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Role of adventitious microflora in proteolysis and lipolysis of Serra cheese: preliminary screening

Abstract Six different types of bacteria (five strains of lactic acid bacterium and one strain of coliform) isolated from 35-day-old Serra cheese were assayed for proteolytic and lipolytic activities on milk agar and tributyrin agar, respectively. Peptidase and lipase activities were further studied via analytical assaying of free amino acids by spectrophotometry and of free fatty acids by HPLC in in vitro curdled milk (previously prepared from heat-sterilized ovine milk coagulated with thistle flower) subject to ripening for 21 days at 5 °C and 95% relative humidity. *Leuconostoc mesenteroides* ssp. *mesenteroides*/*dextranicum* and *Lactococcus lactis* ssp. *lactis* displayed significant proteinase and peptidase activities, where *Leuconostoc lactis* and *Enterococcus faecium* only exhibited peptidase activity. *Leuc. mesenteroides* ssp. *mesenteroides*/*dextranicum*, *Ent. faecium*, and *L. lactis* ssp. *lactis* only showed lipase activity on milk fat after a long incubation period. Short- and medium-chain fatty acid residues were released preferentially by microbial lipases, although long-chain fatty acids were also significantly released by lipases from *Leuc. mesenteroides* ssp. *mesenteroides*/*dextranicum*. *Lactobacillus paracasei* ssp. *paracasei* and *Hafnia alvei* did not display measurable protease, peptidase, or lipase activities. In view of their hydrolase activities, it is suggested that *L. lactis* ssp. *lactis* and *Leuconostoc* spp. (with possible incorporation of *Ent. faecium*) are a potential mixed-strain starter for Serra cheesemaking.

Key words Proteinase · Peptidase · Lipase · Lactic acid bacteria · Coliforms · Ovine milk cheese

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Introduction

Serra da Estrela cheese (or simply Serra) is a semi-soft Portuguese cheese manufactured at the farm level only from raw ovine milk and is coagulated with a plant rennet (the thistle flower, *Cynara cardunculus*, L.). Due to its unique organoleptic characteristics, Serra has earned, over several decades, the reputation of being the best Portuguese cheese and, due to a consequent unusually high demand, its market price has consistently attained very high values ($\approx$US$30/kg). In order to protect the consumer by certifying the geographical origin of the cheese and by guaranteeing its high quality, Serra cheese now has *Appéllation d'Origine Controllée* status for legal protection, which has been enforced through FAPROSERRA (the Portuguese Federation of Manufacturers of Serra Cheese). Marketing of the cheese has been undertaken by ESTRELACOOP (the Regional Company for Trade of Serra Cheese). (FAPROSERRA and ESTRELACOOP are both independent cooperative organizations.)

Although high-quality standards have been tested by FAPROSERRA using randomly selected Serra cheese samples from each producer on a routine basis, maintenance or achievement of such high-quality standards by the cheesemakers will eventually require the development of microbial additives based on the naturally occurring, adventitious microflora. It is well known that the quality of the milk used, in particular its contaminating microflora, is a factor that, in addition to technological parameters of cheesemaking and cheese ripening, determines the physico-chemical characteristics of the final cheese [1–3]. Extremely high numbers of lactic acid bacteria and coliforms were found in Serra cheese ($\approx 10^8$ and 10^6 cfu g^{-1}, respectively, where cfu is colony forming units), as well as quite low numbers of staphylococci and yeasts ($\approx 10^3$ and 10^4 cfu g^{-1}, respectively) [2, 3]. Since proteolysis and lipolysis (brought about by the plant rennet itself,

adventitious milk enzymes, and enzymes contributed by the contaminating microflora upon secretion or lysis) are pathways that are very important for the eventual formation of small molecules that are significant for development of flavour, the monitoring of the progress of such biochemical routes would provide the best indication of the final flavour intensity to be expected (although organoleptic sampling remains the best indication of perceived cheese quality). One way to monitor proteolysis in cheese is via the quantification of free amino acids, which are released from peptides mainly by peptidases synthesized by microorganisms [4]; on the other hand, one way to monitor lipolysis in cheese is via quantification of free fatty acids, which in cheeses ripened for only a short time are released from triglycerides of milk fat mainly by native milk lipase and by lipases synthesized by starter and nonstarter microorganisms [5]. Therefore, studies of the proteinolytic, peptidolytic and lipolytic activities of selected dominant strains from the indigenous microflora of Serra cheese are relevant in attempts to develop a starter or a secondary microflora additive. This work was thus focused on a preliminary screening of dominant pure strains of lactic acid bacteria and coliforms isolated from traditional Serra cheese in terms of their ability to release free amino acids and free fatty acids, using ovine milk curdled by thistle flower as substrate; the major goal was then to identify those strains that may play an important role in cheese ripening.

Materials and methods

Cultures

Dominant bacteria previously isolated from 35-day-old Serra cheese and identified using the API methodology [2] were used in the present study. Such bacteria included *Leuconostoc lactis*, *Leuconostoc mesenteroides* ssp. *mesenteroides/dextranicum*, *Lactobacillus paracasei* ssp. *paracasei*, *Lactococcus lactis* ssp. *lactis*, *Enterococcus faecium*, and *Hafnia alvei*. Experimental inocula of *Lactococcus* and *Enterococcus* for the milk and cheese experiments were obtained after growth in MRS broth (Lab M, Bury, UK) at 37 °C; inocula of *Lactobacillus* and *Leuconostoc* were obtained after growth in MRS broth at 30 °C; and inoculum of *Hafnia alvei* was obtained after growth in nutrient broth (Lab M) at 37 °C.

Milk and curdled milk experiments. Ovine milk from the Bordaleira breed was used and heat processed at 110 °C for 10 min (i.e the strongest type of milk sterilization that does not damage the casein micelle and the fat globule structure of ovine milk). Milk sterility was checked via the absence of microorganisms in plate count agar incubated at 30 °C for 5 days. For the milk experiments, 0.1 ml of single-strain culture was added to 20 ml of sterile milk in a test tube, and the inoculated milk was incubated at the corresponding optimum temperature for 1 and 5 days; the pH value, type of coagulation (strong curd, fine and grainy curd, or no curd formation at all), and gas formation (presence or absence thereof) were followed during the incubation period. Sterile milk samples incubated at the same temperatures were used as controls. For the curdled milk experiments, 0.3 ml of single-strain culture and 0.3 ml of aqueous thistle extract (0.2 g of macerated thistle flowers in 10 ml of water) were added to 60 ml of sterile milk in a test tube. The milk was then incubated at 28 °C until coagulation had occurred ($\approx$2 h). The experimental curdled milk (maintained in the test tube) was then placed in an incubation chamber maintained at 9 °C and 95% relative humidity for 1 and 21 days.

Microbiological assays. Determination of the numbers of viable bacteria in the culture broth and in the 21-day-old milk curdled with thistle flower that was inoculated with a single culture was by plate counting according to details given elsewhere [2, 3]. After 21 days, Gram, catalase, and oxidase reactions were performed using bacteria present in all curdled milk experiments to check for possible microbial contamination. The results were expresssed as cfu/g.

Proteolytic and lipolytic assays. Detection of proteolytic and lipolytic activities of the single-strain cultures was made on 10% (w/v) milk agar (Merck, Darmstadt, Germany) and on tributyrin agar (Merck), respectively, with holes $\approx$3 mm in diameter bored with a sterile Pasteur pipette on the solidified agar, inoculation with the corresponding culture, and incubation for 3 days at the optimum growth temperature; those which maintained a turbid area zone were considered negative for proteolytic or lipolytic activity, whereas those which gave rise to a clear area zone were considered positive. Determination of the concentration of free amino acids in the experimental milk curdled with thistle flower was done according to the method of Folkertsma and Fox [6] using the Cd-ninhydrin reagent after aqueous extraction. Determination of the concentration of free fatty acids in experimental milk curdled with thistle flower was done according to the method of Garcia et al. [7] with modifications, which uses HPLC resolution of individual free fatty acids in milk fat and whose particular application is described in detail elsewhere [8]. The moisture content in cheese was determined according to the method of Case et al. [9].

Statistical analyses. To analyse the contribution of each single culture to the concentrations of free amino acids and free fatty acids in the curdled milk samples, the effects (obtained as the difference between the concentration in the cultured curdled milk sample and the concentration in the control sample) were plotted in normal probability paper, and those effects that were clear outliers from the median straight line were considered as significant [10]; this procedure was followed because an estimator of intrinsic experimental variability (obtained from the average of median effects) was necessary for calculation of the 95% confidence intervals of the (tentatively) significant effects.

Results and discussion

The values of pH, type of coagulation (if any), and presence or absence of gas formation in sterile ovine milk inoculated with each of the bacteria (and corresponding blanks) are tabulated in Table 1. It should be emphasized that a decrease in pH and a fine, grainy curd was observed after 5 days in the control samples incubated at 30 and 37 °C; this observation can probably be accounted for by: (1) thermostable enzymes from *Pseudomonas* spp. that were not destroyed by heat treatment, although the numbers of this bacteria in Bordaleira ovine milk are very low [2]; (2) long-term, thermally induced aggregation of casein micelle/fat globule complexes, which vary widely in size since no homogenization of milk was performed; or (3) proteolytic activity of plasmin specifically upon β-caseins, since this enzyme displays maximum activity at slightly

Table 1 Changes in pH, aspect of casein matrix, and existence or otherwise of gas formation in ovine milk inoculated with strains isolated from 35-day-old Serra cheese and incubated at their optimum temperature

Microorganism	Number[a] (cfu/g)	pH		Coagulation[b]		Gas[c]	
	0 day	1 day	5 days	1 day	5 days	1 day	5 days
Leuconostoc lactis	10^5	4.4	4.1	+ +	+ +	+	+
Leuconostoc mesenteroides ssp. *mesenteroides/dextranicum*	10^2	4.3	4.3	+ +	+ +	+	+
Lactobacillus paracasei ssp. *paracasei*	10^4	3.8	3.8	+ +	+ +	−	−
Lactococcus lactis ssp. *lactis*	10^3	4.0	4.0	+ +	+ +	−	−
Enterococcus faecium	10^4	3.9	3.9	+ +	+ +	−	−
Hafnia alvei	10^3	5.7	5.7	−	+	−	−
Control at 30 °C		6.3	6.0	−	+	−	−
Control at 37 °C		6.3	5.8	−	+	−	−

[a] Numbers of microorganisms in milk after inoculation
[b] + + Strong curd, + fine and grainy curd, − no curd
[c] + Formation of gas, − no formation of gas

alkaline pH and temperatures in the region of 37 °C [11]. It should be noted that although plasmin is inactivated by sterilization at 120 °C for 15 min, it remains partially active in UHT milk heat-processed at 142 °C for 3 s [12], and the characteristics of the thermal processing employed in our experiments (i.e. 110 °C for 10 min) were sufficiently less aggressive than actual sterilization so that it is likely that partial enzymatic activity was maintained and even enhanced [13–15]. The milk in the test tubes inoculated with lactic acid bacteria was coagulated after 1 day: this observation can be explained by isoelectric precipitation of caseins at pH 4.6, which is the basic phenomenon that leads to acid precipitation after in situ production of lactic acid from lactose by a starter culture. *Leuconostoc* spp. are able to produce carbon dioxide as a product of heterofermentative metabolism of glucose to lactate, which is consistent with the formation of gas which was detected in the test tubes inoculated with that bacterium.

The existence, or otherwise, of proteolytic and peptidolytic activities by each selected bacterium is depicted in Table 2. Lactic acid bacteria are nutritionally fastidious and have complex requirements in terms of free amino acids; however, if those nutrients exist at suboptimal levels to support microbial growth, these types of bacteria (especially lactococci) possess a proteolytic system that is able ultimately to hydrolyse milk proteins down to free amino acids [16]. The results listed in Table 2 indicate that *Leuconostoc mesenteroides* ssp. *mesenteroides/dextranicum* and *Lactococcus lactis* ssp. *lactis* were the only lactic acid bacteria able to hydrolyse casein in milk agar, a phenomenon that requires availability of proteinases. Table 2 also includes values for the concentration of free amino acids in the control sample (without culture), as well as the effect produced by each bacteria on that free amino acid concentration. Work with several varieties of cheese manufactured with controlled microflora [15] has emphasized that starter peptidases are primarily responsible for the formation of small peptides and free amino acids. Our results indicated that, from all lactic acid bacteria tested, *Leuconostoc lactis* exhibited the highest peptidolytic activity, followed by *Lactococcus lactis* ssp. *lactis*; *Leuconostoc mesenteroides* ssp. *mesenteroides/dextranicum* and *Enterococcus faecium* are on the edge of statistical significance. Although, in general, lactobacilli display a broad range of peptidase activities [15], our studies did not provide evidence for this type of enzymatic activity in *Lactobacillus paracasei* ssp. *paracasei*. Some Gram-negative psychrotrophic bacteria are also known to possess proteinases that are capable of degrading casein and polypeptides [17], but the curdled milk inoculated with *Hafnia alvei* did not exhibit significantly higher free fatty acid concentrations than the control. It is not possible to directly compare results obtained in vitro from pure, single cultures with those obtained from the cheese itself, because cheese ripening is a rather complex set of phenomena derived from various microenvironmental contributions (e.g. accumulation of free amino acids results from synergistic influences between microorganisms dictated by, e.g. pH and moisture). However, tentative relationships are apparent between the significant increase of free fatty acids by 21 days in milk curdled with thistle flower and inoculated with some of the single cultures, and the increase in the 5% phosphotungstic-acid-soluble nitrogen in Serra cheese after 21 days of ripening reported elsewhere [18].

The existence, or otherwise, of lipolytic activity which releases butyric acid from tributyrin by each selected bacterium is depicted in Table 3. This table also includes information on the effect produced by the selected bacteria on the concentration of free fatty acids in the control sample (curdled milk without culture added). In milk and dairy products, fat hydrolysis is usually effected by the native milk lipase coupled with

Table 2 Proteolytic activity in milk agar, and consequent effect on the overall concentration of free amino acids (*FAA*) in experimental curdled milk, of bacteria isolated from 35-day-old Serra cheese

	Milk agar[a]	Concentration of free amino acids[b] ($mmol_{eq.\,Leu}$ per $kg_{curdled\,milk\,dry\,weight}$)		Viable numbers (cfu/g)
		1 day	21 days	21 days
Control	–	10.3	22.5	
Microorganisms		Effect on FAA concentration[c]		
Leuconostoc lactis	–	0.9	26.4	10^5
Leuconostoc mesenteroides ssp. *mesenteroides/dextranicum*	+	1.3	11.0	10^4
Lactobacillus paracasei ssp. *paracasei*	–	0.3	0.1	10^4
Lactococcus lactis ssp. *lactis*	+	− 1.2	15.3	10^5
Enterococcus faecium	–	− 0.7	10.6	10^6
Hafnia alvei	–	0.7	3.1	10^7

[a] + Clear area zone, − turbid area zone
[b] 95% confidence intervals for the effects on the *FAA* concentration of the control are ± 2.1 and ± 9.5 for 1 day and 21 days respectively
[c] Difference between cultured milk curdled with thistle flower and control sample

Table 3 Lipolytic activity in tributyrin agar, and consequent effect on overall concentration of free fatty acids (*FFA*) in experimental curdled milk, of bacteria isolated from 35-day-old Serra cheese

	Tributyrin agar[a]	Concentration of overall free fatty acids[b] (mg per $kg_{curdled\,milk\,dry\,weight}$)		Viable numbers (cfu/g)
		1 day	21 days	21 days
Control	–	1993	2309	
Microorganisms		Effect on overall FFA concentration[c]		
Leuconostoc lactis	–	220	510	10^5
Leuconostoc mesenteroides ssp. *mesenteroides/dextranicum*	–	570	1590	10^4
Lactobacillus paracasei ssp. *paracasei*	–	160	− 70	10^4
Lactococcus lactis ssp. *lactis*	–	− 10	850	10^5
Enterococcus faecium	–	320	1590	10^6
Hafnia alvei	–	360	140	10^7

[a] + Clear area zone, − turbid area zone
[b] 95% confidence intervals for the effects on control *FFA* concentration are ± 402 and ± 734 for 1 day and 21 days, respectively
[c] Difference between cultured milk curdled with thistle flower and control sample

lipases produced by native or added microflora; since the former is inactivated by pasteurization [19], it will not be considered hereafter in our discussion. Milk fat was significantly hydrolysed only by *Leuconostoc mesenteroides* ssp. *mesenteroides/dextranicum* and *Enterococcus faecium*, and, to a much lesser extent, by *Lactococcus lactis* ssp. *lactis*. These observations agree with those of Mucchetti et al. [20], who found that *Enterococcus* exhibited high lipolytic activity after incubation in whole milk at 32 °C for 5 days, and with those of Fryer et al. [21], who reported that *Lactococcus lactis* ssp. *lactis* are able to hydrolyse milk fat if present in high numbers for a sufficiently long period. Additionally, *Lactobacillus paracasei* ssp. *paracasei* did not exhibit significant release of free fatty acids; Stadhouders and Veringa [22] reported that mesophilic lactobacilli have hardly any effect upon the release of free fatty acids from bovine milk fat during ripening. Although psychrotrophic strains of Enterobacteriaceae exhibit considerable lipolytic activity in refrigerated milk, provided that their numbers are above 10^6 cfu/ml [23], *Hafnia alvei* did not release fatty acids to a significant extent. The relative increase of the concentration of each type of even-numbered free fatty acids in cultured, curdled milk experiments with respect to the control sample is shown in Table 4. In general, lipolysis in milk consists of the preferential release of short- and medium-chain fatty acids; explanations for this observation are that most lipases active in milk readily liberate fatty acids from *sn*-1,3 positions and

Table 4 Detailed lipolytic activity in experimental curdled cultured milk after a period of 21 days of bacteria isolated from 35-day-old Serra cheese

	Concentration of free fatty acid (mg/$kg_{curdled\ milk\ dry\ weight}$)										
	C4:0	C6:0	C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3
Control	302	48	4	120	131	121	418	374	442	205	143
Microorganisms	Relative concentration of FFA[a]										
Leuconostoc lactis	1.4	0.2	3.0	0.7	− 0.2	0.2	0.0	0.1	− 0.1	− 0.1	0.1
Leuconostoc mesenteroides ssp. *mesenteroides/dextranicum*	1.9	0.5	− 1.0	0.5	0.4	0.6	0.4	0.8	0.0	1.4	0.7
Lactobacillus paracasei ssp. *paracasei*	− 0.3	0.1	− 1.0	0.3	− 0.1	− 0.7	0.0	0.2	0.0	− 0.1	0.4
Lactococcus lactis ssp. *lactis*	1.4	0.1	3.3	0.5	0.5	0.1	0.1	0.0	0.1	0.4	0.4
Enterococcus faecium	0.9	2.9	0.0	3.0	0.7	0.2	0.2	0.1	1.0	0.6	0.3
Hafnia alvei	0.0	− 0.1	− 1.0	0.0	0.3	0.0	0.0	0.2	− 0.1	0.2	0.2

[a] Ratio of concentration of each particular *FFA* to the concentration of that *FFA* in the control

those types of fatty acid residues are esterified predominantly in the *sn*-3 position, coupled with postulation that re-esterification of longer-chain free fatty acids by indigenous milk lipases occurs faster than that of short- and medium-chain free fatty acids [5]. Inspection of Table 4 indicates that short- and medium-chain free fatty acids (C4 : 0 to C14 : 0) were preferentially released by 21 days in the experimental, cultured curdled milk. The lipases of microorganisms involved in such preferential release are those from *Leuconostoc mesenteroides* ssp. *mesenteroides/dextrancium* and *Enterococcus faecium*. Long-chain free fatty acids are also released mainly by *Leuconostoc mesenteroides* ssp. *mesenteroides/dextrancium*. These observations provide a clue for the claim by Macedo and Malcata [8] that butyric, caprylic and myristic acids are those fatty acids that are released to the greatest extent during the ripening of Serra cheese, whereas long-chain fatty acids are released to a much lesser extent; however, as mentioned previously, accumulation of free fatty acids in curdled milk inoculated with a single, pure culture must be extrapolated cautiously to a system as complex as that of Serra cheese.

In conclusion, in view of the experimental results produced, it is suggested that the manufacture of actual Serra cheese could proceed from pasteurized milk inoculated with a mixed-strain mesophilic starter composed of *Lactococcus lactis* ssp. *lactis* and *Leuconostoc* spp. (based on the heuristic rule that a good starter should exhibit both proteolytic and lipolytic activities); in the case of Manchego (a similar Spanish cheese manufactured from pasteurized ovine milk), *Leuconostoc* spp. have been successfully added as part of the microbial starter [24]. An alternative would be to include *Enterococcus faecium* as a starter (due to its exhibited proteolytic and lipolytic activities), following claims by Neviani and Mucchetti [25] and Mucchetti et al. [20] that enterococci encourage the growth of lactic acid bacteria, and thus promote caseinolytic activity, in Italian cheeses.

Although relevant, original and useful, the conclusions of this study should be used carefully because the curdled milk system is different from a true cheese system (e.g. in terms of pH and moisture), and because the action of each selected strain was studied independently; it is known that in actual Serra cheese ecological competition between several strains exists, and so the enzyme activities taking place in actual cheese will necessarily mimic those of at least some of the strains studied, but others may not have a chance to display their own enzymatic activity due to the presence of competing strains.

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