaking process, since it is essential for coagulation and the prevention or reduction of the growth of adventitious microflora [27].

3.3 Lipolysis

The extend of lipolysis of experimental cheeses during ripening as indicated by total free fatty acids levels is shown in Fig 2. The lipolysis index increased gradually until 60 d of ripening. It was noticed that lipolysis index were different significantly ($P < 0.05$) among the cheeses inoculated with the four types of strains. The cheeses with adjunct starters, in general, exhibited significantly ($P < 0.05$) higher levels of lipolysis index by the progress of ripening comparing to the control cheese. Cheese made with *Ent. faecalis* and *Ent. faecium* strains involved the highest level of FFA. These results indicated that adjunct enterococci contributed to lipolysis in cheese.

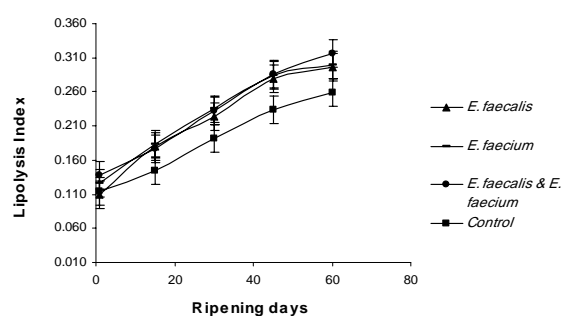


Fig. 2 Formation of total free fatty acids during ripening of UF Iranian white cheese made without (control cheese) or with added *Enterococcus* strains

Milk fat hydrolysis during cheese manufacture and ripening is due to the endogenous milk lipase, the lipolytic enzymes of starter and non-starter bacteria, lipases from psychrotrophic bacteria and exogenous enzyme preparations. Fatty acids can be further converted to methylketones and thioesters, which have been implicated as cheese flavor compounds [27].

3.4 Level of pH 4.6-SN/TN

The levels of pH 4.6-SN/TN in experimental Iranian UF white cheeses during ripening are shown in Fig 3. The concentrations of water-soluble nitrogen showed a gradual increase in all cheeses up to the end of ripening period, with no significant difference ($P < 0.01$) among cheeses made in the presence or absence of enterococci. *Ent. faecium* strains showed higher levels of SN/TN compared to the control or other adjunct-treated cheeses after the 60 days of ripening.

Some authors claim that the enterococci used as adjunct starters in cheese manufacture contribute to increased breakdown of casein and thus to soluble nitrogen production [5]. However, other studies have shown that proteinase activity in enterococci is low, with *Ent. faecalis* being the most proteolytic species [30].

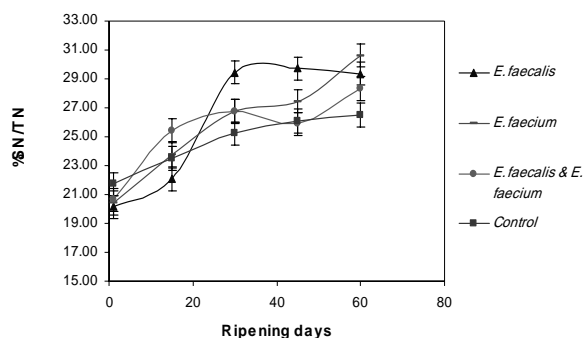


Fig. 3 Formation of pH 4.6-soluble nitrogen as a percentage of total nitrogen (SN/TN) in UF Iranian white cheeses made with *Enterococcus faecalis*, *Ent. faecium*, *Ent. faecalis* and *Ent. faecium*, and control samples during ripening

Centeno [5] reported in all of the batches made with enterococci the percentages of soluble nitrogen were higher than in the control batches, which indicates, in general terms, a greater proteolysis in the batches made with enterococci. The highest values were observed in the samples contained *Ent. faecalis* var *liquefaciens*. The increase in soluble nitrogen over the period of maturation of fermented dairy products made with *E. faecalis* has also been noted by other authors [6].

3.5 Level of non-protein nitrogen (NPN)

The levels of NPN/TN in experimental UF Iranian white cheeses during ripening are shown in Fig 4. The levels of NPN/TN were significantly ($P < 0.01$) different in the four types cheeses. The degree of secondary proteolysis in terms of NPN were higher in cheeses with *Ent. faecium* strains. In samples with *Ent. faecium* strains, NPN/TN increased from 5.03% at the beginning of ripening to 8.42% at 60 d of ripening, while in cheeses made with *Ent. faecalis* and *Ent. faecium* strains, NPN/TN% was lower during ripening. High and medium molecular mass peptides and caseins are gradually broken down by rennet and starter culture enzymes to lower molecular mass peptides and amino acids (2, 25) which are soluble in 12% TCA [17]. Therefore, the amount of 12% TCA-soluble nitrogen increases with the age of cheese [26].

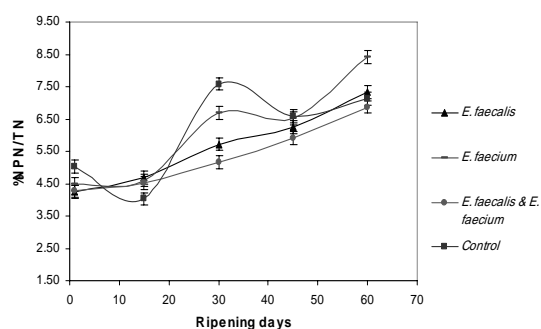


Fig. 4 Formation of non-protein nitrogen (NPN) in 12% TCA as a percentage of total nitrogen (NPN/TN) in UF Iranian white cheeses made with *Enterococcus faecalis*, *Ent. faecium*, *Ent. faecalis* and *Ent. faecium*, and control samples during ripening

The increase in phosphotungstic acid soluble nitrogen levels in cheeses made with enterococci which has been linked with the activity of the peptidases of these microorganisms and associated with low pH levels [7, 18].

3.6 Urea-PAGE

Urea-PAGE electrophoretograms of the pH 4.6-insoluble fraction of experimental UF white cheeses of the first replication after 1, 15, 30, 45 and 60 d of ripening are shown in Fig 5. Results of other trials were similar (not shown). There were observed some differences in electrophoretic patterns among the four cheese types. In samples made with *Ent. faecalis* and *Ent. faecium* strains, α s1-casein had the most hydrolysis, a fact which may be attributable both to a possible action of proteolytic enzymes (intracellular) of strains [21] and to a greater retention of rennet as a result of the less intense whey drainage during the production of these cheeses. The rennet remaining in the curd after whey drainage may be responsible for the initial degradation of the caseins (especially of the α s1-casein) forming high molecular weight peptides [11].

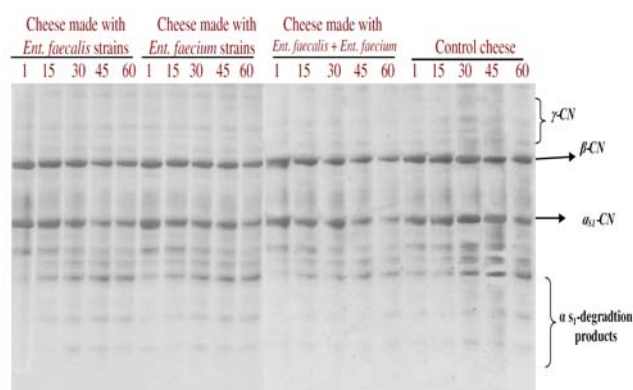


Fig. 5 Urea polyacrylamide gel electrophoretograms of experimental UF Iranian white brined cheeses made without (control cheese) or with added *Enterococcus* strains after 1, 15, 30, 45 and 60 d of ripening.

3.7 Sensory characteristics

The sensory assessment of the experimental Iranian UF white cheeses during ripening is presented in Table 2. There were significant ($P < 0.01$) differences among cheeses inoculated with the four types of strains in terms of sensory characteristics. Cheeses with the *Ent. faecalis* and *Ent. faecium* as adjunct starters received better grades than the other three samples. Cheeses made with *Ent. faecium* received lower scores than cheeses made with other adjuncts. The panelist's comments indicated that cheeses prepared with *Ent. faecium* strains lacked good flavor after two months of ripening and were scored lower due to some inappropriate and pasty texture and weaker aroma at the end of ripening period. In contrast, cheeses made with *Ent. faecalis* and *Ent. faecium* never received any comment about the presence of bitter flavors. The proteolytic and esterolytic activities displayed by some enterococcal strains, as well as their ability to metabolise citrate, may contribute to cheese ripening and flavour development. Because of these interesting metabolic properties, enterococci have been proposed as part of defined starter culture combinations for different European cheeses, such as Feta, water-buffalo Mozzarella and Cebreiro cheeses [12, 22]. Moreover, the significant increase in cheese flavor can be linked to the high levels of free amino groups in cheese made with the adjunct culture that possess considerable levels of aminopeptidolytic

activity. Several studies emphasized that a relationship exists between amino N content and flavor in cheese [23].

IV. CONCLUSIONS

High levels of enterococci are considered to lead to deterioration of some organoleptic characteristics in certain cheeses. On the other hand, many reports indicate the desirable role of enterococci in cheese production and quality. Furthermore, many cheese-related strains display the ability to produce bacteriocins against pathogens or food spoilage bacteria, thus offering a tool for increasing of food safety. Based on the overall evaluation of the results obtained from the physicochemical and sensorial analysis, the most pronounced impact of the enterococci strains on Iranian UF white cheese was observed on the lipolysis index, the levels of NPN/TN and the organoleptic properties of the ripened cheese. The present work demonstrates the technological potential of *Enterococcus* strains to be used as adjunct starters in the production of UF cheese. They appear to have the potential metabolic characters involved in cheese ripening and in aroma and flavor development.

TABLE II SENSORY EVALUATION OF UF IRANIAN WHITE CHEESES MADE WITH *ENT. FAECALIS*, *ENT. FAECIUM*, *ENT. FAECALIS* AND *ENT. FAECIUM*, AND CONTROL SAMPLES DURING RIPENING

Ripening , day	CONTROL SAMPLES DURING RIPENING			Control
	<i>Ent.</i> <i>faecalis</i>	<i>Ent.</i> <i>faecium</i>	<i>Ent.</i> <i>faecalis</i> & <i>Ent.faecium</i>	
1	3.08 ^a	3.45 ^{bd}	4.03 ^c	3.63 ^d
15	3.37 ^{abd}	3.09 ^b	4.00 ^{cd}	3.74 ^d
30	3.39 ^{abd}	3.31 ^{bd}	4.00 ^c	3.62 ^d
45	3.59 ^{abd}	3.70 ^{bd}	4.09 ^c	3.70 ^d
60	3.80 ^{abd}	3.72 ^{bd}	4.23 ^c	3.74 ^d

ACKNOWLEDGMENTS

The authors would like to thank the head of Drug Applied Research Center and Biotechnology Research Center of Tabriz University of Medical Sciences for providing the research facilities of this study.

REFERENCES

[1] Andrews, A. T. (1983) Proteinases in normal bovine milk and their action on casein. *Journal of Dairy Research*, 50, 45-55.
 [2] Ardo, Y. (1997). *Microbiology and biochemistry of cheese and fermented milk*. (Ed. B.A. Law) London, 207-218.
 [3] Blakesley, R. W. and Boezi, J. A. (1977). A new staining technique for proteins in polyacrylamide gels using Coomassie Brilliant Blue G250. *Annal Biochemistry*, 82, 580-581.
 [4] Centeno, J. A., Menendez, S. and Rodriguez-Otero, J. L. (1996). Main microbial flora as natural starters in Cebreiro raw cow's-milk cheese (Northwest Spain). *International Journal of Food Microbiology*, 33, 307-313.
 [5] Centeno, J. A., Menendez, S., Hermida, M. and Rodriguez-Otero, J. L. (1999). Effects of the addition of *Enterococcus faecalis* in Cebreiro cheese manufacture. *International Journal of Food Microbiology*, 48, 97-111.
 [6] El-Samragy, Y. A., Fayad, E. O., Aly, A. A. and Hagrass, A. E. A. (1988). Properties of labneh-like product manufactured using *Enterococcus* starter cultures as novel dairy fermentation bacteria. *Journal of Food Protection*, 51, 386-390.
 [7] Engels, W. J. M. and Visser, S. (1994). Isolation and comparative characterization of components that contribute to the flavour of different types of cheese. *Netherlands Milk and Dairy Journal*, 48, 127140

[8] Foulquie Moreno, M. R., Sarantinopoulos, P., Tsakalidou, E. and De Vuyst, L. (2006). The role and application of enterococci in food and health. *International Journal of Food Microbiology* 106, 1-24.
 [9] Fox, P. F. (1963). Potentiometric determination of salt in cheese. *Journal of Dairy Science* 46, 744-745.
 [10] Fox, P. F., Guinee, T.P. and McSweeney, P. L. H. (2000). *Fundamental of cheese science*. Gaithersburg: Aspen Publisher, Inc.
 [11] Gripon, J. C., Desmazeaud, M. J., Le Bars, D. and Bergere, J. L. (1975). Etude de role des micro-organismes et des enzymes au cours de la maturation des fromages. II. Influence de la pression commerciale. *Le Lait* 55, 502-516.
 [12] Hatzikamari, M., Litopoulou-Tzanetaki, E. and Tzanetakis, N. (1999). Microbiological characteristics of Anevato: A traditional Greek cheese. *Journal of Applied Microbiology* 87, 595-601.
 [13] Hesari, J., Ehsani, M. R., Khosroshahi, A., and McSweeney, P. L. H. (2006) Contribution of rennet and starter to proteolysis in Iranian UF white cheese. *Lait*, 86, 291-302.
 [14] IDF. (1964). Determination of protein content of process cheese products. IDF Standard 25. International Dairy Federation, Brussels, Belgium.
 [15] IDF. (1982). Determination of the total solid content (cheese and processed cheese). IDF Standard 4a, Brussels, Belgium: International Dairy Federation.
 [16] Institute of Standards and Industrial Research of Iran. (1998). Sensory evaluation of cheese. ISIRI Number, 4938, Tehran, Iran.
 [17] Kuchroo, C. N. and Fox, P. F. (1982). Soluble nitrogen in cheddar cheese. Comparison of extraction procedures. *Michwissenschaft* 937, 331-335.
 [18] Litopoulou-Tzanetaki, N., Tzanetakis, A. and Vafopoulou-Mastrogiannaki. (1993). Effect of the type of lactic starter on microbiological, chemical and sensory characteristics of Feta cheese. *Food Microbiology* 10, 31-41.
 [19] Lopez-Diaz, T. M., Alonso, C., Roman, C., Garcia-Lopez, M. L. and Moreno, B. (2000). Lactic acid bacteria isolated from a hand-made blue cheese. *Food Microbiology* 17, 23-32.
 [20] Marshal, R. T. (1992). Standard methods for the examination of dairy products. American Public Health Association, Washington, D.C.
 [21] Menendez, S., Centeno, J. A., Godinez, M. R. and Rodriguez-Otero, J. L. (1998). Algunas propiedades tecnologicas y actividades enzimaticas de cepas de *Enterococcus faecalis* aisladas del queso del Cebreiro. *Alimentaria* 296, 71-76.
 [22] Morandi, S., Brasca, M., Andrighetto, C., Lombardi, A. and Lodi, R. (2006). Technological and molecular characterization of *Enterococci* isolated from north-west Italian dairy products. *International Dairy Journal* 16, 867-875.
 [23] Muir, D. D., Banks, J. M. and Hunter, E. A. (1996). Sensory properties of Cheddar cheese: Effect of starter type and adjunct. *International Dairy Journal* 6, 407-423.
 [24] Nunez, M., Garcia -Aser, C., Rodriguez-Martin, A., Medina, M. and Gaya, P. (1996). The effect of ripening and cooking temperatures on proteolysis and lipolysis in manchego cheese. *Journal of Food Chemistry* 21, 115-123.
 [25] O'keefe, R. B., Fox, P. F. and Daly, C. (1978). Proteolysis in cheddar cheese. Role of coagulants and starter bacteria. *Journal of Dairy Research* 45, 465-477.
 [26] Reville, W. J., and P. E. Fox. 1978. Soluble protein in Cheddar cheese: a comparison of analytical methods. *Irish Journal of Food Science and Technology*, 2, 67-76.
 [27] Sarantinopoulos, P., Kalantzopoulos, G. and Tsakalidou, E. (2002). Effect of *Enterococcus faecium* on microbiological, physicochemical and sensory characteristics of Greek Feta cheese. *International Journal of Food Microbiology* 76, 93-105.
 [28] Shalabi, S. I. and Fox, P. F. (1987). Electrophoretic analysis of cheese: Comparison of methods. *Irish Journal of Food Science and Technology* 11, 135-151.
 [29] Sousa, M. J. and McSweeney, P. L. H. (2001). Studies on the ripening of colony an Irish farmhouse Camembert-type cheese. *Irish Journal of Agricultural Food Research* 40, 83-95.
 [30] Suzzi, G., Caruso, M., Gardini, F., Lombardi, A., Vannini, L., Guerzoni, M.E., Andrighetto, C. and Lanorte, M. T. (2000). A survey of the enterococci isolated from an artisanal Italian goat's cheese (*Semicotto caprino*). *Journal of Applied Microbiology* 89, 267-274.