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Cheese milk low homogenization enhanced early lipolysis and volatiles compounds production in hard cooked cheeses

María A. Vélez *, Erica R. Hynes, Carlos A. Meinardi, Verónica I. Wolf, María C. Perotti

Instituto de Lactología Industrial (INLAIN, Universidad Nacional del Litoral/CONICET), Santiago del Estero 2829, Santa Fe, Argentina

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ABSTRACT

Homogenization applied to cheese milk has shown to increase lipolysis but its use is not spread as it can induce detrimental effects. The aim of this work was to assess the effect of low-pressure homogenization of the cream followed by pre-incubation of cheese milk on the composition, ripening index, lipolysis and volatile profiles of hard cooked cheeses. For that, control and experimental miniature Reggianito cheeses were made and analyzed during ripening (3, 45 and 90 days). Homogenization had no impact on composition and proteolysis. An acceleration of the lipolysis reaction was clearly noticed in cheeses made with homogenized milk at the beginning of ripening, while both type of cheeses reached similar levels at 90 days. We found the level of several compounds derived from fatty acid catabolism were noticeably influenced by the treatment applied: straight-chain aldehydes such as hexanal, heptanal and nonanal and methylketones from C_5 to C_9 were preferentially formed in experimental cheeses.

1. Introduction

Lipolysis is one of the main biochemical events that occurs during cheese ripening, and is caused by hydrolytic enzymes resulting in the hydrolysis of milk lipids to free fatty acids (FFA), partial glycerides and glycerol (McSweeney & Sousa, 2000). Further, FFA undergoes catabolic reactions that conduct to the formation of aroma compounds, such as esters, methylketones, lactones and secondary alcohols; moreover, short-chain fatty acids contribute themselves to cheese flavour. These compounds give characteristic aromatic/taste notes to some cheese varieties, being of great importance in mold and Grana cheeses (Collins, McSweeney, & Wilkinson, 2003). Inhard cooked cheeses as Reggianito, moderate to extensive lipolysis is an advantage for genuine flavour development. Reggianito cheese is an Argentinian product which manufacture has been inspired in Italian cheeses such as Parmigiano Reggiano and Grana Padano (Candioti et al., 2002; Vélez et al., 2011).

Enzymes responsible for liberation of FFA in cheese come from six main sources; the milk itself (mainly the indigenous lipoprotein lipase, LPL), rennet paste, starter bacteria, secondary organisms, non-starter lactic acid bacteria (NSLAB) and exogenous lipases (Collins et al., 2003). In milk, lipolysis does not occurs spontaneously, as the LPL is electrostatically associated to the casein micelle and the substrate is in the form of globules protected by the milk fat globule membrane (MFGM). However, physical treatments applied to milk prior to cheese making (agitation, pumping, homogenization) may decrease the protective action of the MFGM (Evers, 2004).

The basic principle of homogenization consists in the disruption of milk fat globules to smaller ones, achieved by forcing the milk at high pressure through small holes (Walstra, Geurts, Noomen, Jellema, & Boekel, 1999). During this process surface area of fat is considerable increased, which is stabilized by casein micelles and whey proteins. Thus, the homogenization would also favor the accessibility of lipolytic enzymes to fat (Kelly, Huppertz, & Sheehan, 2008). Indeed, homogenization is performed in cheeses where lipolysis is desired to enhance flavour (mainly in blue cheeses). On the other hand, it has also been used for reduced-fat cheese varieties in order to improve texture obtaining higher moisture and smoother or creamier bodied cheeses (Johnson, 2011). However, homogenization is not widespread in cheese making technologies, and no previous reports of this procedure applied in hard cooked cheeses is available. The aim of the present work was to assess the effect of low-pressure homogenization of the cream followed by a pre-incubation step of cheese milk on composition, proteolysis, lipolysis and the volatile profiles of Reggianito type cheeses.

2. Materials and methods

2.1. Pre-treatment of cheese milk

Fig. 1 shows the experimental scheme performed. A volume of 40 L of bulk raw milk (pH 6.7 ± 0.05) supplied by a nearby dairy plant (Milkaut S.A., Santa Fe, Argentine) was centrifuged (500 g, Alfa Laval, Lund, Sweden) at a flow of 40 L/h and at 40 °C, and skim milk (<0.5% of fat content) and cream (>40% fat content) were obtained. These fractions were mixed to obtain a cream with 20% fat which was split into two portions, unhomogenized and one-stage homogenized at 9 MPa

* Corresponding author.
E-mail address: mvelez@fiq.unl.edu.ar (M.A. Vélez).

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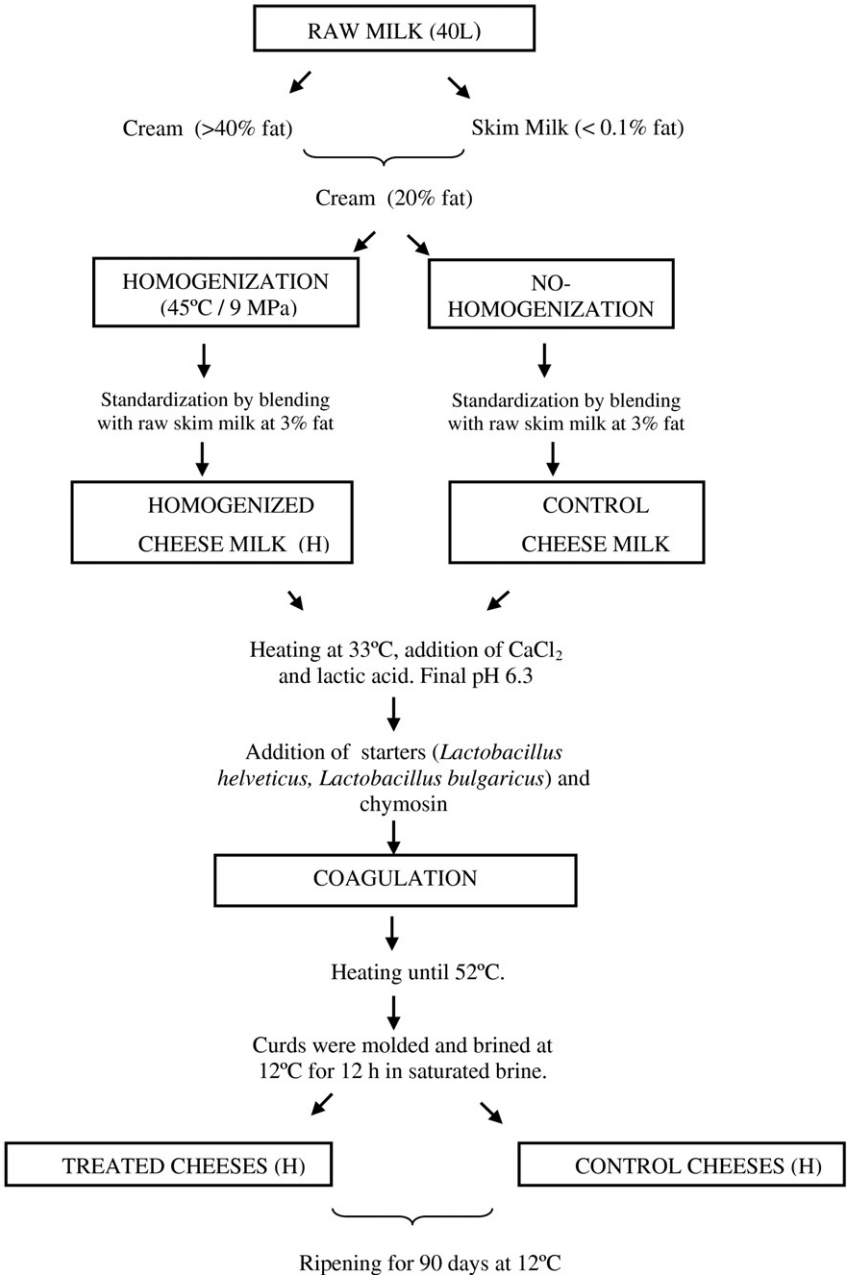

Fig. 1. Flow diagram depicting the process of manufacture of Reggianito cheeses, as well as the experimental treatment added (homogenization).

and 45 °C (Homogenizer 31 M-3TA, Gaulin Corporation, Boston, MA). Each portion was subsequently blended with raw skim milk to obtain cheese milk standardized at 3% fat content. Cheese milks were incubated for 12 h at 12 °C prior to cheese making day and destined to the manufacture of control (C) cheeses and cheeses made homogenized cream (H).

2.2. Total and free fat analysis

Total fat content of cream and skim milk samples were determined by Gerber method (Bradley et al., 1992). Free fat was analyzed on homogenized and unhomogenized cream samples (20% fat) to assess the damage of the MFGM. For that purpose, samples were centrifuged in standardized conditions (600 g 10 min 60 °C) and the layer of free fat was measured with a caliber (Vélez et al., 2011).

2.3. Phase contrast microscopy

Cream samples (20%) were viewed and photographed using a Contrast-phase Microscope (Jenned 2 - Carl Zeiss-Jena, Jena, Germany) attached to a camera (nit-AKS 24 × 36, automatic 2).

2.4. Cheese manufacture

Reggianito-type cheeses were made at laboratory scale (Fig. 1) using an equipment composed by 4 vats of 5 L each, operated in parallel (Vélez, Perotti, Wolf, Hynes, & Zalazar, 2010). C and H raw cheese milks were heated to 33 °C and $CaCl_2$ was added (Merck, Darmstadt, Germany) to final concentration of 0.14 g/L. Lactic acid (15 g/L) was added until milk reached a pH of 6.3–6.4. Then, two DVS commercial starters of *Lactobacillus helveticus* (LH-B02) and *Lactobacillus bulgaricus*

(Lb-12) (Chr. Hansen, Buenos Aires, Argentina) were added in a dose to reach a total starter bacterial count of 10^6 cfu/mL of milk. After manual stirring, 70 mg of chymosin (Maxiren 150, France) previously suspended in 10 mL of distilled water was added. Once coagulum acquired the required firmness (18–20 min), curd was cut to the size of a rice grain. Mixture of curd particles and whey was stirred and heated until 52 °C (approximately 1 °C/min); stirring was stopped to allow the curd grains to go to the bottom and separate whey. Curds were put into molds and pressed for 20 h, brined for 12 h in saturated brine at 12 °C. Cheeses of approximately 500 g were obtained and ripened for 90 days at 12 °C.

After cheese making, whey samples were analyzed for total fat by Gerber method (Bradley et al., 1992).

After two weeks of ripening, cheeses were vacuum wrapped in plastic films. Sampling of cheeses were performed at 3, 45 and 90 days (ISO, 2008b) and samples were stored at −18 °C until analysis. Two cheese makings trials (A and B) were performed in different days: per day, two cheeses of each type (C and H) were performed.

2.5. Cheese composition and ripening index

Samples were analyzed at 3 and 90 days for moisture by oven drying (ISO, 2004) and pH by potentiometric method (Bradley et al., 1992); fat and protein contents were analyzed at the beginning of ripening (3 days) by Gerber-Van Gulik method (ISO, 2008a) and Kjeldahl method (ISO, 2011) respectively. Nitrogen content was also assessed at 90 days in the fraction of the cheese soluble extract at pH 4.6 (SN), and expressed as percentage of total nitrogen (SN/TN, ripening index).

2.6. Microbial counts

Thermophilic lactic bacteria populations were assessed at 3 and 90 days by plating sample dilutions on skim milk agar and counting colonies after 48 h of incubation at 37 °C (Candioti et al., 2002).

2.7. FFA analysis

Extraction of fat matter, isolation of FFA, derivatization to ethyl esters and quantification of FFA by internal standard method (using C7:0 and C17:0 as standards), were performed in duplicate as described by Perotti, Bernal, Meinardi, and Zalazar (2005). A PerkinElmer model GC-9000 series gas chromatograph (PerkinElmer Corp., Waltham, MA) equipped with a flame ionization detector (FID) and with a split/splitless injector was utilized. Ethyl esters FFA were separated on a fused silica capillary column (60 m × 0.25 mm; HP-INNOWax, Agilent J&W, USA) coated with a bonded polyethylene glycol stationary phase (0.25 μm layer thickness); carrier gas H_2 flow at 2 mL/min; 1 μL injection; split mode injection at 1:50 splitting ratio; injector and detector temperatures at 250 and 300 °C, respectively; oven temperatures running from 75 °C (1.5 min) up to 150 °C (10 min) at 8 °C/min, then increased to a final temperature of 245 °C (15 min) at 10 °C/min. The concentrations of FFA were determined at 3, 45 and 90 days and were expressed as μmol of FFA per 100 g of fat. Besides, the degree of lipolysis was calculated as the sum of individual free fatty acids (total free fatty acids TFFA, mg/kg cheese).

2.8. Volatile compounds

Volatile compounds were isolated by headspace solid-phase microextraction (HS-SPME) and analyzed by GC/FID-MS. Samples of 5 g of cheese were weighed in a 40 mL screw-top glass vial closed with a cap with a Teflon-linedsilicone rubber septum (Supelco Inc., Bellefonte, PA). Samples were preheated for 10 min at 40 °C. Then, a 1 cm 50/30 μm DVB/Car/PDMS fiber (Supelco Inc., Bellefonte, PA) was exposed to the vial headspace during 15 min. After that, the fiber was withdrawn and inserted into the injection port of the GC (same equipment above mentioned) for 5 min at 250 °C and the volatile compounds were desorbed in splitless mode. Chromatographic analysis, identification of volatile compounds and semi-quantitative analysis (peak area values) were performed according to Wolf, Vénica, and Perotti (2015).

2.9. Statistical analysis

One-way ANOVA was applied to compositional data, lipolysis and volatile compounds profiles, using a general linear model procedure with least significant difference pairwise comparison at 95% confidence level to test differences between both types of cheeses at each sampling time as well as to determine changes during ripening, if appropriate. Besides, Principal Component Analysis (PCA) was conducted on the values of the individuals FFA through ripening. After that, Discrimant Analysis (DA) was applied to PC scores to establish those variables capable of discriminating the samples from different months and treatment (Hair, Anderson, Tatham, & Black, 1999).SPSS software (v 10.0, SPSS Inc., Chicago, IL) was employed.

3. Results and discussion

3.1. Microscopy and free fat analysis

The microscopic observations of C and H cream samples (20% fat) are shown in Fig. 2. In homogenized cream samples (H), small fat globules joined forming clusters. In this sense, it have been reported that fat globules in cream homogenized can be subject to several kinds of aggregation (Walstra, Geurts, Noomen, Jellema, & Boekel, 1999). By contrast, in unhomogenized samples (C) larger and unclustered fat globules were observed, which is expected for milk fat in its native state.

Free fat was not observed, neither in samples submitted to homogenization treatment nor in C samples, confirming an appropriated performance of the homogenization step. It is known that the formation of visible oil in emulsions - oiling off- can be caused by either an extensive coalescence or ineffectiveness of the homogenization process (Evers, 2004). The validity of our method for assessing free fat was previously checked, as we reported a free fat layer (approx. 8 mm)in cream samples with significant damage of MFGM (by mechanical agitation) and without absorption of surface-active compounds on the fat globule-milk plasma interface agitated samples (Vélez et al., 2010).

On the other hand, H samples were visible whiter than C samples due to an increase in the number of light scattering centers in homogenized samples (Walstra, Geurts, Noomen, Jellema, & van Boekel, 1999).

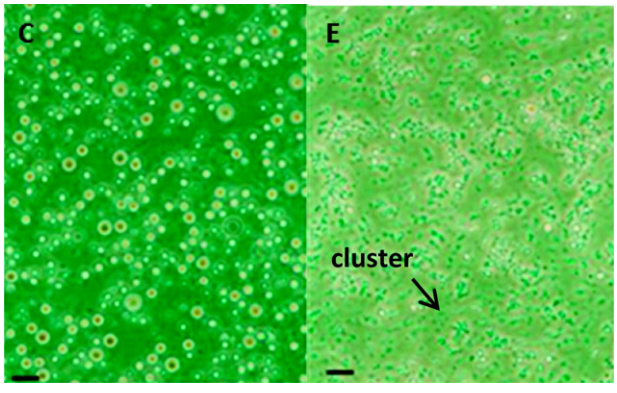

Fig. 2. Microscopic observations of control cream (C) and homogenized (H) samples. Lines represent 10 μm.

Table 1
Composition data of control and experimental cheeses. Significance of the main effect.

	Control samples	Experimental samples	Significance
Moisture (%) (3 days)	42.40 ± 2.57	43.95 ± 0.62	NS
Moisture (%) (90 days)	30.78 ± 1.83	32.44 ± 1.16	NS
FDM% (3 days)	50.64 ± 1.85	49.35 ± 4.45	NS
Protein % (90 days)	29.19 ± 1.29	26.73 ± 1.63	NS
pH (3 days)	5.42 ± 0.03	5.30 ± 0.04	S
pH (90 days)	5.43 ± 0.05	5.33 ± 0.06	S
SN-4.6/TNc (90 days)	15.82 ± 2.07	13.48 ± 0.23	NS

FDM Fat in dry matter.
PDM Protein in dray matter. Soluble nitrogen content (SN) at pH 4.6 as a percentage of total nitrogen.
NS not significant differences ($p > 0.05$).
S Significant differences ($p < 0.05$).

3.2. Global composition, microbial counts and proteolysis

Composition data from cheeses is shown in Table 1. At the end of ripening (90 days) the cheeses accomplished the requirements of Argentinean legislation for moisture (maximum value of 35.9%) and fat content (between 45 and 59% in dry matter) (ANMAT, 2014)for this cheese variety.

All cheeses had similar moisture content at the beginning of ripening, followed by a significant ($p < 0.05$) and equivalent decrease due to water evaporation prior to vacuum packaging. Other authors, as Brito, Manríquez, Molina, and Pinto (2003), Madadlou, Mousavi, Khosrowshahi, Emam-Djome, and Zargaran (2007), Rowney, Hickey, Roupas, and Everett (2003) and Zamora et al. (Zamora, Ferragut, Juan, Guamis, & Trujillo, 2011) found higher moisture in cheeses made with homogenized milk fat, in comparison with control counterparts. They adjudicated the differences to the poor syneresis of curds prepared from homogenized milk as homogenized fat globules interact with para-casein matrix (Kelly et al., 2008).

Considering fat content, H and C cheeses had similar values, although differences ($p < 0.05$) were detected in whey from both types of cheeses. The whey derived from homogenized cheeses had lower content of fat (0.17%) than that from control cheeses (0.5%). Even if differences in fat content of whey were noticed, values were low and this effect was not reflected in cheese fat contents. The lower fat losses in whey from homogenized cheeses could be due to the size reduction of fat globules and to the modified MFGM, which is associated with proteins. Fat content was similar to Vélez et al. (2010) for the same type of cheese, prepared with raw milk (3% fat). Different results were reported in relation to the effect of low-pressure homogenization on fat content of cheeses and no previous reports on hard cooked cheeses are available. Nair, Mistry, and Oommen (2000) found that fat content (dry matter) of Cheddar cheeses made with homogenized cream at 10.4/3.5 MPa was lower than cheeses obtained from cream homogenized at 3.5/3.5 and 6.9/3.5 MPa, but similar to control cheeses. In this case, the results were correlated with whey fat contents. Rowney et al. (2003) and Zamora et al. (2011) studied Mozarella and fresh cheeses manufactured with homogenized milk at 2.6 and 15 MPa, respectively, showing an increase in their fat content compared to unhomogenized samples. Deegan and McSweeney (2013) found a diminution in fat (dry matter) for Cheddar cheeses made using homogenized milk at different pressures (5, 10, 15, 20, 25 MPa) compared to controls, but they reported a decrease in fat content of whey with increasing pressure. Metzger and Mistry (Metzger & Mistry, 1994) described an increase in fat in dry matter in Cheddar cheeses made with homogenized cream at 17.26/3.43 MPa compared to no-treated cheeses; fat in whey decreased for cheeses made with homogenization treatment. Likewise, Madadlou et al. (2007) reported higher fat content in cheeses manufactured with homogenized cream at 6.0/2.5 and 9.0/2.5 MPa.

The protein content of cheeses at the end of ripening showed similar values between treatments, and were comparable to the values reported for Reggianito cheeses (Candioti et al., 2002; Vélez et al., 2010).

As for pH, there were no significant differences between cheeses ($p > 0.05$). Variable results were reported in relation to the effect of milk homogenization on cheese pH. Deegan et al. (2013) found no influence of milk homogenization (10 MPa) in Emmental cheeses. On the contrary, other authors showed an increase in pH values in Cheddar made with homogenized milk (Deegan & McSweeney, 2013; Madadlou et al., 2007; Metzger & Mistry, 1994; Nair et al., 2000).

In respect to microbiology, at 3 days microbial counts were 10^8 CFU/g in all cheeses, and decreased one log order during ripening.

On the other hand, proteolysis levels also kept constant ($p > 0.05$) between cheeses, and they were comparable to those reported for matured Reggianito cheeses (Candioti et al., 2002; Wolf, Perotti, Bernal, & Zalazar, 2010). Similar results were obtained by other authors (Metzger & Mistry, 1994; Nair et al., 2000).

3.3. Lipolysis in cheeses

The degree of lipolysis showed differences between both types of cheeses: for control cheeses at 3, 45 and 90 days of ripening the values were 4595, 9588 and 19,644 mg FFA/kg cheese, respectively; instead, for H cheeses the values were 16,297, 19,740 and 23,013 mg/kg cheese. TFFA average value was significantly ($p < 0.05$) higher in cheeses from homogenized cream than in control cheese sat 3 and 45 d of ripening, but at 90 d their levels matched ($p > 0.05$). In particular, TFFA were 72, 51 and 15% higher in H cheeses than in C cheeses at 3, 45 and 90 days, respectively. At the end of ripening, the global level of lipolysis for all cheeses was around 21,000 mg/kg, which was higher than the values reported for 6 month-old Reggianito cheeses. Perotti et al. (2005) and Wolf et al. (2010) found lipolysis levels of about 1400–3000 mg/kg for Reggianito prepared with pasteurized milk, while values of 6500 mg/kg were detected by Vélez et al. (2010) in cheeses made with milk sanitized by gravitational creaming.

At 3 days, C cheeses had 1.5% fat hydrolyzed, while H cheeses had 5.9%. Through ripening, both types of cheeses reached similar values, approximately 6% hydrolysis. In comparison with grana Italian cheeses, lipolysis progress was higher: Parmigiano Reggiano and Grana Padano cheeses (18–24 months ripening) have approx. 2% fat hydrolyzed (Battistotti & Corradini, 1993) with lipolysis degree between 4993 and 13,697 ppm (Gobetti & DiCagno, 2003). In this type of cheese, raw milk is sanitized by gravitational creaming (milk is left in shallow separation basins for about 8–12 h), which promotes the action of LPL (Wilkinson, 2007). The advance of lipolysis found in the present work is comparable to that in mold cheeses: 5–10% fat hydrolysis in Camembert and up to 20% in blue-vein cheeses (Collins et al., 2003). In particular, in Danablu, a semi-hard cow's cheese prepared with homogenized cream and skim milk and maturated for 3 months (Ardö, 2011) the concentration of FFA can exceed 30,000 ppm.

On the other hand, the accumulation of TFFA followed different rates in both cheese types. In H cheeses, the production of FFA was accelerated immediately after cheesemaking, and then it increased by 1.2 and 1.4 times towards the middle and the end of ripening. In C cheeses, the accumulation - from a much lower starting point - increased progressively up to the end of ripening, by 2 times from 3 days up to 45 d, and by 4.3 times up to 90 d, which allowed to match the TFFA of H cheeses.

The increase trend found for C cheeses over ripening have been described for numerous cheese varieties (Malacarne et al., 2009; O'Mahony, Sheehan, Delahunty, & McSweeney, 2006; Voigt, Chevalier, Qian, & Kelly, 2010). As for cheeses made with low-pressure homogenized milk, an early lipolysis was also reported by Brito et al. (2003), Michalski et al. (2004), Deegan et al. (2013) and Deegan and McSweeney (2013) for Chanco, Emmental and Cheddar cheeses, respectively. In particular, Deegan et al. (2013)and Deegan and McSweeney (2013)incubated the milk after homogenization during 1 h at 37 °C prior to cheese making in order to allow lipolysis to proceed unhindered.

In the present work the results indicate that the technological approach was successful at accelerating hydrolysis of TG. During the incubation of milk (for 12 h at 12 °C) before cheese making, lipolytic enzymes from milk and microbial lipases acted on acylglycerides mainly in H cheeses, as homogenization improved fat accessibility. The progressive increase of lipolysis over 90 d in C cheeses and the fact that C and H cheeses reached similar levels at the end of ripening, show that enzymes remained active during the storage period. Though, fat accessibility seems to be a limiting factor in fat lipolysis, but the extension of the reaction would also depend on the preservation of enzyme activities during ripening and the susceptibility of enzymes to product inhibition. Some authors reported that lipolytic activity of cheeses made with raw and pasteurized milk remained constant during ripening, but the higher activity in raw cheeses was adjudicated to LPL and enzymes from NSLAB (Svensson, Hernández, Virto, & De Renobales, 2006). NSLAB have weak to moderate lipase and esterase activities which contribute to FFA release (Holland et al., 2005). As for LPL, there are no studies of its specific activity in cheese matrix but it is known that is susceptible to product inhibition (Olivecrona, Vilaro, & G, 2003).

As for the percentage of FFA groups in relation to total FFA, some differences were detected between cheeses. For H cheeses, the percentage of SCFFA (C4:0–C8:0) increased from 3% at 3 d to 6.6% at 45 d and it remained constant until the end of ripening, while in C cheeses the percentage increased from 3% at 3 d to 9% at 45 d and then it diminished to 6% at 90 d. Thereby, at 90 d, C and H cheeses had similar SCFFA percentages. The percentage of medium-chain free fatty acids (MCFFA, C10:0–C14:0) was around 25% at the three sampling times for H cheeses, contrary to what happened in C cheeses in which it remained constant up to the middle of ripening and then it increased equalizing the values found in H cheeses (20, 18 and 25% at 3, 45 and 90 days, respectively).For C cheeses, the percentage of long-chain free fatty acids (LCFFA, C16:0–C18:2) represented approx. 77% of TFFA at 3 d and then diminished until 72% and 68% at 45d and 90d, respectively. For H cheeses, lower percentages were obtained, being of about 71% at 3 d and then diminished to 69% at 45 d and remained constant up to 90 days. Again, the values matched for both types of cheeses at the end of ripening. This point could be due to the fact that both, H and C cheeses, have similar enzymatic specificity along ripening.

The FFA groups' percentages during ripening of cheeses subject to low-pressure homogenization have not been reported as such to the best of our knowledge. Yet calculations made from published values indicate that FFA composition does vary in some cases. Deegan and McSweeney (2013) did not find differences in %SCFFA during ripening (180 d) between Cheddar cheeses made with homogenized milk (at 5 and 10 MPa) and their controls; although a significant increase of this fraction was verified for all cheeses during ripening (180 d). A similar trend was found for %MCFFA. As for LCFFA group, no clear pattern was found through ripening time. Meanwhile, Deegan et al. (2013)reported an increase of %SCFFA but a decrease of %MCFFA and %LCFFA through time, for Emmental cheeses manufactured with homogenized milk (at 5 and 10 MPa) and their respective un-treated cheese controls. In particular, at the beginning of ripening, %SCFFA and %MCFFA were lower in homogenized cheeses compared to controls; while %LCFFA were similar among cheeses. In all cases, the percentage values at the end of ripening were matched.

The amounts of individual FFA, in C and H cheeses at each sampling time are shown in Table 2. Palmitic and myristic were the main saturated FFA and oleic was the major unsaturated FFA found in all cheese samples. FFA profile is similar to the distribution found in milk fat (MacGibbon & Taylor, 2006) and in Reggianito cheeses (Perotti et al., 2005; Vélez et al., 2010).

At 3 and 45 d, all FFA concentrations were higher in H than in C cheeses ($p < 0.05$), whereas at 90 d the behavior was similar except for C6:0, C14:0, C18:1 and C18:2, where concentrations remained without differences.

FFA data were subjected to PCA (Fig. 3) using the correlation matrix (mean-centred and scaled). The value of Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy was 0.89 which is greater than the minimum value 0.6 indicating that the size of sample is adequate to consider PCA as appropriate tool to reduce the data and the Bartlett´s test of sphericity was significant indicating that the intercorrelation among the variables was strong to be considered in PCA (Hair et al., 1999). The first two principal components (PC) explained 89% of the total variance, PC1 and PC2 describing 78% and 11% of the variation, respectively. PC1 was positively correlated to all fatty acids analyzed, while PC2 was positively associated with short-chain FFA (butyric, caproic and caprylic), oleic and linoleic acids and negatively associated with medium-chain FFA (capric, lauric and myristic), palmitic and stearic acids. Globally, H cheese samples were clearly distinguished from C samples and within each cheese type were grouped by ripening time. E samples of 3 d were placed on the lower right quadrant characterized with higher concentrations of palmitic and stearic acids, while samples of 45 d and 90 d could not be noticeably separated between them and were grouped along positive PC2 axis. By contrast, unripened C samples appeared in the lower left quadrant, and samples of 45d and 90d were located in the upper left and in the upper right quadrants, respectively. The latter was characterized by higher contents of SCFFA and C18:1 and C18:2.All E samples (E-t3, E-t45, E-t90) and C samples of 90 d (C-t90) were placed in the positive semi-plane of PC1axis, as their lipolysis levels were similar, and differed from the other C samples (C-t3 and C-t45) which were situated in the negative semi-plane of PC1 axis. PC1 separated cheese samples with cheese age, from negative to positive as the ripening progressed, and PC2 differenced samples based on

Table 2
FFA concentrations of control (C) and experimental (E) cheeses during ripening, expressed as μmol/100 g fat. Mean values of two independent cheese making trials.

Free fatty acids	3 days		45 days		90 days	
	C	E	C	E	C	E
C4:0	245[b] ± 45	603[a] ± 92	1838[b] ± 150	2327[a] ± 338	2234[a] ± 168	2025[b] ± 185
C6:0	128[b] ± 34	427[a] ± 97	539[b] ± 42	741[a] ± 31	1046[a] ± 36	1033[a] ± 123
C8:0	105[b] ± 22	508[a] ± 62	357[b] ± 63	618[a] ± 52	571[b] ± 25	730[a] ± 37
C10:0	203[b] ± 62	913[a] ± 118	471[b] ± 55	1175[a] ± 132	687[b] ± 23	1221[a] ± 103
C12:0	209[b] ± 72	601[a] ± 44	480[b] ± 53	907[a] ± 95	542[b] ± 42	845[a] ± 57
C14:0	1030[b] ± 350	5410[a] ± 306	1518[b] ± 58	4558[a] ± 204	5027[a] ± 969	5424[a] ± 1505
C16:0	2124[b] ± 452	8650[a] ± 190	3235[b] ± 144	7411[a] ± 422	6759[b] ± 609	9347[a] ± 1354
C18:0	514[a] ± 42	1828[b] ± 78	834a ± 63	2146[b] ± 150	1097[b] ± 112	1918[a] ± 90
C18:1	1574[b] ± 223	4434[a] ± 289	3016[b] ± 353	4384[a] ± 203	5242[a] ± 250	4826[a] ± 730
C18:2	289[b] ± 71	748[a] ± 93	449[b] ± 39	707[a] ± 40	1044[a] ± 130	853[a] ± 107

[a] Numbers represent mean ± standard deviation.
[b] For each time of ripening, means in a row without common letters differ ($p < 0.05$).

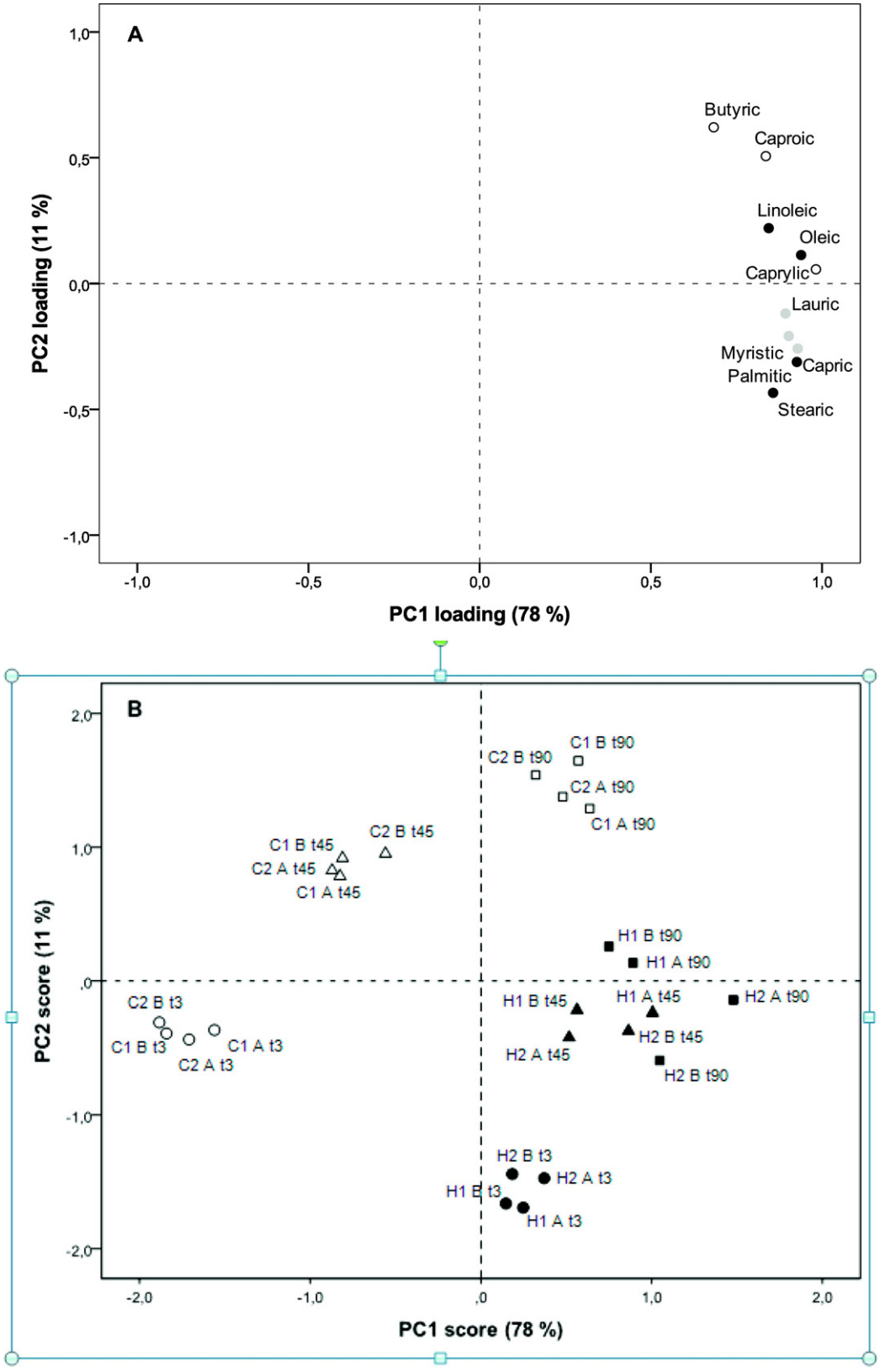

Fig. 3. Loading (A) and score (B) plots of the first and second principal components after Principal Component Analysis (PCA) based on the free fatty acids (FFA) of hard cheeses made from homogenized milk (9 MPa) (Hcheeses) and untreated milk (C cheeses) at 3, 45 and 90 days (t3, t45, t90) of storage at 12 °C. Cheeses were carried out in duplicate (1 and 2) from two independent cheese making trials (A and B).

the relative differences among individual FFA by the treatment applied to cheese milk. In particular, the samples C-t3, C-t45 and C-t90 showed the most visible separation with ripening time, indicating a progressive increase in lipolysis for this type of cheeses; in contrast, the samples E-t3, E-t45 and E-t90 were nearly placed describing, for this case, similar lipolysis patterns.

Finally, in order to validate the visual interpretation of PCA, discriminant analysis (DA) was applied on PC1 and PC2 scores using ripening time and cheese treatment as classification factors. There was no difference between the actual group of cheese sample and assigned group of cheese sample; in fact, all E samples were clearly distinguished of all C samples (100% of cases), while when the time of ripening was used as classification factor, 96% of cases were correctly grouped.

3.4. Volatile profiles

A total of 37 volatile compounds, including 5 aldehydes, 12 alcohols, 8 ketones, 5 esters and 7 acids were identified in cheese samples. They were found in both types of cheeses and have been reported as typical components of volatile profile of grana-type cheeses, including Reggianito (Barbieri et al., 1994; Ceruti, Zorrilla, & Sihufe, 2016; Wolf et al., 2010). Volatiles identified in the analyzed samples and their mean peak area values are listed in Table 3 for individual trials (A and B) at the different ripening times (3, 45 and 90 days). Besides, in order to have an overall impression of the volatile profiles of cheeses, the mean percentage values (average of two trials) of the different chemical groups, calculated with respect to the total peak area values of the identified compounds, were also compared between both types of cheeses (Fig. 4).

3.4.1. Acids

The acidic fraction was mainly composed of even linear-chain (fatty) acids from C_2 to C_{12}. Lipolysis of milk fat seems to be the metabolic pathway of formation of carboxylic fatty acids with >4 carbon atoms. Short- and medium-chain free fatty acids are important contributors to the flavour in a wide variety of cheeses (Addis, Pirisi, Di Salvo, Podda, & Piredda, 2005; Collins et al., 2003). As seen in Fig. 4, regardless of the ripening time, the volatile fraction of both types of cheeses was composed of >50% of acidic components, representing the main group among all volatiles. Butanoic and hexanoic acids were the most abundant throughout ripening, reaching percentual values above 65% of total acidic compounds (data not shown). This finding has also been observed in samples of Reggianito cheese (Ceruti et al., 2016; Wolf et al., 2010).

The comparison of the peak area values of single fatty acids derived from lipolysis revealed that at the beginning of maturation and at 45 days, levels of the major acids such as butanoic, hexanoic and octanoic acids were higher in H than in C cheeses ($p < 0.05$) whereas at the end of ripening, there were not statistical differences among cheeses ($p > 0.05$). Overall, the data confirmed the trends found for lipolysis analysis: both, lipolysis and volatile profiling results showed that the step of homogenization applied to milk had a strong influence on the production of the FFA immediately after cheese making, and this originated great changes with regards to control cheeses. These differences were significant up to middle of ripening, but then decreased and levelled up towards 90 days.

Concerning the behavior of fatty acids over the ripening period, the data in Table 3 revealed that butanoic, hexanoic and octanoic acids shown a trend to increase ($p < 0.05$). Among minority acids, decanoic acid increased after 45 days ($p < 0.05$) whereas dodecanoic acid remained unchanged in almost all samples.

In addition to fatty acids derived from lipolysis, the presence of acetic acid and 3-methylbutanoic acid was detected. Acetic acid reached a higher level in cheeses made from unhomogenized milk than those made from homogenized milk at the beginning of ripening ($p < 0.05$) whereas at 45 days, differences between cheeses were not observed ($p > 0.05$). At 90 days, the data recorded were inconsistent; a higher level in C than in H cheeses was detected ($p < 0.05$) in trial A, while statistical differences were not observed in trial B ($p > 0.05$). On the other hand, the evolution of acetic acid with ripening time did not show a defined trend. These results reveal that the homogenization of milk did not produce in any case an increase in acetic acid content, which is not surprising taking into account that its biosynthesis in cheeses includes pathways such as lactate/citrate metabolism and amino acid catabolism rather than lipolysis (Ott, Germond, & Chaintreau, 2000). For 3-methylbutanoic acid (or isovaleric acid), a typical compound derived from leucine catabolism (McSweeney & Sousa, 2000), the values did not show differences between cheeses at each sampling time and these were not modified during maturation ($p > 0.05$).

3.4.2. Alcohols

Alcohols were the most diversified chemical group of compounds. Among them, linear-chain primary alcohols (from C_2 to C_7), linear-chain secondary alcohols (from C_3 to C_7) and branched-chain alcohols (2-methyl-1-propanol and 3-methyl-1-butanol) were detected. From Fig. 4, it can be observed that alcohols were the second group of volatile compounds in all cheeses at any ripening time, ranging from 11% to 30% and showing a decrease during ripening for both types of cheeses.

Regardless of the sampling time, primary alcohols represented >80% of total compounds in all samples (data not shown). In particular, ethanol was the most abundant (from 50% to 70% of total alcohols) and its level remained almost steady throughout ripening in all cheeses ($p > 0.05$). The prevalence of ethanol has been reported in a wide variety of cheeses (Kondyli, Katsiari, Masouras, & Voutsinas, 2002; Wolf et al., 2010). In individual trials, C cheeses had significantly higher contents of ethanol than H cheeses at each sampling time ($p < 0.05$). As happened in the case of acetic acid, this observation could be explained by the origin of ethanol in cheeses as it is a common terminal end-product in the breakdown of glucose or it is produced from amino acid catabolism such as alanine (Kondyli et al., 2002). For the remaining primary alcohols, statistical differences between cheeses were not detected at any ripening time ($p > 0.05$). No general pattern of increase or decrease of the levels of these compounds with ripening time was detected.

Secondary and branched-chain alcohols represented a minor fraction, among which 2-pentanol and 3-methyl-1-butanol prevailed. The occurrence of linear-chain primary alcohols longer than ethanol and linear-chain secondary alcohols in cheeses seems to be related, at least partially, with the fatty acid catabolism (Barbieri et al., 1994; Curioni & Bosset, 2002) whereas the presence of branched-chain alcohols is clearly associated to amino acid catabolism. In general, no significant changes attributable to homogenization were found, as high variability with the ripening time or the trial was verified.

3.4.3. Aldehydes

Within the chemical family of aldehydes, acetaldehyde, 3-methylbutanal, hexanal, heptanal and nonanal were detected. At any ripening time, the proportion of aldehydes was low with respect to other chemical classes (from 2% to 4%) (Fig. 4). Acetaldehyde constituted the most abundant aldehyde, ranging from 40% to 75% of total aldehydes, depending on the sampling time (data not shown). The levels found were higher in C than in H cheeses until 45 days ($p < 0.05$), and then, statistical differences were not detected ($p > 0.05$). This result is in agreement with the origin of acetaldehyde in cheeses, which does not appear to be related to fatty acid catabolism. In fact, it has been suggested that could derive from the breakdown of threonine, from the lactose metabolism or by the oxidation of ethanol (McSweeney & Sousa, 2000). Besides, at this point, it is important to highlight that for the three metabolically related compounds, namely acetaldehyde, ethanol and acetic acid, homogenized cheeses had similar or lower levels than unhomogenized counterparts, indicating that homogenization does not favor their production. Regarding to the evolution of acetaldehyde during ripening, the levels did not change in C cheeses ($p > 0.05$), whereas in H cheeses an unclear trend was observed.

In relation to 3-methylbutanal, the levels were not affected by the type of cheese or the ripening time, which was in accordance with the origin of this compound; it derives from leucine catabolism (Curioni & Bosset, 2002). Straight-chain aldehydes such as hexanal, heptanal and nonanal were higher ($p < 0.05$) in H than in C cheeses at 90 days.

Table 3
Volatile compounds at different stages of ripening of cheeses made with non-homogenised milk (C cheeses) and homogenised milk (H cheeses).[a]

		Trial A		Trial B	
Volatile compounds	Time of ripening	C	H	C	H
Aldehydes					
Acetaldehyde	3	177.7 ± 8.8aA	93.8 ± 9.1bA	172.5 ± 16.1aA	110.5 ± 11.9bB
	45	201.3 ± 13.6aA	129.0 ± 10.7bA	167.4 ± 10.3aA	118.4 ± 5.5bB
	90	166.5 ± 12.5aA	173.1 ± 8.4aA	143.1 ± 12.1aA	156.9 ± 7.3aA
3-methylbutanal	3	18.3 ± 1.1aA	12.7 ± 0.8bA	19.5 ± 0.6aA	11.0 ± 1.9bB
	45	22.2 ± 6.2aA	18.4 ± 5.4aA	25.2 ± 0.1aA	23.5 ± 3.0aA
	90	15.1 ± 3.1aA	21.2 ± 5.0aA	23.8 ± 2.7aA	20.6 ± 2.4aA
Hexanal	3	9.8 ± 1.5aB	7.7 ± 0.9aB	10.8 ± 1.4aB	8.6 ± 1.4aB
	45	6.8 ± 0.2aB	5.7 ± 1.1aB	7.0 ± 2.7aB	10.8 ± 1.3aB
	90	16.4 ± 1.6aA	29.6 ± 3.4aA	21.9 ± 2.2bA	35.9 ± 3.3aA
Heptanal	3	23.3 ± 2.1aB	19.5 ± 3.0aB	23.4 ± 3.1aB	16.7 ± 1.7aB
	45	25.6 ± 3.3aB	30.6 ± 2.1aB	18.1 ± 3.0aB	16.2 ± 2.9aB
	90	48.2 ± 5.7bA	82.3 ± 4.0aA	51.5 ± 4.5bA	85.8 ± 6.7aA
Nonanal	3	12.6 ± 0.6aB	11.5 ± 1.6aB	12.8 ± 0.6aB	10.9 ± 0.8aB
	45	18.1 ± 3.7aB	21.9 ± 6.9aB	9.5 ± 0.2aB	9.5 ± 1.9aB
	90	51.6 ± 8.9bA	100.3 ± 13.6aA	53.0 ± 7.0bA	101.7 ± 13.5aA
Ketones					
Propanone	3	16.6 ± 0.7aB	13.4 ± 1.5aA	15.7 ± 1.4aB	10.4 ± 2.0aB
	45	51.6 ± 4.7aA	13.0 ± 1.9bA	48.7 ± 4.3aA	30.1 ± 3.0bA
	90	51.0 ± 9.0aA	50.7 ± 2.9aB	43.1 ± 6.2aA	38.1 ± 4.7aA
Butanone	3	13.5 ± 0.6aB	9.2 ± 1.2bA	15.0 ± 0.3aB	8.1 ± 0.5bC
	45	45.9 ± 2.6aA	17.9 ± 3.9bA	52.7 ± 5.4aA	31.8 ± 1.1bA
	90	46.5 ± 2.4aA	14.4 ± 3.4bA	20.3 ± 1.2aB	15.3 ± 0.8bB
2,3-butanedione + 2-pentanone	3	118.2 ± 4.9aA	95.1 ± 8.1aB	109.7 ± 9.6aA	98.2 ± 7.5aA
	45	132.9 ± 7.1aA	129.2 ± 4.3aB	112.9 ± 6.2aA	106.2 ± 2.2aA
	90	112.0 ± 13.5bA	207.0 ± 9.1aA	77.4 ± 7.6bA	117.5 ± 6.5aA
2-hexanone	3	73.1 ± 7.2aA	53.6 ± 3.1aA	70.5 ± 8.0aA	57.2 ± 11.5aA
	45	5.6 ± 1.9bC	30.2 ± 3.2aB	7.6 ± 0.6bC	28.3 ± 4.6aB
	90	30.4 ± 0.9bB	57.3 ± 4.7aA	41.3 ± 6.3bB	65.2 ± 4.1aA
2-heptanone	3	121.6 ± 9.2aA	104.3 ± 7.4aA	133.1 ± 7.7aA	117.2 ± 13.7aA
	45	32.6 ± 2.8bB	167.7 ± 14.6aB	45.0 ± 6.5bB	192.3 ± 7.6aB
	90	55.3 ± 7.8bB	386.6 ± 23.8aC	128.9 ± 12.2bA	334.8 ± 18.6aC
3-hydroxy-2-butanone	3	416.9 ± 30.5aA	274.1 ± 22.6bA	374.8 ± 46.5aA	203.5 ± 18.1bA
	45	123.3 ± 9.1aB	94.8 ± 5.2aB	160.5 ± 12.1aB	52.7 ± 9.7bB
	90	93.7 ± 7.0aB	55.7 ± 4.9bB	122.8 ± 8.5aB	46.6 ± 9.4bB
2-nonanone	3	9.8 ± 0.8aA	7.7 ± 0.9aA	10.2 ± 0.9aA	9.9 ± 0.5aA
	45	6.6 ± 1.4bA	77.2 ± 13.6aB	9.3 ± 1.9bA	39.6 ± 2.7aB
	90	26.5 ± 3.3bB	166.2 ± 11.7aC	44.4 ± 5.0bB	150.0 ± 10.8aC
Alcohols					
2-propanol	3	36.5 ± 2.4aAB	27.1 ± 5.1aAB	31.0 ± 2.8aA	23.4 ± 4.3aA
	45	29.8 ± 1.7aB	36.4 ± 4.6aA	29.6 ± 3.1aA	20.2 ± 2.2aA
	90	49.4 ± 7.3aA	13.0 ± 3.8bB	27.3 ± 1.1aA	26.6 ± 3.7aA
Ethanol	3	1349.7 ± 30.3aA	1098.0 ± 55.1bA	1460.3 ± 65.5aA	996.0 ± 71.8bA
	45	1513.8 ± 91.2aA	1009.7 ± 96.5bA	1365.4 ± 84.8aA	1044.7 ± 77.6bA
	90	1446.3 ± 54.4aA	848.1 ± 85.9bA	1311.9 ± 35.7aA	1076.0 ± 47.6bA
2-butanol	3	9.7 ± 1.9aAB	7.3 ± 1.1aB	9.2 ± 1.4aB	7.7 ± 0.3aB
	45	16.6 ± 2.9aA	15.2 ± 1.9aB	16.1 ± 0.6bA	34.6 ± 2.5aA
	90	5.5 ± 0.1bB	34.0 ± 5.2aA	7.8 ± 1.9bB	33.2 ± 1.4aA
1-propanol	3	85.9 ± 10.4aB	82.1 ± 6.8aA	94.7 ± 4.4aB	86.2 ± 6.7aA
	45	613.4 ± 24.6aA	83.1 ± 1.2bA	276.9 ± 34.7aA	106.0 ± 2.3bA
	90	651.3 ± 49.2aA	64.9 ± 4.3bA	98.2 ± 2.5aB	109.5 ± 4.7aA
2-methyl-1-propanol	3	13.5 ± 1.1aA	14.3 ± 2.0aA	11.4 ± 0.6aA	9.2 ± 0.5aA
	45	17.7 ± 1.5aA	19.4 ± 0.8aA	13.6 ± 3.3aA	12.6 ± 1.4aA
	90	14.7 ± 1.2aA	16.7 ± 2.5aA	15.1 ± 0.4aA	14.4 ± 2.0aA
2-pentanol	3	92.8 ± 3.5 aB	68.6 ± 4.3bB	93.7 ± 2.4aA	80.3 ± 9.6aA
	45	91.0 ± 3.3aB	71.9 ± 2.9bB	57.1 ± 12.5aB	57.8 ± 7.9aA
	90	264.9 ± 28.4aA	116.9 ± 10.0bA	69.3 ± 2.5aAB	73.3 ± 3.4aA
1-butanol	3	128.9 ± 2.2aA	122.9 ± 21.8aA	126.6 ± 6.3aA	113.7 ± 8.2aA
	45	88.7 ± 3.7aB	86.3 ± 8.7aB	48.7 ± 8.1aB	43.7 ± 7.7aB
	90	60.5 ± 14.7aB	59.0 ± 15.0aB	39.7 ± 9.9aB	35.2 ± 9.5aB
3-methyl-1-butanol	3	235.6 ± 5.7aA	202.4 ± 18.4aA	225.9 ± 17.1aA	218.1 ± 17.6aA
	45	143.2 ± 22.0aB	132.9 ± 6.7aB	149.3 ± 13.5aB	155.5 ± 8.6aB
	90	65.1 ± 7.1aC	69.5 ± 7.1aC	83.5 ± 3.0aC	79.1 ± 5.5aC
1-pentanol	3	18.2 ± 1.5aC	14.3 ± 1.1aC	16.1 ± 1.3aB	11.5 ± 2.2aB
	45	38.8 ± 6.6aB	43.6 ± 1.3aB	30.5 ± 2.4aB	23.2 ± 4.0aB
	90	69.8 ± 5.1bA	96.7 ± 1.9aA	87.8 ± 22.4aA	88.4 ± 18.1aA
2-heptanol	3	13.3 ± 2.1aB	7.7 ± 1.8aB	11.8 ± 1.2aB	11.9 ± 0.4aB
	45	25.6 ± 1.6bB	56.2 ± 1.2aA	16.0 ± 4.0aB	17.9 ± 0.4aB
	90	92.2 ± 9.6aA	50.8 ± 5.3bA	31.0 ± 2.6aA	33.7 ± 4.1aA
1-hexanol	3	30.6 ± 1.5aB	32.1 ± 2.4aB	20.9 ± 4.1aB	23.0 ± 3.4aB
	45	27.2 ± 1.5aB	26.6 ± 2.1aB	15.5 ± 3.5aB	11.2 ± 2.2aB
	90	65.6 ± 6.4aA	69.2 ± 10.3aA	55.3 ± 10.4aA	60.2 ± 10.9aA
1-heptanol	3	9.8 ± 1.0aC	7.5 ± 0.8aC	9.1 ± 0.9aC	12.0 ± 0.3aC
	45	38.2 ± 2.3aB	32.6 ± 1.9aB	29.3 ± 2.2aB	29.8 ± 2.7aB

Table 3 (*continued*)

Volatile compounds	Time of ripening	Trial A		Trial B	
		C	H	C	H
	90	81.5 ± 5.7aA	85.4 ± 8.1aA	78.0 ± 0.9aA	84.9 ± 6.2aA
Esters					
Ethyl acetate	3	67.7 ± 4.9aC	39.4 ± 2.7bB	65.9 ± 5.7aC	43.9 ± 2.2bB
	45	160.9 ± 6.2aB	47.8 ± 0.7bB	107.4 ± 6.6aB	61.2 ± 8.1bB
	90	218.0 ± 14.3aA	115.7 ± 5.5bA	169.5 ± 10.1aA	108.6 ± 9.0bA
Ethyl butanoate	3	183.5 ± 9.1aC	123.0 ± 17.6bB	196.0 ± 8.1aC	140.8 ± 12.0bB
	45	504.3 ± 42.9aB	137.6 ± 2.3bB	382.6 ± 16.6aB	232.9 ± 30.7bB
	90	794.6 ± 65.6aA	540.2 ± 8.4bA	659.9 ± 36.1aA	503.1 ± 75.5bA
Ethyl hexanoate	3	87.1 ± 8.6aC	66.0 ± 8.8aB	84.1 ± 6.2aC	70.4 ± 6.3aC
	45	228.1 ± 6.7aB	107.5 ± 9.1bB	180.7 ± 2.5aB	173.8 ± 20.3aB
	90	541.9 ± 70.3aA	282.1 ± 43.2bA	344.4 ± 12.4aA	383.5 ± 33.8aA
3-methylbutyl butanoate	3	6.5 ± 0.8aB	5.4 ± 0.7aB	7.8 ± 2.1aB	6.0 ± 1.4aB
	45	16.3 ± 2.8aB	17.1 ± 3.2aB	8.7 ± 2.2aB	9.7 ± 1aB
	90	50.4 ± 12.9aA	52.4 ± 8.5aA	31.6 ± 3.3aA	36.1 ± 1.7aA
Ethyl octanoate	3	38.1 ± 3.9aB	25.6 ± 3.6aC	34.4 ± 2.2aA	24.9 ± 4.4aA
	45	36.7 ± 4.2aB	45.8 ± 5.1aB	29.6 ± 4.0aA	18.5 ± 2.1aA
	90	59.8 ± 5.5aA	71.3 ± 7.4aA	13.8 ± 1.3aB	16.9 ± 2.7aA
Acids					
Acetic acid	3	886.1 ± 91.3aAB	683.7 ± 61.1bA	862.0 ± 97.2aA	579.2 ± 30.1bB
	45	626.4 ± 21.0aB	649.3 ± 56.7aA	805.3 ± 43.9aA	687.9 ± 27.0aAB
	90	1138.5 ± 115.3aA	654.1 ± 77.7bA	856.2 ± 43.3aA	742.1 ± 46.0aA
Butanoic acid	3	1448.3 ± 108.1bC	1806.7 ± 98.9aC	1898.2 ± 137.2bC	2476.7 ± 60.2aC
	45	2632.7 ± 89.1bB	4075.3 ± 80.9aB	3257.4 ± 210.0bB	4279.9 ± 183.3aB
	90	5111.7 ± 66.4aA	5370.8 ± 93.8aA	5292.0 ± 374.6aA	5977.4 ± 250.9aA
3-methyl butanoic acid	3	22.2 ± 0.7aA	29.4 ± 3.1aA	19.4 ± 1.8aA	13.0 ± 2.1aA
	45	25.3 ± 1.5aA	28.3 ± 2.5aA	17.0 ± 3.0aA	13.1 ± 2.8aA
	90	28.9 ± 2.4aA	31.3 ± 1.9aA	20.3 ± 3.7aA	19.0 ± 4.0aA
Hexanoic acid	3	716.0 ± 67.3bC	1107.9 ± 92.1aC	937.9 ± 41.8bC	1541.4 ± 81.6aC
	45	1444.2 ± 23.5bB	2320.2 ± 14.2aB	1663.4 ± 79.3bB	2281.3 ± 161.6aB
	90	2788.9 ± 79.5aA	3012.4 ± 80.4aA	2826.7 ± 122.4aA	3146.9 ± 62.4aA
Octanoic acid	3	147.6 ± 18.3bC	272.6 ± 28.9aC	170.8 ± 25.7bC	462.1 ± 19.5aC
	45	280.9 ± 31.2bB	422.5 ± 17.7aB	369.4 ± 28.5bB	603.3 ± 27.6aB
	90	592.2 ± 51.9aA	701.1 ± 29.9aA	653.3 ± 37.0aA	727.8 ± 29.8aA
Decanoic acid	3	49.6 ± 9.6bB	108.4 ± 13.4aB	47.9 ± 3.4bB	176.6 ± 14.8aB
	45	66.1 ± 16.6bB	138.6 ± 4.6aB	83.5 ± 12.0bB	193.2 ± 15.8aB
	90	118.5 ± 5.0bA	253.8 ± 7.8aA	152.5 ± 10.3bA	254.3 ± 5.3aA
Dodecanoic acid	3	10.6 ± 1.0aA	16.5 ± 2.0aA	9.8 ± 0.7bB	21.1 ± 2.4aA
	45	11.0 ± 1.1aA	13.2 ± 1.2aA	12.6 ± 0.9bB	23.7 ± 0.6aA
	90	13.2 ± 1.3aA	17.0 ± 1.4aA	19.4 ± 2.3aA	25.2 ± 1.3aA

a,b: type of cheese factor: for each trial, the same lowercase letters within a row denote no significant differences ($p > 0.05$) between both types of cheeses.
A–C: time of ripening factor: For each cheese, the same capital letters within a column denote no significant differences ($p > 0.05$) between values obtained at different days of ripening according to Tukey's ANOVA.
[a] Peak area values ($\times 10^3$) of volatile compounds identified in C and H cheeses from A and B trials. Peak areas are mean values of two cheeses analyzed in duplicate.

They can be formed by β-oxidation of unsaturated fatty acids (Barbieri et al., 1994; Curioni & Bosset, 2002), and our results showed a significant effect of the pretreatment of milk. Differences were only at the end of ripening, suggesting that transformation of FFA followed the initial accelerated lipolysis found in H cheeses.

3.4.4. Ketones

Among ketones group were detected methylketones (propanone, butanone, 2-pentanone, 2-hexanone, 2-heptanone and 2-nonanone), diketones (2,3-butanedione or diacetyl) and hydroxyketones (3-hydroxy-2-butanone or acetoin). As seen in Fig. 4, they ranged from 3% to 11%, depending on type of cheese and ripening time. The most abundant ketones identified in all samples were diacetyl + 2-pentanone (unresolved peak), acetoin and 2-heptanone. At the beginning of ripening, the proportion of acetoin ranged from 40% to 50% of total ketones in both types of cheeses and then, a marked decrease was observed, which was more intense in H cheeses, reaching percentages of 6% at 90 days (data not shown). The minor incidence of acetoin in the volatile profile during ripening of cheeses was offset by the relevant role of diacetyl + 2-2-pentanone and 2-heptanone, whose percentages increased sharply in aged cheeses (data not shown).

Diacetyl and its reduction product, acetoin, derive from lactose and citrate metabolism whereas 2-pentanone is related to β-oxidation of free fatty acids (McSweeney & Sousa, 2000). Higher levels of diacetyl + 2-2-pentanone were found in H cheeses at 90 days, while no statistical differences were detected earlier. The opposite behavior was observed for acetoin level which was higher in C cheeses and decreased in all samples during ripening.

Other ketones derived from free fatty acids catabolism are 2-hexanone, 2-heptanone and 2-nonanone (Collins et al., 2003). They reached similar levels in both types of un-ripened cheeses but in the course of ripening a higher content in H than C cheeses was always recorded ($p < 0.05$). In agreement with our results, Cao, Fonseca, Schoenfuss, and Rankin (2014) reported higher concentrations of methylketones in blue cheeses made from homogenized milk at 7 MPa compared to the unhomogenized samples. On the other hand, the trend observed for each compound during ripening was different depending on the trial and type of cheese. As for propanone and butanone, they are normal constituents of raw milk and are thought to be derived from cow feed rather than from fatty acids metabolism (Toso, Procida, & Stefanon, 2002). According with the hypothesis about their origin, our results showed that the homogenization process does not promote their production. Moreover, the changes throughout ripening were erratic in all cheeses.

3.4.5. Esters

Esters are common products derived of fatty acid catabolism (Collins et al., 2003). They were found in low proportions in all cheeses, ranging from 4% to 9%. Regardless of type of cheese, a slight increase in the percentages of esters was observed throughout ripening. This result was

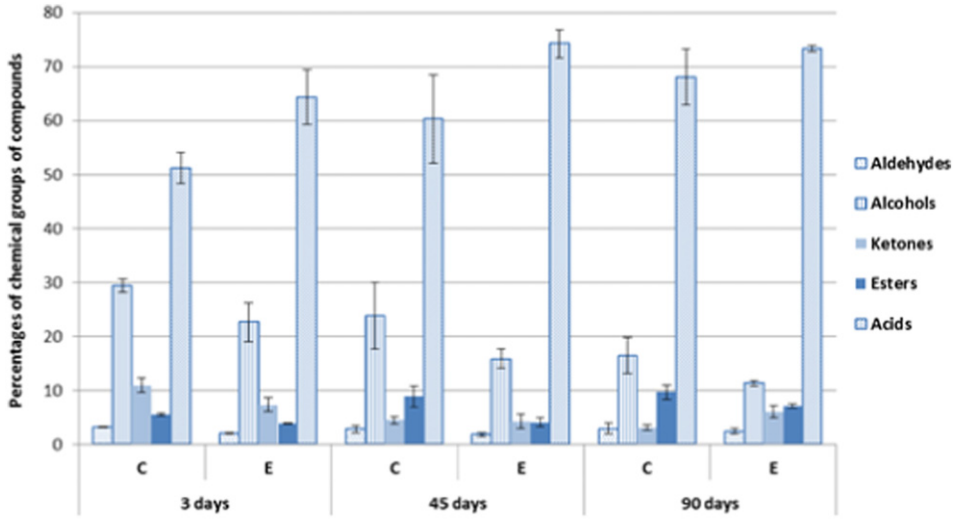

Fig. 4. Percentage of chemical groups of compounds in cheeses made with homogenized milk (H) and unhomogenized milk (C) at each sampling time (3, 45 and 90 days). Values are average for two independent cheese making trials of each type of cheese, analyzed in duplicate.

expected, as esterification is one of the dominant chemical events during cheese ripening (Caporaso, Armento, & Sacchi, 2015). Within this group, mostly ethyl esters (ethyl acetate, ethyl butanoate, ethyl hexanoate and ethyl octanoate) were identified. Esters have a relevant role in the sensory characteristics of grana-type cheeses, providing fruity notes (Liu, Holland, & Crow, 2004). Ethyl acetate and ethyl butanoate showed higher levels ($p < 0.05$) in C than H cheeses for all ripening times, and ethyl hexanoate at 45 and 90 days in cheeses from trial A. Conversely, no differences in ethyl octanoate between cheeses from both trials were found. As for evolution during ripening, the three esters showed a general trend to increase. In cheeses made from homogenized milk a significantly increase was evidenced from 45 to 90 days whereas in C cheeses a progressive increase was detected throughout ripening ($p < 0.05$).

The formation of esters in cheeses is still largely unknown, and the reactions that could be involved are esterification and alcoholysis. Ethyl esters biosynthesis in cheeses is likely due to a combined effect of factors such as the presence of substrates (acids and alcohols), enzymes, and environment conditions. The role of alcohols, mainly ethanol, on ester biosynthesis in cheeses has been extensively investigated (Liu et al., 2004; Richoux, Maillard, Kerjean, Lortal, & Thierry, 2008). Data on the volatiles in different types of cheeses suggest that alcohol availability is the limiting factor of ester biosynthesis. In our study, the highest levels of the major ethyl esters (ethyl acetate, ethyl butanoate and ethyl hexanoate) observed in C cheeses could be attributed, at least partially, to a higher availability of ethanol in these cheeses as was above mentioned.

4. Conclusions

The pre-treatment of cheese milk: cream homogenization followed by an incubation step, showed to have influence on lipolysis and production of volatile compounds in hard cooked cheeses. Indeed, fat accessibility seems to be a limiting factor in lipolysis, as an acceleration of this biochemical event was clearly evidenced in FFA production showing an early lipolysis. Some compounds derived from fatty acid catabolism such as hexanal, heptanal and nonanal and methylketones from C_5 to C_9 were preferentially formed in homogenized cheeses at different ripening times. Homogenization did not cause detrimental effects on cheese composition and proteolysis index.

Based on these results, the innovative treatment of milk low homogenization/incubation applied to hard cooked cheese technology has proved to be effective in lipolysis acceleration and FFA catabolism. It could be easily scaled up; however, further studies should be carried out in order to evaluate sensory and textural attributes of the products.

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