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# Proteolytic activity of some *Lactobacillus paracasei* strains in a model ovine-milk curd system: Determination of free amino acids by RP-HPLC

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**Abstract**

Curd slurries were prepared from ovine milk to study the proteolytic properties of various strains of *Lactobacillus paracasei*. A commercial industrial starter and the strains to be tested in this study, *Lb. paracasei* strains Pa1, Pa2, Pa3, were inoculated in separate slurries. Free amino acids were analysed on days 0, 2, 5, 7, and 10. As ripening progressed, total free amino acids increased significantly ($P < 0.01$); content ranged from 150 mg/100 g dry matter (DM) on day 0 to 600 mg/100 g DM on day 10. Generally speaking, CIT, GLN, LEU, ASN, PRO, and 4-HYPRO were the main free amino acids in all four slurries tested, accounting for 60–82% of the total free amino acids. The slurries made using strains Pa1 and Pa3 were similar to the control slurry, and these three slurries exhibited the highest proteolysis levels. Differences between the strains of the species tested were observed.


*Keywords:* *Lactobacillus paracasei*; Proteolysis; Slurry; Amino acids

## 1. Introduction

Proteolysis is the most complex of all the conversion processes taking place during cheese maturation and may be the most important in terms of aroma, taste, and texture development (Grappin, Rank, & Olson, 1985; Sousa, Ardö, & McSweeney, 2001; Urbach, 1993). The contribution of proteolysis to taste and aroma may be direct, by releasing peptides and amino acids, or indirect, by catabolizing amino acids to amines, acids, thioles, thioesters, etc. (Law & Wigmore, 1983; Visser, Hup, Exterkate, & Stadhouders, 1983). Proteolysis in cheeses is catalyzed by: (a) residual rennet, (b) indigenous milk enzymes, (c) starter bacteria and the enzymes they produce, (d) adjunct cultures and the enzymes they produce and (e) adventitious non-starter microflora and the enzymes they produce (Sousa et al., 2001).

Microorganisms, other than those making up the starter culture, which play a significant role in developing the aroma and flavour attributes of cheeses, have been observed to be present in raw milk (Martley & Crow, 1993; McSweeney, Fox, Lucey, Jordan, & Cogan, 1993). Mesophilic lactobacilli are one of the most common groups of non-starter microorganisms present in cheeses. They are normally found in all types of cheese and are extremely important during ripening, when they attain high counts in such cheeses as Roncal, Fiore Sardo, Cheddar, Los Ibores, Comté, Dutch-type cheese, and Swiss cheese (Arizcun, Barcina, and Torre, 1997; Bouton, Guyot, and Grappin, 1998; Demarigny, Beuvier, Dasen, and Duboz, 1996; Fitzsimons, Cogan, Condon, and Beresford, 1999; Jordan and Cogan, 1993; Mannu, Comunian, and Scintu, 2000; Mas and González-Crespo, 1992; McSweeney and Fox, 1993; Williams and Banks, 1997).

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*Lactococcus* is the predominant genus of lactic acid bacteria found in milk samples, followed by *Lactobacillus*. This relationship is inverted in cheese, with *Lactobacillus* taking over as the predominant group in Cheddar (Fitzsimons et al., 1999; McSweeney et al., 1993; Williams & Banks, 1997) and Fiore Sardo (Mannu et al., 2000) cheese. Within this genus, *Lactobacillus casei* and *Lactobacillus paracasei* are quantitatively the most abundant species in many cheese varieties, such as Roncal and Idiazábal (Arizcun et al., 1997; Ortigosa, 2002), Manchego (Núñez & Martínez-Moreno, 1976), La Serena (Fernández del Pozo, Gaya, Medina, Rodríguez-Marín, & Núñez, 1988), Serra (Macedo, Malcata, & Oliveira, 1993), Arzúa-Ulloa (Centeno, Cepeda, & Rodríguez-Otero, 1996), La Armada (Prieto, Franco, Urdiales, Fresno, & Carballo, 1998), Majorero (Fontecha, Peláez, Juárez, Requena, & Gómez, 1990), Cheddar (Jordan & Cogan, 1993), and Fiore Sardo (Mannu et al., 2000).

Isolating native strains from milk and from artisanal cheeses for subsequent use as starters for pasteurized milk helps preserve certain taste, aroma, and texture characteristics in the resulting cheeses. Native strains of the lactobacilli *Lb. casei*, *Lb. paracasei*, *Lb. plantarum*, and *Lb. curvatus* have been used in previous studies (Lynch, McSweeney, Fox, Cogan, & Drinan, 1996, 1997; Lynch, Muir, Banks, McSweeney, & Fox, 1999; Muehlenkamp-Ulate & Warthesen, 1999; Ortigosa, 2002; Trépanier, El Abboudi, Lee, & Simard, 1992).

Assessing the activity of individual bacteria in cheeses is costly and slow because of the protracted ripening periods needed. The use of model cheeses or slurry systems undergoing accelerated ripening allows rapid examination of the proteolytic potential of bacteria. Systems of this kind have been used in a number of studies to evaluate the impact of different lactic acid bacteria on cheese quality (Antonsson, Ardö, Nilsson, & Molin, 2002; Crow, Curry, & Hayes, 2001; Farkye, Madkor, & Atkins, 1995; Hannon et al., 2003; Muehlenkamp-Ulate & Warthesen, 1999; Parra, Requena, Casal, & Gómez, 1996).

The object of the present study was to examine the proteolytic properties of three strains of native *Lb. paracasei* within a short time frame, using ovine-milk curd slurries as substrate.

## 2. Materials and methods

### 2.1. Strain selection

The bacterial strains tested had previously been isolated from raw ewe's milk and had been identified to species level by the polymerase chain reaction (PCR) using *Lb. paracasei*-specific oligonucleotide primers and to strain level by the randomly amplified polymorphic DNA (RAPD) method. Three strains (designated Pa1, Pa2, and Pa3) were selected on the basis of three attributes, namely, acid-producing ability, isolation from source milks which had yielded cheeses that earned high sensory scores, and persistence of the strain in the cheeses until advanced stages of ripening.

At the same time, a control cheese was manufactured using a freeze-dried industrial starter culture composed of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* in the amount of 1 U/100 kg of slurry ($1\ U = 5 \times 10^{11}$ cfu).

### 2.2. Manufacture of the model cheese slurries

Curd slurries were prepared according to a slightly modified version of the method published by Parra et al. (1996). Pasteurized ovine milk was used, the pH was adjusted to 6.3, $CaCl_2$ (Laboratorios Arroyo S.A., Santander, Spain) was added (1 l/4.000 l), and coagulation was achieved using industrial rennet (1/10,000). The curd was then cut, pressed, homogenized, and transferred to sterile screw-cap bottles and sterilized (110 °C/10 min). The slurries were aseptically homogenized with a sterile NaCl solution (pH 5.4) to a concentration of 2.4 g NaCl per kg of slurry. The selected strains and the industrial starter were inoculated in separate slurries in the amount of $10^6$–$10^8$ cfu/ml. The slurries then underwent accelerated ripening at 30 °C for 10 d, with samples being taken on days 0, 2, 5, 7, and 10. The slurries were designated SC for the control slurry made using the industrial starter and SPa1, SPa2, and SPa3, respectively, for the slurries made using the added *Lb. paracasei* strains Pa1, Pa2, and Pa3.

### 2.3. Physicochemical analysis

#### 2.3.1. Dry matter and pH

Dry matter (DM) was determined according to IDF-FIL (1958) standard no. 4.

The pH was measured using a model 507 Crison® pH-meter with a Xerolyt® penetration electrode, catalogue No. 52-32 Crison® (Crison Instruments, S.A., Alella, Barcelona, Spain).

#### 2.3.2. Analysis of the free amino acids (FAAs)

RP-HPLC analysis of the FAAs was performed according to the method of Izco, Torre, and Barcina (2000). Samples were analysed on a Waters HPLC system consisting of two model 515 pumps, a model 717 PLUS injector, a temperature control module, and a model 996 photodiode array detector at the 254 nm setting, operated using Millennium 2010 software. The column used was a Waters Pico-Tag C18 reversed-phase column (300 mm × 3.9 mm i.d., 60 A pore size and 4 μm particle size) (Waters, Milford, MA, USA), held at 46 °C. A master solution of amino acids (Sigma, St. Louis, MO, USA), to which methionine sulfone (Sigma) had been added as an internal standard, was used for FAA identification and quantification.

A two-solvent gradient was used to run the samples: solution A comprised 70 mM sodium acetate and 2.5% acetonitrile adjusted to pH 6.55 with acetic acid, and solution B was 45% acetonitrile, 40% water, and 15% methanol.

Before each injection the column was equilibrated with solvent A for 20 min.

## 3. Results and discussion

Fig. 1 depicts the chromatograms of the FAA analysis of the control slurry (SC) and the cheese slurries (SPa1, SPa2, and SPa3) inoculated with the respective *Lb. paracasei* strains on day 10 of ripening, showing the FAAs identified and considered. Together with the peaks for intact FAAs, the cheese extraction and derivatization process also yielded a number of unidentified peaks, although these seldom interfered with identification of the other peaks.

Tables 1 and 2 set out the results obtained for the cheese slurries.

The total free amino acid (TFAA) content (calculated as the sum of all the individual amino acids considered), as determined by RP-HPLC, ranged from 150 mg/100 g DM on day 0 to 600 mg/100 g DM on day 10.

Comparing the *Lb. paracasei* cultures, cheese slurries SPa1 and SPa3 exhibited a behaviour similar to that of the control slurry and had the highest TFAA concentrations. The starter cultures for these three slurries yielded the highest proteolysis levels at the end of ripening. The behaviour of slurry SPa2 was less similar to that of the control slurry, and lower TFAA levels were attained. This sort of variation between strains of the same species has also been observed in the past (Lynch et al., 1999; Muehlenkamp-Ulate & Warthesen, 1999; Sasaki, Bosman, & Tan, 1995).

Studying Cheddar slurries made with a control strain of *Lc. lactis* subsp. *cremoris* or with the control strain plus *Lb. casei* and *Lb. paracasei* strains, Muehlenkamp-Ulate and Warthesen (1999) found no statistical differences in the TFAA contents of the slurries made with this last-mentioned species and the control slurry. Lynch et al. (1999) reported similar findings on comparing a control cheese with experimental cheeses made with *Lb. paracasei* after three months of ripening. In this study, no statistical differences were recorded between slurry SC and slurries SPa1 and SPa3 but, in contrast, as already indicated, slurry SPa2 did display TFAA levels statistically different from the control.

While slurries SC, SPa1, and SPa3 all had similar TFAA values, higher than those for slurry SPa2, the behaviour of each slurry varied over the ripening period. In this respect, slurry SC attained its peak TFAA value on day 2, while slurries SPa1 and SPa2 both reached their peak levels later, on day 5. Slurry SPa3 was the last to reach its peak value, on day 10. Therefore, as far as TFAA levels are concerned, slurry SPa1 was most similar in behaviour to that of slurry SC, in that it reached the same level a little later, whereas slurry SPa3 was the slowest, needing the entire ripening period considered to reach the maximum TFAA value (see Figs. 2 and 3).

As can be seen in Fig. 1, for the most part citrulline, glutamine, leucine, asparagine, proline, and 4-hydroxyproline were the main FAAs in all the slurries, that is, both in the control slurry (SC) and in the three slurries (SPa1, SPa2, and SPa3) made with the respective added *Lb. paracasei* strains. These FAAs accounted for between 60% and 82% of the total FAAs. The first two alone accounted for nearly 40–60% of the total FAAs in all the slurries at all ripening times considered (Table 1).

Certain of these amino acids, more specifically asparagine, leucine, and glutamine, have also been reported to be the main amino acids in other types of cheese, e.g., Ossau-Iraty (Izco et al., 2000), Idiazábal (Ordoñez, Ibáñez, Torre, & Barcina, 1998; Mendía, Ibáñez, Torre, & Barcina, 2000) and Roncal (Muñoz, Ortigosa, Torre, & Izco, 2003). Leucine, in particular, is one of the main amino acids in many types of cheese, including Idiazábal (Vicente, Ibáñez, Barcina, & Barron, 2001), Ossau-Iraty (Izco et al., 2000), Cheddar (Lynch et al., 1996; McSweeney & Sousa, 2000), Emmental (Thierry, Salvat-Brunaud, Madec, Michel, & Maubois, 1998), Picante (Freitas, Pintado, Pintado, & Malcata, 1999), Mahón (García-Palmer, Serra, Palou, & Gianotti, 1997), and Fossa (Gobbetti et al., 1999). In the present study using curd slurries, this amino acid was one of the major amino acids but was less predominant than in other types of cheese such as those referred to above. By the same token, both asparagine and glutamine are typical amino acids in cheeses made from pasteurized milk, in which they tend to be present in higher quantities (García-Palmer et al., 1997; Lau, Barbano, & Rassmusen, 1991), as was the case for the experimental slurries made in this study.

A comparison of the amino acid profiles shows that, while slurry SC exhibited TFAA levels similar to those for slurries SPa1 and SPa3, various amino acids, e.g., glutamic acid, proline, valine, methionine, cystine, and isoleucine, were present in larger amounts in the slurries made with the *Lb. paracasei* strains than in slurry SC. Other amino acids, such as asparagine and glutamine, were present in higher amounts in slurry SC than in slurries SPa1 and SPa3. This agrees with the findings of Lynch et al. (1996), who examined the amino acid profiles of Cheddar cheeses and observed that certain amino acids were present in larger amounts in cheeses made with a *Lactococcus* starter culture plus added strains of *Lb. casei* subsp. *casei* and *Lb. casei* subsp. *pseudoplantarum* than in control cheeses made using the starter alone. Some of these amino acids were the same as those recited above.

As already mentioned, concentrations of methionine and cystine, both sulfur-containing amino acids, were higher in slurries SPa1 and SPa3 than in slurry SC, made using the industrial starter, consisting of *Lc. lactis*. Williams, Noble, and Banks (2001) noted that strains of this latter species catabolized these amino acids to a greater extent than did *Lb. paracasei* strains; hence it is no surprise that levels of these amino acids were lower in the control slurry in this study, since rates of breakdown would be expected to be higher in that slurry.

Various studies (Laht, Kaska, Eliasc, Adamberg, & Paalme, 2002; Mendía et al., 2000; Muñoz et al., 2003)

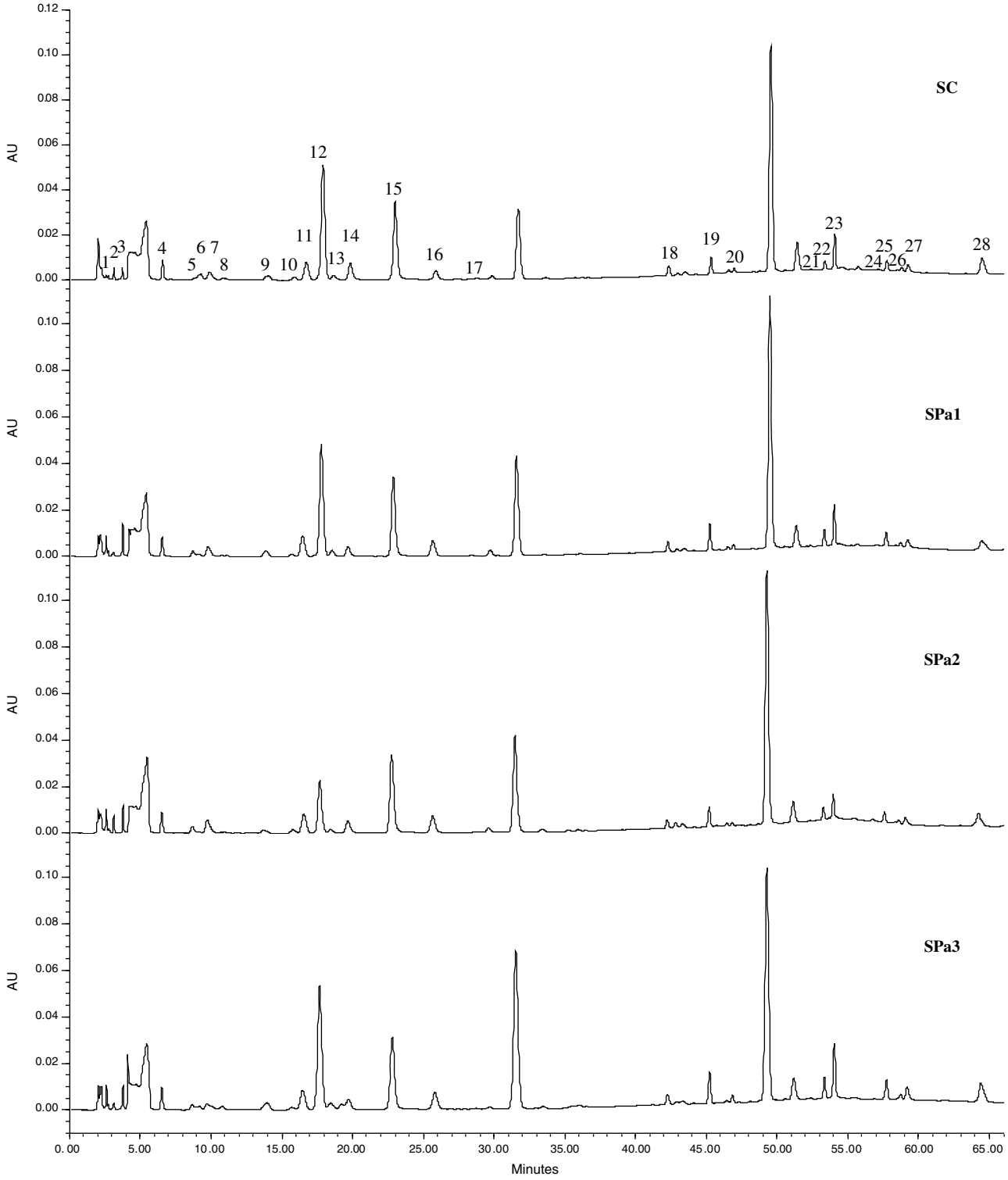


Fig. 1. Chromatograms from the free amino acid analysis for the control (SC) and *Lb. paracasei* (SPa1, SPa2, and SPa3) inoculated cheese slurries on day 10 of ripening. Peaks: 1 = PSER, 2 = ASP, 3 = GLU, 4 = 4-HYPRO, 5 = SER, 6 = ASN, 7 = GLY, 8 = GLN, 9 = TAU, 10 = HYS, 11 = GABA, 12 = CIT, 13 = THR, 14 = ALA, 15 = I.S. (methionine sulfone), 16 = PRO, 17 = M-HYS, 18 = TYR, 19 = VAL, 20 = MET, 21 = CYS, 22 = ILE, 23 = LEU, 24 = H-LYS, 25 = PHE, 26 = TRP, 27 = ORN, 28 = LYS.

have reported a relationship between ornithine levels and the development of the non-starter lactic acid bacteria (NSLAB), with these bacteria producing Orn from arginine. In the present study, this finding held only for slurry SPa3. This type of variation in strain behaviour is consistent with the observations published in various other studies, indicating that not all *Lb. paracasei* strains are able to employ that pathway. For instance, Williams et al. (2001)

Table 1
Free amino acid (FAA) contents expressed as mg FAA/100 g dry matter in the control (SC) and *Lb. paracasei* (SPa1, SPa2, and SPa3) inoculated cheese slurries on days 0, 2, and 5 of ripening

| Amino acid | Day 0 | | | | | Day 2 | | | | | Day 5 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SC | SPa1 | SPa2 | SPa3 | *P* | SC | SPa1 | SPa2 | SPa3 | *P* | SC | SPa1 | SPa2 | SPa3 | *P* |
| PSER | 5.3$^{b}$ | 6.2$^{b}$ | 13.5$^{a}$ | 4.9$^{b}$ | *** | 2.8$^{b}$ | 1.8$^{b}$ | 12.2$^{a}$ | 7.7$^{ab}$ | * | 2.7$^{b}$ | 2.7$^{b}$ | 13.5$^{a}$ | 11.7$^{a}$ | *** |
| ASP | 0.2 | 0.2 | n.d. | 0.2 | NS | 0.6 | 0.2 | 0.4 | 0.9 | NS | 0.2 | 0.7 | 2.0 | 0.8 | NS |
| GLU | 1.0 | 1.2 | 0.5 | 0.7 | NS | 3.4 | 5.7 | 6.5 | 3.9 | NS | 5.5 | 8.7 | 11.1 | 8.0 | NS |
| 4-HYPRO | 14.4 | 16.3 | 15.4 | 14.1 | NS | 16.1 | 14.2 | 14.7 | 17.1 | NS | 18.0 | 12.3 | 16.1 | 15.4 | NS |
| SER | 1.6 | 1.1 | 0.9 | 0.4 | NS | 3.2$^{a}$ | 2.1$^{b}$ | 1.3$^{b}$ | 1.4$^{b}$ | ** | 3.7 | 3.6 | 3.1 | 2.7 | NS |
| ASN | 0.9 | 2.1 | n.d. | n.d. | NS | 42.9$^{a}$ | 8.5$^{b}$ | 6.2$^{b}$ | 4.5$^{b}$ | *** | 50.7$^{a}$ | 26.7$^{b}$ | 6.8$^{c}$ | 21.8$^{b}$ | *** |
| GLY | 8.1$^{a}$ | 6.8$^{a}$ | 5.9$^{a}$ | 3.2$^{b}$ | ** | 5.0 | 7.6 | 7.6 | 5.5 | NS | 6.7$^{b}$ | 9.8$^{a}$ | 10.0$^{a}$ | 6.9$^{b}$ | ** |
| GLN | 37.5 | 37.4 | 28.9 | 55.5 | NS | 129 | 47.5 | 39.0 | 63.0 | NS | 148$^{a}$ | 50.5$^{b}$ | 60.6$^{ab}$ | 97.8$^{ab}$ | * |
| TAU | 5.8 | 6.7 | 5.5 | 4.9 | NS | 6.8 | 3.7 | 4.6 | 4.5 | NS | 8.7 | 4.1 | 3.8 | 7.0 | NS |
| HYS | 1.0 | 0.2 | 0.2 | 0.6 | NS | 5.0 | 2.6 | 5.0 | 1.6 | NS | 5.8$^{ab}$ | 4.0$^{bc}$ | 6.6$^{a}$ | 2.9$^{c}$ | ** |
| GABA | 0.2$^{ab}$ | n.d.$^{b}$ | 0.3$^{a}$ | n.d.$^{b}$ | ** | 23.6$^{a}$ | 1.9$^{b}$ | 1.6$^{b}$ | 0.8$^{b}$ | *** | 27.1 | 21.7 | 19.6 | 18.0 | NS |
| CIT | 55.9$^{b}$ | 57.4$^{b}$ | 65.1$^{ab}$ | 81.0$^{a}$ | * | 254$^{a}$ | 165.0$^{b}$ | 71.1$^{d}$ | 126$^{c}$ | *** | 171$^{a}$ | 200$^{a}$ | 76.9$^{b}$ | 143$^{ab}$ | *** |
| THR | 3.9$^{b}$ | 4.7$^{ab}$ | 5.6$^{a}$ | 3.2$^{b}$ | * | 9.2$^{a}$ | 6.5$^{ab}$ | 5.0$^{b}$ | 4.7$^{b}$ | * | 8.5 | 8.0 | 6.0 | 7.9 | NS |
| ALA | 4.6 | 2.4 | 3.0 | 3.6 | NS | 6.5 | 3.0 | 5.3 | 5.8 | NS | 10.6$^{a}$ | 8.9$^{ab}$ | 5.7$^{b}$ | 7.8$^{ab}$ | * |
| PRO | 1.9$^{a}$ | 0.1$^{b}$ | 0.2$^{b}$ | 0.0$^{b}$ | *** | 21.2 | 18.0 | 23.0 | 17.4 | NS | 21.6$^{b}$ | 36.1$^{a}$ | 32.2$^{a}$ | 32.6$^{a}$ | *** |
| M-HYS | 4.8 | 6.0 | 3.7 | 3.5 | NS | 4.3 | 6.1 | 6.6 | 4.1 | NS | 8.3$^{ab}$ | 10.3$^{a}$ | 4.9$^{b}$ | 4.5$^{b}$ | ** |
| TYR | 1.3 | 2.4 | 1.3 | 3.7 | NS | 7.4 | 5.1 | 5.6 | 6.1 | NS | 11.2 | 11.2 | 8.2 | 10.0 | NS |
| VAL | 0.5 | 0.9 | 1.2 | 0.7 | NS | 7.1$^{b}$ | 6.3$^{bc}$ | 9.2$^{a}$ | 6.0$^{c}$ | *** | 8.3$^{b}$ | 18.3$^{a}$ | 17.1$^{a}$ | 16.5$^{a}$ | ** |
| MET | 0.7 | n.d. | n.d. | 0.3 | NS | 1.1$^{b}$ | 2.4$^{a}$ | 2.6$^{a}$ | 2.5$^{a}$ | * | 1.8 | 5.8 | 3.7 | 4.4 | NS |
| CYS | n.d. | n.d. | n.d. | n.d. | NS | n.d. | n.d. | n.d. | n.d. | NS | n.d. | 1.6 | n.d. | 0.9 | NS |
| ILE | 1.0 | 1.8 | 1.5 | n.d. | NS | 3.9 | 3.8 | 4.6 | 4.1 | NS | 4.4$^{b}$ | 12.2$^{a}$ | 8.4$^{ab}$ | 10.8$^{a}$ | ** |
| LEU | 2.4 | 1.3 | 0.6 | 1.5 | NS | 9.6 | 12.2 | 13.0 | 12.9 | NS | 13.2 | 20.7 | 22.5 | 32.7 | NS |
| H-LYS | 0.8 | 1.9 | 1.5 | 1.1 | NS | 1.3 | 8.1 | 1.7 | 0.9 | NS | 2.3 | 5.0 | 1.9 | 2.1 | NS |
| PHE | 0.8$^{a}$ | 0.3$^{b}$ | 0.3$^{b}$ | 0.3$^{b}$ | * | 4.1 | 5.6 | 5.9 | 6.2 | NS | 5.4 | 16.9 | 10.7 | 14.4 | NS |
| TRP | 1.4 | 1.6 | 0.9 | 1.2 | NS | 3.3 | 3.3 | 3.1 | 2.4 | NS | 3.6 | 3.4 | 2.8 | 3.3 | NS |
| ORN | 0.6 | 0.0 | 0.0 | 0.2 | NS | 2.1 | 1.2 | 1.2 | 1.8 | NS | 3.2 | 2.2 | 2.0 | 4.4 | NS |
| LYS | 0.4 | 0.2 | 0.5 | 0.2 | NS | 7.9 | 3.7 | 8.3 | 4.1 | NS | 9.3$^{ab}$ | 11.5$^{ab}$ | 8.2$^{b}$ | 26.4$^{a}$ | * |
| TFAAs | 157$^{b}$ | 159$^{b}$ | 157$^{b}$ | 185$^{a}$ | * | 581$^{a}$ | 346$^{b}$ | 265$^{c}$ | 316$^{bc}$ | *** | 561$^{a}$ | 517$^{ab}$ | 364$^{b}$ | 514$^{ab}$ | * |

n.d.: not detectable.
Different superscripts in the same row on the same sampling date indicate significant differences between the mean values.
*P*, level of significance; NS, non-significant; *, significant at the level of 0.05; **, significant at the level of 0.01; ***, significant at the level of 0.001.

showed that 45% of 22 *Lb. paracasei* strains isolated from cheese were able to catabolize arginine, while Laht et al. (2002) showed that arginine could be used by three of six *Lb. paracasei* strains.

Differences among strains of the species *Lb. paracasei* have also been observed in aminotransferase activity, especially on leucine and phenylalanine (Williams, Noble, Tamman, Lloyd, & Banks, 2002). This could explain the findings of the present study, in which the values for these two amino acids varied for the different strains from day 7 on.

The opposite is true for amino acids such as asparagine and glutamine, and slurry SC made using the commercial starter generally had higher values for these amino acids than had the slurries made using the *Lb. paracasei* strains. In a study in which strains of *Lactococcus lactis* were modified to express peptidases of lactobacillus strains, levels of such amino acids as asparagine, glutamine, and proline increased more than 3.5-fold (Courtin et al., 2002). This suggests that certain peptidases of lactobacillus strains help free these amino acids specifically. This finding contradicts the results for asparagine and glutamine observed in this study. On the other hand, there have been studies of the catabolic activity of microorganisms in which *Lb. paracasei* strains have been observed to be capable of breaking down these amino acids (Kieronczyk, Skeie, Olsen, & Langsrud, 2001). This enhanced catabolic action could account for the lower levels of these amino acids recorded here for the slurries made using the *Lb. paracasei* strains, particularly pronounced in the case of asparagine in slurry SPa2.

GABA (γ-aminobutyric acid) is another important amino acid, because it is related to low-quality cheeses (Choisy et al., 1990). In fact, it is an amine produced by decarboxylation of glutamate by the enzyme glutamate decarboxylase. There were no differences in the levels of this amino acid in the different slurries for the intermediate ripening times. On the other hand, on day 10 slurry SPa2 had lower levels than slurry SC. The values recorded, around 2–4% of the TFAAs, were similar to or lower than had levels that have been recorded in Idiazábal (Mendía et al., 2000; Ordoñez et al., 1998; Vicente et al., 2001), Gouda (Nomura, Kimoto, Someya, Furukawa, & Suzuki, 1998), and Babia-Laciana (Franco, Prieto, Bernardo, González Prieto, & Carballo, 2003) cheese but higher than those reported for certain other cheeses, such as Ossau-Iraty (Izco et al., 2000) and Camembert, Edam, and Emmental (Nomura et al., 1998).

The amino acids ASP, 4-HYPRO, TAU, and TRP displayed no significant differences between the slurries at any of the ripening times considered.

Table 2
Free amino acid (FAA) contents expressed as mg FAA/100 g dry matter in the control (SC) and *Lb. paracasei* (SPa1, SPa2, and SPa3) inoculated cheese slurries on days 7 and 10 of ripening

| Amino acid | Day 7 | | | | | Day 10 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | SC | SPa1 | SPa2 | SPa3 | P | SC | SPa1 | SPa2 | SPa3 | P |
| PSER | 3.9 | 6.0 | 7.9 | 10.6 | NS | $5.9^{ab}$ | $3.6^{b}$ | $9.4^{a}$ | $7.4^{ab}$ | * |
| ASP | 0.1 | 0.7 | 1.7 | 0.7 | NS | 0.1 | 1.1 | 1.4 | 1.3 | NS |
| GLU | 5.6 | 10.8 | 11.4 | 7.8 | NS | $5.6^{b}$ | $12.7^{a}$ | $13.6^{a}$ | $9.0^{ab}$ | ** |
| 4-HYPRO | 16.6 | 14.1 | 15.0 | 15.3 | NS | 17.5 | 13.8 | 16.1 | 16.3 | NS |
| SER | 2.3 | 3.1 | 3.0 | 4.0 | NS | $2.0^{b}$ | $3.7^{ab}$ | $3.5^{ab}$ | $5.6^{a}$ | * |
| ASN | $54.0^{a}$ | $24.6^{b}$ | $7.1^{c}$ | $19.4^{b}$ | *** | $68.1^{a}$ | $32.4^{b}$ | $8.0^{c}$ | $28.0^{b}$ | *** |
| GLY | $5.6^{c}$ | $11.9^{a}$ | $9.7^{b}$ | $6.9^{c}$ | *** | $6.6^{b}$ | $18.1^{a}$ | $9.3^{b}$ | $6.7^{b}$ | * |
| GLN | 140 | 57.5 | 75.8 | 93.9 | NS | 156.8 | 62.3 | 72.7 | 115.0 | NS |
| TAU | 8.7 | 5.4 | 4.0 | 7.6 | NS | 9.4 | 4.5 | 4.0 | 8.5 | NS |
| HYS | 6.0 | 7.1 | 5.2 | 4.4 | NS | 6.1 | 8.8 | 5.3 | 5.0 | NS |
| GABA | 25.6 | 22.1 | 19.0 | 18.3 | NS | $28.5^{a}$ | $25.8^{ab}$ | $16.9^{b}$ | $20.7^{ab}$ | * |
| CIT | $208^{a}$ | $202^{a}$ | $72.5^{c}$ | $119^{b}$ | *** | $176^{a}$ | $195.6^{a}$ | $73.4^{b}$ | $147^{ab}$ | ** |
| THR | 8.6 | 8.2 | 6.5 | 7.7 | NS | 6.6 | 11.2 | 6.4 | 8.9 | NS |
| ALA | 11.2 | 8.7 | 6.7 | 9.9 | NS | $12.5^{a}$ | $9.7^{ab}$ | $6.3^{b}$ | $11.5^{a}$ | ** |
| PRO | $18.7^{b}$ | $32.2^{a}$ | $30.1^{a}$ | $27.6^{ab}$ | * | 19.8 | 31.7 | 28.5 | 29.0 | NS |
| M-HYS | $7.3^{b}$ | $13.2^{a}$ | $6.0^{b}$ | $4.7^{b}$ | ** | 7.2 | 5.6 | 5.8 | 4.6 | NS |
| TYR | $11.4^{a}$ | $7.7^{b}$ | $7.8^{b}$ | $10.8^{a}$ | ** | $14.0^{a}$ | $9.3^{b}$ | $8.0^{b}$ | $13.7^{a}$ | *** |
| VAL | $8.3^{c}$ | $24.9^{a}$ | $15.9^{bc}$ | $17.6^{ab}$ | * | $10.5^{b}$ | $27.4^{a}$ | $15.0^{ab}$ | $21.6^{ab}$ | * |
| MET | $1.9^{c}$ | $5.8^{a}$ | $3.2^{bc}$ | $4.8^{ab}$ | * | $3.2^{b}$ | $9.1^{a}$ | $2.5^{b}$ | $6.5^{ab}$ | * |
| CYS | $0.0^{c}$ | $3.6^{a}$ | $0.0^{c}$ | $1.4^{b}$ | *** | $0.0^{b}$ | $4.5^{a}$ | $0.0^{b}$ | $3.4^{a}$ | *** |
| ILE | $4.5^{c}$ | $17.3^{a}$ | $7.0^{bc}$ | $12.0^{ab}$ | ** | $6.2^{b}$ | $18.7^{a}$ | $6.1^{b}$ | $16.0^{a}$ | * |
| LEU | $12.8^{c}$ | $29.1^{ab}$ | $19.4^{bc}$ | $35.2^{a}$ | * | $18.1^{b}$ | $31.0^{ab}$ | $19.7^{b}$ | $41.7^{a}$ | ** |
| H-LYS | $1.7^{b}$ | $7.9^{a}$ | $2.0^{b}$ | $2.4^{b}$ | ** | 3.5 | 8.9 | 1.9 | 3.9 | NS |
| PHE | $5.9^{b}$ | $6.8^{b}$ | $8.0^{b}$ | $15.4^{a}$ | *** | $6.9^{b}$ | $9.9^{b}$ | $8.5^{b}$ | $17.5^{a}$ | *** |
| TRP | 3.8 | 4.2 | 2.8 | 3.9 | NS | 3.6 | 9.0 | 2.4 | 5.1 | NS |
| ORN | 3.3 | 2.4 | 2.0 | 4.8 | NS | $2.9^{b}$ | $2.5^{b}$ | $1.6^{b}$ | $5.3^{a}$ | * |
| LYS | $10.3^{b}$ | $19.9^{a}$ | $21.8^{a}$ | $18.0^{a}$ | * | 11.0 | 20.3 | 19.3 | 22.2 | NS |
| TFAAs | $586^{a}$ | $557^{a}$ | $371^{c}$ | $484^{b}$ | *** | $608^{a}$ | $591.3^{a}$ | $366^{b}$ | $581^{ab}$ | * |

n.d., not detectable.
Different superscripts in the same row on the same sampling date indicate significant differences between the mean values.
*P*, level of significance; NS, non-significant; *, significant at the level of 0.05; **, significant at the level of 0.01; ***, significant at the level of 0.001.

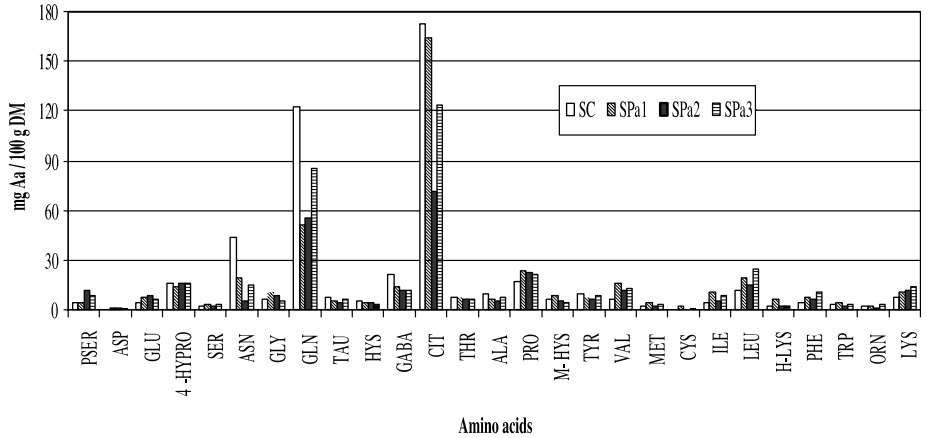


Fig. 2. Mean free amino acid concentrations in the control (SC) and *Lb. paracasei* (SPa1, SPa2, and SPa3) inoculated cheese slurries on day 10 of ripening.

The general trend was for the TFAAs to increase significantly ($P < 0.01$) as the ripening time increased. As a rule, all the amino acids increased with ripening time, as has been reported for many other cheeses (Izco et al., 2000; Lynch et al., 1999; Muehlenkamp-Ulate & Warthesen, 1999; Vicente et al., 2001).

At the same time, levels of some amino acids did not change with ripening time, and the values for certain others even decreased. Similar results have been published for Mahón cheese (García-Palmer et al., 1997). 4-Hydroxyproline levels held constant over the ripening period considered in all four slurries tested (SC, SPa1, SPa2, and

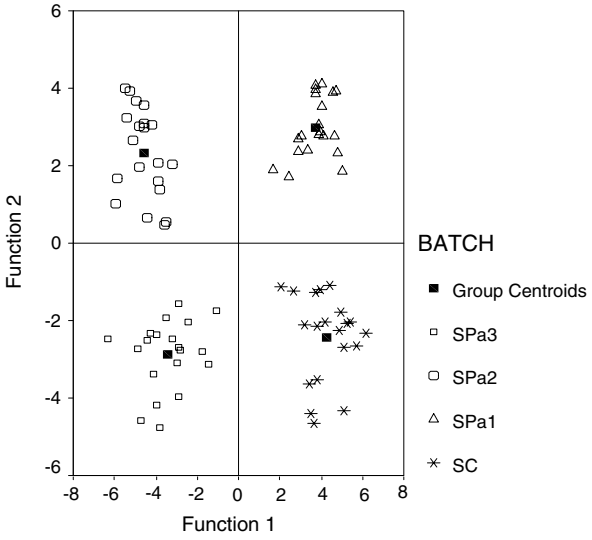


Fig. 3. Plot of the canonical discriminant functions obtained using the FAA analysis results classifying the control (SC) and *Lb. paracasei* (SPa1, SPa2, and SPa3) inoculated cheese slurries.

SPa3). Taurine, aspartic acid, and methyl-histidine also held steady over the ripening period in all the slurries made using the *Lb. paracasei* strains. Similarly, phosphoserine, glutamine, cysteine and tryptophan remained constant in the control slurry. In a study on Urbasa cheese, which is quite similar to Roncal cheese, tryptophan levels also stayed constant over a 120-day ripening period (Guindeo, Astiasarán, & Bello, 1990).

Glutamic acid is an amino acid that is regarded as an index of cheese ripening (Farkye & Fox, 1990; McSweeney et al., 1993; Rosenberg & Altemueller, 2001), and hence the trend for this amino acid during the ripening period is of special importance. For this reason, concentrations of this amino acid in the different slurries were regressed linearly on ripening time, and the coefficient values of the regressions appear in Table 3.

Table 3
Coefficient values for the linear regressions for glutamic acid

| Cheese slurry | $a_0$ | $a_1$ | $r$ |
|---|---|---|---|
| SC | −2.42 | 1.71 | 0.88 |
| SPa1 | −1.88 | 0.85 | 0.97 |
| SPa2 | −1.32 | 0.71 | 0.94 |
| SPa3 | −1.40 | 1.05 | 0.93 |

SC, slurry made using an industrial starter; SPa1, slurry made using *Lb. paracasei* strain Pa1; SPa2, slurry made using *Lb. paracasei* strain Pa2; SPa3, slurry made using *Lb. paracasei* strain Pa3.

The results show that coefficient values were higher for all the slurries made using the *Lb. paracasei* strains than for the control slurry made using the commercial starter, which can be interpreted as indicating that the ripening process was more pronounced in the slurries made with the *Lb. paracasei* strains than in the control slurry.

Table 4 lists the amino acids selected by discriminant analysis. Plotting discriminant functions 1 and 2 yielded good classification of the cheeses with a 100% correct classification rate. Phosphoserine, citrulline, asparagine, and threonine were the main amino acids contributing to function 1. The first two of these amino acids were present at significantly lower concentrations in slurries SC and SPa1 than in the other two slurries, which also exhibited higher levels of asparagine and threonine. Using either of the two functions, slurry SPa2 was the furthest from the control slurry (SC). As has already been discussed, this finding was the result of both the TFAA content and the levels of each of the individual amino acids.

## 4. Conclusions

Differences between strains of the single species considered, *Lb. paracasei*, were observed. Both the TFAA levels and the profile of the individual amino acids observed depended on the strain employed in the starter, since performance by strains of the same species varies. This means

Table 4
Discriminant analysis: summary of parameter (FAA) selection and standardized canonical discriminant function coefficients

| Step | Parameter entered | Wilk's $\lambda$ | Significance | Function 1 | Function 2 | Function 3 |
|---|---|---|---|---|---|---|
| 1 | ASN | 0.527 | 0.0000 | 1.707 | 1.359 | **−1.876** |
| 2 | VAL | 0.275 | 0.0000 | −4.691 | 0.109 | **−1.912** |
| 3 | LEU | 0.182 | 0.0000 | 3.656 | −1.590 | **0.817** |
| 4 | PSER | 0.121 | 0.0000 | **−1.734** | 0.580 | −0.243 |
| 5 | ORN | 0.062 | 0.0000 | −0.643 | **−0.777** | 0.629 |
| 6 | GLU | 0.046 | 0.0000 | −1.633 | **1.325** | −0.221 |
| 7 | GLN | 0.034 | 0.0000 | −1.137 | **−1.466** | 0.318 |
| 8 | H_LYS | 0.026 | 0.0000 | 0.408 | 1.517 | **0.635** |
| 9 | M_HYS | 0.021 | 0.0000 | 1.653 | **1.017** | 0.440 |
| 10 | GLY | 0.016 | 0.0000 | 4.471 | **−0.040** | −0.237 |
| 11 | ALA | 0.011 | 0.0000 | −1.302 | **−1.814** | 0.106 |
| 12 | CIT | 0.006 | 0.0000 | **1.508** | −1.017 | 0.677 |
| 13 | TAU | 0.005 | 0.0000 | 2.224 | **−0.006** | 0.900 |
| 14 | ASP | 0.004 | 0.0000 | **1.753** | 0.453 | 0.198 |
| 15 | THR | 0.003 | 0.0000 | **−1.250** | 1.127 | −0.084 |
| 16 | PHE | 0.002 | 0.0000 | 0.880 | 1.165 | **−0.361** |
| 17 | MET | 0.002 | 0.0000 | −2.337 | −0.486 | **2.178** |

that, when setting out to select a starter for use in cheese-making, it is necessary to study the behaviour of each individual bacterial strain rather than generalizing results for a given species as a whole.

Model systems of ovine-milk curd slurries can be used to screen the proteolytic abilities of potential starter bacteria as well as of non-starter bacteria for use as adjuncts in cheese making.

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