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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.