PEPTIDASE PROFILING OF LACTOBACILLUS CASEI

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SUMMARY
Fifty four Lactobacillus casei strains were investigated and compared for their peptidase profiling by statistical analysis of aminoacids released from milk proteins. Forty one strains formed a homogeneous group; only two strains, not included in the above group, resulted the most suitable for grana cheese production either for their aminoacidic pattern or total aminoacid amount.

INTRODUCTION
Proteolytic activity in lactic acid bacteria is important for cell growth, but also for the flavour developed during cheese ripening (Law, 1982; Thomas, 1987). Proteases and, particularly peptidases are responsible for accumulation of the appropriate aminoacids in ripened cheese (Olson, 1990) and microbial catabolism of released aminoacids, produces characteristic volatile components of the finished cheese flavour (Hemme et al., 1982).

Lactobacillus species posses more varied peptidolytic profiles than lactococci (Hickey et al., 1983; Ezzat et al., 1986) and attempts to detect the number of peptidases were made by some authors (Abo-Elnaga and Plapp, 1987; Frey et al., 1982).

Lactobacillus casei displays high peptidolytic activities against a wide range of substrates. Little is known however about the complexity of peptidase enzymatic system in this organism (El Soda et al., 1978; El Soda and Desmazeaud, 1981; Hegazi and Abo-Elnaga, 1987; Abo-Elnaga and Plapp, 1987; Arora et al., 1990). To improve our understanding of the role of L. casei in cheese ripening, a study has been conducted on the pattern of aminoacids released from milk proteins by its peptidolytic enzymes.

MATERIAL AND METHODS
Strains and culture conditions. Fifty four L. casei strains, isolated from natural whey cultures for grana cheese of different areas, were employed in this study. All were subcultured in MRS(DIFCO) broth at 37°C and maintained at -80°C.

Cell free crude extract (CFCE) preparation. Cells from 500 mL MRS broth of a 12h culture, were collected by centrifugation, washed twice in saline and appropriately resuspended in 100 mM phosphate buffer pH 7 to give comparable optical density, then mechanically disintegrated in a Braun disintegrator, using 0.11 mm diameter ballottini beads. Supernatants from sedimentation and washes of glass beads, were pooled and made to a final volume of 50 ml; CFCE were stored at 80°C until use.

Enzymatic assay. Amounts of CFCE, corresponding to 50 mg cells (dry weight), were mixed with 7 ml of renneted cold milk and NaCl to a final concentration of 2% (W/V); 1% thimerosal was employed as antimicrobial. The suspensions were incubated at 37°C; samples were taken daily, for three days, and analyzed as described below. Rennet was added to produce peptide as substrates for bacterial peptidases.

Monitoring of proteolysis. Hydrolysis products were evaluated spectrophotometrically using orthophthalaldehyde (OPA) as fluorescent dye, according to the method of Church et al. (1983). Casein breakdown was detected by urea PAGE as described by Seibert et al. (1997), employing
Tris-glycine (pH 8.4) as running buffer and Tris-HCl (pH 8.9) as electrode buffer. At the end of incubation samples for free aminoacids analysis were ultrafiltered on membranes (500 Da Molecular Weight Cut Off) and the permeates mixed with equal volumes of OPA solution prepared according to Lindroth and Mopper (1979) for derivatization. Free aminoacid analysis was performed on a HPLC Ultra Wisp 715 sample processor. Derivatized aminoacids were injected into a Nova-Pak C18 columns (Water Associates) and eluted with an optimized solvent gradient of two eluents with the following composition: Eluent A: 5.4 mM Na2HP04 and 5.4 mM CH3COOH adjusted to pH 6.5 with acetic acid. Eluent B: 90% methanol and 10% eluent A.

Eluted aminoacids were detected by Spectrofluorimetry, operating at 330 nm and 440 nm as excitation and emission wavelength respectively; raw data were quantified by Maxima 820 chromatography Workstation vers. 3.3 programme.

Statistical analysis. Relative percentages of aminoacids for each of the 54 strains, were subjected to multivariate statistical analysis: cluster and principal component analysis, both performed by Stat Graphic vers. 2.1 programme. Principal component analysis is an useful technique for reducing the number of variables. Principal components must fulfill the condition of orthogonality.

Distance indexes were calculated from a reference aminoacidic composition, which was the average of several analysis of good quality Parmesan cheese (Resmini et al., 1985).

RESULTS AND DISCUSSION

The results obtained from OPA test showed a high correlation with the total aminoacid concentration by HPLC analysis (R2=0.92); this agrees with the weak proteolytic activity detected in these bacteria. Infact no strain exhibited any appreciable proteinase activity, probably masked by the relevant proteolytic activity of chimosine. As regards the total aminoacid concentration, the strains could be grouped into four classes: 21%, 42% and 25% of strains produced total amounts of aminoacids included between 2,8-5, 5-10, 10-15 mg/ml whey respectively, while only 12% more than 15 mg/ml whey. Free aminoacids from control samples containing only rennet, were less than 0.25 mg/ml whey.

Among the different distance indexes evaluated, the most appropriate were the Euclidean distances defined by the formula: \( d_i = \sqrt{\sum_j (S_{ij} - R_i)^2} \), where \( S_{ij} \) is the relative ith aminoacid concentration of the jth strain and \( R_i \) the same for the reference. The results indicate that the strains 3, 13 and 29 are the nearest to the reference, while the strains 48 and 51 were the most distant (Fig. 1).

Clustering process, based on relative percentages of aminoacids, permitted to quantify the similarity rate among the strains as cluster levels. Fortyeone strains resulted closely related by low cluster level (Fig. 2); the strains 48 and 51 clustered together at the highest cluster level. The strains 3 and 29 appeared similar and more closely related to the principal group than the strain 13.

The normalized quantities of the aminoacids, subjected to multivariate statistical analysis of the principal components, gave a distribution of the strains as shown in the bidimensional space of the two principal components representing the 65% of total variance (Fig. 3).

The origin of such space is the centroid of the point configuration and represents the characteristic profile of the species; strain dislocation is in accordance with cluster analysis, except for strains 2, 11, 43 which would require a three dimensional representation to demonstrate their distance from the baricenter. Also by this analysis the strains 29 and 3 resulted the nearest to the reference (Fig. 3); from the whole, such strains appear to be the most