

New Proteolytic Pathway with Probable Hypoallergenic Properties of *Lactobacillus* Isolated from Dromedary Milk

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Abstract Cow milk is a nutritious product that could be the first food introduced into infant's diet when breastfeeding is not possible. However, ingestion of cow milk can cause allergic reaction in subjects which adverse immunologic response to bovine milk proteins. Clinical trials demonstrated that the microbial fermentation of bovine milk could be a possible strategy to reduce allergic symptoms in childhood. During fermentation process, some epitopes of milk proteins are destroyed by digestive enzymes of lactic acid bacteria. The antigenicity reduction depends on the specificity of the proteinases from these bacteria. In this study, functional three autochthonous *Lactobacillus plantarum*, *Lactobacillus brevis* and *Leuconostoc mesenteroides ssp dextranicum* isolated from Algerian dromedary milk were tested for their proteolytic and hypoallergenic properties. Hydrolysis of cow milk proteins by isolates was assayed using different techniques. Results revealed that *Lactobacillus plantarum* C22P was associated with rapid growth and pronounced proteolytic activity when cultured in the presence of cow milk proteins. A total degradation of beta lactoglobulin and k-casein was obtained by this strain. Results presented in this manuscript suggest that *Lb plantarum* C22P could be used as new potential adjunct bacteria with interesting proteolytic and hypoallergenic activities.

Keywords Camel milk · *Lb plantarum* · Proteolytic activity · Hypoallergenic properties

1 Introduction

During centuries lactic acid bacteria (LAB) have been involved in human diets. In foods, LAB are mainly associated with lactic acid production and proteins hydrolysis which promote product preservation, flavour elaboration and texture amelioration [1]. Through their proteolytic system, this group of bacteria plays also a significant role in reduction of cow milk allergenicity by immune response modulation and allergen hydrolysis [2,3]. The growth of LAB in milk is supported by various proteinases and peptidases which degrade caseins into smaller peptides and free amino acids essential for the good development of such auxotroph bacteria [2]. The reduction of cow milk allergenicity suggests that during proteolysis, epitopes of proteins allergens are digested [3]. Among cow milk proteins, it is believed that beta lactoglobulin is the most potent allergen for children and infants [3,4]. The allergenic potential of this peptide has been attributed to its high resistance to proteases and acidic hydrolysis and the fact that β -lactoglobulin is not present in human milk [5]. Therefore, the difference of antigenicity reduction is due to the diversity in the hydrolytic ability of LAB strains [3]. In this context, the aim of this study is to evaluate the enzymatic proteolytic activities of autochthonous LAB isolated from the Algerian dromedary milk in order to constitute local collection of new bacteria with potentially interesting technologic and hypoallergenic properties.

2 Materials and Methods

2.1 Bacterial Strains and Culture Conditions

Strains tested in this study were autochthonous *Lb plantarum* C22P, *Lb brevis* C21B and *Leuconostoc mesenteroides ssp*

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dextranicum C8M isolated from Dromedary milk collected in *Tindouf* and *Bechar* areas (southwest, Algeria). Isolates were identified in previous study by the phenotypic characteristics and 16S rDNA sequencing fragment and were selected on the basis of their technologic properties [6]. Stock cultures were maintained at 80 °C in Man–Rogosa–Sharpe broth (Merck, Oxoid) containing 20% (v/v) glycerol. The working cultures were pre-cultured from the stock cultures by two consecutive transfers in MRS broth. The *Lb brevis* CHTD27 belonging to the laboratory collection [7] was previously isolated from camel milk and used as positive proteolytic control. This strain was cultured in the same conditions as described above.

2.2 Proteolytic Activity

2.2.1 The Cell-Surface Proteolytic Activity

The extracellular protease activity (EPA) of isolates was noted by transparent halos around colonies grown on caseinate calcium agar (Conda) supplemented by 10 g L⁻¹ of skimmed cow milk UHT powder Asturiana (Asturias, Spain).

2.2.2 Proteolytic Activity in Milk

The proteolytic activity in skim cow milk after 24h of incubation was detected using the Folin–Ciocalteu reagent [8]. Isolates were used individually or in mixed cultures according to the following combinations (C8M+C21B, C8M+C22P, C21B+C22P and C8M+C21B+C22P). Reconstituted 10% (w/v) skim cow milk Asturiana (Asturias, Spain) was inoculated at 1% (v/v) with overnight at 30 °C MRS broth cultures. After milk clotting, 1 mL of each sample was homogenized to 1 mL of sterile distilled water and 10 mL of 0.72 M trichloroacetic acid (TCA) solution. The suspension was left to stand at room temperature and then filtered through Whatman filter paper (Whatman Ltd., Maidstone, UK). For the reaction mixture, 2.5 mL of the filtrate was added by 5 mL sodium carbonate polyphosphate solution (75 g of anhydrous sodium carbonate and 10 g of sodium tetraphosphate in 500 mL of distilled water) and 1.5 mL of Folin–Ciocalteu commercial reagent (panreac) diluted twice with distilled water. The absorbance was measured at 650 nm against blank (mixture without milk filtrate). Results were expressed as mg of tyrosine/L using tyrosine (0–50 mg L⁻¹) curve.

2.2.3 Cow Milk Proteins Hydrolysis Profile

Sample preparation Caseins and whey protein were extracted from clotted cow milk using 1 M ammonium acetate buffer pH 4.0 and repeated cycles of centrifugation (3500g/15 min/20 °C). Pellets were resuspended in the same buffer for the same treatment which was repeated thrice [9]. After the last centrifugation, pellets were dissolved in 0.2 M NaOH

solution and vigorously stirred and then 20 µg of proteins from each sample were prepared in 76 µL of Laemmli commercial buffer sample (Bio Rad, Spain) containing 4 µL of β-mercaptoethanol. Before electrophoresis, samples were boiled for 3 min.

Electrophoresis (SDS-PAGE) The proteolytic patterns of cow milk proteins after coagulation were analysed by polyacrylamide-SDS gel electrophoresis using Mini-PROTEAN II Tetra Vertical Electrophoresis Cell (Bio Rad, Spain). The polyacrylamide gels consisted of 12% resolving gel and 4% stacking gel. The electrophoresis buffer was a solution of pH 8.3 containing 3 g L⁻¹ Tris, 14.5 g L⁻¹ glycine and 1 g L⁻¹ SDS. Proteins samples and NYZ colour protein Marker II (Bio Rad, Spain) were separated at a total running time of 45 min at 220 V constant voltage. The gels were fixed in a solution of methanol/acetic acid/water (50:7:43 v/v) for 15 min and then stained for 2 h with 0.05% (w/v) Coomassie Brilliant Blue R-250 staining solution in methanol/acetic acid/water (50:5:40 v/v). The gels were de-stained with a solution containing methanol/ethanol/acetic acid/water (20:10:5:65 v/v). Resulting gels were scanned using the Universal Hood II Molecular Imager Gel Doc XR System (Bio Rad, Spain). Protein band densities were analysed and compared to the no inoculated milk profile using the Quantity One[®] software package (Bio Rad, Spain). Comparative peak areas estimation was carried out.

2.3 Statistical Analysis of Results

Data were subjected to the variance analysis using IBM SPSS Statistics 20.0 program (IBM Corporation, Somers, NY, USA). ANOVA was carried out so as to infer on the significance of differences between the tested isolates. Duncan's post hoc test was used for comparison at a significance degree of $p = 0.05$.

3 Results and Discussion

Data concerning the proteolytic activity of isolates are reported in Table 1 and Figs. 1 and 2. After the overnight incubation at 30 °C, C22P was easily identified for its good growth and extracellular proteolytic activity indicated by the clear halo around colonies on the caseinate calcium plate (Fig. 1); however, both C21B and C8M showed weak growth and no proteolytic activity on the same medium (Table 1; Fig. 1).

Variability in the ability of the isolates to hydrolyse the cow milk caseins was observed (Fig. 2). In general, isolates used in simple or combined cultures showed significant ($p < 0.05$) higher proteolytic activity than the control *Lb brevis* CHTD27 (Fig. 2). Results of the measurement of



Table 1 Proteolytic activity of isolates on caseinate calcium agar medium

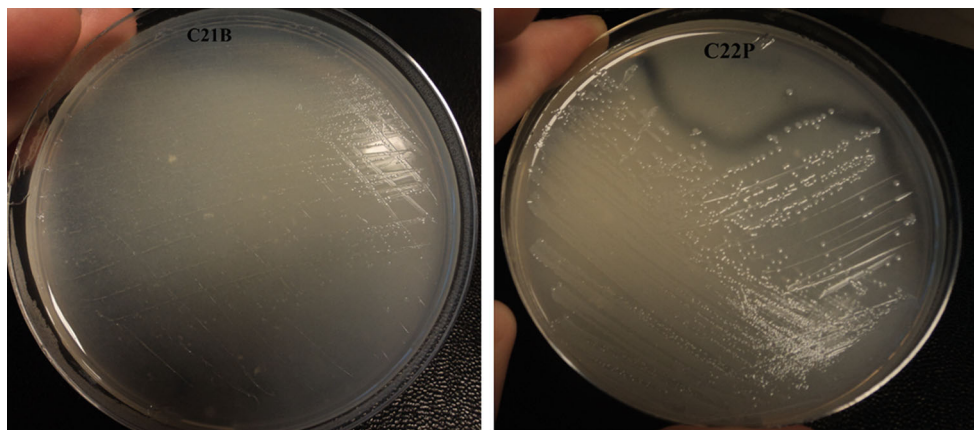
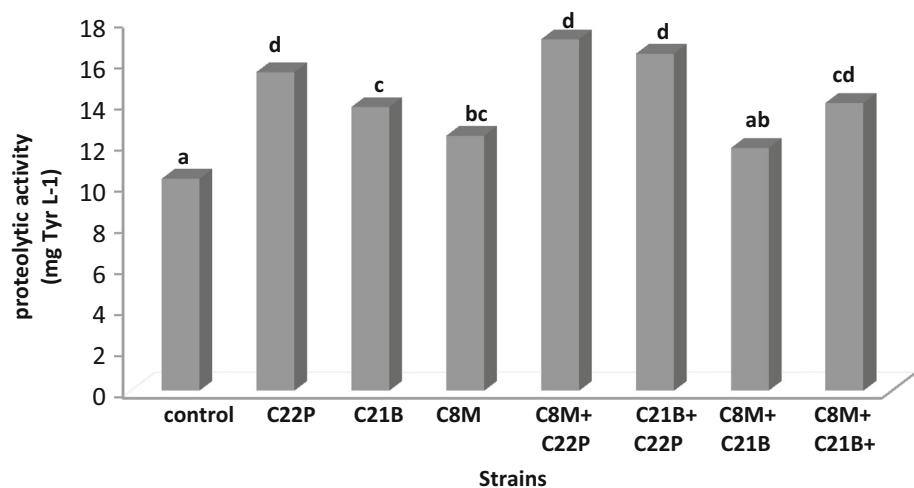
Culture	Control	C22P	C21B	C8M
Growth ^a				
24h	+	+	–	–
48	+	+	+	+
EPA ^b	+	++	–	+

^a Growth on milk caseinate calcium agar (– absence; + presence)

^b Extracellular proteolytic activities on milk caseinate calcium agar, noted by transparent halos around bacterial colonies (– absence of halo, + presence of halo)

the soluble tyrosine and tryptophan in milk ranged between 11.8 and 17.1 mg L⁻¹ tyrosine. As shown in Fig. 2, the *Lb plantarum* C22P isolate showed the highest ($p < 0.05$) proteolytic activity (15.5 ± 2.2 mg L⁻¹ tyrosine) when used alone or in combined culture with *Leuconostoc mesenteroides ssp dextranicum* C8M (17.1 ± 1.5 mg L⁻¹ tyrosine) or with *Lb brevis* C21B (16.4 ± 1.2 mg L⁻¹ tyrosine), respectively.

Values obtained in this study are lower than 57.3 mg L⁻¹ tyrosine reported for a strain of *Lactobacillus plantarum* isolated from Beyaz cheese [10]. In cheese manufacturing *Lactobacillus plantarum* species are usually used as adjunct bacteria which exhibited a number of intracellular peptidases but contribute poorly to casein hydrolysis [11, 12]. No significant differences ($p < 0.05$) was observed between *Lb brevis* C21B and *Leuconostoc mesenteroides ssp dextranicum* C8M isolates which showed the same weak proteolytic activity in milk cultures ($p < 0.05$) (Fig. 2). Same low proteolytic activities were noted for strains of *Lb brevis* and *Leuconostoc mesenteroides* isolated from different origin [13, 14]. Several works reported that the growth and metabolic activities of LAB could be affected by both biochemical and biophysical environments [15]. In the case of this study, the slow proteolytic activity of isolates can be explained by the no adaptation of these bacteria to the microenvironment of the cow milk since they were isolated from the camel milk. The low proteolytic activity of the studied isolates could be a suitable trait if they are used as adjunct bacteria. Such strains would be related to the formation of several organic acids

**Fig. 1** Proteolytic activity of *Lb brevis* C21B and *Lb plantarum* C22P on caseinate calcium agar**Fig. 2** Proteolytic activities in milk cultures expressed as mg Tyr L⁻¹. a–d Mean values not followed by the same letter in the same row are significantly different ($p < 0.05$) by Duncan's post hoc test

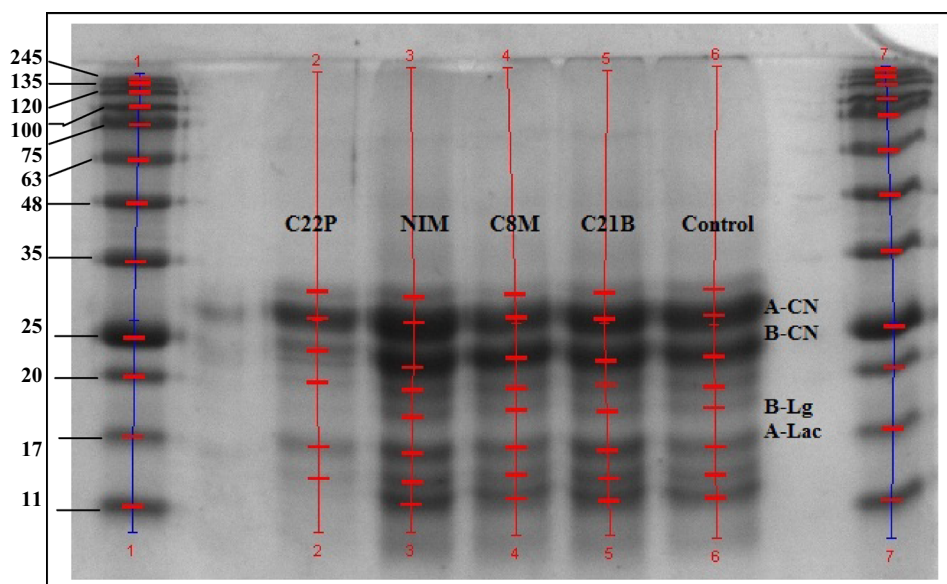


Fig. 3 Proteolytic profile of casein by isolates in simple cultures: (1,7) NYZ colour protein Marker II; (2) *Lb plantarum* C22P; (3) no inoculated milk; (4) *Ln mesenteroides ssp dextranicum* C8M; (5) *Lb brevis* C21B; (6) control (*Lb brevis* CHTD27)

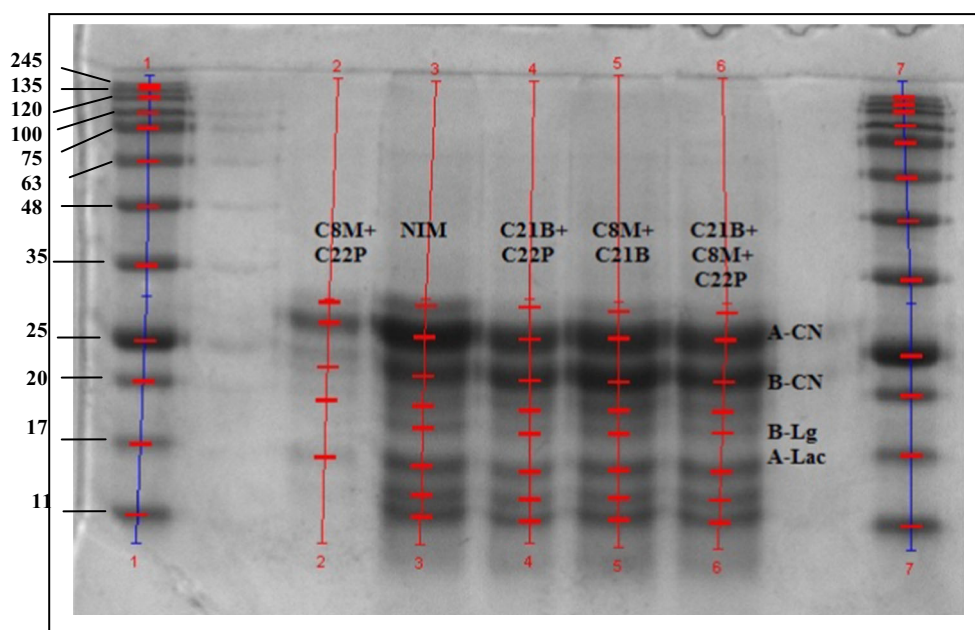


Fig. 4 Proteolytic profile of casein by isolates in combined cultures: (1,7) NYZ colour protein Marker II; (2) *Lb plantarum* C22P+ *Ln mesenteroides ssp dextranicum* C8M; (3) no inoculated milk; (4) *Lb brevis*

C21B+ *Lb plantarum* C22P. (5) *Lb brevis* C21B+ *Ln mesenteroides ssp dextranicum* C8M; (6) *Lb brevis* C21B+ *Lb plantarum* C22P+ *Ln mesenteroides ssp dextranicum* C8M

and volatiles compounds which influence positively the textures and the flavours of many fermented products without development of bitterness or too-soft final product defaults. *Lactobacillus brevis* C21B isolate was also related to an important aminopeptidase activity towards leucine and lys-p-nitroanilide derivatives [6]. This high aminopeptidase activity may have significant interest, especially when such enzymes have been related to the bitterness reduction after the bitter peptides degradation in dairy cow products [16].

The proteolytic pattern of proteins in cow milk cultures was analysed by SDS-PAGE (Figs. 3, 4; Table 2). Protein bands were identified on the basis of their molecular weight and by comparison with the no inoculated milk sample. Beta casein and kappa casein showed peaks with molecular mass of 21.07 and 19.2 kDa, respectively. Only one peak (26.1 kDa) was obtained for alpha casein, which means that on a 12% acrylamide gel α s1-CN and α s2-CN could not be separated as reported by Ong et al. [17].

Table 2 Hydrolysis of milk proteins by isolates in simple and combined cultures after 72 h of incubation. Results are expressed as percentage (%) of non-hydrolyzed casein and whey proteins

Cultures	Control	C22P	C21B	C8M	C8M+C22P	C21B+C22P	C8M+C21B	C8M+C21B+C22P
α s-CN	72.55	57.82	71.21	82.68	20.2	41.14	60.5	53.04
β -CN	80.52	32.6	100	69.0	13.5	65.98	80.29	100
κ -CN	48.65	0	61.71	100	4.3	17.6	10.8	12.5
β -Lactoglobulin	91.1	0	95	86	0	16.7	84.8	50.6

Results showed that caseinolytic activities of isolates used alone were higher than those of the *Lb brevis* CHTD27 control strain (Fig. 3). α s-CN was relatively more susceptible to proteolysis than β -CN (Table 2). The lower rate of degradation of the β -CN compared with α s-CN may be attributed to the inaccessibility of β -CN in the micelle structure [18]. However, *Lb plantarum* C22P which was the most proteolytic among isolates, showed preference for β -CN (67.4%) than α -CN (42.18%). Similar previous results reported also the fast degradation of β -CN than α -CN by strains of *Lb plantarum* [19]. α S1-casein is the major cow milk casein allergen. Experiments showed that several sequential epitopes are distributed along α S1-casein. However, mainly the intact form of this molecule is allergen. In general, caseins are easily digested in the gut; therefore, data on their allergenic activity are rare [5].

Visible bands of about 18.0 kDa of β -lactoglobulin were detected in all cultures except in those prepared with *Lb plantarum* C22P used either individually (Fig. 3) or in combinations with *Leuconostoc mesenteroides ssp dextranicum* C8M (Fig. 4), suggesting its complete degradation into small peptides and/or amino acids with molecular weight <11,000 kDa no detected on gels. Many previous results showed a partial degradation of cow milk whey protein by lactic acid bacteria and particularly by *Lactobacillus* species [3,20]. This partial degradation of allergens peptides in cow milk was usually related to a reduction without a total abolishment of antigenicity/allergenicity [3,4]. The present study demonstrates a complete degradation of β -lactoglobulin by *Lactobacillus plantarum* which could eliminate completely this allergenic peptide in milk preparations. According to the American Academy of Pediatrics, 90% of infants do not manifest any clinical symptoms to “extensively hydrolyzed milk formula” those containing only oligopeptides with molecular weight <3000 Da [21,22]. This new isolate could be used in the preparation of substitute formulas, especially when cow milk-based formula supplemented with probiotics (mainly *Lactobacillus* species) has shown beneficial effect in patients suffering from milk allergies not only after hydrolysis of allergens but also by immune system modulation [23].

4 Conclusion

Proteolysis has been identified as the key process influencing the rate of flavour and texture development in most fermented food. It was also related to the reduction of cow milk allergy. According to the present study, the three selected isolates showed different proteolytic properties. Weak proteolytic activities were noted for both *Lb brevis* C21B and *Ln mesenteroides ssp dextranicum* C8M when used alone; however, rapid growth and interesting extracellular proteolytic activity were observed for *Lb plantarum* C22P used alone or in combination with *Lb brevis* C21B or with *Leuconostoc mesenteroides ssp dextranicum* C8M suggesting the potential interest of this strain and combinations. This new bacterium seems also a complete degradation of beta lactoglobulin. Such new characteristic is probably linked to anti-allergenic properties.

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