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Full Length Research Paper

Technologically important properties of lactic acid bacteria isolated from raw milk of three breeds of Algerian dromedary (*Camelus dromedarius*)

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A total of 9 samples of individual dromedary raw milks from N'ajjer (3), Targui (3) and Reguibi (3) breeds were collected from 3 camels nomad herd in south Algeria and were analysed for bacterial load. A total of 23 strains of lactic acid bacteria were isolated, out of which 12 strains were cocci and 11 strains were facultatively heterofermentative lactobacilli. Lactic acid bacteria were identified on the basis of phenotypic characters as *Lactococcus lactis* subsp*. lactis, Enterococcus faecalis, Enterococcus faecium, Enterococcus durans, Lactobacillus paracasei* subsp*. paracasei, Lactobacillus plantarum* and *Lactobacillus rhamnosus.* Whole cells of lactococci, enterococci and lactobacilli showed proteolytic activity and were found to differed in terms of their acidifying activities. Proteolytic and autolytic activity were generally higher for most lactobacilli compared to other isolates and none of the strains produced biogenic amines in the method applied. A wide variety of this 23 lactic acid bacteria strains isolated from Algerian dromedary milks that showed potentially important properties suggest that they are good candidate for camels milk processing or other dairy fermentation process.

Key words: Algerian dromedary milks, lactic acid bacteria, identification, acidification, proteolysis, autolysis.

INTRODUCTION

Development and research activities on domestic animals are mostly concentrated on species and breeds of animals available in the temperate zones of Europe and North America (Simoons, 1974; Ingram and Mount, 1975; Saint-Martin, 1990; Ruane, 2000). This results in a relative neglect of several species of animals native to the tropics and subtropics. The camel (*Camelus dromedarius*) is certainly one of the most neglected species of the domestic animals (Saint-Martin, 1990; Lhoste, 2004). The majority of the studies conducted on camels concentrate mainly on its anatomical features and physiological adaptations to desert conditions (Emmanuel, 1979; Engelhardt and Rubsamen, 1979; Schmidt-Nielsen, 1997). Information about camels as milk animal is very limited (Yagil, 1982; Saint-Martin, 1990; Lhoste, 2004). Camel milk is an important part of the human diet in many parts of the world (Dahl and Hjort, 1979; Yagil, 1982; Yagil et al., 1998; Lhoste, 2004). People unfamiliar with camels are surprised to learn that a normal camel on good feed can produce 2000 litres of milk per lactation period (Yagil, 1982; Kamoun, 1995; Lhoste, 2004), and even higher milk yields have been recorded (Knoess, 1980; Yagil et al., 1998).

In pastoral societies, milk is traditionally consumed predominantly in the form of fermented milk. Fermentation is the only means of preserving milk under warm condition (Mohamed et al., 1990; Farah, 1993; Kamoun, 1995). In many arid areas, camels play a central role as milk suppliers where they are either home-consumed or sold (Yagil, 1982; Kamoun, 1995; Lhoste, 2004). To prepare fermented camel milk, containers of calabash, clay pots, plant fibre vessels or hollowed wood vessels are smoked by burning chips of *Olea Africana* or *Acacia busia*. The daily residual fresh milk is poured into the milk container.

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No starters are used and acidification develops after a few days, either from natural flora of milk when it is not boiled, or from the bacteria growing on the sides of the vessel. The milk is left in a quite place, often in a covered container sheltered from dust for usually 24 - 48 h until it becomes sour. The ambient temperature is normally between 25 and 35°C. Due to spontaneous nature of the fermentation, this traditional method results in a product with varying taste and flavour and often of poor hygienic quality.

To improve the spontaneous traditional fermentation, controlled fermentation using mesophilic lactic acid bacteria starter culture is a very important strategy for camel milk processing (Farah et al., 1990; Mohamed et al., 1990; Abu-Tarboush, 1994, 1996; Kamoun, 1995; Abu-Tarboush et al., 1998; Lhoste, 2004). This work was therefore aimed for isolation and characterization of lactic acid bacteria from raw milks of three breeds of Algerian dromedary and evaluated for their technologically important properties.

MATERIALS AND METHODS

Sampling

A total of 9 individual dromedary raw milks from N'ajjer (3), Targui (3) and Reguibi (3) breeds were collected aseptically from 3 camels' nomad herd in the regions of Tin-Guentourin (Illizi), Tin-Zaiatin (Ain Amenas) and Tindouf in South Algeria. Each sample representing the pooled milk from one single milking of each herd, were immediately cooled and brought to the laboratory in an isotherm container, and analysed on arrival.

Isolation and identification of strains

10 ml of milk sample were vigorously homogenized with 9 volumes of sterile diluent's [0.1% (w/v) bacteriological peptone, 0.85% (w/v) NaCl] and serial 10-fold dilutions (10^{-1} to 10^{-8}) were prepared using the same diluents. 1 ml of these dilutions was pour-plated in the media for lactic acid bacteria, M17 (Terzaghi and Sandine, 1975) and MRS (de Man et al., 1960) adjusted to pH 5.5. After incubation at 30°C for 24 h and 3 days, representative strains of lactic acid bacteria were obtained from M17 and MRS plates of highest sample dilutions. Colonies were either randomly picked up or when the plate contained less than 10 colonies (Leisner et al., 1997). The purity of the isolates was checked by streaking again to fresh agar plates, followed by macroscopic and microscopic examinations. The strains displaying the general characteristics of lactic acid bacteria were chosen from each plate for further studies. The strains of lactic acid bacteria were stored without appreciable loss of properties in skimmed milk at –20°C. Working cultures were also kept on MRS agar or M17 agar slant at 4°C and streaked every 4 weeks (Samelis et al., 1994; Herrero et al., 1996).

Cell morphology and mobility of all isolates of lactic acid bacteria were observed using a phase contrast microscope following the method of Harrigan (1998). Isolates were Gram-stained and tested for catalase production. Preliminary isolation and grouping was on the basis of cell morphology and phenotypic properties using gas production from glucose, determined in M17 and MRS broth containing inverted Durham; growth at different temperatures (10, 15, 37 and 45°C), and at pH 9.6 as well as the ability to grow in the different concentrations of NaCl (2, 3, 4 and 6.5%, w/v); Sherman test and survival after heating of 60°C for 30 min (Samelis et al., 1994); hydrolysis of arginine, tested on M17 and MRS with bromocresol purple (Thomas, 1973); and production of acetoin from glucose, determined by using the Voges-Prokauer test (Zourari et al., 1991).

The fermentation of carbohydrates was determined in MRS and M17 broth without glucose and meat extract with 0.04 g/l bromocresol purple and phenol red respectively as pH indicator, supplemented with 1% (w/v) of the following carbohydrates: glucose; arabinose; cellobiose; galactose; sucrose; lactose; maltose; mannitol; melizitose; melibiose; raffinose; rhamnose; ribose; trehalose; D-xylose; sorbitol (Sigma). To ensure anaerobic conditions, each tube was topped up with two drops of sterile liquid paraffin after incubation (Samelis et al., 1994). Tests for phenotypic characterization were conducted twice for each strain.

Acidification ability

The strains were initially grown in MRS (rods) or M17 (cocci) broth and then in sterile reconstituted skim milk supplemented with yeast extract (0.3%) and glucose (0.2%) for two successive subcultures. Sterile reconstituted skim milk (100 ml) was inoculated with 1% of a 24 h activated culture and pH changes were determined using pH meters (glass electrode, HANNA instruments, Padova, Italy) during incubation at 30°C after 6 h for lactococci and lactobacilli and after 5 h for enterococci. The pH of the culture was also measured after 18 h for lactococci and/or 24 h for lactobacilli and enterococci (Durlu-Ozkaya et al., 2001).

Proteolytic activity

Surface-dried plates of milk agar (Gordon et al., 1973) were streaked with 24 h old culture, incubated at 30°C for 4 days, and examined for any halo of proteolysis around and underneath the growth for assessment of proteolytic activity. After 72 h of incubation in the skimmed milk, the proteolytic activity (expressed as free amino acids in the medium) was evaluated at 507 nm after reaction with Cd-ninhydrin (Folkertsma and Fox, 1992) with a carry IE UV-visible spectrophotometer (Varian, Australia). Free amino groups were quantified as standard mM equivalency of glycine per litre of milk (meq Gly/l) (Bouton et al., 1993).

Autolytic activity

The biomass pellet was resuspended in potassium phosphate buffer (10 mM, pH 5.5) containing 1 M NaCl and diluted to OD_{650} equal 1.0. The rate of autolysis was determined according to the method described by Thiboutot et al. (1995). The cell suspension was subjected to one cycle of freezing (-20°C for 24 h) and thawing then incubated at 37°C. The autolytic activity was determined as the percentage decrease in the absorbance at 650 nm at different time intervals as described by Boutrou et al. (1998), which was defined as follows: $(A_0 – A_t) \times 100/A_0$ where A_0 = initial absorbance, and A_t = absorbance measured after t days of incubation. Autolysis was ranked in accordance to the activity level of each genus: lactococci; good 25 – 37, fair 15 – 24, poor 1 – 14; lactobacilli; good 70 – 96, fair 40 – 69, poor 0 – 39; and enterococci; good 35 – 66, fair 24 – 34, poor 0 - 22 as described by Ayad et al. (2004).

Decarboxylase activity

The ability of the test strains to decarboxylate histidine, tyrosine, lysine and ornithine, was detected as suggested by Joosten and Northolt (1989).

RESULTS AND DISCUSSION

Identification of isolates

The physiologic characteristics of the strains are shown in Table 1. Out of a total of 37 isolates obtained from raw dromedary milks from tree local breeds [N'ajjer (14), Targui (12) and Reguibi (11)], twenty-tree strains showed positive Gram reactions, absence of mobility, absence of spore formation, absence of catalase activity cocci which produced no gas from glucose (12 isolates), and/or rods (11 isolates). Among the cocci, 5 isolates were able to grow at 10 and 37°C, but none at pH 9.6 broth, and 45°C, except for DN11. In addition, they did not survive at 60°C for 30min. All of the 5 strains could grow in 4%, but not in 6.5% NaCl broth and produced NH_3 from arginine. The strains formed acid from lactose and ribose but acid production from mannitol, sucrose and xylose was strain dependent. The phenotypic characteristics of the strains (Table 1) suggest their close resemblance to *Lc. lactis* subsp. *lactis* (Sharpe, 1979; Schleifer et al., 1985; Balows et al., 1991). Seven isolates of cocci were able to grow at 10 and 45°C in 6.5% NaCl and pH 9.6 broth. They also survive at 60°C for 30 min and form NH_3 from arginine, but not CO_2 from glucose; and were characterrized as enterococci. Three of them seemed to be *Ec. faecalis*, as suggested by their ability to survive at 60°C for 30 min and to ferment sorbitol (Sharpe, 1979; Shleifer and Kilpper-Balz, 1984; Devriese et al., 1991; Manero and Blanch, 1999). Four enterococci strains were differentiated by their ability to form acid from sugars. Thus, 3 strains producing acid from mannitol and arabinose were characterized as *Ec. faecium*, one strain is characterized by inability to ferment melibiose and sucrose and unable, in general, to ferment sugars as *Ec. durans* (Schleifer and Kilpper-Balz, 1984; Devriese et al., 1991, Manero and Blanch, 1999).

The 11 isolates of Gram-positive rods grew at 15°C and did not form either CO_2 from glucose or NH_3 from arginine. These characteristics suggest their classification as facultatively heterofermentative lactobacilli (Sharpe, 1979; Balows et al., 1991). Two out of 11 isolates did not form acid from arabinose, melibiose, raffinose and rhamnose and were characterized as *Lb. paracasei* subsp. *pracasei* (Collins et al., 1991; Balows et al., 1991). A total of 5 isolates of rods were classified as *Lb. plantarum*, as suggested by their sugar fermentations patterns. All these strains fermented arabinose, cellobiose, lactose, maltose, melibiose, raffinose, ribose, sucrose and trehalose (Sharpe, 1979; Balows et al., 1991). These strains did not form acid from rhamnose and acid production from sorbitol and xylose was variable and strain dependant. The last 4 strains were unable to ferment melibiose, raffinose, xylose, sucrose and arabinose but were able to form acid from rhamnose and classified as *Lb. rhamnosus.*

This diversity of species is very relative and dependent primarily on the nature of the material isolated and the different criteria used for each study, as reported by Fitzsinimons et al. (1999) and Bissonnette et al. (2000).

Technological properties

With respect to the acidifying activity of the strains (Figure 1) it seems that none of the *Lc. lactis* subsp. *Lactis* strains can be characterized as fast, as they did not reach a pH of 5.0 ± 0.2 in 6 h at 30°C (Huggins and Sandine, 1984). All strains were faster initially acidifying (6 h) and the Δ pH (18 h) was ranged generally between 1.65 and 2.10 pH units, except for strain DT11, which had shown the higher acidifying activity estimated at 2.32 pH unit (Δ pH 18 h). *Lactobacillus* strains differed in their ability to reduce the pH of milk initially and there were strains that did not change the pH of milk at 6 h. Nevertheless, after 24 h incubation the Δ pH (24 h) of the strains were similar and ranged between 0.88 and 1.35, except for strain DT18, which had a Δ pH (24h) of 1.61. *Lactobacillus casei* and *Lb. plantarum* may ferment lactose through a β-galactosidase activity, but some strains also show a β-phospho-galactosidase activity (Herrero et al., 1996). The acidifying abilities of enterococci at 30°C were, in general, low and only cultures did not lower the pH of milk to pH < 5.0 after 24 h incubation. The ΔpH (24 h) was ranged between 1.05 and 1.51. Nevertheless, the Δ pH (5 h) of enterococci was, in general, higher than that of lactobacilli at 6 h, but there was a tendency for the strains to become slow after 5 h.

A rapid decrease in pH during the initial step of cheese preparation is of crucial importance in cheese manufacture, since it is essential for coagulation and prevention or reduction of the growth of adventitious microflora. The fast acidifying strains are good candidate in the dairy fermentation process as primary starter organisms, whereas, the poor acidifiers strains can be used as adjunct cultures depending on their other important properties, e. g., proteolytic and autolytic activity.

The strains were characterized by different caseinolytic breakdown ability (Figure 2). All strains exhibit proteolytic activities as revealed by clear halos on milk agar (showing > 2 mm hydrolysis zones in milk agar plate). These activities ranged between 2.30 - 5.51 meq Gly/l for *Lc. lactis* subsp. *lactis* strains. The proteolytic activity of the enterococci strains was measured at levels between 1.85 (*Ec. faecium* strain DN5) and 5.95 meq Gly/l (*Ec. feacium* strain DT2). The proteolytic activity of lactobacilli ranged between 1.70 (*Lb. plantarum* strain DR22) and 5.7 meq Gly/l (*Lb. rhamnosus* strain DR19). The data reported here on proteolytic activity suggest that there was no relationship between the proteolytic and acidifying activities of the strains, as also suggested by Bottazi (1962) and Fontina et al. (1998) for strains of lactobacilli.

Thus, strains with the strongest acidifying abilities (Lactobacilli strains DT18, DR22 and DT23 and enter-

Table 1. Phenotypic characteristics of lactic acid bacteria isolated from dromedary raw milks.

Gram	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	-
Morphology	Cocci	Cocci	Cocci	Cocci	Rods	Rods	Rods
Production of CO_2 from glucose	-	-	-	-	-	-	-
Growth at pH 9.6	-	+	+	+			
Growth at							
10°C	+	+	+	+			
15°C	+	+	+	+	+	+	+
37°C	+	+	+	+			
45°C	-	+	+	+	+/-	-	+
Growth at							
2% NaCl	+	+	+	+			
3%	+	+	+	+			
4%	+	+	+	+			
6.5%	-	+	+	+	-	-	-
Sherman test	+	+	+	+			
Hydrolyse of Arginine (ADH)	+	+	+	+	-	-	-
Survival at 60°C for 30 min	-	+	+	+			
Acetoin (VP)	+	+	+	+			
Acid from:							
Glucose	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+
Sorbitol	-	+	-	-	+	+	+
Melibiose	-	-	+	-	-	+	-
Raffinose	-	-	-	-	-	+	-
Xylose	+	-	-	-	-	+	-
Sucrose	+	+	+	-	+	+	-
Arabinose	-	+	+	-	-	+	-
Melezitose	-	-	-	-	+	+	+
Rhamnose	-	+/-	-	-	-	-	+
Maltose	+	+	+	+	+	+	+
Ribose	+	+	+	+	+	+	+
Mannitol	+	+	+	-	+	+	+
Trehalose	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+
Identified as	*Lc. lactis* ssp. *lactis* (n =.5)	*Ec. faecalis* (n = 3)	*Ec. faecium* (n = 3)	*Ec. durans* (n=1)	*Lb. paracasei* ssp. *paracasei* (n = 2)	*Lb. plantarum* (n = 5)	*Lb. rhamnosus* (n = 4)

+: positive reaction
-: negative reaction
-/+: variable reaction

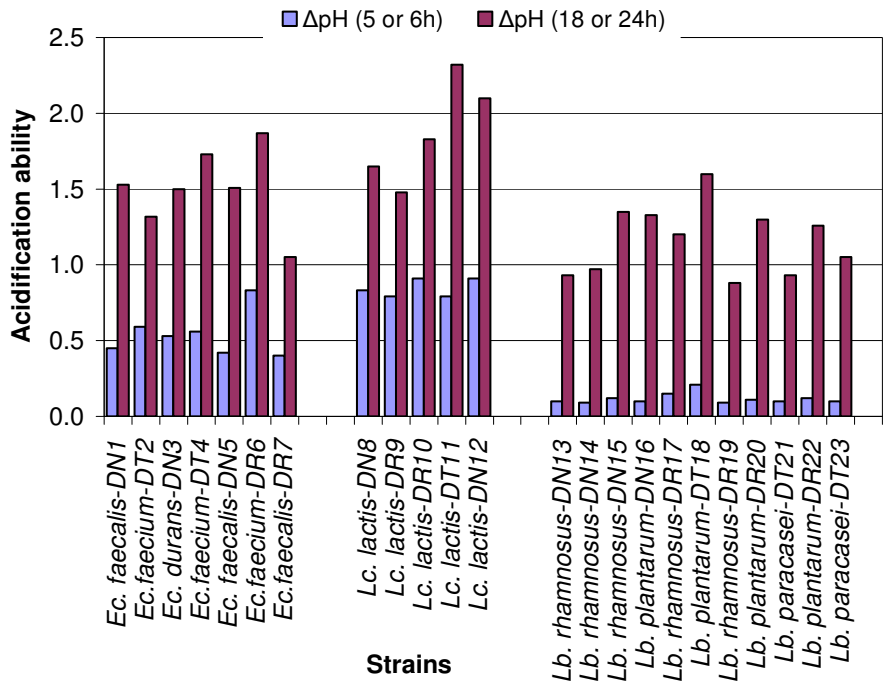

Figure 1. Acidifying activities of lactic acid bacteria strains isolated from raw dromedary milks tested in this study. The pH of the culture was also measured after 6 h for lactococci and lactobacilli and after 5 h for enterococci and after 18 h for lactococci and/or 24 h for lactobacilli and enterococci. **DR**, Dromedary milk of Reguibi breed; **DT**, Dromedary milk of Targui breed; and **DN**, Dromedary milk of N'ajjer breed.

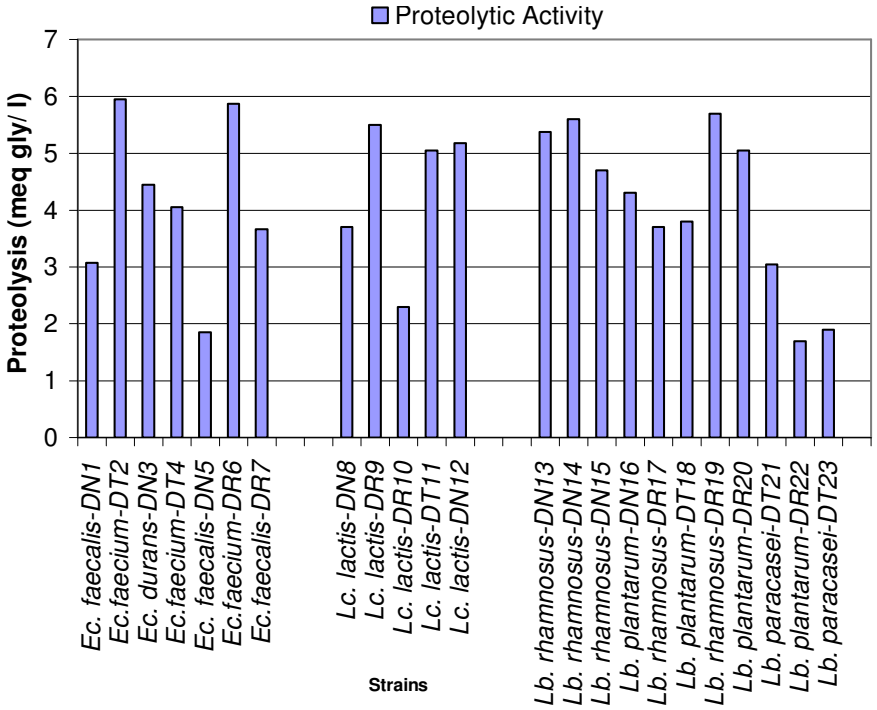

Figure 2. Proteolytic activities of lactic acid bacteria strains isolated from raw dromedary milks tested in this study and evaluated in the skimmed milk after 72 h of incubation at 507 nm following reaction with Cd-ninhydrin (Folkertsma and Fox, 1992). The proteolytic activity was expressed as free amino groups with mM equivalency of Glycine per litre of milk (meq Gly/l). **DR**, Dromedary milk of Reguibi breed; **DT**, Dromedary milk of Targui breed; and **DN**, Dromedary milk of N'ajjer breed.

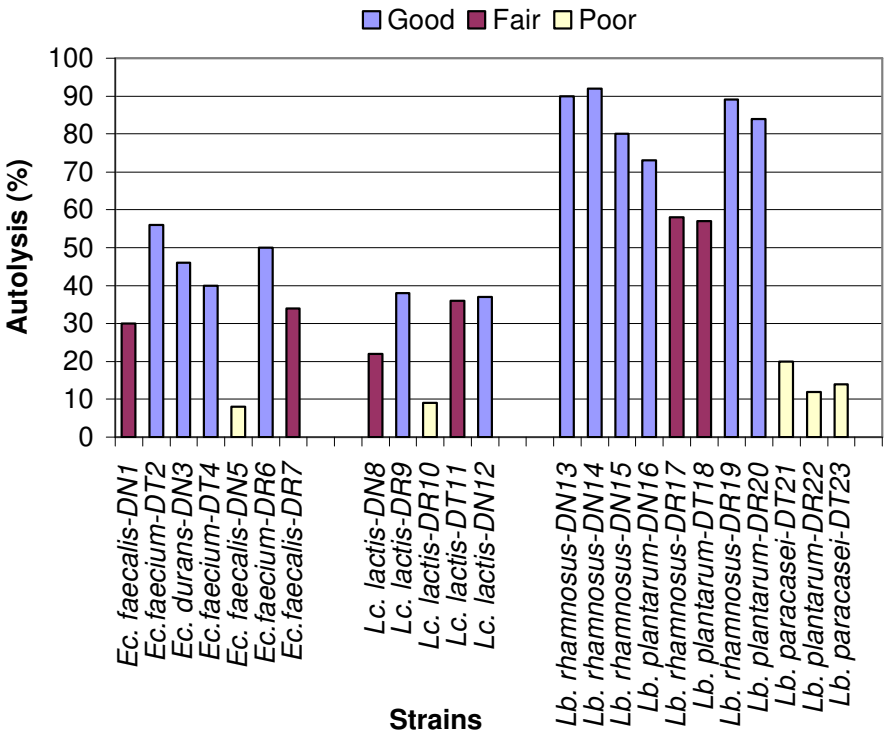

Figure 3. Autolytic activities of lactic acid bacteria strains isolated from raw dromedary milks tested in this study. The autolytic activity was determined as the percentage decrease in the OD_{650}, and was ranked in accordance to the activity level of each genus; Good, Fair and Poor. **DR**, Dromedary milk of Reguibi breed; **DT**, Dromedary milk of Targui breed; and **DN**, Dromedary milk of N'ajjer breed.

ococci strains DN1, DT4 and DN5) did not exhibit the highest proteolytic activities. Hence there were strains with very low acidifying but high proteolytic activity (e.g. *Lb. plantarum* DR19) and strains with high acidifying and protreolytic activities (for example, *Lc. lactis* subsp. *lactis* strains DT11, DN12 and *Ec. faecium* strain D06). The protease system of *Lactococcus* has been studied for several years and consists of cell wall-bound proteinases and several peptidases (Bockelmann, 1995). Enzymes formed by *Lactobacillus* strains were studied in detail and many authors have described enzymes that were biochemically similar to those of *Lactococcus* and their importance for cheese ripening is obvious (Bockelmann 1995). The proteolytic activity and acid production of enterococci during growth in milk are sometimes comparable to those of *Steptococcus thermophilus* (Gatti et al., 1994).

The proteolytic activity of dairy lactic acid bacteria is essential for the bacterial growth in milk and involved in the development of organoleptic properties of different fermented milk products (Axelsson, 1998; Christensen et al., 1999). The production of high quality fermented dairy products is dependent on proteolytic systems of starter bacteria, since peptidase and amino acids formed have a direct impact on flavour or serve as flavour precursors in these products. Several peptidases with different specificities have been identified in lactic acid bacteria; all peptidases have been found to be intracellular and liberated in fermented milk products after cell lysis (Law and Haandrikman, 1997; Axelsson, 1998)

The ability of strains to lyse and subsequent release of their intracellular enzymes is a desirable trait during the ripening of cheese; the degree of autolysis is strain dependent (Wilkinson et al., 1994; El-Soda et al., 2000). The autolytic activities of strains were found among various strains and were classified into three groups; poor, fair, and good (Figure 3) according to the autolytic capacity of each genus as described by Ayad et al. (2004). *Lb. rhamnosus* DN13, DN14, DN15, DR19; *Lb. plantarum* DR20, DN16; *Lc. lactis* DN12, DR9; *Ec. faecium* DT2, DT4, DR6 and *Ec. durans* DN3 exhibited good autolysis. We noticed that the autolysis rate ranging from 73 to 92% for Lactobacilli, 37 to 38% for lactococci and 40 to 56% for enterococci. The fair group, *Lb. rhamnosus* DR17; *Lb. plantarum* DT18; *Lc. lactis* DT11, DN8 and *Ec. faecalis* DN1, DR7, showed levels of autolysis ranging from 57 to 58% for lactobacilli, 22 to 36% for lactococci and 30 to 34% for enterococci. The poor autolysis strains, *Lb. paracasei* subsp. *paracasei* DT21, DT23; *Lb. plantarum* DR22; *Lc. lactis* DR10 and *Ec. faecalis* DN5, showed an autolytic rate ranging from 12 to 20% for lactobacilli, 9% for lactococci and 8% for enterococci.

Lactobacilli showed higher autolysis rate compared to enterococci and lactococci strains (Dako et al., 1995; El-Soda et al., 1995). The authors indicated that *Lactobacillus* autolysed more rapidly than *Lactococcus* strains. These results are comparable with the findings of Ayad (2001) who reported that several wild lactococci strains were found to be stable in milk cultures and during cheese ripening in contrast to industrial strains. The differences in the autolytic rate of some strains (Figure 3) indicate the wide diversity among the strains. Cultures can be of interest during cheese manufacture because of faster release of their intracellular proteolytic and lipolytic enzymes which will contribute in flavour formation in the manufacture of fermented dairy products (Wouters et al., 2002; Ayad et al., 2003). The autolytic properties of several cheese related microorganisms have been reviewed (El-Soda et al., 1995). It was shown that one of the most effective ways to accelerate cheese ripening was addition of adjunct cultures, mainly *Lactobacillus* sp. and the selection of these cultures should be based on enzymes profiles and autolytic properties.

All lactic acid bacteria strains tested in this study were screened for their ability to produce biogenic amines. None of them produced biogenic amines during the investigation period. Decarboxylating bacteria can find suitable conditions to proliferate and produce biogenic amines during ripening of cheese because tyramine is the only biogenic amine produced after growth in milk by *Ec. faecalis* and *Ec. faecium* in the presence of a pool of free amino acids as precursor (Giraffa et al., 1995).

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