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# Effect of high pressure homogenisation of milk on cheese yield and microbiology, lipolysis and proteolysis during ripening of Caciotta cheese

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The principal aim of this work was to compare Caciotta cheeses obtained from cow milk previously subjected to high pressure homogenisation (HPH) at 100 MPa with those produced from raw (R) or heat-treated (P) cow milk. HPH had both direct and indirect effects on cheese characteristics and their evolution during ripening. In particular, HPH treatment of milk induced a significant increase of the cheese yield; moreover, it affected the microbial ecology of both curd and cheese. Compared with the thermal treatment, the HPH treatment resulted in a decrease of about one log cfu/g of yeast and lactobacilli cell loads of the curd. The initial milk treatment also affected the evolution over time and the levels attained at the end of ripening of all the microbial groups studied. In fact, lactobacilli, microstaphylococci and yeast cell loads remained at lower levels in the cheeses obtained from HPH milk with respect to the other cheese types over the whole ripening period. Moreover, HPH of milk induced marked and extensive lipolysis. Cheeses from HPH milk showed the presence of high amounts of free fatty acids immediately after brining. The electrophoretic patterns of the different cheese types showed that Caciotta made from HPH-treated milk was characterized by a more extensive and faster proteolysis as well as a significant modification of its volatile molecule profile. The results obtained and the sensory analysis indicated that HPH treatment of milk was able to differentiate Caciotta cheese or to modify its ripening patterns.

**Keywords:** High pressure homogenisation, Caciotta cheese, proteolysis, lipolysis, ripening.

High pressure homogenisation (HPH) is one of the most promising alternatives to traditional thermal treatments for fluid food decontamination and diversification. Its effectiveness in the inactivation of pathogenic and spoilage microorganisms in model and real systems is well documented (Lanciotti et al. 1994, 1996; Kheadr et al. 2002; Vachon et al. 2002; Wuytack et al. 2002; Diels et al. 2003; Thiebaud et al. 2003). Moreover, continuous or semi-continuous application of this technique to improve safety and microbiological quality of milk and whole liquid eggs has been proposed (Guerzoni et al. 1997, 2002). Cavitation and viscous shear have been identified as the primary mechanisms of microbial cell disruption during HPH (Middelberg, 1995; Kleinig & Middelberg, 1998). In addition to the effects on microbial cells, HPH treatment acts on food constituents, especially proteins, including enzymes, leading to modifications in their functional properties and activities (Kheadr et al. 2002; Vannini et al. 2004). More specifically, HPH treatment of skim and whole milk has been reported to modify the ratio of the nitrogen fractions and the soluble forms of calcium and phosphorous, to improve the coagulation characteristics of milk as well as to increase the cheese yields (Humbert et al. 1980; Guerzoni et al. 1999; Kheadr et al. 2002; Hayes & Kelly, 2003; Lanciotti et al. 2004a; Hayes et al. 2005). Moreover, the HPH treatment of milk was associated with an enhancement and an acceleration of both proteolytic and lipolytic activities of goats' cheese during ripening (Guerzoni et al. 1999). Accelerated lipolysis has also been observed in Crescenza, a traditional Italian soft cheese, when produced using milk HPH-treated at 100 MPa (Lanciotti et al. 2004a).

The effects of HPH of milk on the microbiological and rheological characteristics of Cheddar and goat cheeses and yoghurt have been studied by Kheadr et al. (2002) and

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Guerzoni et al. (1999), respectively. The results of these studies suggested that HPH treatment of milk can be used to develop dairy products with different rheological and sensory properties. For example, HPH treatment of milk, in combination with fat and soluble solids addition, provides a route to obtain yoghurts with a great variety of viscosity values (Lanciotti et al. 2004b). The indirect effects of HPH on the rheological and microstrucutral properties of cheeses may offer a source of innovation or reduction of ripening costs for traditional cheese products.

This work examined the effects of HPH treatment of milk on microbiological, enzymatic, microstructural and sensory aspects in Caciotta cheese. Caciotta is a semi-hard cheese of cooked paste, typical of middle of Italy, produced from pasteurised ovine or bovine milk, with rennet added as enzymatic coagulant; furthermore, starter cultures can also be included. Ripening time can range from 10 to 40 d at temperatures between 15 and 18 °C. Although there is a great demand for this kind of cheese, it is not characterized by pronounced flavour and its market value is quite stable. For these reasons, a differentiation as well as a reduction of ripening time can positively influence the commercial success of this product.

## Material and Methods

### *Cheesemaking*

Fresh raw cows' milk was obtained from a local dairy farm. Three different types of Caciotta cheese were produced using 300 l milk treated at 100 MPa with HPH, 300 l milk pasteurised at 72 °C for 30 s and 300 l raw milk, respectively. A total of 9 batches (3 replicates for each cheese type) were produced in a cheese dairy on 3 consecutive days with milk from the same farm. The technological and microbiological factors were kept as similar as possible for the 9 batches.

For HPH treatment, milk was homogenised using a one-stage continual high pressure homogeniser PANDA (Niro Soavi, Parma, Italy) equipped with a PNSA valve with a flow rate of 10 l/h. Milk was previously refrigerated and subjected to HPH treatment at an inlet temperature of ca. 5–7 °C; the temperature increase during HPH treatment was monitored at the outlet product point. The milk outlet temperature did not exceed 30±2 °C and was then collected in receiving containers and immediately heated to 37 °C.

Also raw milk was heated to 37 °C while pasteurised milk was cooled until it reached the same temperature.

The nine batches were inoculated with milk culture. The milk cultures were prepared incubating at room temperature (20–25 °C) for 10 h raw cow milk previously heated at 60 °C for 30 min. The pH of milk cultures, after 10 h, was 5·4±0·2.

Milk cultures and commercial rennet (1 : 10 000 – 25% pepsin : 75% chimosyn; Bellucci, Modena, Italy) were added to all batches, brought at 37 °C. The coagulation occurred within 35 min. After curd cutting and resting (15 min), the curds were separated from whey, cooked at 90 °C for 5 min and placed into perforated hoops (plastic cheese moulds) for 12 h at 37 °C. During this period the cheeses were pressed by means of a Perspex disc weighted to 100 g and every 20 min turned and further 100 g weights added until a total 500 g rested on each cheese. Then they were salted at 20 °C in NaCl (100 g/l) brine and ripened for 27 d at 16 °C. For each cheese type 15 cheeses were produced per replicate. Before ripening each cheese weighed 2·0 kg. Cheeses were sampled immediately after salting and after 6, 13 and 27 d for microbiological analysis and assessment of proteolysis and lipolysis as well as aroma compound determination and cheese microstructure analysis, by scanning electronic microscope (SEM).

At each sampling time during ripening, the microbiological and chemico-physical analyses were performed in duplicate on 3 samples (produced on 3 different days) for each cheese type (i.e. R cheese, made from raw milk, HPH cheese, from homogenised milk, and P cheese, from pasteurised milk).

### *Cheese yields*

The cheese yields were calculated as the percentage ratio between the weights of the milk used and the cheeses obtained.

### *Compositional analysis*

Grated cheese sample (<1 mm particle size) were analysed for fat (International Dairy Federation (IDF), 1996), protein (IDF, 1993) and moisture (IDF, 1982).

### *Microbiological analysis*

For all curds and cheeses, 10 g samples, taken at the surface and in the inner part of each cheese, were placed in 90 ml sterile saline water (9 g/l) and homogenised in a stomacher (Lab-blender 80, Pbi International, Milan, Italy) for 2 min. Decimal dilutions of the homogenate were made in sterile saline water and 0·1 ml of appropriate dilutions were spread onto the surface of Sabouraud dextrose agar (Oxoid, Basingstoke, UK); the plates were incubated at 28 °C for 72 h, after which the yeast colonies were counted. Counts of the lactobacilli were made by plating appropriate dilutions of the samples on MRS agar (Oxoid) and incubating at 32 °C for 48 h in anaerobic jar containing $H_2$ and $CO_2$ gases (generated by an Oxoid BR38 kit). Lactococci were counted on M17 agar plates after aerobic incubation at 37 °C for 48 h. Micrococci and enterococci were counted by surface plating on Baird-Parcker (with added Egg yolk Tellurite Emulsion) and Slanetz and Bartley medium incubated at 37 °C for 48 h and 44 °C for 24 h, respectively.

The microbiological analyses of cheeses were performed at 0 time (immediately after salting) and after

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6, 13 and 27 d storage. The results are mean of three replicates (3 cheeses produced on different days) and two repetitions (2 cheeses produced in the same day).

### *Lipolysis assessment*

The presence of free fatty acids (FFAs) in the three cheese types was assessed immediately after salting, after 13 and 27 days of ripening.

Cheese lipids extractions were performed as described by de Jong & Badings (1990). Each sample was prepared as follows: cheese (1·0 g) was ground with anhydrous $Na_2SO_4$ (3·0 g) and then 0·3 ml 2·5 M-$H_2SO_4$ was added. This mixture was extracted three times with 3 ml diethyl ether/heptane (1 : 1, v/v). After each extraction, the solution was clarified by centrifugation (500 ***g*** for 2 min at room temperature), and the upper solvent layer was transferred to a screw-capped tube containing anhydrous 1 g $Na_2SO_4$. The pooled diethyl ether/heptane extract was applied to a Bond Elut aminopropyl column (3 ml, containing 500 mg silica modified with aminoprolyl groups, IST, Mid Glamorgan, UK) that was conditioned with 10 ml heptane. The FFAs were eluted with 10 ml diethyl ether containing 2% (v/v) formic acid.

For GC analyses, an Agilent Hewlett-Packard 6890GC gas-chromatograph equipped with a MS detector (Hewlett-Packard 5970 MSD) and a 30 m × 0·32 i.d. fused silica capillary column coated with a 0·2 um film of Carbowax (Supelco) as a stationary phase was used. The conditions were as follows: injection temperature, 220 °C; detector temperature, 220 °C; carrier gas (He) flow rate, 1·5 ml/min; splitting ratio, 1 : 50 (v/v). The oven temperature was programmed from 60 to 220 °C at 4 °C/min. The identification of the individual FFAs of cheese samples was based on comparison of the retention times of the unknown FFAs with those obtained from the known FFA standards (Sigma, Stheiheim, Germany). Moreover, the identification of FFAs was carried out by computer matching of their mass spectral data with those of the compounds contained in the Agilent Hewlett-Pakard NIST 98 and Wiley vers.6 mass spectral database. The quantification of FFA level of cheese samples was performed using C9 : 0 as internal standard at concentration of 1 mg/ml. The results are means of three replicates (3 cheeses produced on different days) and two repetitions (2 cheeses produced on the same day).

### *Aroma profiles*

Aroma compounds were monitored during ripening by using a gas-chromatographic-mass spectrometry coupled with solid phase micro extraction (GC-MS-SPME) technique. For each cheese type, 5 g sample were sealed in sterilized vials. Samples were heated at 70 °C for 25 min and volatiles adsorbed for 20 min on a fused silica fibre covered by polydimethylsiloxane-divinyl benzene (PDMS-DVB), 65 µm (Supelco, Stheiheim, Germany). Adsorbed molecules were desorbed in the gas-chromatograph for 5 min. For peak detection, an Agilent Hewlett-Packard 6890 GC gas-chromatograph equipped with a MS detector (Hewlett-Packard 5970 MSD) and a 50 m × 0·32 i.d. fused silica capillary column coated with a 1·2 µm polyethylenglycole film (Chrompack CP-Wax 52 CB) as stationary phase were used. The condition were as follows: injection temperature, 250 °C; detector temperature, 220 °C; carrier gas (He) flow rate, 1 ml/min; splitting ratio, 1 : 20 (v/v). The oven temperature was programmed as follows: 50 °C for 2 min; from 50 °C to 65 °C, with a 1 °C/min rate of increase; from 65 °C to 220 °C, with a 5 °C/min increase, then holding for 22 min. For volatile peak identification, the same method previously described for FFAs was employed. The quantification of volatile compounds was performed on the basis of calibration curves obtained by adding pure standards to 5 g ground cheeses from raw, homogenised and pasteurised milk and treating as previously described for aroma compounds analysis.

### *Proteolysis assessment*

The evolution of proteolysis during ripening was monitored by SDS-PAGE electrophoresis. A Vertical System Hoefer SE 600 SERIES (Amersham Pharmacia Biotech, UK) was used. Sample loading volume and concentrations of separating and stacking gels were 30 µl, 15% and 5% acrylamide-bisacrylamide, respectively.

Protein and peptide extracts were prepared, according to the method of Kuchroo & Fox (1982), as follows: 5 g cheese were homogenised with 20 ml water for 3 min at 20 °C and incubated for 1 h at pH 4·6 at 40 °C. The sample was centrifuged at 3000 ***g*** for 20 min at 5 °C and the pellet was suspended in 5 ml 7 M-urea and stored in freezer at −20 °C until SDS-PAGE analysis. Before analysis, 150 mg of the suspension were added with 2·5 ml 0·166 M-Tris – 1 mM-EDTA, pH 8 and 2·5 ml 7% SDS and centrifuged at 5000 ***g*** for 20 min at 4 °C. Protein and large peptide solutions were prepared by heating 1 ml supernatants for 5 min at 95 °C and adding 0·2 ml β-mercaptoethanol. Glycerol (0·2 ml) and 0·2 ml bromophenol (0·02%) were added to the sample before loading on the gel.

The standards used were made of SDS-PAGE Molecular Weight Standard Broad Range (BioRad Laboratories, München, Germany), Polypeptide SDS-PAGE Molecular Weight Standard (BioRad Laboratories) and a mixture of α-casein, β-casein and lysozyme (3 mg/ml). The analysis was performed three times (corresponding to the samples produced on the different days) for each ripening time and each cheese type.

### *Scanning Electron Microscopy*

In order to evaluate the microstrucutral differences among the three different types of Caciotta cheese immediately

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**Table 1.** Composition of Caciotta chesees made from raw, high pressurised or pasteurised cow milk

| Composition | Cheese type | | | | | |
|---|---|---|---|---|---|---|
| | 0 days | | | 27 days | | |
| | R† | HPH‡ | P§ | R | HPH | P |
| Moisture (%, w/w) | 56±0·90 | 59±0·60 | 55±0·70 | 38±0·6 | 38±0·5 | 37±0·6 |
| Fat (%, w/w) | 22±0·11 | 17±0·45 | 21±0·23 | 33±0·27 | 30±0·32 | 31·5±0·19 |
| Protein (%, w/w) | 16±0·30 | 18±0·40 | 17±0·30 | 28±0·40 | 27±0·30 | 24±0·40 |

†R: cheese produced using raw milk
‡HPH: cheese produced using homogenised milk
§P: cheese produced using pasteurised milk

after brining and at the end of ripening, the samples were prepared for Scanning Electron Microscopy (SEM), using the following conditions: 5 $mm^3$ Caciotta samples were fixed in glutaraldehyde solution (2·5%, v/v) for 12 h at 4 °C and post fixed in an osmium tetraoxide solution (1%, v/v) at 4 °C for 1 h. After fixation, samples were dehydrated in a series of increasing concentration water/acetone solutions (50, 70, 95 and 100%), dried by using liquid $CO_2$ as drying fluid (Emitech LDT K 850, Kent, UK), and covered with gold (Emitech, Sputter, Coatter K550). Cheese sample microstructure was viewed and examined with a Scanning Electron Microscope (Philips SEM 505, Oregon, USA).

For each cheese type five SEM micrographs per replicate were subjected to image analysis. Images of SEM micrographs were obtained by digitalisation using a colour plane scanner (Scan Jet 6300 C). After acquisition, digitalised images were converted into grey scale and evaluated with an advance Image Analysis Software (Image Pro-Plus v4.1, Media Cybernetics, USA) selecting area corresponding to empty and full spaces (i.e. cavities and lipo-proteic matrix, respectively). The software, examining all the pixels in the image, calculated the percentage of each area.

### *Organoleptic Analysis*

In order to evaluate and compare the sensory attributes of each cheese type produced, a not-structured panel test was performed at the end of ripening. Samples were served at 25 °C to 25 evaluators during sessions in which 3 samples per replicate were considered. Appearance and colour, flavour, softness, firmness, bitterness, piquant, sweetness, saltiness and aftertaste were evaluated. The assessors were asked to rate each attribute on a 0 to 5 point scale. In addition, they were asked to rate each product in terms of overall acceptability.

### *Statistical Analysis*

Significant differences in microbiological data, total FFAs and organoleptic analysis were tested using a two-tailed paired t-test (Statistica for Windows, Statsoft Inc, Tulsa, UK). The level of significance was determined at $P<0·05$.

## Results

### *Cheese yield and composition*

According to t-student test, the milk treatment at 100 MPa induced a significant increase in cheese yield. In fact, the yields of the cheeses produced from HPH milk were 12·5±0·5% while those of curds obtained with raw and pasteurised milk were 10·2±0·3% and 9·5±0·2%, respectively.

Also the main chemical composition of cheeses were affected by the technological treatment applied to milk. In fact, moisture content of HPH cheese was significantly higher than those of raw and heat-treated cheeses at the beginning of ripening; moreover, HPH cheeses showed the highest protein content (Table 1).

### *Evaluation of microbial population*

The three batches of milk used for cheese-making were unusually contaminated as shown by microbiological data of raw milk reported in Table 2. The HPH treatment induced slight reductions of all the microbial groups which were significantly lower than those induced by thermal treatment. However, cell loads of the microbial groups taken into consideration, and particularly of microstaphylococci and enterococci, for P cheeses immediately after salting and during ripening were higher than those of HPH and R cheeses. This result could be due to a systematic post-contamination after milk thermal treatment.

HPH of milk affected the microbial ecology of both curds and cheeses. Treatment of milk at 100 MPa only slightly reduced the initial cell loads of lactococci in HPH cheeses analysed immediately after brining. On the contrary, the treatment caused a decrease of about one log cfu/g of the yeast and lactobacilli cell loads, compared with the raw milk. The initial milk treatment also affected the evolutions over time and the levels attained at the end of ripening of all the microbial groups considered. Microstaphylococci and yeasts remained at lower levels in cheeses obtained from HPH milk with respect to the other cheese types over the whole ripening period. In particular, microstaphylococci and yeasts did not exceed 6·18 and 3·90 log cfu/g, respectively, in HPH cheeses. These two

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**Table 2.** Evolution of microbial populations (log cfu/g)* of Caciotta cheeses during ripening at 16 °C

| | | Cheese ripening time (days) | | | | |
|---|---|---|---|---|---|---|
| Cheese type | | Milk | 0 | 6 | 13 | 27 |
| Raw† | Lactobacilli | 3·54 ± 0·17$^{a}$ | 8·86 ± 0·25$^{a}$ | 8·10 ± 0·32$^{a}$ | 7·14 ± 0·35$^{a}$ | 7·91 ± 0·32$^{a}$ |
| | Lactococci | 3·99 ± 0·25$^{a}$ | 9·17 ± 0·38$^{a}$ | 8·45 ± 0·25$^{a}$ | 7·54 ± 0·28$^{a}$ | 7·80 ± 0·40$^{a}$ |
| | Yeasts | 3·26 ± 0·45$^{a}$ | 4·25 ± 0·17$^{a}$ | 5·25 ± 0·26$^{a}$ | 6·25 ± 0·15$^{a}$ | 6·03 ± 0·23$^{a}$ |
| | Microstaphylococci | 3·59 ± 0·30$^{a}$ | 6·38 ± 0·20$^{a}$ | 6·20 ± 0·30$^{a}$ | 6·26 ± 0·25$^{a}$ | 7·57 ± 0·15$^{a}$ |
| | Enterococci | 3·05 ± 0·20$^{a}$ | 6·57 ± 0·30$^{a}$ | 6·35 ± 0·30$^{a}$ | 6·40 ± 0·18$^{a}$ | 6·90 ± 0·34$^{a}$ |
| HPH‡ | Lactobacilli | 3·47 ± 0·25$^{a}$ | 8·00 ± 0·40$^{b}$ | 7·50 ± 0·35$^{b}$ | 6·86 ± 0·27$^{a}$ | 7·55 ± 0·17$^{b}$ |
| | Lactococci | 3·18 ± 0·30$^{b}$ | 8·80 ± 0·45$^{a}$ | 8·00 ± 0·25$^{a}$ | 7·20 ± 0·35$^{a}$ | 8·00 ± 0·25$^{a}$ |
| | Yeasts | 2·98 ± 0·18$^{a}$ | 3·35 ± 0·18$^{b}$ | 3·50 ± 0·17$^{b}$ | 3·77 ± 0·15$^{b}$ | 3·90 ± 0·15$^{b}$ |
| | Microstaphylococci | 3·07 ± 0·25$^{b}$ | 6·52 ± 0·20$^{a}$ | 5·80 ± 0·25$^{a}$ | 5·90 ± 0·20$^{a}$ | 6·18 ± 0·28$^{b}$ |
| | Enterococci | 3·02 ± 0·38$^{a}$ | 6·40 ± 0·20$^{a}$ | 6·35 ± 0·20$^{a}$ | 6·29 ± 0·24$^{a}$ | 6·66 ± 0·30$^{a}$ |
| Pasteurised§ | Lactobacilli | 2·07 ± 0·34$^{b}$ | 9·12 ± 0·45$^{a}$ | 8·40 ± 0·40$^{a}$ | 7·83 ± 0·46$^{a}$ | 8·28 ± 0·38$^{a}$ |
| | Lactococci | 2·01 ± 0·23$^{c}$ | 8·80 ± 0·38$^{a}$ | 8·05 ± 0·32$^{a}$ | 7·93 ± 0·35$^{a}$ | 7·75 ± 0·35$^{a}$ |
| | Yeasts | 2·17 ± 0·18$^{b}$ | 4·29 ± 0·14$^{a}$ | 5·05 ± 0·20$^{a}$ | 5·99 ± 0·23$^{a}$ | 6·83 ± 0·30$^{c}$ |
| | Microstaphylococci | 2·46 ± 0·28$^{c}$ | 6·92 ± 0·20$^{b}$ | 6·70 ± 0·26$^{b}$ | 6·63 ± 0·20$^{a}$ | 7·17 ± 0·27$^{c}$ |
| | Enterococci | 2·19 ± 0·15$^{b}$ | 7·38 ± 0·25$^{b}$ | 7·30 ± 0·35$^{b}$ | 7·23 ± 0·38$^{b}$ | 7·55 ± 0·32$^{b}$ |

*Within a column, values corresponding to the same microbial group with common superscript do not differ significantly ($P > 0·05$)
† cheese produced using raw milk
‡ cheese produced using homogenised milk
§ cheese produced using pasteurised milk

microbial groups attained levels higher than 7 and 6 log cfu/g in the P and R cheese types at the end of ripening.

### *Lipolysis and aroma profiles*

In order to evaluate whether HPH of milk was able to induce significant modifications of lipolytic profiles in cheese, analysis of free fatty acids (FFAs) was performed immediately after brining and during the ripening. As shown by Table 3, the HPH of milk was associated with an extensive lipolysis in Caciotta cheese. In fact, the presence of significant amounts of FFAs was observed in the cheeses from HPH milk immediately after brining. In particular, the mean total level of FFAs was 1154·5 ppm in the HPH cheeses. On the other hand, the mean total FFAs released in cheeses from raw or heat treated milk were 126·6 or 217·7 ppm, respectively. A specificity for fatty acids was observed in the lipolysis. Indeed, after 27 d ripening the HPH cheeses were characterized by a higher level of saturated FFAs. Moreover, the ratio between unsaturated and saturated FFAs was 0·86 and 0·78 for R and P cheeses, respectively while it was 0·47 for HPH cheeses.

An intense lipolysis occurred also in R cheeses. However, their FFAs profiles revealed a late occurrence of FFAs and the prevalence of oleic (C18 : 1Δ9) acid at the end of ripening. On the contrary, the free fatty acid profiles of P cheeses showed a minor number of FFAs whose contents, however, increased over time.

HPH cheeses were characterised by a high and specific level of branched-chain FFAs such as C15 : 0iso, C15 : 0anteiso and C17 : 0anteiso. The latter FFA was not detected in the other cheeses after 27 days.

The HPH treatment of milk induced modifications of the volatile compound profiles of Caciotta cheese. As shown by Table 5 the cheeses produced using HPH milk were characterised, from the beginning of ripening, by higher occurrence of short- and medium-chain fatty acids such as butanoic (C4 : 0), caproic (C6 : 0), caprylic (C8 : 0) and capric (C10 : 0) acids. However, methyl-ketones, such as 2-heptanone, 2-nonanone and 2-undecanone, which are formed by β-oxidation of FFAs (Marilley & Casey, 2004), attained the highest levels in R-cheeses (Table 4) and were not detected in HPH-cheeses (Table 5). The contents of higher alcohols and the corresponding esters, which are associated with yeasts and LAB metabolism, i.e. isoamyl acetate, phenylethyl ethanol and acetoin, attained the lowest levels in HPH-cheeses (Table 5). Potent odorants such as dimethyl disulphide and dimethyl trisulphide deriving from methionine by Strecker degradation were detected only in cheeses made from pasteurised milk immediately after salting and also during ripening (Table 6).

### *Proteolysis profiles*

The electrophoretic patterns obtained for the different cheese types showed that Caciotta made from HPH-treated milk was characterized by a more extensive proteolysis (Fig. 1). A number of bands corresponding to low molecular weight peptides were present in the profiles of the HPH-cheeses immediately after brining. In particular, a band with a molecular weight between 1000 and 2000 Da and a band with a molecular weight of about 6500 Da were observed. An analogous band, although having lower intensity, was present also in P-cheeses, but only at the end of ripening period.

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**Table 3.** Evolution of free fatty acids (FFAs)*, expressed as ppm, in different types of Caciotta cheese as affected by initial milk treatment and ripening time

| | Ripening time (days) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | 13 | | | 27 | | |
| FFAs (ppm) | Raw† | HPH‡ | Past§ | Raw | HPH | Past | Raw | HPH | Past |
| C12 : 0 | —¶ | 57·6 ± 1·2 | 25·3 ± 0·2 | 29·1 ± 0·2 | 55·9 ± 0·3 | 26·8 ± 0·8 | 44·9 ± 0·2 | 52·8 ± 1·0 | 33·0 ± 0·2 |
| C14 : 0 | — | 121·0 ± 1·5 | 31·2 ± 0·1 | 43·7 ± 0·3 | 116·5 ± 1·8 | 45·5 ± 0·5 | 91·5 ± 0·7 | 110·8 ± 1·5 | 62·1 ± 0·4 |
| C15 : 0iso | — | 27·9 ± 0·1 | — | — | 27·9 ± 0·2 | 15·2 ± 0·2 | 28·0 ± 0·1 | 27·4 ± 0·2 | 24·1 ± 0·1 |
| C15 : 0anteiso | — | 23·9 ± 0·2 | — | — | 23·8 ± 0·1 | — | 24·2 ± 0·2 | 23·7 ± 0·9 | — |
| C15 : 0 | — | 32·5 ± 0·1 | — | 24·1 ± 0·1 | 31·7 ± 0·1 | 12·3 ± 0·2 | 27·3 ± 0·2 | 30·7 ± 0·3 | 25·3 ± 0·1 |
| C16 : 0 | 24·4 ± 0·2 | 326·3 ± 6·3 | 54·6 ± 1·5 | 94·8 ± 2·1 | 336·8 ± 6·2 | 75·3 ± 0·4 | 205·9 ± 2·5 | 320·4 ± 5·2 | 146·8 ± 0·7 |
| C16 : 1Δ9 | — | 29·9 ± 0·2 | — | 24·0 ± 0·1 | 29·6 ± 0·1 | — | 32·9 ± 0·4 | 29·1 ± 0·3 | 26·2 ± 0·1 |
| C17 : 0anteiso | — | 24·1 ± 0·3 | — | — | 23·9 ± 0·4 | — | — | 23·7 ± 0·3 | — |
| C17 : 0 | 26·8 ± 0·4 | 25·4 ± 0·4 | — | — | 25·4 ± 0·2 | — | 23·8 ± 0·1 | 25·1 ± 0·1 | — |
| C18 : 0 | 23·4 ± 0·2 | 116·7 ± 3·1 | 34·2 ± 0·3 | 45·2 ± 0·4 | 135·5 ± 2·4 | 50·2 ± 1·0 | 76·1 ± 0·1 | 126·2 ± 3·4 | 61·4 ± 0·2 |
| C18 : 1 (Δ9 trans) | — | 36·7 ± 0·7 | — | 23·6 ± 0·1 | 34·9 ± 0·2 | — | 30·8 ± 0·2 | 33·0 ± 0·4 | 26·4 ± 0·1 |
| C18 : 1 (Δ9 cis) | 25·6 ± 0·4 | 259·0 ± 3·2 | 47·5 ± 1·2 | 92·6 ± 2·4 | 241·2 ± 2·8 | 98·2 ± 1·1 | 302·0 ± 3·4 | 222·6 ± 2·2 | 157·6 ± 2·0 |
| C18 : 2 (Δ9,12) | — | 46·6 ± 0·5 | 24·9 ± 0·3 | 32·5 ± 0·4 | 45·2 ± 2·1 | 27·2 ± 0·4 | 59·1 ± 1·2 | 43·7 ± 0·3 | 40·8 ± 0·5 |
| C18 : 3 | 26·4 ± 0·3 | 26·9 ± 0·1 | — | — | 26·5 ± 0·5 | 12·3 ± 0·2 | 26·2 ± 1·1 | 26·0 ± 0·8 | 24·3 ± 0·2 |
| Total FFAs (ppm)¥ | 126·6[a] | 1154·5[b] | 217·7[c] | 409·6[a] | 1154·8[b] | 363[a] | 972·7[a] | 1095·2[b] | 628[c] |
| U/S†† | 0·69 | 0·52 | 0·49 | 0·73 | 0·48 | 0·61 | 0·86 | 0·47 | 0·78 |

* Data are means of three repetitions and two replicates ± standard deviation
† Caciotta cheese from raw milk
‡ Caciotta cheese from homogenised milk
§ Caciotta cheese from pasteurised milk
¶ data not detected
†† ratio between mean unsaturated and mean saturated FFAs
¥ mean value of total FFAs. Within a raw, values corresponding to the same ripening time with common superscript do not differ significantly ($P > 0·05$)

**Table 4.** Evolution of volatile compounds (mg/kg) during ripening of Caciotta cheese made from raw milk

| | Ripening time (days) | | |
|---|---|---|---|
| Volatile compounds | 0 | 13 | 27 |
| Ethanol | 6·1 ± 0·3 | — | — |
| 2-Heptanone | —* | — | 10·5 ± 0·6 |
| Isoamyl alcohol | 4·5 ± 0·3 | 6·9 ± 0·4 | 5·3 ± 0·3 |
| Acetoin | 31·1 ± 2·5 | 8·9 ± 0·7 | 6·0 ± 0·5 |
| 2-Nonanone | — | 7·0 ± 0·4 | 40·2 ± 2·4 |
| Acetic acid | 32·0 ± 1·9 | 8·0 ± 0·5 | 30·3 ± 1·8 |
| 2-Heptanol | — | — | 7·0 ± 0·4 |
| 2-Undecanone | — | 5·5 ± 0·3 | 8·5 ± 0·4 |
| Butanoic acid | 6·5 ± 0·4 | 37·7 ± 2·3 | 92·2 ± 5·5 |
| Phenylethyl acetate | — | 5·0 ± 0·3 | — |
| Caproic acid | 7·6 ± 0·5 | 59·98 ± 3·6 | 225·4 ± 13·5 |
| 2-Phenylethyl ethanol | 2·7 ± 0·2 | 16·1 ± 0·8 | 8·6 ± 0·4 |
| Caprylic acid | 12·6 ± 0·8 | 70·1 ± 4·2 | 195·3 ± 11·7 |
| Capric acid | 11·7 ± 0·7 | 73·2 ± 4·4 | 10·9 ± 0·6 |

* data not detected

**Table 5.** Evolution of volatile compounds (mg/kg) during ripening of Caciotta cheese made from homogenised milk

| | Ripening time (days) | | |
|---|---|---|---|
| Volatile compounds | 0 | 13 | 27 |
| Ethanol | 5·5 ± 0·3 | 10·1 ± 0·5 | 11·2 ± 0·6 |
| Isoamyl alcohol | 6·2 ± 0·3 | 16·3 ± 0·9 | 3·5 ± 0·2 |
| Acetic acid | 4·4 ± 0·3 | 16·0 ± 0·9 | 15·8 ± 0·9 |
| Butanoic acid | 88·9 ± 5·3 | 195·7 ± 11·7 | 177·7 ± 9·9 |
| Caproic acid | 242·1 ± 13·9 | 311·9 ± 15·6 | 344·3 ± 16·8 |
| Caprylic acid | 245·7 ± 14·2 | 330·1 ± 16·5 | 325·5 ± 15·8 |
| Capric acid | 116·2 ± 6·9 | 172·2 ± 8·6 | 160·0 ± 7·9 |

### *Microstructural features*

In Figs 2a, b & c the SEM micrographs of cheeses produced from raw, HPH-treated and pasteurised milk after 27 d ripening are shown. The microstructure of R-cheeses showed a more compact and continuous structure, including large cavities having regular dimensions (Fig. 2a). The HPH-cheeses were characterised by a protein network with irregular cavities having different diameters including also very small ones (Fig. 2b). On the other hand, the structure of P-cheeses seemed to be less packed and with large and continuous cavities. Moreover, fat globules were also evident for this cheese type (Fig. 2c).

For each cheese type, both after brining and after 27 d, five SEM micrographs per replicate were analysed with an advanced Image Analysis Software (Fig. 2a, b & c). The samples presented significant differences also after brining. In fact, the percentage areas of cavities (empty space) and

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**Table 6.** Evolution of volatile compounds (mg/kg) during ripening of Caciotta cheese made from pasteurised milk

| | Ripening phase (days) | | |
|---|---|---|---|
| Volatile compounds | 0 | 13 | 27 |
| Dimethyl disulphide | 14·1±1·1 | — | — |
| 2-Heptanone | —* | 3·2±0·2 | 5·4±0·3 |
| Isoamyl alcohol | 5·5±0·4 | 5·3±0·4 | 5·0±0·4 |
| Acetoin | 12·6±1·0 | 6·4±0·5 | 5·2±0·4 |
| 2-Nonanone | — | 5·7±0·5 | 31·4±548 |
| Dimethyl trisulphide | 4·7±0·4 | 15·7±1·2 | 40·2±322 |
| Acetic acid | 12·6±0·8 | 14·5±0·8 | 19·9±1·29 |
| 2-Heptanol | — | 3·3±0·2 | 5·0±0·3 |
| 2-Undecanone | — | 1·1±0·1 | 2·6±0·2 |
| Butanoic acid | 10·7±0·6 | 12·1±0·7 | 25·3±1·5 |
| Caproic acid | 16·9±1·0 | 20·0±1·2 | 82·7±4·9 |
| 2-Phenylethyl ethanol | 3·9±0·2 | 3·5±0·2 | 2·5±0·2 |
| Caprylic acid | 13·9±0·7 | 18·1±0·9 | 29·9±1·5 |
| Capric acid | 9·3±0·5 | 16·8±0·8 | 23·4±1·2 |

* data not detected

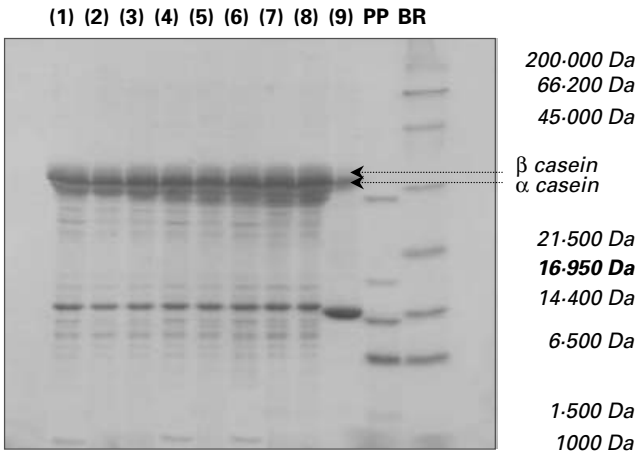


**Fig. 1.** Protein profile during ripening of cheeses made with different milk pre-treatments. (1) Cheese from HPH milk after salting; (2) Cheese from P milk after salting; (3) Cheese from R milk after salting; (4) Cheese from HPH milk after 13 days; (5) Cheese from R milk after 13 days; (6) Cheese from HPH milk after 27 days; (7) Cheese from P milk after 27 days; (8) Cheese from R milk after 27 days; (9) Mixture of α-casein, β-casein and lysozyme; **PP** Polypeptide (marker); **BR** Broad Range (marker).

solid matrix were respectively: 41±1·5 and 58±2 for R-cheese type; 46±0·9 and 53±1·2 for HPH-cheese type and 34±2·1 and 65±0·8 for P-type (see Fig. 2a, b & c). These data outline the differences in microstructure of the 3 cheese type: in particular, R-cheeses were characterised by the lowest level of empty spaces compared with P- and HPH-cheeses.

### *Sensory assessment*

The sensory assessment of the three cheese types is reported in Table 7; the organoleptic properties were evaluated in 27 day-old cheeses. The HPH-cheeses received a significantly higher overall grade than the other samples. The colour and appearance, the flavour, the texture and the sweet taste were the sensory attributes that mostly contributed to the rating of the HPH-cheeses. No significant differences, according to the t-test, were observed between R- and P-cheeses, except for piquant taste; this characteristic was significantly more pronounced in the R-cheeses.

## Discussion

All the cheese features taken into consideration, in particular cheese yield, volatile profile, proteolysis and lipolysis, were significantly affected by the initial milk treatment.

Many techniques have been assessed for increasing cheese yields including heat and hydrostatic HPH of milk (Huppertz et al. 2004). However, cheeses from heat treated milk often show, in addition to an increased moisture content, textural and flavour defects as well as increases of rennet coagulation time and a reduced gel strength (Singh & Waugana, 2001). On the contrary, high pressure treatment of milk increased the cheese yield without detrimental effects on milk coagulation properties and sensory defects (Huppertz et al. 2004). The results of the present research demonstrated how the use of a continuous or semi continuous high pressure homogeniser can increase Caciotta cheese yield by 3% with respect to that of milk heat treatment without detrimental effects on technological and sensorial features.

The increase of the water binding capacity of proteins and the higher retention in the curd of whey proteins have been identified as the key factors for yield increase in cheeses obtained from goat or cow milk processed by HPH (Guerzoni et al. 1999; Garcia-Risco et al. 2002; Kheadr et al. 2002; Lanciotti et al. 2004a). In addition, HPH of milk is reported, in contrast with hydrostatic pressure treatment, to improve also the coagulation characteristics of milk due the modification of the balances between soluble and insoluble forms of calcium, phosphorus and nitrogen (López-Fandiño et al. 1998; Guerzoni et al. 1999; Kheadr et al. 2002).

HPH of milk induced significant and selective changes in the microbial ecosystem; in particular, yeasts and microstaphylococci did not grow in HPH-cheeses during ripening. A shift in microbial population in cheeses obtained from HPH goat and cow milks has already been observed by Guerzoni et al. (1999) and Lanciotti et al. (2004a), respectively. This effect on the microbial population could be the result of a direct selection of the milk population, due to pressure sensitivity of different species (Lanciotti et al. 1994, 1996; Vachon et al. 2002; Vannini et al. 2004). Moreover, conformational modifications of milk proteins resulting in cheeses microstructure changes could have an indirect effect on the growth potential of

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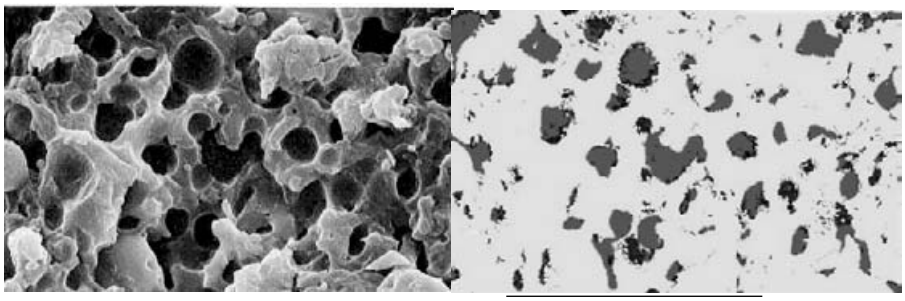
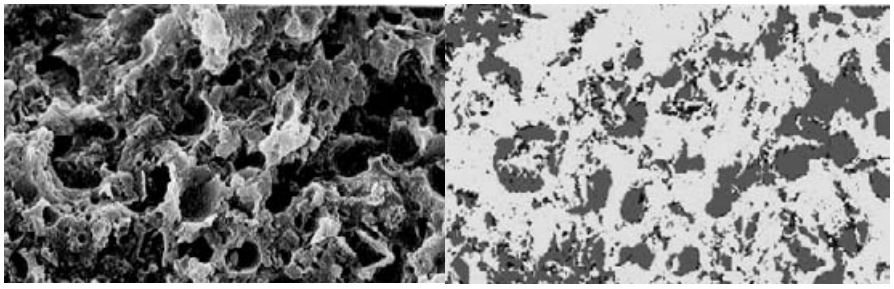
a



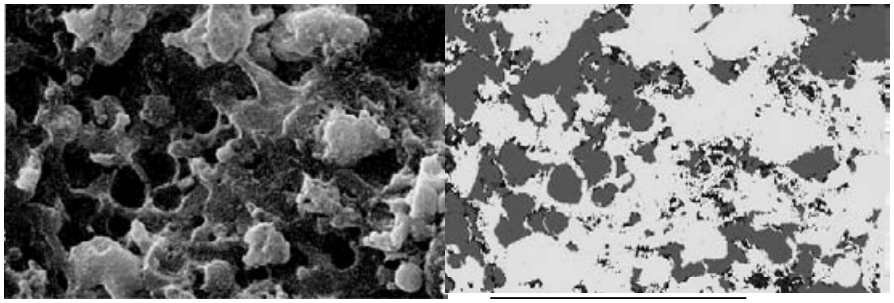
| Spaces | Area (%) |
|---|---|
| Full | 88·7 ± 1·5 |
| Empty | 11·3 ± 1·1 |

b



| Spaces | Area (%) |
|---|---|
| Full | 72·5 ± 1·1 |
| Empty | 27·5 ± 0·9 |

c



| Spaces | Area (%) |
|---|---|
| Full | 69·4 ± 1·4 |
| Empty | 30·6 ± 1·3 |

**Fig. 2.** SEM micrographs of 27 day-old cheeses made from raw (a), homogenised (b), pasteurised (c) milk.

various microbial groups. Guerzoni et al. (1997) observed that HPH can be used as a tool to modulate the size of water droplets in the emulsions or the cavities included in the lipid/protein matrices of model light dairy products. The growth extent of pathogenic species such as *Listeria monocytogenes* or yeasts like *Yarrowia lipolytica* was proportional to the droplets aqueous cavity sizes. Also, the more extensive release of medium- and long-chain free fatty acids in HPH cheeses could contribute also to the microbial selection as already observed in caprine cheeses made from HPH milk (Desmazeaud, 1994; Guerzoni et al. 1999). It is well known that the secondary microflora of cheeses, constituted mainly of enterococci, micrococci, NSLAB and yeasts, contribute to proteolysis and lipolysis of cheese (Beresford et al. 2001). Although a deeper investigation at species level has not been performed, the

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**Table 7.** Sensory evaluation scores* of 27 day-old Caciotta cheeses made from raw, pressurised or pasteurised cow milk

| | Cheese type | | |
|---|---|---|---|
| Sensory criteria | Raw | HPH | Pasteurised |
| Appearance and colour† | 2·4 ± 1·1[a] | 3·7 ± 1·0[b] | 2·5 ± 1·1[a] |
| Flavour† | 2·5 ± 1·3[a] | 3·0 ± 1·6[b] | 2·5 ± 1·3[a] |
| Taste† | | | |
| Bitterness | 3·2 ± 1·5[a] | 2·9 ± 1·3[b] | 3·0 ± 1·1[a] |
| Piquant | 2·1 ± 1·3[a] | 1·8 ± 1·2[b] | 1·4 ± 1·0[b] |
| Sweetness | 1·7 ± 1·2[a] | 1·4 ± 1·1[a] | 1·8 ± 1·1[a] |
| Saltiness† | 3·1 ± 1·1[a] | 3·1 ± 1·2[a] | 3·12 ± 1·2[a] |
| Texture† | | | |
| Softness | 1·3 ± 1·0[a] | 1·9 ± 1·2[b] | 1·3 ± 1·1[a] |
| Firmness | 3·5 ± 1·2[a] | 3·7 ± 0·9[b] | 3·6 ± 1·0[a] |
| Aftertaste† | 2·8 ± 1·3[a] | 2·7 ± 1·0[a] | 2·7 ± 1·3[a] |
| Overall assessment‡ | 2·8 ± 1·4[a] | 3·2 ± 1·1[b] | 3·0 ± 1·3[a] |

* Values are means ± SE for analyses performed by 25 assessors. Within a raw, values with common superscript do not differ significantly ($P > 0·05$)
† Five-point sensory scale (from 0 = low intensity to 5 = high intensity)
‡ Five-point scale (from 0 = poor to 5 = excellent)

results obtained in this work suggested differences in the quantitative ratios between these different microbial groups in relation to the initial milk treatment, whose effects on ripening patterns can not be underestimated. Therefore, it is difficult to establish a precise cause/effect relationship between the different phenomena observed. However, the low level of yeasts and microstaphylococci, which are reported to have extra-cellular enzymatic activities (Guerzoni et al. 2001), and the low content of primary metabolites and fatty acid transformation products (methyl ketones) in HPH cheeses suggest that the early accumulation of FFAs can be attributed to an activation of the native lipases. Infact, HPH treatment has been reported to enhance the activity of naturally-occurring enzymes such as lysozyme, lactoferrin and lactoperoxidase (Vannini et al. 2004). The specific occurrence of branched chain FFA (BCFA) suggests that also the activity of native protease was enhanced in HPH-chesses. In fact, amino acid catabolism is one plausible explanation for the occurrence of BCFA in cheeses because milk fat lacks BCFA (Ganesan & Weimer, 2004). FFAs can originate from lipolysis, proteolysis and lactose fermenation. Endogenous or microbial esterases and lipases may cause the release of linear-chain FAs, whereas proteolytic enzymes are responsible for the formation of C15 : 0iso, C15 : 0anteiso and C17 : 0anteiso originating from breakdown of isoleucine and leucine (Berdagué et al. 1987).

In R- and P- cheeses the lipolysis was late and mainly attributable to microbial lipases. An increase (compared with thermal treatment) of proteolytic and lipolytic activities due to the high pressure homogenization of milk has already been observed in caprine, Crescenza and Cheddar cheeses (Guerzoni et al. 1999; Kheadr et al. 2002; Lanciotti et al. 2004a). However, although low molecular weight bands occurred in the electrophoretic patterns of HPH-cheeses immediately after brining, no enhancement of both endogenous and exogenous plasmin activity was observed during a preliminary experiment on cow raw whole milk after HPH treatment at 80 MPa (Iucci, 2004).

The microstructural differences observed in the 3 cheese types can have an indirect effect on lipolysis. In fact, it is well established that lipase is active at the aqueous–lipid interface (Derewenda & Sharp, 1993; Verger, 1997; Guerzoni et al. 2001) and that the reaction rate of lipase varies directly with the surface area of the substrate available and the exposure of tryglycerides to the enzyme activity (Hadeball, 1991). The SEM micrographs, and particularly the empty-full ratio, indicated that the exposed inner surface of HPH-cheeses, also immediately after brining, was more particulate, presumably giving rise to a higher contact surface. Moreover, HPH treatment is reported to modify the size of fat globules, thereby increasing their exposure to enzyme activities (Kheadr et al. 2002). On the other hand, according to Hayes et al. (2005) to cover the greatly increased exposed fat interface in HPH milk, part of the casein in milk is adsorbed onto the newly-formed globules. Also Paquin (1999) reported that HPH treatment favours protein adsorption on the fat globules. This protective layer of casein micelle fragments can explain the lack of further release of FFAs in HPH cheeses during ripening. The initial lipolytic activity in Caciotta made from pasteurised milk can also be attributable to a different composition of microbial population; for example, high levels of enterococci and microstaphylococci were observed.

The different proteolytic and lipolytic patterns observed, as well as the differences in microbial population, may explain the sensory differences evidenced for the three Caciotta types. In fact, the HPH treatment of milk induced a significant modification of the volatile compound profiles of the cheeses. In particular, much more higher levels of short- and medium-chain FFAs were detected in HPH cheeses also immediately after salting. Their levels remained significantly higher than those of the other cheese types for the whole ripening period. Analogous results have been found for long-chain FFAs. The early release of FFAs, which can be attributed to a HPH activation of the endogenous LPL, was not accompained by a further conversion in methyl-ketones, such as 2-heptanone, 2-nonanone and 2-undecanone, and secondary alcohols such as 2-heptanol, which are reported to be β-oxidation products of FFAs (Marilley & Casey, 2004). These metabolites were found only in 13 and 27 day-old R and P cheeses. The formation of these potent odorants, generally associated with blue cheeses or ripened cheeses including Emmenthal, is attributed prevalently to β-oxidation activity of yeasts (*Candida* spp. and *Geotrichum* spp.) and fungi (Jelen & Wasowicz, 1998). The absence in HPH cheeses of methyl ketones, secondary and higher alcohols, such as phenethyl alcohol and isoamyl alcohol, can be due to the significantly lower level of yeasts during the whole ripening period.

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Despite the minor complexity of the volatile profiles, the HPH cheese received a higher sensory grade than did the other samples for attributes such as colour and appearance, flavour, texture and sweet taste. These characteristics can be associated, in addition to the absence of methyl ketones and sulphur compounds, with an enhanced hydrolysis of protein and the modification of cheeses microstructure induced by milk HPH treatment. The different microstructure induced by HPH treatment presumably influenced the taste perception. In fact, as reported by Taylor & Linforth (1998), factors such as the physicochemical properties of the flavour compounds, the food matrix characteristics (i.e. microstructure, rheology and composition), as well as the mastication of the food, influence flavour release during consumption.

The results obtained in this experimentation indicated the HPH treatment of milk has good potential to differentiate Caciotta cheese or to accelerate its ripening. However, the complexity of the milk system makes it difficult to study the effects of HPH on casein water binding capacity and activation mechanisms of endogeous enzymes. Thus, further researches, based on small angle X-ray scattering investigations, in model systems, could clarify the direct effect on proteins of HPH and provide information useful for a better exploitation of this process and for an appropriate temperature modulation.

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