Lait 82 (2002) 289–304
© INRA, EDP Sciences, 2002
DOI: 10.1051/lait:2002011

Original article

Proteolytic, lipolytic and autolytic activities of enterococci strains isolated from Egyptian dairy products

Bassem Bahy EL-DIN[a], Morsi EL-SODA[b]*, Nihal EZZAT[b]

[a] Quality Control Department, Mashreq des Produits Laitiers (Bongrain Egypte), Borg El-Arab City, Alexandria, Egypt
[b] Laboratory of Microbial Biochemistry, Department of Dairy Science and Technology, Faculty of Agriculture, Alexandria University, Alexandria, Egypt

(Received 16 January 2001; accepted 14 September 2001)

Abstract – Enterococci were isolated from Egyptian Domiatti and Mish cheeses made from raw milk. The proteolytic, lipolytic and autolytic activities of the isolated strains were studied to select the most appropriate cultures to be used for the enhancement of the organoleptic properties of cheese. The intracellular peptide hydrolase activities of enterococci strains were determined using various synthetic aminopeptidase and dipeptidyl-aminopeptidase substrates. 4-nitrophenyl and 2-nitrophenyl derivatives of fatty acids were used to measure the esterolytic activity of the different strains while emulsions of various triglycerides and butter fat were used to evaluate their lipolytic activities.The autolytic properties of the cultures were also considered. Three strains of *Enterococcus faecium* were freeze-shocked and evaluated as adjunct cultures in a cheese slurry system.

All the strains exhibited an aminopeptidase activity hydrolysing leucyl 4-nitroanilide faster than the arginyl, alanyl, prolyl or glycyl derivatives. An active dipeptidyl-aminopeptidase activity was also detected.

The *Enterococcus faecium* strains showed an active esterase system. The 4-nitrophenyl derivatives of the fatty acids were hydrolysed faster than the 2-nitrophenyl. The lipase substrates were hydrolysed according to the following order: tributyrin > tricaproin > tricaprylin > butterfat. The autolytic properties varied according to the strains tested.

Cheese slurries ripened with adjunct *Enterococcus faecium* exhibited considerably higher levels of water-soluble nitrogen (WSN) and total free amino acids (FAA) when compared to the control after incubation for 5 d at 32 °C. With this finding, a high variability of some technologically relevant properties was found and could be the basis for the selection of specific cultures to be used as adjunct cultures in the production of traditional cheeses.

***Enterococcus* / aminopeptidase / esterase / autolysis / culture adjunct**

---

* Correspondence and reprints
Tel.: (02) 03 590 3915; fax: (02) 03 590 3915; e-mail: morsi_elsoda@hotmail.com

Résumé – **Activités protéolytique, lipolytique et autolytique de souches d'entérocoques isolées de produits laitiers égyptiens.** L'activité peptide hydrolase intracellulaire d'*Enterococcus* isolée des fromages égyptiens Domiatti et Mish a été déterminée en utilisant différents substrats synthétiques d'aminopeptidases et de dipeptidylaminopeptidases.
Des dérivés 4-nitrophenyl et 2-nitrophenyl des acides gras ont été utilisés pour mesurer l'activité esterolytique des différentes souches, tandis que des émulsions de différents triglycérides et de la matière grasse du beurre ont été utilisés pour évaluer leurs activités lipolytiques. Les propriétés autolytiques des cultures ont aussi été mesurées. Trois souches d'*Enterococcus faecium* ont été soumises à un choc thermique par congélation et évaluées comme « levains atténués » dans un système de pâtes fromagères.
Toutes les souches ont montré une activité aminopeptidase en hydrolysant le leucyl 4-nitroanilide plus rapidement que les dérivés arginyl, alanyl, prolyl et glycyl. Une forte activité dipeptidyl aminopeptidase a été mise en évidence.
Enterococcus faecium présente un système esterasique actif. Les dérivés 4-nitrophenyl des acides gras ont été hydrolysés plus rapidement que les dérivés 2-nitrophenyl. Les substrats de la lipase ont été hydrolysés dans l'ordre suivant : tributyrine, tricaproine, tricapryline, matière grasse du beurre. Les propriétés autolytiques variaient d'une souche testée à l'autre.
Les pâtes de Cheddar affinées et inoculées avec différentes souches atténuées *Enterococcus faecium,* montrent une teneur plus élevée en azote soluble (WSN) et en acides aminés libres totaux (FAA) quand on les compare au contrôle après incubation 5 j à 32 °C.

***Enterococcus* / aminopeptidase / esterase / autolyse / levain atténué**

1. INTRODUCTION

Enterococci are Gram-positive bacteria and fit within the general definition of lactic acid bacteria [30]. They occur and grow in a wide variety of artisanal cheeses produced in southern Europe (Portugal, Spain, Italy and Greece) from raw or pasteurised goat's, ewe's, water-buffalo's or bovine milk. High levels of contaminating enterococci usually result from poor hygienic practices during cheese manufacture [48, 51, 66] and lead to deterioration of sensory properties in some cheeses but they play a major role in ripening and aroma development in other cheese varieties [11, 13, 48, 54, 56, 67, 68]. Moreover, for some *Enterococcus* species there is no evidence at present for a role in human disease [15].

The dominance or persistence of enterococci in some cheeses during ripening can be attributed to their wide range of growth temperatures, their high tolerance to heat, salt and acid [31, 48, 49, 56, 72] and their production of proteolytic enzymes involved in casein degradation [68, 72]. Also, salt concentration increases during cheese ripening. This is an important selection factor for growth of salt-tolerant enterococci and *Lb. plantarum* during the late stages of cheese ripening [31, 48, 56]. The beneficial effect of enterococci in cheesemaking has been attributed to hydrolysis of milk fat by esterases [69]. In addition, enterococci produce typical flavour components such as acetaldehyde, acetoin and diacetyl [11, 68]. This beneficial role of enterococci in the development of cheese aroma has led to inclusion of enterococcal strains in certain starter cultures. For example, enterococci were suggested for use as a starter in the production of Cebreiro cheese [11]. Similarly, *E. durans* was shown to be important for aroma development in Feta cheese when used in a starter together with other lactic acid bacteria (LAB) [50]. For Mozzarella cheese made from raw water-buffalo milk, a strain of *E. faecalis* was selected together with other LAB for use in a starter culture preparation [13, 58]. A food company sought clearance from the British "Advisory Committee on Novel Foods and Processes" (ACNFP) for the use of

E. faecium strain K77D as a starter culture in fermented dairy products [1], and the committee decided that the culture was acceptable for such use. Clearly, the enterococci play an important role in the manufacture of cheeses typical to some regions, and their use has a major impact in the flavour development of cheeses.

The aim of the present contribution is therefore to describe the lipolytic, proteolytic and autolytic properties of the enterococci and to follow enzyme activities during cheese ripening in a slurry system made with various freeze-shocked cultures. The results of this work should provide further information needed to select *Enterococcus* cultures to be used as adjuncts in cheesemaking.

2. MATERIALS AND METHODS

2.1. Cultures and growth conditions

The microorganisms used in this study were: *E. durans* AU149, *E. faecium* AU152, *E. faecium* MES27#16, *E. durans* AU171, *E. faecium* AU237, *E. faecium* AU336, *E. durans* AU601, *E. faecium* AU778, *E. faecium* AU1123, *E. faecium* AU232, *E. faecium* MES26, *E. faecium* AU728 and *E. faecium* AU1247 obtained from the culture collection of the laboratory of microbial biochemistry (LBDM). For the preparation of cells, 1000 mL of MRS broth [14] was inoculated with 100 mL of an active culture of the *Enterococcus* strain. Cell growth phases were monitored by measuring the absorbance at 650 nm using a LKB (Pharmacia Nova Spectrophotometer II, Cambridge, England). After 8–12 h, early stationary phase cells were harvested by centrifugation at 2 800 *g* for 20 min at 4 °C. The pellet was then washed twice with potassium phosphate buffer, pH 7.0. The resultant pellet was ground for 20 min in a mortar using alumina powder (Sigma type A-5, St. Louis, USA). The extract was resuspended in 1.36 $g \cdot L^{-1}$ potassium phosphate buffer. The suspension was than centrifuged at 4 000 *g* for 1 h at 4 °C. The supernatant containing the intracellular enzymes was stored at –20 °C.

2.2. Enzyme assay

2.2.1. Aminopeptidase and dipeptidyl aminopeptidase

The aminopeptidase activity was measured according to the procedure described by El-Soda and Desmazeaud [21]. L-leucyl, L-lysyl, L-alanyl, L-glycyl, L-arginyl and L-prolyl 4-nitroanilide were used as substrates. The same procedure was also used to follow the dipeptidylaminopeptidase activity but the substrates used for this purpose were arginyl-proline, glycyl-proline, glycyl phenylalanine and alanyl-alanine 4-nitroanilide, all substrates obtained from Sigma chemical CO., St. Louis, USA. One unit of enzymatic activity was defined as the variation of 0.01 unit of absorbance at 420 nm in 1 min for 1 mL enzyme of the different strains of enterococci under previously described assay conditions. The specific activity was defined as the number of activity $units \cdot mg^{-1}$ of protein, which is present in the supernatant.

2.2.2. Esterases

The esterase activity was measured according to the procedure described by Brandl and Zizer [8], using 4 and 2-nitrophenyl derivatives of acetate, butyrate, caproate, caprylate and caprate as substrates and 1.36 $g \cdot L^{-1}$ phosphate buffer, pH 7.0. A unit of esterase activity was defined as the variation of 0.01 unit of absorbance at 420 nm in 1 min for 1 mL enzyme of the different strains of enterococci under the previously described assay conditions. The specific activity was defined as the number of activity $units \cdot mg^{-1}$ of protein.

2.2.3. Assay of lipase activity

The lipase activity was measured according to the procedure described by Umemoto et al. [71].

2.3. Protein determination

The protein concentration was estimated according to the method of Lowry et al. [53], using the folin-phenol reagent with bovine serum albumin as standard.

2.4. Measurement of the rate of autolysis

The rate of autolysis was measured according to Thiboutot et al. [65] and could be summarised as follows: cells of different cultures were harvested by centrifugation and washed twice; the resulting bacterial pellet was then resuspended in 1/10 of the original volume of the growth medium, and then frozen at –20 °C. The frozen cell suspension was exposed to two cycles of freezing and thawing. A portion of cell suspension (~10%,v/v) was added to 27.2 $g \cdot L^{-1}$ potassium phosphate buffer pH 5.5 containing 58.5 $g \cdot L^{-1}$ sodium chloride to obtain an optical density of 0.8 to 1.0 at 650 nm and incubated at both 10 °C and 37 °C. After set time intervals the percentage decrease in optical density was measured and expressed as % autolysis.

2.5. Preparation of Cheddar cheese slurry

2.5.1. Cheddar cheese curd

Cheddar cheese curd was manufactured using small cheesemaking laboratory scale equipment, according to Kosikowski [43]. To twenty-four liters of warm (30 °C) pasteurised (72 °C for 15 s) cow's milk, 240 mL of a pure starter culture of *Lactococcus lactis* ssp. *lactis* ESO11 and commercial rennet (26 mL) were added. Then, rennet-treated milk was left to coagulate under quiescent conditions. The coagulum was then cut and cooked to 38 °C over 30 min (1 °C raise per 5 min) after which the whey was completely drained. Cheddaring was accomplished by cutting the curd; the curd was then left for 15 min and turned over. This step was repeated at 15-min intervals until the end of the cheddaring. At this stage, the pH of the curd reached a value of 5.2 to 5.3 and was used for slurry preparation.

2.5.2. Cheddar cheese slurry

Cheese slurry was prepared by modification of the method of Kristoffersen et al. [44] as described by Farkye et al. [27]. Unsalted curd (85 g), freeze-shocked suspension of *E. durans* AU149 (2.25 mL) or *E. faecium* AU336 (2.7 mL) or *E. faecium* AU1123 (4.25 mL), 3 g sterile NaCl and sterile distilled water (10 mL) were blended into a slurry in a sterile blender. The slurry was transferred aseptically into a sterile wide mouth bottle which was capped loosely and incubated at 32 °C for 5 d under anaerobic conditions using a Gas Pack (Generbox anaer + indicator, bio Mérieux, Lyon, France). Control slurry was prepared without the cell suspension. Each slurry preparation was replicated three times using freshly made starter cheese curd.

2.6. Extent of proteolysis

Extent of proteolysis of cheese slurry samples was monitored by the determination of water-soluble nitrogen (WSN) [45] and free amino acids.

2.6.1. Standard curve of leucine

Standard curve was carried out using L-leucine (L. light, CO. LTD. Colnbrook, England) at concentrations from 0.026 $g \cdot L^{-1}$ to 0.16 $g \cdot L^{-1}$.

2.6.2. Analysis of Cd-ninhydrin reactive amino groups

The concentrations of free amino acids in the WSN extracts were determined in duplicate by the modified [29] Cd-ninhydrin method of Doi et al. [16]. Lyophilised WSN extracts were reconstituted in distilled water [w·v^{-1}] and adjusted to pH 7.0 with 4 g·L^{-1} NaOH. A portion (0.2 mL) of the reconstituted extract was diluted to 1.0 mL with distilled water and 2.0 mL Cd-ninhydrin was added. The solution was heated at 84 °C for 5 min, cooled, and the absorbance measured to 507 nm (A_{507}) on a LKB Pharmacia Nova spectrophotometer. The Cd-ninhydrin reagent was prepared by dissolving 0.8 g ninhydrin in a solution consisting of 80 mL denatured ethanol, 10 mL acetic acid and 1 mL of aqueous $CdCl_2$ (1 g·mL^{-1}). The A_{507} was converted to g·L^{-1} leucine from a standard curve prepared with leucine.

3. RESULTS AND DISCUSSION

3.1. Aminopeptidase activity

Results in Table I reveal the presence of activity on aminopeptidase substrates in the crude cell free extract of most of the *Enterococcus* strains tested. Enterococci are in that respect comparable to other lactic acid bacteria [41, 42, 46, 60]. The enzyme system of the *Enterococcus* strains studied was most active on leu-4NA when compared to the other 4-nitroanilide derivatives. For instance, the specific activity for the hydrolysis of leu-4NA was 0.6 for *Enterococcus faecium* AU336 while it was 0.3, 0.3, 0.1, 0.22 and 0.01 in the case of arg-4NA, lys-4NA, alan-4NA, glyc-4NA and prol-4NA, respectively. A rather similar trend could also be noticed for the other strains.

Very little activity could be measured in all the strains on pro-4NA, indicating weak proline iminopeptidase activity. This was consistent with the findings of Khalid et al. [40] who reported that strains of *Lactobacillus helveticus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* slightly hydrolysed L. pro-4NA, indicating weak proline iminopeptidase activity. The proline iminopeptidase activity of *Pediococcus* sp., *Lactobacillus casei* and *Lactobacillus plantarum* was also very low compared to their aminopeptidase activity [24, 33]. On the other hand, they differed from *Propionibacterium freudenreichii*, *Propionibacterium* sp., *Bifidobacterium infantis* and *Bifidobacterium longum,* which showed high levels of proline iminopeptidase [23, 59]. *E. faecium* AU232, *E. faecium* AU1123 and *E. faecium* AU1247 were distinguished from the other studied strains by their higher aminopeptidase activities with most substrates.

3.2. Dipeptidyl aminopeptidase activity

A dipeptidyl aminopeptidase was also detected in the crude cell free extract of the different *Enterococcus* strains. Most of the strains tested were capable of hydrolysing: arginyl-proline-4NA, glycyl-proline-4NA, alanyl-alanine 4NA and glycyl-phenylalanine 4NA. Results in Table I show higher activity for most of the strains on glycyl-phenylalanine 4NA when compared to the other substrates. It was also of interest to notice that glycyl-phenylalanine 4NA was the only substrate hydrolysed by *E. faecium* AU778 while the latter substrate and alanyl-alanine 4NA were the only substrates hydrolysed by *E. faecium* AU152, *E. faecium* MES 27#16 and *E. faecium* MES26. *E. faecium* AU232, *E. faecium* AU1123 and *E. faecium* AU1247 differed from the other *Enterococcus* strains because of their higher activity on arginyl-proline 4NA and glycyl-proline 4NA when compared to glycyl-phenyl alanine 4NA. In fact, the specific activity for the hydrolysis of arginyl-proline-4NA was 1.7, 5.8 and

Table I. Specific activity of the aminopeptidase and dipeptidyl aminopeptidase from *Enterococcus* species (activity units·mg^{-1} protein).

Strain #	L-Arg 4NA	L-Lys 4NA	L-Ala 4NA	L-Leu 4NA	L-Gly 4NA	L-Pro 4NA	Ala-Ala 4NA	Gly-Phe 4NA	Arg-Pro 4NA	Gly-Pro 4NA
Enterococcus durans AU 149	0.14	0.02	0.02	0.1	0.02	0.01	0.03	0.12	0.02	0.02
Enterococcus faecium AU 152	0.14	0.1	0.03	0.1	0.04	0	0.03	0.1	0	0
Enterococcus faecium MES27#16	0.11	0.04	0.1	0.03	0.1	0	0.1	0.2	0	0
Enterococcus durans AU 171	0.3	0.1	0.1	0.4	0.04	0.02	0.1	0.25	0.2	0.16
Enterococcus faecium AU 232	3.6	1.7	3.5	15	0.1	0.3	0.2	0.02	1.7	1.8
Enterococcus faecium MES 26	0.1	0.01	0.1	0.1	0.02	0.01	0.04	0.26	0.001	0.001
Enterococcus faecium AU 237	0.23	0.03	0.04	0.2	0.02	0.01	0.1	0.17	0.01	0.02
Enterococcus faecium AU 336	0.3	0.3	0.1	0.6	0.22	0.01	0.01	0.02	0.01	0.01
Enterococcus durans AU 601	0.01	0.01	0.11	0	0.04	0.01	0.01	0.01	0	0.001
Enterococcus faecium AU 728	0.1	0	0.04	0.01	0.1	0.021	0.01	0.03	0.001	0
Enterococcus faecium AU 778	0.03	0.1	0.01	0.01	0.01	0.003	0	0.02	0	0.01
Enterococcus faecium AU 1123	3.4	2.1	5	18	0.1	0.21	0.5	0.22	5.8	7
Enterococcus faecium AU 1247	2.2	2	3.6	12	0.1	0.2	0.4	0.18	4	6

4NA: para nitroanilide.
Arg: arginine; Lys: lysine; Leu: leucine; Ala: alanine; Gly: glycine; Pro: proline.
One unit of enzymatic activity: the variation of 0.01 unit of absorbance at 420 nm in 1 min for 1 mL enzyme.
#: Number.

Table II. Specific activity of the esterase and lipase from *Enterococcus* species (activity units·mg^{-1} protein).

Strain #	Acetate		Butyrate		Caproate		Caprylate		Caprate		T.C2	T.C4	T.C6	G.M.A	G.M.B	M.F	O.O
	4	2	4	2	4	2	4	2	4	2							
Enterococcus durans AU 149	10.8	0.25	30	0.6	33.3	0.34	12.5	0.3	4.45	0.2	1.8	2.45	0.54	0.6	1.4	0.3	0.4
Enterococcus faecium AU 152	8.8	0.59	26.6	0.74	17	0.4	7.4	0.3	4	0.1	1.7	1.9	0.44	1.1	1.5	0.36	0.22
Enterococcus faecium MES27#16	10.13	0.67	25	1	21	0.6	10.15	0.6	4.5	0.3	1.9	2.1	0.5	1.4	1.7	0.45	0.44
Enterococcus durans AU 171	6.5	0.3	22	1	24.5	0.6	9.6	0.3	4.21	0.18	0.99	1.4	0.18	0.4	1.1	0.13	0.1
Enterococcus faecium AU 232	8	0.4	1.05	0.9	3	0.3	4	0.27	2.2	0.5	0.96	1.8	0.35	0.71	0.43	0.54	0.43
Enterococcus faecium MES 26	3	0.14	8	0.5	11	0	4	0.13	0.8	0.13	0.81	1.3	0.05	0.3	0.67	0.27	0.5
Enterococcus faecium AU 237	2.7	0.27	19	0.5	21	0.2	10	0.2	4	0.1	0.83	2.23	0.5	1.5	2	0.16	0.3
Enterococcus faecium AU 336	7	0.3	38	0.7	41	0.5	15	0.12	6	0.1	1.5	2.25	0.43	0.6	1.65	0.42	0.33
Enterococcus durans AU 601	4.2	0.32	29.5	0.74	22	0.12	15	0.16	6	0.04	0.54	0.98	0.2	0.21	0.8	0.14	0.25
Enterococcus faecium AU 728	5	0.21	32	0.78	28.5	0.5	9	0.31	7.7	0.12	0.93	1.41	0.18	0.3	1.1	0.2	0.11
Enterococcus faecium AU 778	5	0.1	28.5	0.72	24	0.33	12	0.22	4.3	0.04	0.6	1.6	0.26	0.95	1.1	0.4	0.19
Enterococcus faecium AU 1123	0.9	0.1	0.5	0.1	0.6	0.1	0.2	0.4	0.1	0.1	0.09	0.5	0.13	0.19	0.3	0.08	0.14
Enterococcus faecium AU 1247	0.6	0.1	0.1	0.06	0.4	0.02	0.1	0.1	0.1	0.01	0.081	0.53	0.18	0.06	0.7	0.03	0.02

2: ortho derivatives; 4: para derivatives.
T.C2: Tri-acetin; **T.C4**: Tri-butyrin; **T.C6**: Tri-caprylin; **G.M.A**: glycerol monoacetin; **M.F**: milk fat; **G.M.B**: glycerol monobutyrin; **O.O**: olive oil.
One unit of enzymatic activity: the variation of 0.01 unit of absorbance at 420 nm in 1 min for 1 mL enzyme.
#: Number.

4 while it was 0.02, 0.22 and 0.18 for glycyl-phenylalanine-4NA for *E. faecium* AU232, *E. faecium* AU1123 and *E. faecium* AU1247, respectively. It is therefore suggested to consider the enzyme a X-propyl dipeptidyl peptidase. Dipeptidyl aminopeptidase activity was detected in several genera of lactic acid bacteria [7, 10, 23, 24, 28, 34, 39, 55, 64].

On the other hand, this is the first report describing the presence of a dipeptidyl aminopeptidase in the enterococci.

3.3. Detection of the esterase activity

The results (Tab. II) describing the esterolytic activity of the enterococci indicate that all the enterococci tested showed an active esterolytic system. As a general rule, the thirteen strains tested showed similar behaviour with all substrates. They were active on all 4- and 2-nitrophenyl derivatives of the fatty acids containing up to 10 carbon atoms. Propionibacteria seemed to be different from the enterococci in this respect since they hydrolysed the naphthyl derivatives of lauric, myristic, palmitic and stearic acids. *E. faecium* AU152, *E. faecium* MES27#16, *E. durans* AU601, *E. faecium* AU728 and *E. faecium* AU778 showed the highest activity on 4-nitrophenyl butyrate, while *E. durans* AU149, *E. durans* AU171, *E. faecium* AU232, *E. faecium* MES26 and *E. faecium* AU336 were most active on 4-nitrophenyl caproate. A lower rate of activity could be measured for 4-nitrophenyl caprylate and 4-nitrophenyl caprate. Similar observations were reported by Tsakalidou et al. [70] who found that the purified esterase from *E. faecium* ACA.DC 237 hydrolysed the 2-nitrophenyl derivatives of fatty acids in the order C6>C8>C10>C12. Also, similar observations were made by Hosono et al. [35]. In fact, the authors found that ethyl butyrate production was higher when compared to ethyl caproate production by 3 lactococci and 2 lactobacilli. *E. faecium* AU232 was distinguished from all the other species by the fact that it was more active than all the other strains on the rest of the substrates on 4-nitrophenyl acetate. A similar observation was reported by Khalid et al. [40] who found that *Lb. helveticus* ATCC10797 hydrolysed 4-nitrophenyl acetate faster than 4-nitrophenyl butyrate. Table II indicates that differences exist between strains of the same species: *E. faecium* AU336 shows higher activity if compared to *E. faecium* AU152, *E. faecium* MES27#16, *E. faecium* AU237, *E. faecium* AU778 and *E. faecium* AU1123 for most of the 4-nitro derivatives. From the data illustrated in Table II, it can also be noticed that the hydrolysis of the 4-nitrophenyl derivatives of the fatty acids was always significantly higher when compared to 2-nitrophenyl derivatives. The most significant differences could be observed in the case of *E. durans* AU601 where the specific activity for 4-nitrophenyl caproate hydrolysis was 22, while it was only 0.12 for the ortho-derivative and *E. faecium* AU336 which showed a specific activity of 15 and 0.12 for the para- and 2-nitrophenyl caprylate, respectively. This is probably due to the differences in the steric hindrance of the two substrates. The straight configuration of the 4-derivatives seems to be more accessible to the active site of the enzyme. *E. faecium* AU1123 and *E. faecium* AU1247 can be distinguished from the other strains by their lower esterase activities. A similar trend can also be observed with the hydrolysis for 2-derivatives.

3.4. Detection of the lipase activity

The substrates used during this investigation for the evaluation of the lipolytic activity of the enterococci could be grouped as follows: the synthetic monoglycerides: glycerol monoacetin-glycerol monobutyrin, the synthetic triglycerides: triacetin-tributyrin-tricaprylin and the natural fats: milk fat-olive oil. The results illustrated in Table II show that among the synthetic monoglycerides, glycerol monobutyrin

was the most rapidly hydrolysed by the majority of the *Enterococcus* strains tested, and the rate of hydrolysis decreased with glycerol monoacetin. It also appears that differences exist between the rate of hydrolysis of the triglycerides studied. Tributyrin was the best substrate for all the strains tested. A lower rate of hydrolysis could be measured in the case of triacetin and tricaprylin. Also, similar observations were obtained in the hydrolysis of natural fats. When comparing the rate of hydrolysis of the different classes of substrates, it was possible to reach the following conclusions:

1. Tributyrin is the best substrate among all the substrates tested.

2. Glycerol monobutyrin is the best substrate among the monoglycerides.

3. There are no significant differences in the rate of hydrolysis between milk fat and olive oil. Similar observations were reported by Chander et al. [12] who found that the purified lipase from *E. faecalis* hydrolysed the synthetic triglycerides in the order: tributyrin > tricaproin > tricaprylin > triolein. Among the synthetic triglycerides, *E. faecalis* lipase hydrolysed tributyrin more rapidly than other simple triglycerides. Concerning the specificity of the lipase system, the general conclusion reached by most authors was that lactic acid bacteria hydrolyse triglycerides composed of short chain fatty acids more rapidly than those composed of higher chain fatty acids. The same authors [12] also found that *E. faecalis* lipase hydrolysed butter oil to a lower degree than tributyrin. Singh et al. [61] also found that intracellular lipase from *Lactobacillus lactis* hydrolysed tributyrin whereas tripalmitin and triolein hydrolysis was very limited. Umemoto et al. [71] showed that in the case of *Lb. casei*, *Lb. plantarum*, *Lb. helveticus* and *Lactococcus lactis* subsp. *diacetylactis* that tributyrin emulsions were hydrolysed more rapidly than butter fat and olive oil. A similar trend can also be found in the work of Carini et al. [9] and El-Soda et al. [22]. Comparable results are also reported in the work of Dovat et al. [17] with lactic streptococci. These authors concluded that enterococci were more active than the lactic streptococci on tributyrin and tripropionin. Tricaproin and tricaprylin were less frequently hydrolysed and triolein was not attacked at all. Stadhouders et al. [63] examined the rate of lipolysis of partially hydrolysed fat by the lactic streptococci. It was found that more fatty acids were formed from mixtures of tri-, di- and monoglycerides than from the pure triglycerides, which led the authors [63] to conclude that di- and monoglycerides are better substrates for starter bacteria. It was also demonstrated that the rate of tributyrin hydrolysis by the pediococci was higher than the rate of hydrolysis of triglycerides containing long chain fatty acids [4, 57].

3.5. Autolytic properties of different strains of enterococci

The autolytic properties of the studied strains were compared in 27.2 $g \cdot L^{-1}$ potassium phosphate buffer, pH 5.5 containing 58.5 $g \cdot L^{-1}$ sodium chloride at 37 °C and 10 °C. The obtained results (Fig. 1) indicate the rate of autolysis for all the strains tested at 37 °C. It is noticeable that there was a gradual increase in the percentage of autolysis with time. Most of the tested strains exhibited an autolysis rate lower than 25% after 120 h at 10 °C. From the previous results, it is obvious that the autolysis rate of the studied strains incubated at 37 °C was higher when compared to the autolysis measured at 10 °C. This is in agreement with the work of El-Soda et al. [25] who studied the influence of temperature on cell autolysis on a wide range of cheese-related microorganisms, including the following genera: *Leuconostoc,* pediococci, lactococci, lactobacilli, *Bifidobacterium* and *Propionibacterium*. Autolysis was measured at 10, 20, 30, 40 and 50 °C. It was found that the maximum autolysis occurred at

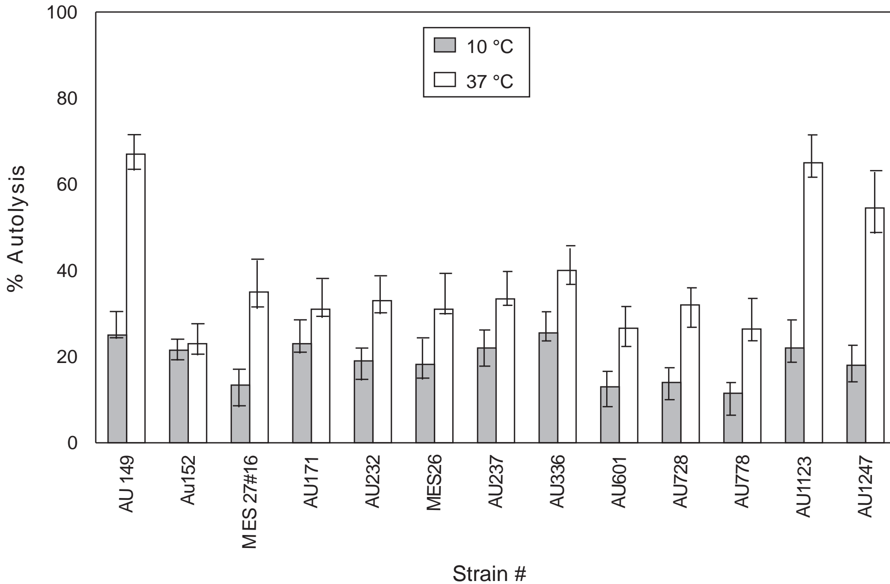

Figure 1. Autolytic properties of different *Enterococcus* strains after 120 h.

temperatures similar or close to the optimum growth temperatures of the microorganisms. Higher autolysis at 40 °C was also detected by Lemee et al. [47] for *Propionibacterium freudenreichii* CNRZ 725. These authors [47] noticed very little autolysis at 10 °C. Similar observations were also made by Lortal et al. [52] in the case of *Lactobacillus helveticus* CNRZ 414.

The previously described results indicate that *Enterococcus faecium* AU1123 is the highest in peptidase and autolysis while strains AU149 and AU336 showed high levels of lipolysis and autolysis when compared with other strains. These strains were therefore selected to be added as adjunct for the slurry experiments.

3.6. Characteristics of Cheddar cheese slurries made using attenuated adjunct cultures of *Enterococcus* strains

Table III describes the chemical composition of control cheese slurry and cheese slurries with added freeze-shocked cells. Cheddar cheese slurries show that the addition of freeze-shocked cells did not affect the chemical composition of Cheddar cheese slurries through the ripening period. It appears that the chemical composition of *E. durans* AU149, *E. faecium* AU336 and *E. faecium* AU1123 treated slurry was rather close to the control. For instance, the mean values for the protein content of the slurries at the end of the incubation time was as follows: 21.6 for the untreated cheese, 21.04 for *E. durans* AU149, 20.79 for *E. faecium* AU336 and 20.86 for *E. faecium* AU1123. It was of interest to notice that the slurries made with the freeze-shocked cells show higher rates of acid development than the control. The pH was 4.82, 4.89, 4.92 and 5.03 in the case of *E. durans* AU149, *E. faecium* AU336, *E. faecium* AU1123 and the untreated cheese, respectively, which may indicate that the freeze-shocking treatment did not affect the acid-producing ability of the enterococci.

Table III. Composition of Cheddar cheese slurries inoculated with different adjunct cultures.

Strain #	% Moisture		pH		% Dry matter		% Fat		% Protein	
	A	B	A	B	A	B	A	B	A	B
Lactococcus lactis ssp. *lactis*ESO11 (control)	51.12	47.15	5.19	5.03	48.8	52.85	22.5	24.2	18.51	21.6
Enterococcus durans AU 149	51.46	46.6	5.18	4.82	48.54	53.4	22.5	24.4	18.06	21.04
Enterococcus faecium AU 336	52.15	47.3	5.18	4.89	47.85	52.7	22.5	24.1	18.15	20.79
Enterococcus faecium AU 1123	53	47.8	5.2	4.92	47	52.2	22.4	24	17.8	20.86
Mean	53	47.8	5.2	4.92	47	52.2	22.4	24	17.8	20.86
S.D.	0.83	0.49	0.01	0.09	0.81	0.49	0.05	0.17	0.29	0.37
t	9.7		6.2		10.03		19.106		12.51	
p	0.00003*		0.0004*		0.0002*		0.00006*		0.00007*	

A: Zero time; B: After 5 d (32 ºC); t = student t-test; p = probability; S.D: Standard deviation; Mean: Mean value; #: Number.
The experiment were characterised by high significance between Zero time and after 5 d.

3.6.1. Ripening indexes

The increase in soluble nitrogen (Tab. IV) during the ripening period indicates that the slurries treated with the *Enterococcus* strains showed higher values of water-soluble nitrogen (WSN) if compared to the control. It was also of interest to notice that *E. faecium* AU1123 showed the highest increase in water-soluble nitrogen when compared to *E. durans* AU149, and *E. faecium* AU336. The percentage increase in water soluble nitrogen was 57.9, 43.3 and 67 higher than the control, in the slurry treated with *E. durans* AU149, *E. faecium* AU336 and the slurry treated with *E. faecium* AU1123, respectively.

The results in Table IV illustrating the liberation of free amino acids during the ripening of the slurries indicate that the addition of freeze-shocked cells led to higher values of free amino acids in the WSN if compared to the control. Cheese slurry made with *E. faecium* AU1123 developed higher free amino acids in the WSN than both the *E. durans* AU149 and *E. faecium* AU336 treated cheese slurries. In fact, the values for free amino acids in the WSN extracts after 5 d were 0.054 $g \cdot L^{-1}$, 0.065 $g \cdot L^{-1}$, 0.06 $g \cdot L^{-1}$ and 0.072 $g \cdot L^{-1}$ leucine equivalents for the control, *E. durans* AU149, *E. faecium* AU336 and *E. faecium* AU1123 cells, respectively. This suggests that freeze-shocked cells of *E. faecium* may be added to cheese to increase the release of free amino acids during ripening, provided that the small peptides from which the amino acids are derived are not limiting.

Our results in that respect are comparable with the work of previous authors for other genera of lactic acid bacteria. Bartels et al. [6] used whole cells of *Lb. helveticus* CNRZ32 which were freeze-shocked at –24 °C before being added to milk for Gouda cheese manufacture in an attempt to enhance flavour development. Substantial increases in water-soluble peptides and amino acids were observed in experimental

Table IV. Formation of free aminogroups and soluble nitrogen content in Cheddar cheese slurries inoculated with different adjunct cultures.

Strain #	Cd-ninhydrin reactive amino groups ($g \cdot L^{-1}$ leucine)		% soluble nitrogen	
	Zero time	After 5 d (32 °C)	Zero time	After 5 d (32 °C)
Lactococcus lactis ssp. *lactis* ES011 (Control)	0.043	0.054	3.800	5.900
Enterococcus durans AU 149	0.046	0.065	4.100	10.100
Enterococcus faecium AU 336	0.045	0.060	4.000	9.200
Enterococcus faecium AU 1123	0.046	0.072	5.000	10.600
Mean	0.046	0.072	5.000	10.6
S.D.	0.0014	0.0076	0.53	2.11
t	4.57		4.33	
p	0.001*		0.002	

t = student t-test; p = probability; S.D: Standard deviation; Mean: Mean value; #: Number .
The experiment were characterised by high significance between Zero time and after 5 d.

cheese compared to controls. Flavour strength was greater and bitterness was reduced throughout the ripening period. El-Shafei et al. [20] studied the effect of adding freeze-shocked cells from *Pediococcus halophilus* on the reduction of the curing time of Ras cheese, where they noticed higher values of soluble nitrogen in the experimental cheese compared to the control cheese. Our work is also comparable to the findings of Aly [2], Aly [3], El-Shafei [18], El-Shafei [19], Ezzat and El-Shafei [26], Johnson et al. [38] and Spangler et al. [62]. These authors reported that the incorporation of freeze-shocked cells of lactobacilli, lactococci, *Leuconostoc* and *Bifidobacterium* increased the levels of proteolysis in the cheese.

We would also like to point out that the model system comprising cheese slurries containing freeze-shocked cells gave promising results. Addition of freeze-shocked cells to starter cheese curd gave a good indication of their contribution to proteolysis during ripening. However, cheesemaking using conventional procedures is still needed to confirm these findings.

4. CONCLUSION

This study reveals that *Enterococcus* strains had proteolysis and lipolysis activities. Also, the strains demonstrated autolytic properties under cheesemaking conditions. Addition of freeze-shocked adjunct enterococci to cheese curd made with starter culture gave a clear indication of their potential contribution to proteolysis during cheese ripening. Ripening indices (WSN and FAA) revealed that adjunct enterococci increased the extent of protein degradation in model cheese slurry. Actually, addition of adjunct cultures with high autolytic properties and high enzyme activity such as *E. faecium* strain AU1123 led to higher concentration of FAA in cheese slurries when compared to control slurry. The presence of high peptidase and esterase activity in extracts from slurries made with adjunct strains of enterococci with enhanced autolytic characteristics reflects the importance of cell lysis during cheese ripening. From these findings and previous work described by Arihara et al. [5], Franz et al. [30], Giraffa [32] and Jett et al. [37], it is possible to select adjunct strains of *E. faecium* for the stability of their enzymes as well as for their improved autolytic properties in the cheese environment. It is also being accepted as a starter by IDF [36]. Devriese and Pot [15] reported that *E. faecium* is among the predominant microorganisms in raw milk, which has important implications for the dairy industry. Giraffa [32] reported that strains of enterococci isolated from dairy products do not produce haemolysin, and it was suggested that absence of haemolytic activity should be a selection criterion for starter strains for dairy use. On the other hand, Jett et al. [37] explained that the pathogenic potential of *E. faecalis* is considered to be greater than that of *E. faecium,* because more than 80% of enterococci associated with human infections are *E. faecalis.* Even though *E. faecalis* seems to have a greater pathogenic potential than *E. faecium,* the association of either of these species with food may not be considered desirable. The incidence of enterococci in human disease, however, does not appear to correlate with the incidence of these organisms in foods, especially when their use as starter cultures or as probiotics is taken into consideration.

REFERENCES

[1] ACNFP, Report on *Enterococcus faecium,* strain K77D. MAFF Advisory Committee on Novel Foods and Processes, Report, Ergon House c/o Nobel House, 17 Smith square, London SW1 3JR, UK, 1996.

[2] Aly M.E., Utilization of freeze-shocked lactobacilli for enhancing flavour development of Ras cheese, Nahrung 34 (1990) 329–35.

[3] Aly M.E., Evaluation of some freeze-shocked lactic starters for accelerated ripening of Domiati cheese made at various salt levels, Egypt. J. Dairy Sci. 24 (1996) 47–60.

[4] Angeles A., Marth E., Growth and activity of lactic acid bacteria in say milk: III. Lipolytic activity, J. Milk Food Technol. 34 (1971) 69: 73.

[5] Arihara K., Cassens R., Luchansky J., Characterization of bacteriocins from *Enterococcus faecium* with activity against *Listeria monocytogenes*, Int. J. Food Microbiol. 19 (1993) 123–134.

[6] Bartels H.J., Johnson M.E., Olson N.F., Accelerated ripening of Gouda cheese. Part II. Effect of freeze-shocked *Lactobacillus helveticus* on proteolysis and flavor development, Milchwissenschaft 42 (1987) 139–144.

[7] Bockelmann W., Fobker M., Teuber M., Purification and characterization of the X-prolyl-dipeptidylaminopeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus acidophilus*, Int. Dairy J. 1 (1991) 51–56.

[8] Brandl E., Zizer T., On the hydrolysis of aromatic esters by microbial preparation with lipolytic and proteolytic activity, Osterr. Milchwirtsch., Wiss. Beil. 28 (1973) 15–24.

[9] Carini S., Kadaravek G., Lodi R., Lipolisi microbica: I lattici. Sci. Tecn. Latt.-Casearia 23 (1972) 377–388.

[10] Casey M.G., Meyer J., Presence of X-prolyl-dipeptidyl-peptidase in lactic acid bacteria, J. Dairy Sci. 68 (1985) 3212–3215.

[11] Centeno J.A., Menéndez S., Rodriguez-Otero J.L., Main microbial flora present as natural starters in Cebreiro raw cow's-milk cheese (Northwest Spain), Int. J. Food Microbiol. 33 (1996) 307–313.

[12] Chander H., Ranganathan R., Singh J., Purification and some properties of lipase from *Streptococcus faecalis*, J. Food Sci. 44 (1979) 1747–1751.

[13] Coppola S., Parente E., Dumontet S., La Peccerella A., The microflora of natural whey cultures utilized as starters in the manufacture of Mozzarella cheese from water-buffalo milk, Lait 68 (1988) 295–310.

[14] De Man J., Rogosa M., Sharpe E., A medium for the cultivation of lactobacilli, J. Appl. Bacteriol. 23 (1960) 130–135.

[15] Devriese L.A., Pot B., The genus *Enterococcus,* in: Wood B.J.B., Holzapfel W.H. (Eds.), The genera of lactic acid bacteria,Vol. 2, Blackie Academic, London, UK, 1995, pp. 327–367.

[16] Doi E., Shibata D., Matoba T., Modified colorimetric methods for peptidase assay, Anal. Biochem. 118 (1981) 173–184.

[17] Dovat A., Reinbold G., Hammond E., Vedamuthu E., Lipolytic and proteolytic activity of enterococci and lactic group streptococci isolated from young Cheddar cheese, J. Milk Food Technol. 33 (1970) 365–372.

[18] El-Shafei H., Accelerated ripening of Ras Cheese using cell free extract, freeze-and heat-shocked *Leuconostoc* spp. cultures, Nahrung 38 (1994) 599–605.

[19] El-Shafei H., Manufacture of Ras cheese with cell free extract, freeze and heat shocked strains of *Bifidobacterium* spp., Indian J. Dairy Sci. 47 (1994) 774–779.

[20] El-Shafei H., Hantira A., Ezzat N., El-Soda M., Characteristics of Ras cheese made with freeze-shocked *Pediococcus halophilus*, Lebensm. Wiss. Technol. 25 (1992) 438–441.

[21] El-Soda M., Desmazeaud M., Les peptide hydrolases des *Lactobacillus* du groupe *Thermobacterium.* I. Mise en évidence de ces activités chez *Lactobacillus helveticus*, *L. acidophilus, L. lactis* et *L. bulgaricus,* Can. J. Microbiol. 28 (1982) 1181–1188.

[22] El-Soda M., Korayem M., Ezzat N., The esterolytic and lipolytic activities of lactobacilli. Part III. Detection and characterization of the lipase system, Milchwissenschaft 41 (1986) 353–355.

[23] El-Soda M., Macedo A., Olson N.F., The peptide hydrolase system of *Bifidobacterium* species, Milchwissenschaft 47 (1992) 87–90.

[24] El-Soda M., Ezzat N., El-Houby T., Zeidan I., The peptide hydrolase system of pediococci: cell bound enzymatic activities. Microbiol. Aliment Nutr. 12 (1994) 145–154.

[25] El-Soda M., Farkye N., Vuillemard J., Simard R., Olson N., El-Kholy W., Dako E., Medrano E., Gaber M., Lim L., Autolysis of lactic acid bacteria: Impact on flavour development in cheese, in: Charalambous G. (Ed.), Food flavour: Generation, analysis and process influence, Elsevier Science B.V. Amsterdam, The Netherlands, (1995), pp. 2205–2223.

[26] Ezzat N., El-Shafei H., Acceleration ripening of Ras cheese using freeze and heat-shocked *Lactobacillus helveticus*, Egypt. J.Dairy Sci. 19 (1991) 347–358.

[27] Farkye N.Y., Madkor S.A., Alkins H.G., Proteolytic abilities of some lactic acid bacteria in a model cheese system, Int. Dairy J. 5 (1995) 715–725.

[28] Fernàndez-Esplà M.D., Fox P.F., Purification and characterization of X-prolyl-dipeptidyl-aminopeptidase from *Propionibacterium shermanii* NCDO 853, Int. Dairy J. 7 (1997) 23–29.

[29] Folkertsma B., Fox P.F., Use of the Cd-ninhydrin reagent to assess proteolysis in cheese during ripening, J. Dairy Res. 59 (1992) 217–224.

[30] Franz C., Holzapfel W., Stiles M., Enterococci at the crossroads of food safety?, Int. J. Food Microbiol. 47 (1999) 1–24.

[31] Freitas A.C., Pais C., Malcata F.X., Hogg T.A., Microbiological characterization of Picante de

Beira Baixa cheese, J. Food Protect. 59 (1996) 155–160.

[32] Giraffa G., Enterococcal bacteriocins: their potential as antilisteria factors in dairy technology, Food Microbiol.12 (1995) 291–299.

[33] Hegazi F.Z., Abo-Elnaga I.G., Proteolytic activity of crude cell-free extract of *Lactobacillus casei* and *Lactobacillus plantarum*, Nahrung 31(1987) 225.

[34] Hiroshi Miyakawa I., Hashimoto I., Nakamura T., Ishibashi N., Shimamura S., Igoshi K., Purification and characterization of an X-proly/dipeptidyl aminopeptidase from *Lactobacillus helveticus* LHE-511, Milchwissenschaft 49 (1994) 670–673.

[35] Hosono A., Elliott J.A., McGugan W.A., Production of ethylesters by some lactic acid and psychrotrophic bacteria, J. Dairy Sci. 57 (1974) 535–539.

[36] IDF, Dairy starter cultures lactic acid bacteria (LAB), Standard 149A, Int. Dairy Fed., Brussels, Belgium (1997).

[37] Jett B.D., Huyke M.M., Gilmore M.S., Virulence of enterococci, Clin. Microbiol. Rev. 7 (1994) 462–478.

[38] Johnson J.A.C., Etzel M.R., Chen C.M., Johnson M.E., Accelerated ripening of reduced-fat Cheddar cheese using four attenuated *Lactobacillus helveticus* CNRZ-32 adjuncts, J. Dairy Sci. 78 (1995) 769–776.

[39] Khalid N.M., Marth E.H., Purification and partial characterization of a prolyl-dipeptidyl aminopeptidase from *Lactobacillus helveticus* CNRZ 32, Appl. Environ. Microbiol. 56 (1990) 381–388.

[40] Khalid N.M., El-Soda M., Marth E.H., Peptide hydrolases of *Lactobacillus helveticus* and *Lactobacillus debrueckii* ssp. *bulgaricus*, J. Dairy Sci. 74 (1991) 29–45.

[41] Kok J., Proteinase genes of cheese starter cultures, Biochem. Soc.Trans. 19 (1991) 670–674.

[42] Kok J., Microbiology of cheese flavor development, genetics of proteolytic enzymes of lactococci and their role in cheese flavor development, J. Dairy Sci. 76 (1993) 2056–2064.

[43] Kosikowski F., Cheese and fermented milk foods, Edwards Brothers, Inc, Ann Arbor, Michigan, USA, 1977.

[44] Kristoffersen T., Mikolajcik E.M., Gould I.A., Cheddar cheese flavor, Part IV. Directed and accelerated ripening process, J. Dairy Sci. 50 (1967) 292–297.

[45] Kuchroo C.N., Fox P.F., Soluble nitrogen in Cheddar cheese: Comparison of extraction procedures, Milchwissenschaft 37 (1982) 331–335.

[46] Law J., Haandrikman A., Review article: Proteolytic enzymes of lactic acid bacteria, Int. Dairy J. 7 (1997) 1–11.

[47] Lemée R., Lortal S., Cesselin B., van Heijenoort J., Involvement of an *N*-acetylglucosaminidase in autolysis of *Propionibacterium freudenreichii* CNRZ 725, Appl. Environ. Microbiol. 60 (1994) 4351–4358.

[48] Litopoulou-Tzanetaki E., Changes in numbers and kinds of lactic acid bacteria during ripening of Kefalotyri cheese, J. Food Sci. 55 (1990) 111–113.

[49] Litopoulou-Tzanetaki E., Tzanetakis N., Microbiology of white brined cheese made from raw goat milk, Food Microbiol. 9 (1992) 13–19.

[50] Litopoulou-Tzanetaki E., Tzanetakis N., Vafopoulou-Mastrojiannaki A., Effect of type of lactic starter on microbiological, chemical and sensory characteristics of Feta cheese, Food Microbiol. 10 (1993) 31–41.

[51] López-Diaz T.M., Santos J.A., González C.J., Moreno B., Garcia M.L., Bacteriological quality of traditional Spanish blue cheese, Milchwissenschaft 50 (1995) 503–505.

[52] Lortal S., Boyaval P., van Heijenoort J., Influence de plusieurs facteurs sur l'autolyse de *Lactobacillus helveticus* CNRZ 414, Lait 69 (1989) 223–231.

[53] Lowry O., Resebrough A., Forr L., Randall R., Protein measurements with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.

[54] Macedo A.C., Malcata F.X., Hogg T.A., Microbiological profile in Serra ewe's cheese during ripening, J. Appl. Bacteriol. 79 (1995) 1–11.

[55] Meyer J., Jordi R., Purification and characterization of an X- prolyl-dipeptidyl aminopeptidase from *Lactobacillus lactis* and from *Streptococcus thermophilus*, J. Dairy Sci. 70 (1987) 738–745.

[56] Ordonèz J.A., Barneto R., Ramos M., Studies on Manchego cheese ripened in olive oil, Milchwissenschaft 33 (1978) 609–612.

[57] Oterholm A., Ordal Z., Witter L.D., Glycerol ester hydrolase activity of lactic acid bacteria, Appl. Microbiol. 16 (1968) 524–527.

[58] Parente E., Villani F., Coppola R., Coppola S., A multiple strain starter for water-buffalo Mozzarella cheese manufacture, Lait 69 (1989) 271–279.

[59] Perez Chaia A., Pesce de Ruiz Holgado A., Oliver G., Peptide hydrolases of propionibacteria: effect of pH and temperature, J. Food Protect. 53 (1990) 237–240.

[60] Pritchard G., Coolbear T., The physiology and biochemistry of the proteolytic system in lactic acid bacteria, FEMS Microbiol. Rev. 12 (1993) 179–206.

[61] Singh A., Srinivasan R., Dudani A., Studies on exocellular and endocellular lipases of some of the lipolytic bacteria, Milchwissenschaft 28 (1973) 164–166.

[62] Spangler P.L., El-Soda M., Johnson M.E., Olson N.F., Amundson C.H., Hill C.G., Accelerated ripening of Gouda cheese made from ultrafiltered milk using liposome entrapped enzyme and freeze-shocked lactobacilli, Milchwissenschaft 44 (1989) 199–203.

[63] Stadhouders J., Veringa H.A., Fat hydrolysis by lactic bacteria in cheese, Neth. Milk Dairy J. 27 (1973) 77–91.

[64] Tan P.S.T., Chapot-Chartier M.P., Pos K.M., Rousseau M., Boquien C.Y., Gripon J.C., Konings W.N., Localization of peptidases in lactococci, Appl. Environ. Microbiol. 58 (1992) 285–290.

[65] Thiboutot H., Dako E., El Soda M., Vuillemard J.C., Power N., Simard R.E., Influence of heat and freeze shocking on the autolysis and peptidase activities of *Lactobacillus casei*, Milchwissenschaft 50 (1995) 448–452.

[66] Thompson T.L., Marth E.H., Changes in Parmesan cheese during ripening: Microflora coliforms, enterococci, anaerobes, propionibacteria and staphylococci, Milchwissenschaft 41 (1986) 201–204.

[67] Torri Tarelli G., Carminati D., Giraffa, G., Production of bacteriocins active against *Listeria monocytogenes* and *Listeria innocua* from dairy enterococci, Food Microbiol. 11 (1994) 243–252.

[68] Trovatelli L.D., Schiesser A., Identification and significance of enterococci in hard cheese made from raw cow and sheep milk, Milchwissenschaft 42 (1987) 717–719.

[69] Tsakalidou E., Manolopoulou E., Tsilibari B., Georgalaki M., Kalantzopoulos G., Esterolytic activities from *Enterococcus durans* and *Enterococcus faecium* strains isolated from Greek cheese, Neth. Milk Dairy J. 47 (1993) 145–150.

[70] Tsakalidou E., Dalezios I., Kalantzopoulos G., Isolation and partial characterization of an intracellular esterase from *Enterococcus faecium* ACA-DC 237, J. Biotechnol. 37 (1994) 201–208.

[71] Umemoto Y., Umeda H., Sato Y., Studies on lipolysis of dairy lactic acid bacteria. Part II. On the lipolytic activities of cell free extracts of lactic acid bacteria, Agric. Biol. Chem. 32 (1968) 1311–1317.

[72] Wessels D., Jooste P.J., Mostert J.F., Technologically important characteristics of *Enterococcus* isolates from milk and dairy products, Int. J. Food Microbiol. 10 (1990) 349–352.

To access this journal online:
www.edpsciences.org