Phenotypic identification and technological properties of lactic acid bacteria isolated from three breeds dromedary raw milks in south Algeria

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Abstract: A total of 9 samples of individual dromedary raw milks from N'ajjer (3 samples), Targui (3 samples) and Reguibi (3 samples) breeds were collected from 3 camels nomad herd in south Algeria and were analysed for bacterial load. A total of 23 isolates of lactic acid bacteria were obtained and characterized; 12 isolates were cocci and 11 isolates 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. *paracsei, Lactobacillus plantarum and Lactobacillus rhamnosus*. Lactococci, enterococci and lactobacilli showed proteolytic activity and were found that differ in their acidifying activities. Proteolytic and autolytic activities were generally higher for most lactobacilli compared to other isolates and none of the isolates produced biogenic amines under the conditions tested. A number of the 23 lactic acid bacteria isolates from Algerian dromedary milks that were characterized here showed properties suggest that they are good candidates for camels milk processing or other dairy fermentation process.

Keywords: Algerian dromedary milks, Lactic acid bacteria, Identification, Acidification, Proteolysis, Autolysis.

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التعريف النوعي والخصائص التكنولوجية لبكتريا حامض اللاكتيك التي تم عزلها من الحليب الخام لثلاثة سلالات من الابل بجنوب الجزائر

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الملخص: تم جمع تسعة عينات من حليب الإبل الخام (ثلاثة عينات من ثلاثة أنواع من الجمال وحيدة السنام بجنوب الجزائر (Reguibi – Targui -N'ajjer) وتحليلها لمعرفة حملها البكتيري. تم عزل وتصنيف 23 نوع من بكتريا حامض اللاكتيك، 12 من البكتريا الكروية و11 من البكتريا العصوية الاختيارية مختلطة التخمر. تم التعرف على بكتريا حامض اللاكتيك اعتمادا على الخصائص النوعية وتشمل: *Lactococcus lactis subsp. Lactis, Enterococcus faecalis, Enterococcus faecium, Enterococcus durans, Lactobacillus paracasei subsp. Paracsei, Lactobacillus plantarum and Lactobacillus rhamnosus*. تتشابه بكتريا حامض اللاكتيك الكروية والعصوية في خصائص تحليل البروتينات وتختلف في قدرتها لإنتاج الاحماض. قدرة بكتريا حامض اللاكتيك العصوية على تحليل البروتينات والتحلل الذاتي مرتفعة بالمقارنة مع البكتريا الأخرى. كل البكتريا المعزولة ليس لها القدرة على انتاج الأمينات الحيوية في ظروف الاختبار. تم عزل وتصنيف 23 نوع من بكتريا حامض اللاكتيك من حليب الجمال وحيدة السنام الجزائرية وأظهرت خصائص تجعلها صالحة للاستخدام في تصنيع حليب الإبل والتخمرات اللبنية الأخرى.

الكلمات المفتاحية: حليب الابل الجزائري، بكتريا حامض اللاكتيك، تعريف، تحمض، تحلل البروتين، تحلل الذاتي.

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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. 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. The majority of the studies conducted on camels concentrate mainly on its anatomical features and physiological adaptations to desert conditions. Information about camels as milk animals is very limited. Camel milk is important to the human diet in many parts of the world. 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), and even higher milk yields have been recorded (Knoess, 1980).

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. In many arid areas camels play a central role as milk suppliers, there is either home-consumed or sold.

To prepare fermented camel milk, containers of calabas, cly potes, 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. 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 quiet place, often in a covered container sheltered from dust for usually 24-48 hours 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.

They improve this spontaneous traditional fermentation, controlled fermentation using mesophilic lactic acid bacteria starter cultures that originated from this ecosystem is a very important strategy for camel milk processing. 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 nine individual dromedary raw milk samples from N'ajjer (3 samples), Targui (3 samples) and Reguibi (3 samples) breeds were collected aseptically from three camels' nomad herd in the regions of Tin-Guentourin (Illizi), Tin-Zaiatin (Ain Amenas) and Tindouf in south Algeria. The nipples of the female camel were rinsed with some water chlorinated and rinsed then with some sterile water. The first jets of milk are eliminated and then samples of 150 to 200 ml were collected in a sterile flask, 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, being analyzed on arrival.

Isolation and Identification of Strains

Ten millilitres of milk sample were vigorously mixed with nine volumes of sterile diluent [1 g l^{-1} bacteriological peptone, 8.5 g l^{-1} NaCl] and serial 10-fold dilutions (10^{-1} to 10^{-8}) were prepared using the same diluent.

One millilitre of these dilutions was pour-plated in the media for lactic acid bacteria i.e. 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 three days, respectively, representative isolates of lactic acid bacteria were obtained from M17 and MRS plates of highest sample dilutions.

Colonies were either randomly picked up or all sampled 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 isolates of lactic acid bacteria were stored without appreciable loss of properties in skimmed milk at − 20°C. The working cultures were also kept on MRS agar or M17 agar slant at 4°C and streaked every four 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°C, 15°C, 37°C 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 % NaCl); 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 acetoïn from glucose, determined by using the Voges-Proskauer test (Zourari et al., 1991).

The fermentation of carbohydrates was determined in MRS and M17 broth without glucose and meat extract and with 0.04 g l^{-1} bromocresol purple and phenol red respectively as pH indicator,

and supplemented with 10 g l^{-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.

Technological Properties
Acidification Ability
The isolates were initially grown in MRS (rods) or M17 (cocci) broth and then in sterile reconstituted skim milk supplemented with yeast extract (3 g l^{-1}) and glucose (2 g l^{-1}) for two successive subcultures. Sterile reconstituted skim milk (100 ml) was inoculated with 1 % (v/v) 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 four days, and examined for any halo of proteolysis around and underneath the growth for assessment of proteolytic activity. After 72h 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 UV-visible spectrophotometer (Varian, Australia). Free amino groups were quantified as standard m mol l^{-1} equivalency of

Glycine per litre of milk (meq Gly/l) (Bouton et al., 1993).

Autolytic Activity

The isolates were cultivated in MRS (rods) or M17 (cocci) broth and the obtained pellets by centrifugation were washed twice in potassium phosphate buffer (10 m mol l^{-1}, pH 7.0) and then resuspended in potassium phosphate buffer (10 m mol l^{-1}, pH 5.5) containing 1 mol l^{-1} 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 24h) 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: $(A_0 - A_t)$ x $100/A_0$ 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 determined as described by Joosten and Northolt (1989).

Results and Discussion

Identification of Isolates

The physiologic characteristics of the strains (which we studied) are shown in Table 1. Out of a total of 37 Isolates obtained from raw dromedary milks from three local breeds [N'ajjer (14 isolates), Targui (12 isolates) and Reguibi (11 isolates)], twenty-three strains showed positive Gram reactions, absence of mobility, absence of spore formation, absence of catalyse activity cocci which produced no gas from glucose (12 isolates), and/or rods (11 isolates). Among the cocci, five isolates were presumptively identified as *L. lactis* subsp. *lactis*, they were able to grow at 10 and 37°C, but not at 45°C, and not in pH 9.6 broth. In addition all of the five isolates could grow in 4% NaCl broth but not in 6 %, they did not also survive at 60°C for 30 min, and produced NH_3 from arginine. All the isolates formed acid from lactose, and ribose but acid production from mannitol, sucrose and xylose was strain dependent. (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 formed NH_3 from arginine, but not CO_2 from glucose and were characterized as enterococci. Three of them seemed to be *E. 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 isolates differed in their ability to form acid from sugars. Three isolates produced acid from mannitol and arabinose and were thus characterized as *E. faecium*. One isolate was characterized by inability to ferment melibioze and sucrose and unable, in general, to ferment sugars and was thus classified as *E. 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 five isolates of rods were classified as *Lb. plantarum*, as suggested by their sugar fermentations patterns. All these isolates fermented arabinose, cellobiose, lactose, maltose, melibiose, raffinose, ribose, sucrose and trehalose (Sharpe, 1979; Balows et al., 1991). These isolates did not form acid from rhamnose and acid production from sorbitol and xylose was variable and strain dependant. The last four isolates were unable to ferment melibiose, raffinose, xylose, sucrose and arabinose and able to form acid from rhamnose and were classified as *Lb. rhamnosus*.

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

Identified as	*L. lactis* ssp. *lactis* (n =.5)	*E. faecalis* (n = 3)	*E. faecium* (n = 3)	*E. durans* (n=1)	*Lb. paracasei* ssp. *paracasei* (n = 2)	*Lb. plantarum* (n = 5)	*Lb. rhamnosus* (n = 4)
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	+	+	+	+	+	+	+

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

Acidification Ability and Proteolytic Activity

With respect to the acidifying activity of the strains (Figure 1) it seems that none of the *L. lactis* subsp. *lactis* strains can be characterized as fast fermenters, as it took > 6 h at 30 °C for them to reach a pH of 5.0 ± 0.2 (Huggins and Sandine, 1984). All isolates were faster initially acidifying (6 h) and the pH drop after 18 h ranged from 1.65 and 2.10 pH units, except for isolate DT11, which showed higher acidifying activity (a drop of 2.32 pH units after 18 h).

Lactobacillus isolates differed in their ability to reduce the pH of milk initially and there were isolates that did not change the pH of milk at 6 h. Nevertheless, after 24 h incubation the drop in pH after 24 h of the isolates were similar and ranged between 0.88 and 1.35, except for isolate DT18, which had a drop in pH after 24 h of 1.61. *Lactobacillus casei* and *Lactobacillus 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 isolates at 30°C were in general low and only isolates did not lowered the pH of milk after 24 h of incubation to pH < 5.0. The drop in pH after 24 h was ranged between 1.05 and 1.51. Nevertheless, the drop in pH after 5 h of incubation of enterococci isolates 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 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.

Our test isolates were characterized by different caseinolytic breakdown ability (Figure 2). All isolates 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 *L. lactis* subsp. *lactis* isolates. The proteolytic activity of the enterococci isolates was measured at levels between 1.85 (*E. faecium* strain DN5) and 5.95 meq Gly/l (*E. feacium* isolate DT2).

The proteolytic activity of lactobacilli ranged between 1.70 (*Lb. plantarum* isolate DR22) and 5.7 meq Gly/l (*Lb. rhamnosus* isolate DR19).

The data reported here on proteolytic activity suggest that there was no relationship between the proteolytic and acidifying activities of the isolates, as also suggested by Bottazi (1962) and Fontina et al. (1998) for isolates of lactobacilli. Thus, isolates with the strongest acidifying abilities (lactobacilli isolates DT18, DR22 and DT23 and enterococci isolates DN1, DT4 and DN5) did not exhibit the highest proteolytic activities. Hence there were isolates with very low acidifying but high proteolytic activity (e.g. *Lb. plantarum* DR19) and isolates with high acidifying and protreolytic activities (for example, *L. lactis* subsp. *lactis* isolates DT11, DN12 and *E. faecium* isolate D06). The proteolytic 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).

Autolytic Activity

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 isolates 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). For isolates with "good autolysis"(*Lb. rhamnosus* DN13, DN14, DN15, DR19; *Lb. plantarum* DR20, DN16; *L. lactis* DN12, DR9; *E. faecium* DT2, DT4, DR6 and *E. durans* DN3) the autolysis rate ranged from 73% to 92% for lactobacilli isolates, from 37% to 38% for lactococci isolates, and from 40% to 56% for enterococci isolates. For isolates with "fair autolysis" (*Lb. rhamnosus* DR17; *Lb. plantarum* DT18; *L. lactis* DT11, DN8 and *E. faecalis* DN1, DR7) autolysis levels ranged 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; *L. lactis* DR10 and *E. 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), they indicated that *Lactobacillus* autolysed more rapidly than *Lactococcus* strains. These results are comparable with the finding 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 with high autolysis rate can be of importance for cheese manufacture because of faster release of their intracellular proteolytic and lipolytic enzymes, which will contribute to flavour formation in the manufacture of fermented dairy products (Wouters et al., 2002; Ayad et al., 2003). As reviewed by El-Soda et al. (1995), one of the most effective ways to accelerate cheese ripening can be the addition of adjunct cultures, mainly *Lactobacillus* ssp. and the selection of these cultures should be based on enzymes profiles and autolytic properties.

Decarboxylase Activity

All lactic acid bacteria strains tested in this study were screened for their ability to produce biogenic amines. None of them produced biogenic amines in our hands. Decarboxylating bacteria can find suitable conditions to proliferate and produce biogenic amines during ripening of cheese. Tyramine is the only biogenic amine produced after growth in milk by *E. faecais* and *E. faecium* in the presence of a pool of free amino acids as precursor (Giraffa et al., 1995).
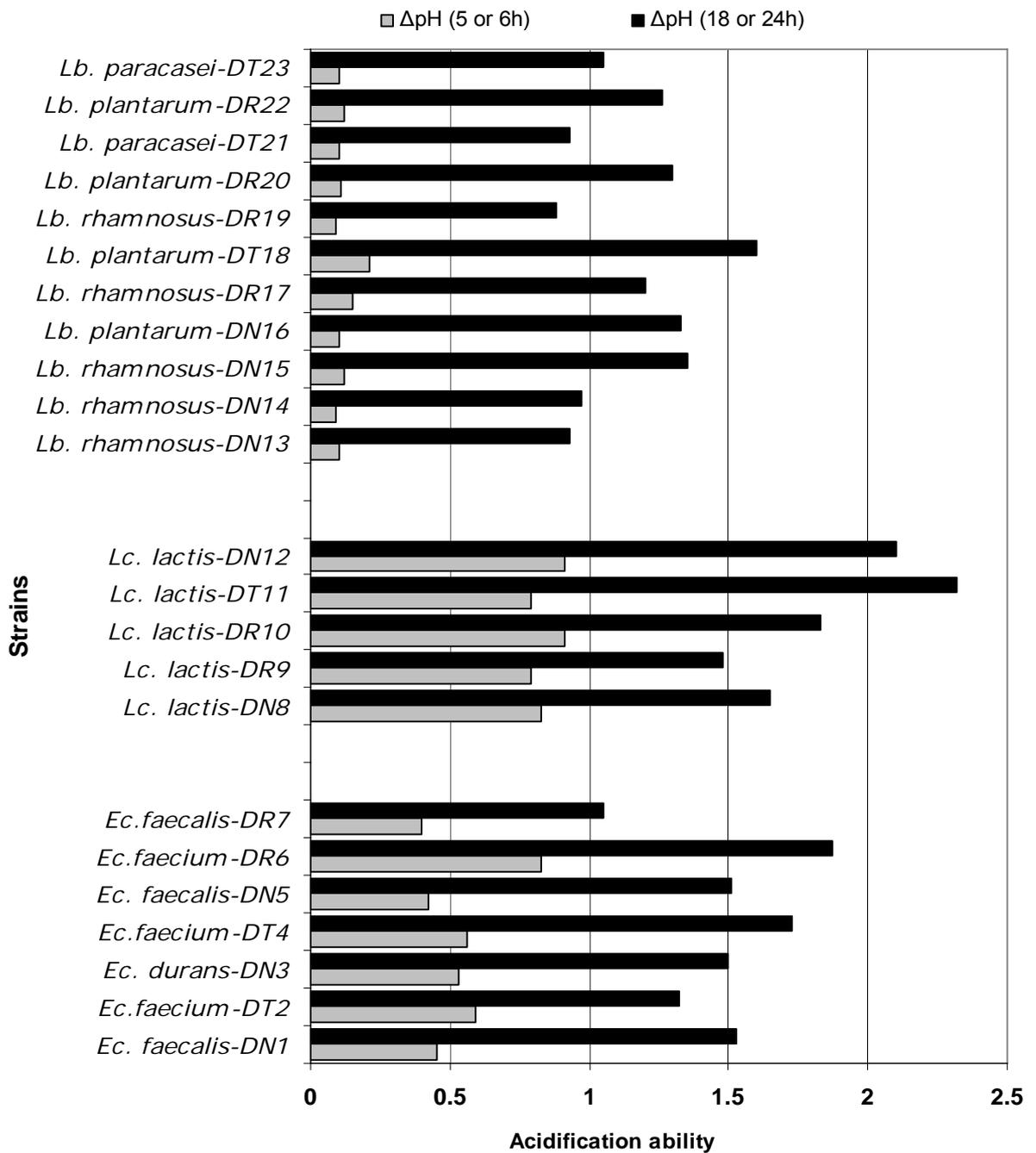
- The pH of the each isolate cultivated in skimmed milk was measured after 6 h for lactococci and lactobacilli and after 5h for enterococci and after 18 h for lactococci and/or 24 h for lactobacilli and enterococci.
- DR: Dromedary milk of Reguibi breed; DT: Dromedary milk of Targui breed; DN: Dromedary milk of N'ajjer breeds.

Figure 1. Acidifying activities of lactic acid bacteria strains isolated from raw dromedary milks tested in this study.
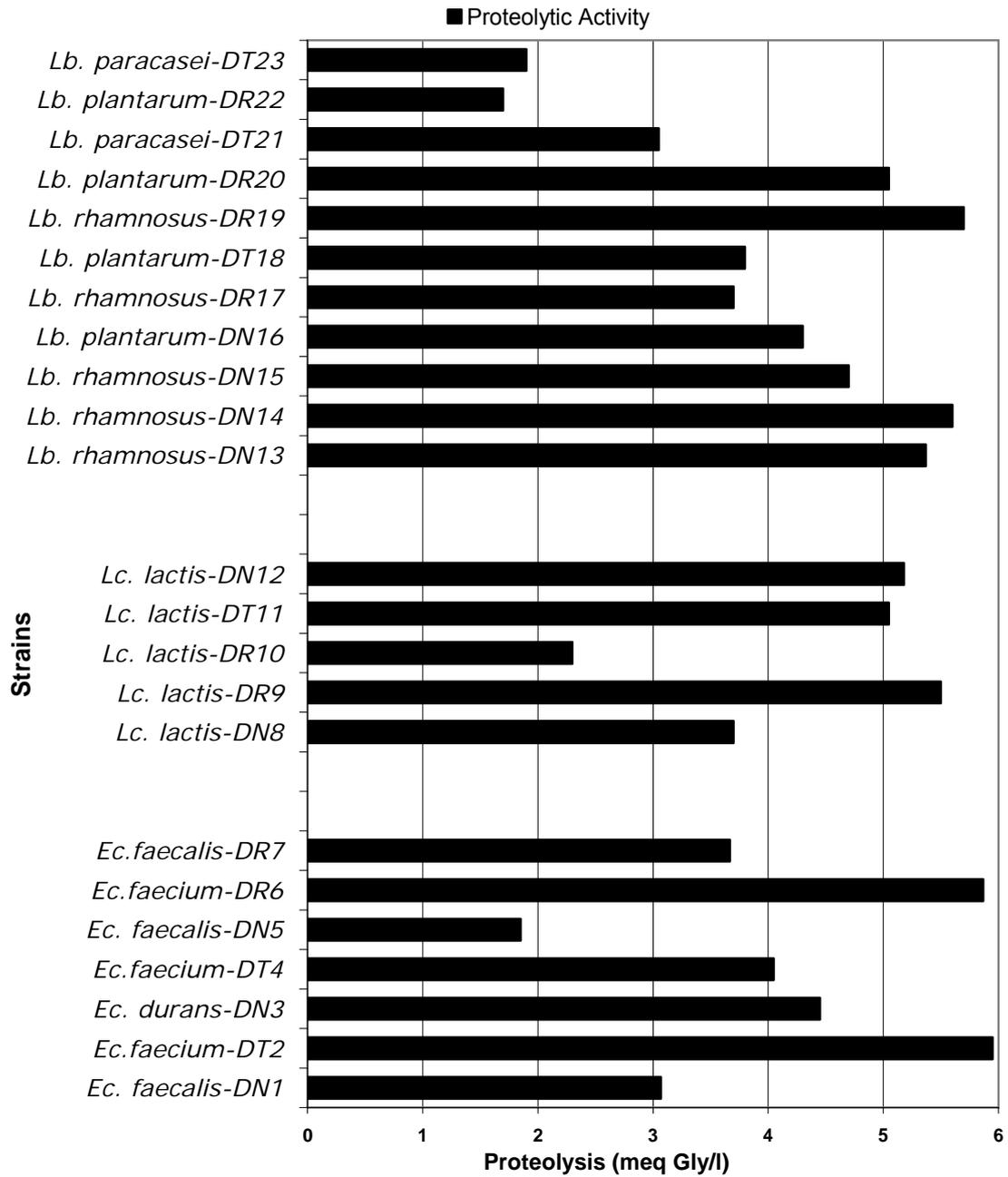
- The proteolytic activity was expressed as free amino groups with mM equivalency of Glycine per litre of milk (meq Gly/l).
- DR: Dromedary milk of Reguibi breed; DT: Dromedary milk of Targui breed; DN: Dromedary milk of N'ajjer breeds.

Figure 2. Proteolytic activities of lactic acid bacteria strains isolated from raw dromedary milks tested in this study, and were evaluated in the skimmed milk after 72h of incubation at 507 nm after reaction with Cd-ninhydrin (Folkertsma and Fox, 1992).
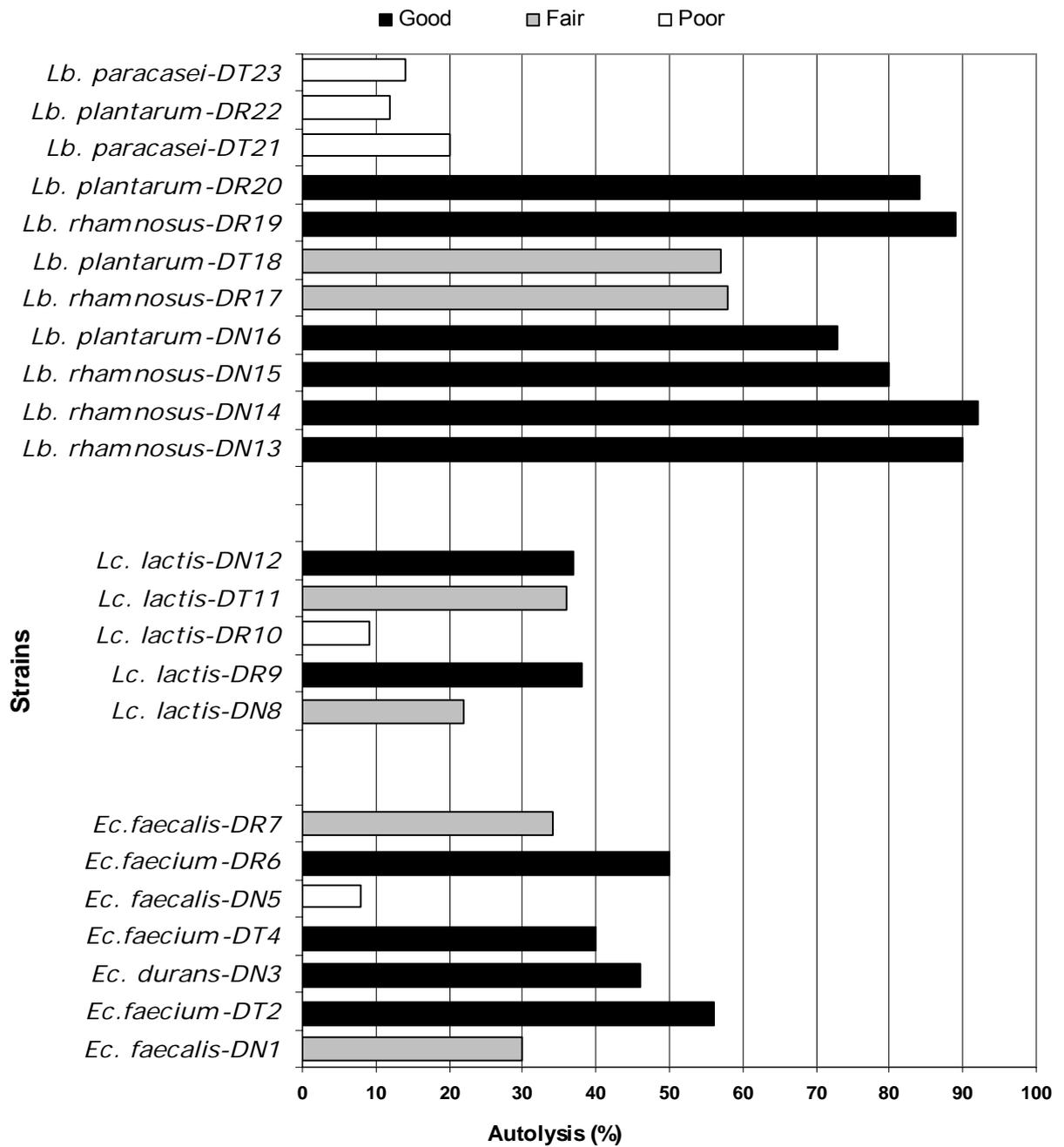
- The autolytic activity was determined as the percentage decrease in the OD_{650}, and was ranked in accordance to the activity level of each genus; Good, Fair and Poor.
- DR: Dromedary milk of Reguibi breed; DT: Dromedary milk of Targui breed; DN: Dromedary milk of N'ajjer breeds.

Figure 3. Autolytic activities of lactic acid bacteria strains isolated from raw dromedary milks tested in this study.

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