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Changes in the pool of free fatty acids in ovine, bovine and caprine milk fats, effected by viable cells and cell-free extracts of *Lactococcus lactis* and *Debaryomyces vanrijiae*

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Abstract

Lipolysis catalysed by lipases, native or released by natural microflora in milk, plays a key role in development of aroma and flavour throughout cheese ripening. This research effort was aimed at a deeper understanding of the action of two wild strains used in traditional ewe's milk cheesemaking in Portugal, viz. *Lactococcus lactis* and *Debaryomyces vanrijiae*. They were both tested as viable cells and cell-free extracts – using bovine, ovine and caprine milk fat emulsions as model substrates. Hydrolysis reactions were carried out at 30 and 37 °C, in the case of *L. lactis* and *D. vanrijiae*, respectively; the contents of short- and medium-chain fatty acids were determined by high pressure liquid chromatography. Our experimental results showed general trends, viz. preferential depletion of medium chain fatty acids throughout the whole reaction time. However, distinct patterns were observed towards different substrates, depending on the source and form of the biocatalyst at stake.

Keywords: Lipolysis; Microorganism; Dairy food; Flavour generation

1. Introduction

Cheese is a complex food matrix, in which a large variety of microorganisms play key roles in flavour development (McSweeney, 2004). Some of those microorganisms are native to raw milk, whereas others are deliberately added as initiators – with the aim of promoting cheese ripening. These may include bacteria, yeasts and moulds, which thus form an intricate micro-ecosystem (Ogier, Son, Gruss, Tailliez, & Delacroix-Buchet, 2002). Flavour development in cheese typically encompasses carbohydrate fermentation, lipid breakdown and protein (and peptide) hydrolysis – followed by chemical and enzyme-mediated transformations of some of their products into volatile compounds (Bruna et al., 2003; McSweeney, 2004).

A number of previous studies have tackled the impact of living cells and nonviable (or attenuated) cells on cheese flavour. For instance, Madkor, Tong, and el Soda (2000) studied the effect of adjunct cultures of *Lactobacillus* spp. in Cheddar cheese. Furthermore, Williams, Beattie, and Banks (2004) monitored the activities of hydrolytic enzymes of microbial origin, that are putatively involved in formation of cheese flavour compounds – using cell-free lysates of 25 bacterial isolates, recovered from the surface population of smear-ripened cheeses. On the other hand, Dahl, Tavaria, and Malcata (2000) reported that semi-volatile fatty acids (and their ethyl esters) are present in traditional Serra da Estrela cheese, probably as a result of the activity of lipases synthesized by yeasts. It is generally believed that, for the role of the adjunct cultures in cheese

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to be maximized, intracellular enzymes should previously be released into the cheese matrix – which explains much of the attention paid to cell autolysis during ripening.

The main objective of this research effort was thus to gain a more in-depth understanding of the processes brought about by microorganisms, that can be associated with cheese flavour formation. For simplicity, the substrate selected was a cheese substitute consisting of emulsified milk fat – from several animal origins, viz.: cow's, ewe's and goat's. The microorganisms investigated were *Lactococcus lactis* and *Debaryomyces vanrijiae*, both in the form of viable cells and cell-free extracts. The former is a mesophilic lactic acid bacterium, often found in several ripened Portuguese traditional cheeses – which is also sometimes intentionally added to milk, as part of a starter culture (Swearingen, O'Sullivan, & Warthesen, 2001), owing to its ability to ferment lactose to lactic acid (Kuipers, 2001; Tungjaroenchai, Drake, & White, 2001). *Debaryomyces vanrijiae* was originally isolated from traditional Serra da Estrela cheese (Tavaria & Malcata, 1998). A closely related species, *Debaryomyces hansenii*, is commonly found as part of the surface microflora of other ripened cheeses (Leclercq-Perlat, Oumer, Bergere, Spinnler, & Corrieu, 2000; van den Tempel & Jakobsen, 2000).

Depending on the intrinsic properties and actual concentrations of the microorganism(s) present, they may have a positive effect (*e.g.* reduction in acidity and aroma development) or a negative effect (*e.g.* generation of off-flavours or poor appearance) – that builds up throughout ripening (Martin, Berger, le Du, & Spinnler, 2001). Better knowledge of the fate of short and medium-chain fatty acids – which contribute directly to aroma (or are precursors thereof), and of how they are affected by individual wild strains will back up educated attempts to control and optimize cheesemaking.

2. Experimental approach

2.1. Materials

2.1.1. Chemicals

Orthophosphoric acid (85%, v/v), potassium carbonate, sodium hydroxide and anhydrous sodium sulphate were obtained from Merck (Darmstadt, Germany); 18-crown-6-ether was purchased from Merck (München, Germany); butylated hydroxyanisole, *p*-bromophenacyl bromide and potassium phosphate were from Sigma (St. Louis, USA); chloroform, formic acid, acetonitrile (190™) and methanol (205™) were from ROMIL Chemicals (Leicester, UK). All free fatty acid standards (>99.9% pure) were obtained from Sigma. Fermentation ethanol (96%, v/v) was obtained from AGA (Lisboa, Portugal). Dry nitrogen (C-55™) (<1 ppm H_2O and <1 vpm O_2) was purchased from Carburos Metalicos (Barcelona, Spain). All chemicals purchased were reagent-grade, and were used without further purification. Tap water was subjected to successive steps of reverse osmosis, adsorption, deionization, microfiltration and photo-oxidation in a Milli-Q Plus 185 water purification system (Molsheim, France) – down to a final conductivity of 18.2 $M\Omega\,cm^{-1}$.

2.1.2. Substrates

Salt-free, pasteurized butter produced from cow's milk was purchased from AGROS (Vila do Conde, Portugal), and aliquots of ca. 250 g were kept in sealed plastic bags at −30 °C until required. Salt-free butter produced from ewe's and goat's milks was supplied by ANCOSE (Oliveira do Hospital, Portugal), and also stored in sealed plastic bags at −30 °C until required.

2.1.3. Microorganisms

A lactic acid bacterium (*L. lactis*) and a yeast (*D. vanrijiae*), previously isolated from ripened Serra da Estrela cheese (Tavaria & Malcata, 1998), were used as biological vectors to bring about lipolysis of the aforementioned milk fat substrates.

2.1.4. Processing apparata

A 200-mL round bottom flask, inserted within a jacketed Schott glass beaker (from Duran, Mainz, Germany), was used to carry out the hydrolysis reactions. The setup comprised a constant temperature bath, equipped with a mechanical agitator and a digital temperature controller (from Julabo Labortechnik, Seelbach, Germany), with external recirculation through said jacketed beaker.

2.1.5. Analytical equipment

Nylon membrane filters (NALGENE™, 0.45 μm) were purchased from Nalge (New York, NY, USA). Disposable cuvettes for spectrophotometric readings were purchased from Kartell (Milan, Italy). The HPLC equipment (from MERCK, Darmstadt, Germany) consisted of a programmable autosampler (model L-7250), a LichroCART® 250-4 C-18 reversed-phase column (25 cm × 4 mm × 5 μm Lichrospher®100) coupled with a pre-column cartridge (4 mm LichroCART®, Manu-CART® "4"), a programmable solvent delivery system with a quaternary pump (model L-7100), a programmable multiwavelength UV spectrophotometer (model L-7400), an interface (model D-7000), and a software package for system control and data acquisition (model D-7000 Chromatographic Data Station Software) – all from Merck (San Jose, CA, USA).

2.2. Methods

2.2.1. Preparation of anhydrous butterfat

Milk fat (from either cow's, ewe's or goat's milks) was pretreated by the procedure of Kalo, Huotari, and Antila (1990), with slight modifications (Balcão and Malcata, 1997, 1998a, 1998b; Balcão, Kemppinen, Malcata, & Kalo, 1998a, 1998b). Water was removed from butter in a separation funnel at 60 °C; fat was then filtered out using filter paper, and dried in a vacuum of ca. 800 mbar for 1 h, in a boiling water bath. Anhydrous dry nitrogen was then bub-

bled in the melted butterfat for ca. 5 min (to help in removal of residual oxygen and water), and the dried fat thus obtained was poured into 500-mL Schott flasks and duly stored under nitrogen at −30 °C until further required.

2.2.2. Culture of lactic acid bacterium

Lipolytic *L. lactis* had been previously isolated from Serra da Estrela Cheese (Tavaria & Malcata, 1998), and maintained in glycerol at −80 °C. This culture was inoculated on M17 agar medium (MERCK), and incubated for 24 h at 37 °C (this incubation temperature was selected because its optimal growth temperature range is 30–45 °C). After this time, the bacterium colonies were removed, further inoculated on fresh M17 medium, and allowed to grow for an extra 24 h at 37 °C. Subsequently, using a 2% (v/v) inoculum in 2 L of the appropriate liquid medium, they were allowed to grow at 37 °C for another 18 h. The medium was then centrifuged at 13,000*g* for 20 min. The resulting cell-rich pellet was finally washed three times with phosphate buffer (0.01 mol/L, pH 7).

2.2.3. Culture of yeast

Lipolytic *D. vanrijiae* had been previously isolated from Serra da Estrela Cheese (Tavaria & Malcata, 1998), and maintained in glycerol at −80 °C. This culture was inoculated on Petri dishes of PDA (Potato Dextrose Agar, from Lab M, Amersham, UK), and incubated for 24 h at 25 °C (this incubation temperature was selected because its optimal growth temperature range is 25–30 °C). After this time, the yeast colonies were removed, further inoculated on fresh PDA medium, and allowed to grow for an extra 24 h at 25 °C. Subsequently, using a 2% (v/v) inoculum in 2 L of yeast extract-mannitol (YM) broth medium, they were allowed to grow at 25 °C for another 24 h. The medium was then centrifuged at 13,000*g* for 20 min. The resulting cell-rich pellet was finally washed three times with phosphate buffer (0.01 mol/L, pH 7).

2.2.4. Preparation of cell-free extracts

The appropriate pellet (obtained as described in the previous two subsections) was ground with alumina type 305 (Sigma) – using a mortar and pestle, in the proportion 1:5 (v/v) of pellet:alumina. The resulting extracts were then suspended in 50 mL of buffer, and centrifuged for 20 min at 13,000*g* to remove alumina and cell debris. The supernatant – which will hereafter be designated as cell-free extract (CFE), was kept frozen at −30 °C until use. The amount of protein in the CFE was evaluated using the Protein Assay Kit from BioRad (Munich, Germany).

2.2.5. Performance of hydrolysis reactions

All hydrolysis reactions were carried out using the experimental setup described above. For each reaction with a given type of milk fat, anhydrous milk fat (40 mL) – obtained as detailed before, was maintained at 37 °C for *L. lactis* and at 25 °C for *D. vanrijiae,* and kept at that temperature for 10 min prior to addition of 8 mL of viable cells, or 12 mL of CFE of either microorganism. A 750-μL aliquote of the reaction mixture was withdrawn, and poured into a vial containing 2.25 mL of an internal standard solution – 1.0 g/L of margaric (C17:0) and nonanoic (C9:0) acids, in 1:1 (v/v) methanol–chloroform, containing 0.05% butylated hydroxyanisole, and 2 g of sodium sulphate; this sample was labeled as $t = 0$ min. The biomass suspension was then added to the melted milk fat in the thermostatted reactor, and the chronometer was started; the reaction was allowed to proceed, under continuous stirring provided by a magnetic stirrer (Variomag Electronicruhrer, Daytona Beach, USA) set at 500 rpm, up to 7 h. Samples (750 μL) from the reaction mixture were withdrawn at pre-selected time intervals, poured directly into Eppendorf vials containing either of the internal standard solution, immediately frozen at −30 °C, and kept at this temperature until assaying for free fatty acids was in order.

2.2.6. Assay for free fatty acids

Free fatty acids in plain milk fat and in the reaction mixture were analyzed by HPLC, according to the procedures described in detail by Garcia, Reyes, Malcata, Hill, and Amundson (1990) and Malcata (1991), with modifications (Balcão & Malcata, 1997, 1998a, 1998b; Balcão et al., 1998a, Balcão, Kemppinen, Malcata, & Kalo, 1998b). For calibration, stock solutions of 12 different free fatty acid standards, viz.: C4:0 (butyric acid), C6:0 (caproic acid), C8:0 (caprylic acid), C10:0 (capric acid), C12:0 (lauric acid), C14:0 (myristic acid), C16:0 (palmitic acid), C18:0 (stearic acid), C18:1ω-9 (oleic acid), C18:2ω-6 (linoleic acid), C18:3ω-3 (γ-linolenic acid) and C20:0 (arachidic acid), were obtained by weighing given amounts of fatty acid standards and dissolving them in a 1:1 (v/v) methanol/chloroform mixture – so as to achieve a final (known) concentration of ca. 0.2 mol/L of each free fatty acid. Aliquots (100 μL) of every stock solution of fatty acid standards were taken and made up to 6 mL with methanol/chloroform (1:1, v/v). A volume of 4.5 mL of an internal standard solution containing both 0.00316 mol/L of C9:0 and 0.00185 mol/L of C17:0 in a 1:1 (v/v) methanol/chloroform mixture – prepared according to a similar procedure, and further stabilized with 0.05% butylated hydroxyanisole, was then added to obtain a final 4:3 (v/v) ratio of sample to internal standard solution. This procedure was independently used for other aliquots of the fatty acid standard stock solutions, viz. 200, 300, 400 and 500 μL. Aliquots (100 μL) of each resulting solution were withdrawn and added to 4.0 mL of a 1 g/L solution of *p*-bromophenacyl bromide in acetonitrile. To the resulting solution, 80 μL of a 5 g/L solution of 18-crown-6-ether in acetonitrile was added, and this procedure was followed by addition of 0.2 g of potassium carbonate. After thorough mixing, the solution was incubated at 75–80 °C for 30 min (in order to promote derivatization), allowed to cool to near room temperature before adding 40 μL of a 40 g/L solution of

formic acid in acetonitrile (so as to quench the derivatization reaction), and finally incubated at 75–80 °C for an additional 5 min. Following refrigeration (at ca. 4 °C) for at least 1 h, samples were cold-filtered through 0.45 μm nylon membrane filters.

For samples withdrawn from the reaction medium, the procedure was similar: aliquots (750 μL) were taken at predetermined time intervals, and poured into vials containing 2.25 mL of the appropriate internal standard solution, and ca. 2 g of sodium sulphate; from this solution, 500 μL was withdrawn, and added to another vial containing 4 mL of *p*-bromophenacyl bromide; after this point, the procedure was the same as for calibration.

In either situation, 20-μL aliquots of the filtered samples were injected into the reverse-phase column, connected to a pre-column cartridge; separation was effected at 33 °C using a mobile phase of water, methanol and acetonitrile, under a gradient pattern – which is described in detail elsewhere (Garcia et al., 1990); the flow rate of eluant was 1 mL/min, and absorbance of the eluate was read at 254 nm. Duplicate determinations were carried out for every sample.

2.3. Statistical analysis

A full factorial analysis of variance – ANOVA, was performed using the Statistical Package for the Social Sciences v. 11.5 (SPSS, Chicago, IL, USA). The statistical analysis included type of microorganism, source of milk fat and reaction time as main factors. Multiple comparisons were further carried out, using Bonferroni's test (at a level of significance of 5%).

3. Results

The evolution with time of normalized free fatty acid concentrations in bovine, ovine and caprine anhydrous milk fats, subjected to hydrolysis brought about by viable cells and CFE of *L. lactis* and *D. vanrijiae*, are displayed in Figs. 1–3. Normalization of the values in these figures used their initial ($t = 0$) concentrations. Normalized concentrations of free fatty acids above unity (Figs. 1–3) indicate net release of fatty acid moieties brought about by the microorganism or the enzyme added to the reaction media, whereas values below unity imply net consumption of such fatty acid moieties by the microorganism, or esterification effected by the enzyme.

Specific trends can be highlighted in our data. First, medium-chain fatty acids (e.g. C10:0, C12:0 and C14:0) were in general consumed; the only exceptions were observed with CFE of *L. lactis* and viable cells of *D. vanrijiae* when acting upon ovine milk fat. Second, and regarding short-chain fatty acids (e.g., C4:0, C6:0 and C8:0), one perceives no variation in concentration of free C6:0 throughout reaction time, in all cases. An interesting observation can be made regarding C8:0: this fatty acid was released by both CFE and viable cells of *D. vanrijiae* acting on bovine milk fat; however, in the case of its ovine and caprine counterparts, the viable cells of that microorganism led to release, whereas the corresponding CFE led to consumption.

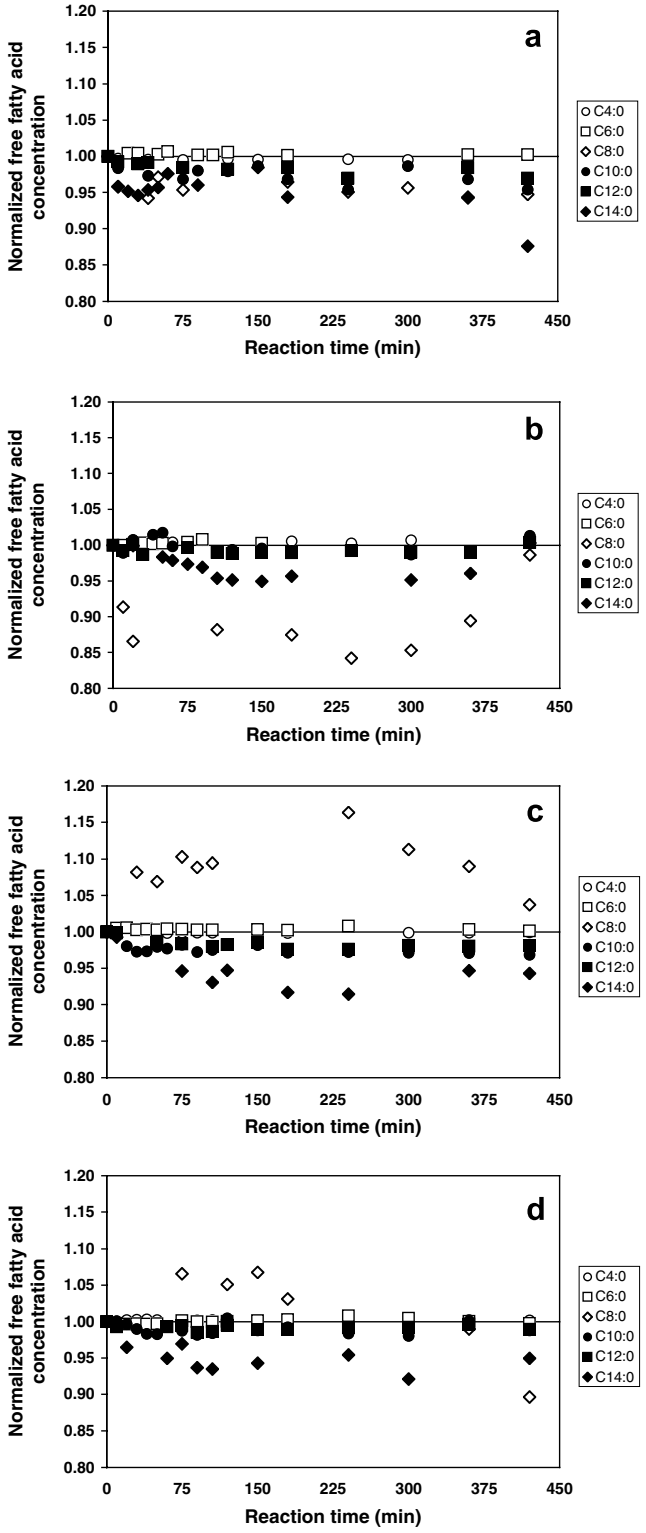

Fig. 1. Evolution of normalized free fatty acid concentrations in lipolyzed bovine milk fat with reaction time, effected by *Lactococcus lactis* as (a) viable cells and (b) cell-free extract, and by *Debaryomyces vanrijiae* as (c) viable cells and (d) cell-free extract.

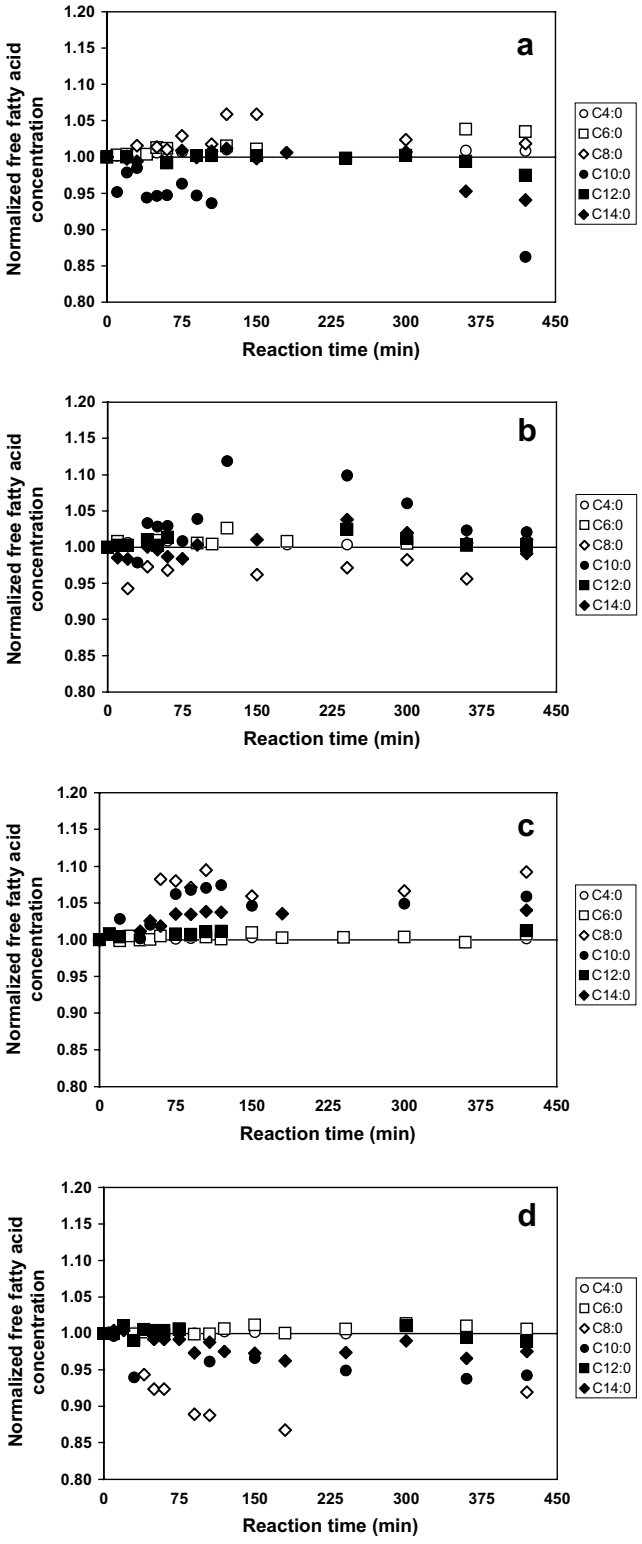

Fig. 2. Evolution of normalized free fatty acid concentrations in lipolyzed ovine milk fat with reaction time, effected by *Lactococcus lactis* as (a) viable cells and (b) cell free extract, and by *Debaryomyces vanrijiae* as (c) viable cells and (d) cell-free extract.

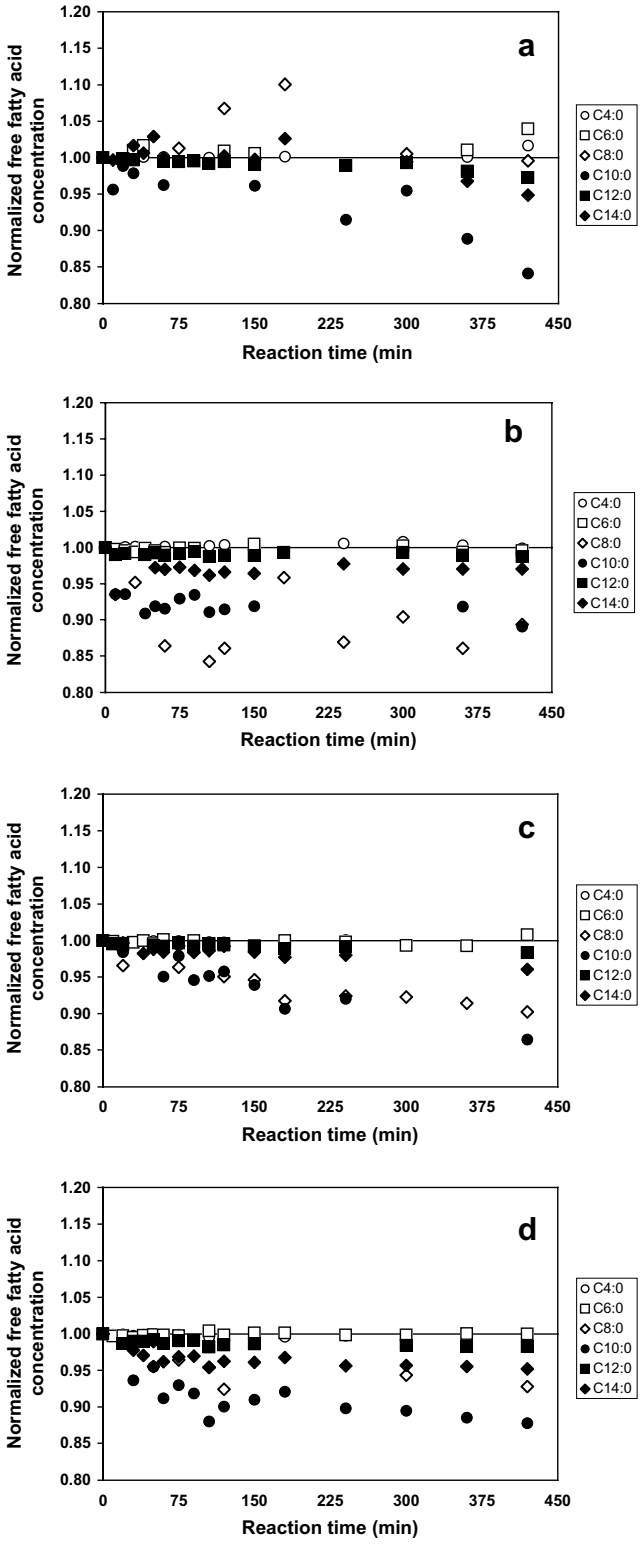

Fig. 3. Evolution of normalized free fatty acid concentrations in lipolyzed caprine milk fat with reaction time, effected by *Lactococcus lactis* as (a) viable cells and (b) cell-free extract, and by *Debaryomyces vanrijiae* as (c) viable cells and (d) cell-free extract.

The average overall fatty acid compositions of milk fats were reported by Jensen (2002), Nawar (1985), Alonso, Fontecha, Lozada, Fraga, and Juárez (1999), indicating average molecular weights of the fatty acids esterified in bovine, ovine and caprine milk fats of ca. 236.5, 220.0 and 227.2 g/mol, respectively. Assuming that milk fat is

Table 1
Final degree of hydrolysis (in duplicate) of caprine, ovine and bovine milk fats, effected by *Lactococcus lactis* and *Debaryomyces vanrijiae*, as viable cells (VC) and cell-free extracts (CFE).

Biological system		Milk fat	Degree of hydrolysis (%)	
			1st replicate	2nd replicate
L. lactis	CFE	Bovine	1.35	1.36
		Ovine	1.33	1.35
		Caprine	1.36	1.38
	VC	Bovine	1.36	1.38
		Ovine	1.29	1.20
		Caprine	1.34	1.36
D. vanrijiae	CFE	Bovine	1.38	1.38
		Ovine	1.30	1.30
		Caprine	1.33	1.32
	VC	Bovine	1.37	1.37
		Ovine	1.30	1.30
		Caprine	1.33	1.35

composed only by triglyceride molecules, and considering that the density of milk fat at 40 °C is 0.905 g/mL (Kurtz, 1965), then the theoretical amount of fatty acids released from milk fat, in the case of complete hydrolysis, would lead to a total concentration of ca. 3632, 3890 and 3773 mmol/L, for bovine, ovine and caprine milk fat, respectively. Combining these figures with the data depicted in Figs. 1–3, one can estimate the fractional hydrolysis by 7 h of reaction – as shown in Table 1. Control experiments were performed in parallel, with dispersions held at the same temperature and stirring speed, but without addition of biocatalyst. Results obtained indicated that no hydrolysis whatsoever occurred in the absence of biocatalyst.

4. Discussion and conclusions

Cheese flavour development is a consequence of a combination of specific enzyme activities, metabolic pathways by microorganisms and uncatalysed chemical transformations, which typically produce a large number of compounds – including free (short and medium-chain) fatty acids.

In the analytical procedure by HPLC, odd-carbon chain free fatty acids were used as internal standard – because the derivatization reactions may not proceed to completion, and given that naturally occurring fatty acid residues in animal fats are all even-carbon chain ones. Although there is evidence that C17:0 may appear directly from the diet, originating in bacteria that are high in silage feeds, its presence in the original milkfat was negligible – as expected, since our animals were not fed on silage. On the other hand, its synthesis by the experimental microorganisms studied is not relevant – as confirmed using a similar approach (results not shown). Finally, an alternative gas chromatographic method was also used to check that both C9:0 and C17:0 did not exist to significant amounts in our milk fat feedstocks – so that it would not interfere with the choice of that odd-carbon fatty acid for internal standard. Use of one or the other internal standard in peak area quantification arose because of the better accuracy of the liquid chromatography assays expected when the carbon chain is close to the median of the C4:0–C12:0 range, or the C14:0–C20:0 range, respectively.

Although direct analysis of the actual milk fat feedstocks experimented with would have been a better choice, estimation of the average mole concentration of free fatty acids released upon full hydrolysis was sufficient to ascertain the order of magnitude of the fractional hydrolysis carried out by *L. lactis* and *D. vanrijiae*; being so small (below 1.5%), putative corrections of the former would lead to negligible change in the actual figures.

Among many other species, *L. lactis* appears as part of the natural bulk microflora in traditional Portuguese cheeses; it is also a common component of commercial starter cultures. It has been shown (Collins, McSweeney, & Wilkinson, 2003) that lactic acid bacteria possess lipases and esterases, capable of hydrolyzing mono-, di- and triglycerides. On the other hand, *D. vanrijiae* – isolated in previous work by Tavaria and Malcata (1998) – has to date received no scientific attention pertaining to its action *per se* on dairy substrates. However, another species of the same genus, viz. *D. hansenii*, is often recovered as part of the natural surface microflora in traditional cheesemaking (Leclercq-Perlat et al., 2000; van den Tempel & Jakobsen, 2000). Therefore, selection of these two microorganisms as model species in our research effort is easily and fully justified.

The differences observed in the concentration profile of caprylic acid (C8:0) among the three milkfat substrates, when acted upon by *D. vanrijiae* cells or CFE, deserves further analysis in the future – as unfortunately no similar data exist in the available literature, to aid in meaningful comparative discussion. Whether any mechanistic relationship holds – based on the fact that the (wild) microorganisms employed were previously isolated from ripened ewe's milk cheese, remains to be proven. It might even be hypothesized that bursting of the cells of said yeast activates its otherwise intracellular lipolytic system – which might, in addition, have a particularly high affinity to ovine milk fat; conversely, an analytical artifact could as well have arisen, hardly with statistical significance. Furthermore, it will be interesting to know whether other microorganisms will exhibit similar behaviors, if they are exposed to substrates analogous to those used in this study.

Our work has shown that milk fat emulsions are feasible and useful model substrates for studying the action of lipolytic enzymes and microorganisms involved in cheese flavour development. In fact, both viable cells and their CFE act on those model substrates – should the source of milk fat be bovine, ovine or caprine. Our choice of milk fats from ovine and caprine origins, besides the (classical) bovine one, was deemed relevant – not only because of their importance in traditional cheesemaking in Mediterranean countries, but also because they are still poorly studied substrates of lipase-mediated pathways in foods.

Based on ANOVA of our experimental data concerning the duplicate determinations of the final degree of

hydrolysis (Table 1), one concludes that there are no statistically significant differences between viable cells of the two microorganisms tested, or between viable cells and CFE of each microorganism. However, the type of milk fat affected significantly ($p < 0.05$) the degree of hydrolysis.

In either viable cell or CFE form, the overall degree of hydrolysis observed is within the range expected for slightly lipolyzed cheeses; Olivecrona and Bengtsson-Olivecrona (1991) reported that hydrolysis of as little as 1–2% of the whole milk fat triglycerides gives an easily perceptible "lipolyzed" flavour to milk. On the other hand, the level of lipolysis should not exceed 2% in order to mimick Gouda, Gruyère or Cheddar cheeses (Gripon, 1993).

In conclusion, the procedure proposed and tested in this work constitutes a successful route to produce lipolyzed milk fat products – even from small ruminants feedstocks, with a somewhat promising market demand envisaged by the food industry (Kilcawley, 2001).

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