How three adventitious lactic acid bacteria affect proteolysis and organic acid production in model Portuguese cheeses manufactured from several milk sources and two alternative coagulants

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ABSTRACT

Model cheeses were manufactured according to a full factorial experimental design to help shed light on the individual and combined roles played by 3 native lactic acid bacteria (*Lactococcus lactis* ssp. *lactis*, *Lactobacillus brevis*, and *Lactobacillus plantarum*) upon proteolysis and organic acid evolution in cheese. The model cheeses were manufactured according to a generally representative Portuguese artisanal protocol, but the (ubiquitous) adventitious microflora in the cheesemaking milk were removed via sterilization before manufacture; therefore, the specific effects of only those lactic acid bacteria selected were monitored. In addition, 2 types of coagulant (animal and plant) and 3 types of cheesemaking milk (cow, sheep, and goat) were assessed to determine their influence on the final characteristics of the model cheeses. The nature of the coagulant appeared to be essential during the first stage of proteolysis as expected, whereas the contribution of those bacteria to the pools of total free AA and organic acids was crucial afterward. This was especially so in terms of the differences observed in the metabolisms of lactic acid (in the case of *Lactococcus* spp.) as well as acetic and citric acids (in the case of *Lactobacillus* spp.).

Key words: *Lactococcus*, *Lactobacillus*, sheep milk, plant and animal coagulant

INTRODUCTION

Most Portuguese traditional cheeses bearing a Protected Denomination of Origin status are manufactured from raw whole sheep or goat milks or mixtures thereof. This permits their indigenous microflora, mainly lactic acid bacteria (**LAB**), to play a role during cheesemaking, mainly during ripening. However, this is also a major cause of variability in the organoleptic and overall quality of the final products because several poorly

understood (and thus difficult to control) biochemical changes brought about by microorganisms occur in the cheese matrix. Therefore, some degree of standardization of the manufacturing practices is urged; this goal will eventually require cheesemakers to choose well-defined, specific starter cultures based on their established technological performance.

Addition of tailor-made starter cultures contributes to a higher uniformity at all stages of manufacture and ripening. The primary roles of starter cultures are to convert lactose to lactic acid, to break caseins down into medium and small peptides (and to eventually break these down into free AA), and to hydrolyze milk fat, leading to release of free fatty acids (Fox and Wallace, 1997). All of these functions contribute to the unique characteristics of traditional cheeses. Although some attention has been paid to the process of transformation of cheese curds to distinctively flavored cheeses, the ripening process of most traditional Portuguese cheeses has not yet been fully elucidated. In fact, their intrinsic and relevant variability often hampers statistical significance of the experimental data generated in loco (Macedo and Malcata, 1997a,b; Macedo et al., 1997; Tavaria and Malcata, 1998, 2000; Dahl et al., 2000; Tavaria et al., 2003).

The biochemical reactions that occur in a typical cheese matrix include lipolysis, glycolysis, and proteolysis (Fox, 1993), and the enzymes involved therein, especially those that are active in proteolysis, play a crucial role upon the final textural and sensory characteristics of each type of cheese. Proteases and peptidases are present in the coagulant or are otherwise released by microorganisms upon their lysis, and the activities thereof depend on the environmental conditions prevailing in the curd (i.e., water activity, pH, mineral contents, and redox potential), as well as on the conditions provided throughout ripening (i.e., temperature, level and mode of salt addition, and nature of the secondary microflora added or allowed to grow; Law, 1984).

The objective of this study was to characterize in depth the performance of 3 adventitious LAB via use of a model system that essentially mimics traditional

cheesemaking practices so as to better understand proteolysis and production of organic acids in Portuguese traditional cheeses. Toward this goal, model cheeses were independently produced from sterilized sheep, goat, and cow milks using either animal or plant coagulant coupled with deliberate addition of either one or both adventitious LAB strains. These have been found to be ubiquitous in the bulk of Portuguese traditional cheeses, so their characterization (either alone or as a coculture) was in order. Glycolysis and proteolysis were monitored throughout ripening and correlated with intrinsic and extrinsic parameters that characterize cheesemaking and ripening. This research represents a considerable advance regarding previous work in the field (Pereira et al., 2008a), which pertained to cow milk solely (and thus could not be used to parallel cheesemaking of most traditional Portuguese cheeses) and which did not consider the role of adventitious strains of *Lactobacillus plantarum.*

MATERIALS AND METHODS

Cheese Manufacture

Model cheeses were manufactured from autoclaved (110°C for 10 min) cow, sheep, or goat milk, as appropriate. Thirty-six 2-L batches were thus prepared, according to a factorial design encompassing those 3 types of milk and 2 types of coagulant [from a regular animal source (i.e., an aqueous extract of calf abomasa, supplied by Lusocoalho, Montes da Senhora, Portugal) or from a commercial plant source (i.e., an aqueous extract of dried flowers of *Cynara cardunculus* L., supplied by Formulab, Maia, Portugal)] and 3 microorganisms [i.e., wild strains of *Lactococcus lactis* ssp. *lactis* (LMG S 19870), *Lactobacillus brevis* (LMG 6906), and *Lactobacillus plantarum* (LMG S 19557)]. These strains had been previously isolated from Portuguese traditional cheeses and duly deposited in the Laboratorium voor Microbiologie en Microbiele Genetica, Rijksuniversiteit (Gent, Belgium). Such microorganisms were added either as plain cultures or as 1:1 cocultures of the *Lactococcus* strain and one of the *Lactobacillus* strains, and absence of inoculum was used as control; this added up to 6 inocula. Each set of experimental conditions was made available in duplicate.

Starter cultures were added to the aforementioned 2 L of autoclaved milk at a 1% (vol/vol) level. As for the remaining process, a typical traditional manufacture protocol was followed (Macedo et al., 1993; Freitas and Malcata, 2000) as much as possible, except that the entire process was carried out in a sterile flow chamber using sterile tools and equipment. Sterile common table salt was added to bulk milk at a rate of 20 g/L, and in-

oculation was followed by addition of a solution (Orange Scientific, Belgium) of animal or plant coagulant (0.6 or 4 mL, respectively), sterilized by filtration through a 0.22-µm filter, as appropriate. The resulting mixture was distributed into 200-mL sterile flasks, each with a diameter of 6 cm, and incubated for 1 h at 30°C to bring about clotting. Perpendicular vertical cuts were then made in the curd, and another incubation period of 90 min followed. Finally, the curd was pressed to help in removing residual whey. Cheeses were kept for up to 60 d at 8°C under controlled air humidity (85%).

Physicochemical Characterization

Model cheeses were assayed for moisture throughout ripening by oven drying (IDF, 1982). The pH of cheeses was measured directly with a pH meter (Micro pH 2002, Crison, Barcelona, Spain). Fat, salt, and total protein contents were determined by Fourier-transform infrared spectroscopy using a LactoScope Advanced FTIR (Delta Instruments, Drachten, the Netherlands) after previous calibration.

Microbial Enumeration

Model cheeses were sampled at 0, 7, 14, 30, 45, and 60 d. *Lactobacillus brevis* and *L. plantarum* were enumerated on Rogosa agar (Merck, Whitehouse Station, NJ), and *Lc. lactis* ssp. *lactis* were enumerated on M17 agar (Merck). In the case of the mixed culture, a defined differential medium (which produces colonies with different colors for lactobacilli and lactococci) was used (i.e., lactose sulfite agar, which is often used to enumerate bacteria in yogurt; Imprensa Nacional—Casa da Moeda, 1998). Plates were incubated aerobically at 30°C for 48 h; in the case of Rogosa agar, they were incubated at the same temperature for 5 d.

Glycolysis Assessment

Cheese samples were taken at 0, 7, 14, 30, 45, and 60 d of ripening. A 2-g aliquot was added to 10 mL of 13 mmol/L sulfuric acid and homogenized with an Ultra-Turrax homogenizer (LaboControle, Linda-a-Velha, Portugal) for 3 min at $16,128 \times g$. The mixture was centrifuged (Universal 32R, Hettich, Kirchlengern, Germany) at $3,584 \times g$ and 4°C for 10 min and then filtered through no. 42 filter paper (Whatman, Kent, UK). Immediately before HPLC analysis, samples were sterilized through 0.22-µm filters (Orange Scientific). The HPLC system (Lachrom, Merck Hitachi, Darmstadt, Germany) was composed of an ion exchange aminex HPX 87H column (300 × 7.8 mm), which was maintained at 65°C, and 2 detectors (refractive index