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Characterization of liposomes and their effect on the properties of Cheddar cheese during ripening

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Abstract — This study was undertaken to observe the location and structure of liposomes in the cheese matrix and to identify their effect on the characteristics of Cheddar cheese during ripening. Composition, proteolysis, microstructure and texture of Cheddar cheeses treated with either empty liposomes or liposomes containing cell-free extracts of *Lactobacillus casei* ssp. *pseudoplantarum* were analysed during two months of ripening. Results were compared with control Cheddar cheeses. Observations on cheese microstructure showed that some liposomes were included in the casein matrix, but most vesicles were located in areas close to fat globules and appeared destabilized as early as the first day after manufacture, inducing some changes in the cheese microstructure. The presence of liposomes greatly increased the moisture of cheeses, modified the microstructure and the rheological characteristics of ripened cheese. Cheeses containing liposomes were softer, less cohesive and elastic and more brittle than control cheeses. After two weeks of ripening and until the end of the study, proteolysis, particularly peptidolysis, was higher in cheeses treated with liposomes containing cell-free extracts. HPLC-analysis confirmed intensive peptidolysis between one and two months of ripening. © Inra/Elsevier, Paris

liposome / cheese / microstructure / ripening

Résumé — Caractérisation des liposomes et de leur effet sur les propriétés du fromage cheddar au cours de l'affinage. Cette étude a été réalisée dans le but d'observer la distribution et la structure des liposomes dans la matrice fromagère, ainsi que le rôle des liposomes sur les caractéristiques du fromage cheddar au cours de l'affinage. La composition, la protéolyse, la microstructure et la texture de fromages cheddar, contenant des liposomes vides ou des liposomes dans lesquels ont été encapsulés des extraits cellulaires de *Lactobacillus casei* ssp. *pseudoplantarum*, ont été analysées durant deux mois d'affinage et comparées à des fromages cheddar témoins. Les analyses de microstructure ont montré que quelques vésicules sont incluses dans la matrice caséique, mais la plupart des liposomes sont localisés à proximité des globules de matière grasse et paraissent déstabilisés dès le premier jour d'affinage. La présence de liposomes augmente significativement l'humidité des fromages, modifie la microstructure et les caractéristiques rhéologiques des fromages affi-

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nés. Les fromages contenant des liposomes sont plus mous, moins cohésifs et élastiques et plus friables que les fromages témoins. Les extraits cellulaires immobilisés dans des liposomes favorisent la protéolyse, particulièrement la peptidolyse, dès la deuxième semaine d'affinage et ce, jusqu'à la fin de l'expérience. Les analyses HPLC confirment une peptidolyse intensive entre le premier et le deuxième mois d'affinage. © Inra/Elsevier, Paris

liposome / fromage / microstructure / affinage

1. INTRODUCTION

The organoleptic properties and texture of a firm cheese are greatly dependent on the action during ripening of starters and rennet as well as of enzymatic activities contained in the milk [19]. However, this process is long and many techniques have been proposed to accelerate the ripening process and thus reduce the costs of cheese production. The addition of extra amounts of ripening enzymes is a solution of preference [7]. Among the different methods of enzyme addition proposed, the encapsulation of enzymes in liposomes has recently become the subject of renewed interest [8, 24, 26, 28].

The use of liposomes containing enzyme extracts of *Lactobacillus helveticus* accelerated the formation of the typical flavor of Taleggio cheese without the flavor defects detected in cheeses to which free protease was added [26]. However, cheese texture could be affected by the introduction of lipid vesicles [18, 24]. Another drawback is that most of the methods used for liposome preparation require organic solvents and are difficult to use on a large-scale in the food industry [27]. Other methods for preparing large quantities of liposomes without organic solvents have been proposed [15, 18], but they generally require the use of energy-expensive processes.

A new method for preparing liposomes was proposed by Perrett et al. [23]. This method, based on the transformation of a Pro-Lipo® mixture into a liposome suspension by a simple two-step dilution has been successfully tested for immobilizing proteolytic enzymes [4]. This method is simple, does not require the use of organic solvents and could be used to prepare large amounts of liposomes.

In a previous study, the enzyme complement of *Lactobacillus casei* ssp. *pseudoplantarum* UL137 showed a high potential for Cheddar cheese ripening [5]. Cell-free extracts (CFE) of this strain were immobilized in liposomes obtained from Pro-Lipo®. The main objectives of this study were to quantify the retention of liposome-immobilized CFE in the cheese curd and to follow the changes in these liposomes, as well as in cheese characteristics, during ripening.

2. MATERIALS AND METHODS

2.1. Preparation of cell-free extracts

A cell-free extract (CFE) of *Lactobacillus casei* ssp. *pseudoplantarum* UL137 (*Lb. casei* UL 137) was produced as described by Laloy et al. [16]. Then, a two-step precipitation (40 % and 85 % saturated ammonium sulphate) was carried out on the crude CFE solution. The solution obtained was dialysed overnight, lyophilized and stored at –18 °C. For liposome preparation, CFE solutions were prepared by suspending lyophilized CFE in Tris-HCl buffer (50 mmol·L^{-1}, pH 7.2) to a final protein concentration of 1.3 g·L^{-1}.

2.2. Preparation of liposomes

Liposomes were prepared from the Pro-Lipo® S mixture (Lucas Meyer France, Chelles, France), the solution being immobilized according to the method described by Dufour et al. [4]. Three different liposome preparations were produced

depending on the protein concentration in the CFE solutions being immobilized (CFE solutions containing 0.6 or 1.3 $g \cdot L^{-1}$ protein or Tris-HCl buffer).

2.3. Immobilization efficiency

A sample (about 1 mL) of each liposome suspension was centrifuged at 100 000 *g* for 1 h (L8-70M ultracentrifuge, Beckman, Palo Alto, CA, USA). The amount of CFE in the supernatant (unimmobilized CFE) and in the pellet (immobilized CFE) was determined, using a sandwich ELISA test, as described by Laloy et al. [16].

2.4. Cheese production and experimental design

Cheddar cheeses were made from 8 L of pasteurized milk according to the conventional method [13], using a laboratory computer-controlled cheese plant (Inra, Poligny, France). After production, Cheddar cheeses were divided into equal parts corresponding to the sampling times, vacuum packaged and ripened for 60 days at 12 °C.

The experimental design used was a randomized complete block. Four cheeses were prepared in two replicate blocks. The same volume of liposome suspension (25 mL) was added to each milk vat just before adding the rennet. Sample codes, according to the type of liposome added, were given to the cheeses as follows: NL (cheeses without liposomes); L0 (cheeses with liposomes containing Tris-HCl buffer); L1 (cheeses with liposomes containing the 0.6 $g \cdot L^{-1}$ protein CFE); L2 (cheeses with liposomes containing the 1.3 $g \cdot L^{-1}$ protein CFE).

Three additional Cheddar cheeses containing high quantities of liposomes (150 mL of liposome suspension immobilizing CFE solution containing 0.6 $g \cdot L^{-1}$ protein) were produced. The latter cheeses (HL) were used for the determination of liposome location in the curd during ripening.

2.5. Retention of liposome-immobilized CFE in cheese

Samples (5 g) of NL, L0, L1 and L2 cheeses were taken immediately after pressing and homogenized with 50 mL PBS (10 $mmol \cdot L^{-1}$, pH 7) in a sterile bag, for 2 × 45 s using a Lab-Blender 400 Stomacher (Seward Laboratory, London, England). After heating for 1 h at 50 °C, the cheese suspension was allowed to sediment and the supernatant filtered through Whatman #1 filter paper. The resulting filtrate was used for the determination of CFE concentration by the sandwich ELISA assay, as described by Laloy et al. [16]. This analysis was repeated twice on each cheese sample.

2.6. Chemical analyses

Total nitrogen (N), fat and moisture were determined in duplicate in one-month old NL, L0, L1 and L2 cheeses following a standard procedure [2]. Cheese pH was measured using a Spear Tip combination electrode (Corning, VWR Scientific, Ville Mont-Royal, Canada).

2.7. Proteolysis during cheese ripening

Samples of cheeses NL, L0, L1 and L2 were taken after 1, 7, 14, 30 and 60 days of ripening and then were frozen until evaluation of proteolysis. Thawed cheese (10 g) was homogenized with 40 mL of distilled water, using a Lab-Blender 400 Stomacher, as described above. The cheese suspensions were centrifuged at 3 000 *g* for 20 min at 4 °C. The supernatant was filtered twice through Whatman #42 filter paper. Five mL of the filtrate were analysed for water-soluble nitrogen (WSN) according to the method of Kuchroo and Fox [14]. Trichloroacetic acid-soluble nitrogen (TCA-SN) for the quantification of large peptides and phosphotungstic acid-soluble nitrogen (PTA-SN) for the quantification of small peptides and free amino acids were determined on aliquots of the water-soluble fraction (10 mL), according to Gripon et al. [9]. One gram of cheese was also analysed for total nitrogen content. All nitrogen determinations were made in duplicate by the Kjeldahl method [11].

2.8. HPLC analysis

Samples (~100 g) of NL, L0, L1 and L2 cheeses were taken after 30 and 60 days of ripening, sliced, frozen and freeze-dried. Bitter and astringent fractions were isolated and then analysed by size-exclusion HPLC chromatography, as described by Harwalkar and Elliott [10].

2.9. Transmission electron microscopy

HL cheeses, containing high quantities of liposomes, were observed by electron microscopy after 24 h, 2, 4 and 8 weeks of ripening. Cheese samples were cut into small cubes of about 0.8–1.0 mm side and fixed for 15 h at 4 °C in 3% (w/v) glutaraldehyde in 0.1 mol·L^{-1} sodium cacodylate buffer (pH 7.2) and then for 10 h at 4 °C in 1 % (w/v) osmium tetroxide (pH 7.2). Samples were then dehydrated in a graded ethanol series (30, 50, 70, 80 and 95 %) and embedded in Epon 812. Ultrathin sections (0.1 μm) were cut with an ultramicrotome, collected on formvar-coated nickel grids and stained with uranyl acetate and lead citrate before examination with a JEOL 1 200 EX (Tokyo, Japan) electron microscope at 80 kV [17].

At least three cheese cubes per treatment and sampling time were cut, and 5 to 10 ultrathin sections per cube were observed.

2.10. Textural measurements

The texture of NL, L0, L1 and L2 cheeses was evaluated by a double compression test using a TA-XT2 Texture Analyser (Texture Technologies Corp, Carry, NY, USA), after 2, 4 and 8 weeks of ripening. The compression speed was set at 2 cm·min^{-1}. Before testing, cylindrical cheese samples (1 cm high and 1 cm diameter, exactly measured) were held at room temperature (20–22 °C) in airtight boxes for 2 h. Six cylinders per experimental unit were compressed to 80 % of their original height. The following factors, defined according to Van Vliet [30], were determined from the Texture Profile Analysis: firmness (N), the force necessary to reach 80 % deformation; fracturability or brittleness (N), the breaking strength of the sample; cohesiveness (no dimension), the quantity to simulate the strength of the internal bonds making up the body of the cheese; springiness (no dimension), the extent to which the deformed cheese returned to its undeformed condition after the first compression.

2.11. Statistical analysis

Analysis of variance and multiple comparisons were performed using the General Linear Model procedure of SAS [25]. The significance of the blocks, the treatments (NL, L0, L1 and L2), the sampling time and the interaction between the treatments and the sampling time were tested using the analysis of variance and orthogonal contrasts. When the interaction between the treatments and the sampling time was significant or when a specific analysis was necessary, the Least Significant Difference (LSD) test was performed for each stage of ripening to evaluate the significance of the treatments [29].

3. RESULTS AND DISCUSSION

3.1. Efficiency of enzyme immobilization and liposome retention in the cheese curd

The efficiency of immobilizing CFE in liposomes obtained from Pro-Lipo® was determined by a sandwich ELISA test. Immobilization efficiency of CFE in liposomes was 56.8 ± 3.1 % and 58.4 ± 3.0 % for CFE solutions containing 1.3 or 0.6 g·L^{-1} protein, respectively. For liposomes containing Tris-HCl buffer, the ELISA response was not significant. The high potential of Pro-Lipo® for immobilizing enzymes [4] was confirmed in the present study, since the immobilization efficiency of CFE was similar to the best values recently reported by Fresta et al. [8]. These authors immobilized Neutrase in freeze-thawed and extruded multilamellar vesicles, with an encapsulation efficiency of 65 %.

The retention of liposome-immobilized CFE in the cheese curd was found to be independent of the concentration of CFE solution and reached 62.8 ± 3.6 % (ELISA responses for NL and L0 cheeses were negligible). This retention value was similar to that, 60 %, obtained for multilamellar-vesicle-encapsulated ^{14}C-Neutrase [1], but was lower than the retention of radiolabelled liposomes [12].

When considering the immobilization efficiency and the retention load of liposomes in the cheese curd, about 9.3 mg and 4.4 mg of CFE were retained per kg of L2 and L1 cheeses, respectively. The initial quantities of CFE solution added to milk were 26 and 12 mg for L2 and L1 cheeses, respectively. Therefore, the final yield of

liposome-encapsulated CFE in the curd was about 36 %, which is 7 to 8 times greater than the value obtained with free CFE [16] and 1.2-fold higher than the yield reported by Kirby et al. [12].

3.2. Cheese composition

Compared with the NL and L0 cheeses, the addition of liposome-encapsulated CFE did not significantly influence the fat, ash, protein content or pH of the cheeses (*table I*). Similar results have been observed in Cheddar cheese [18] and in Manchego cheese [24]. However, these authors detected a slightly (but not significant) lower protein content in cheeses supplemented with liposome-encapsulated enzymes. Even though this difference was not found to be significant, *table I* shows the same trend, which suggests a slight, early proteolysis, inducing a decrease in protein retention in the curd. This observation is consistent with the lower dry matter content ($P < 0.05$) in cheeses containing liposomes (*table I*). An increase in moisture in liposome-treated cheeses has been reported [18, 28]. In this study (*table I*), cheeses treated with empty liposomes showed an increase in moisture similar to those treated with liposome-encapsulated CFE ($P < 0.05$). It is thus possible that liposome membranes bind water at their surface. Another hypothesis could be that the syneresis process is influenced by the presence of liposomes in the curd.

3.3 Soluble nitrogen

Ripening time was the most significant factor ($P < 0.001$) influencing changes in WSN, TCA-SN and PTA-SN (*figure 1*). The linear effect of time ($P < 0.001$) always accounted for most of the variability in this factor. Although changes in WSN with time were similar for the four different cheeses, differences between L1, L2 and L0 as well as NL cheeses became significant ($P < 0.01$) only after two months (*figure 1a*).

Compared to L0 and NL cheeses, the highest WSN in L1 and L2 cheeses, after two months of ripening, could be due to greater peptidolysis which probably increased the amount of WSN. Another explanation could be the stimulation of the proteolytic indigeneous microflora by a higher availability of free amino acids, resulting in an increase in WSN.

The rate of TCA-SN and PTA-SN production depended on the treatment that was applied to the cheese ($P < 0.01$). Although the increase of TCA-SN was similar for the four cheeses during the first two weeks, it subsequently became higher in L1 and L2

Table I. Chemical composition of one-month old experimental cheeses.
Tableau I. Composition des fromages expérimentaux après un mois d'affinage.

Cheese	Dry matter (%)	Ash (%)	Fat (%)	Protein (%)	pH
NL	60.5 (0.6†) a*	2.6 (0.1) a	32.4 (1.2) a	23.5 (0.7) a	5.1 (0.1) a
L0	57.9 (0.6) b	2.6 (0.1) a	32.0 (1.1) a	22.6 (0.6) a	5.0 (0.1) a
L1	56.8 (0.6) b	2.4 (0.1) a	32.0 (1.1) a	21.3 (0.6) a	5.0 (0.1) a
L2	57.1 (0.7) b	2.3 (0.1) a	32.1 (1.1) a	21.1 (0.6) a	5.1 (0.1) a

NL, control cheeses; L0, cheeses with empty liposomes; L1, cheeses with liposome-encapsulated cell-free extracts (CFE solution: 0.6 g·L^{-1} protein); L2, cheeses with liposome-encapsulated cell-free extracts (CFE solution: 1.3 g·L^{-1} protein). Data are means of duplicate determinations on two replicates. *In the same column, results with different letters are significantly different ($P < 0.05$). †Standard deviation.
NL, fromages témoins ; L0, fromages avec liposomes vides ; L1, fromages avec liposomes contenant des extraits cellulaires (0,6 g·L^{-1}) ; L2, fromages avec liposomes contenant des extraits cellulaires (1,3 g·L^{-1}). Les données sont la moyenne de deux duplicata effectués sur deux répétitions. *À l'intérieur d'une même colonne, les valeurs associées à une lettre différente sont significativement différentes ($p < 0,05$). †Écart type.

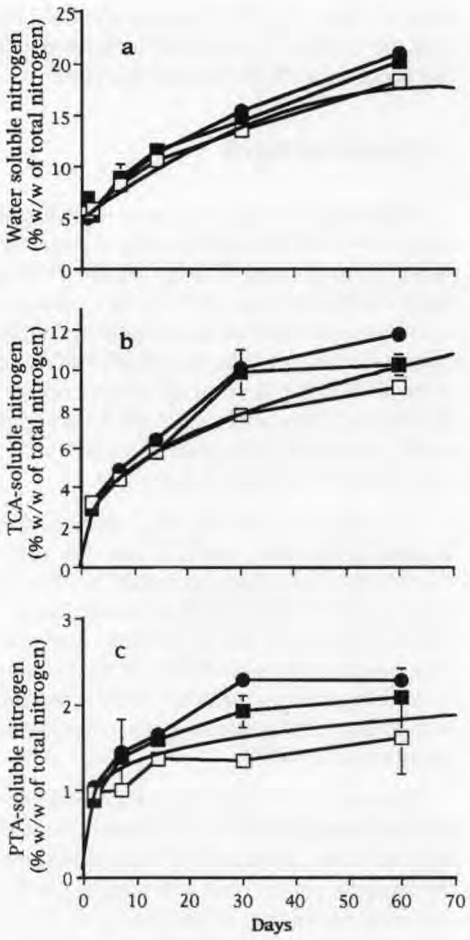

Figure 1. Production of water-soluble nitrogen (**a**), TCA-soluble nitrogen (**b**) and PTA-soluble nitrogen (**c**) during ripening. NL (—); L0 (□); L1 (■); L2 (●) cheeses. The response for the control cheeses (NL) was represented by the best fitted curve for water-soluble nitrogen (r = 0.996), TCA-soluble nitrogen (r = 0.998); PTA-soluble nitrogen (r = 0.957).

Figure 1. Production d'azote soluble dans l'eau (**a**), d'azote soluble dans le TCA (**b**), d'azote soluble dans le PTA (**c**) au cours de l'affinage. Fromages NL (—) ; fromages L0 (□) ; fromages L1 (■) ; fromages L2 (●). Les courbes des fromages témoins (NL) ont été construites à l'aide de modèles ayant le meilleur coefficient de corrélation pour l'azote soluble dans l'eau (r = 0,996) ; l'azote soluble dans le TCA (r = 0,998) ; l'azote soluble dans le PTA (r = 0,957).

cheeses than in NL and L0 cheeses ($P < 0.05$) (*figure 1b*). The same phenomenon was observed for PTA-SN changes, particularly in L2 cheeses (*figure 1c*).

After two months of ripening, values for WSN, TCA-SN and PTA-SN were, respectively, 20, 22 and 35 % higher in L2 cheeses than in NL cheeses ($P < 0.001$). These results show that cell-free extracts of *Lb. casei* UL137 significantly increased the hydrolysis of large peptides into smaller peptides and free amino acids, which confirms their high potential for peptidolysis [6]. This strain clearly exhibited a wide spectrum of peptidase activities with a particularly high level of aminopeptidase activity, which was active for up to two months. This could explain why liposome-encapsulated CFE was detected only after two weeks of ripening. At this stage of ripening, large quantities of peptides resulting from the hydrolysis of α_{s1}-casein [20] were available for hydrolysis by CFE peptidases.

3.4. Level of bitterness

It should be pointed out that cell-free extracts of *Lb. casei* UL137 are especially well adapted for accelerating cheese ripening because of their high debittering activity, which has been correlated to a high level of aminopeptidases [6]. HPLC analyses were carried out to verify the possible presence of bitter peptides in cheeses (*figure 2*). The bitter fractions extracted from NL cheeses and cheeses treated with encapsulated CFE (L2) showed the same pattern. The profiles had the same number of peaks but their shape was different. The first peak (elution time 24.5–27 min) corresponds to the largest peptides (~5 000 to 2 000 Da), and was higher in liposome-treated cheeses than in the control cheeses (*figure 2*). Lemieux et al. [21] did not identify bitterness in this first peak. The peak with an elution time of 31–34 min (~180 to 110 Da) corresponds to free amino acids, which were present at higher concentrations in liposome-treated cheeses than in control cheeses. As

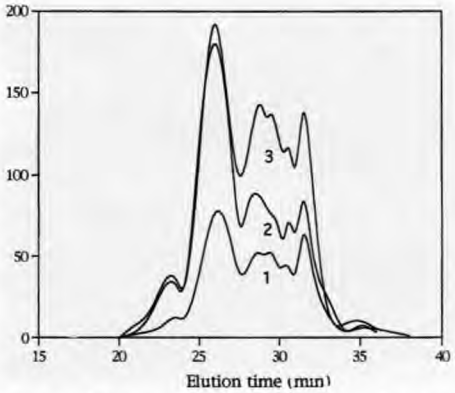

Figure 2. Elution profiles of bitter fractions extracted from NL cheeses after one month of ripening (1); L2 cheeses after one month of ripening (2); L2 cheeses after two months of ripening (3).

Figure 2. Profils d'élution des fractions amères extraites des fromages NL après un mois d'affinage (1) ; des fromages L2 après un mois d'affinage (2) ; des fromages L2 après deux mois d'affinage (3).

well, liposome-treated cheeses contained more free amino acids after two months than after one month of ripening. This confirms the high peptidolytic activity of *Lb. casei* UL137 cell-free extracts. The peak with an elution time between free amino acids and large peptides corresponds to the fraction containing most of the bitter peptides [21]; this fraction was about 2.7 times higher in L2 than in the control cheeses after two months of ripening.

3.5. Microstructure of cheeses

Electron micrographs of NL and HL cheeses are shown in *figures 3* and *4*, respectively. Differences were apparent as early as the first week of ripening. In liposome-treated cheeses, the granular aspect of the casein matrix was more visible than in the control (NL) cheeses, suggesting a less dense structure. This phenomenon was especially apparent at the periphery of fat globules, where sheets and threads systematically interrupted the cohesiveness of the casein matrix.

In cheeses treated with immobilized enzymes, some black spots appeared around the fat globules (*figure 4*) or in the inner face of the fat globule membranes (*figure 8*). Since these inclusions were not observed in NL and L0 cheeses, they could be, at least partially, made up of liposome material. As the external surface of the fat globule membrane is rich in polar groups, some CFE elements may have interacted and bound to these groups.

The addition of high quantities of liposomes has been shown to induce a great alteration in the microstructure of HL cheeses (*figures 4* to *7*). However, apart from the black spots observed, cheeses containing low amounts of liposomes (L0, L1 and L2 cheeses) showed a microstructure similar to control cheeses (micrographs not shown). In this case, the quantity of liposomes added was insufficient to cause any detectable change in the observed microstructure.

3.6. Determination of liposome location in the cheese microstructure

Electron microscopic observations of

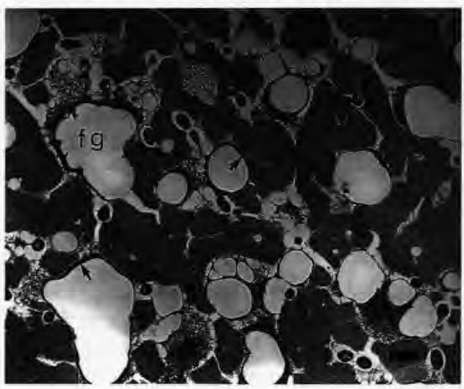

Figure 3. Microstructure of NL cheeses after one week of ripening. Bar represents 2 µm. fg: fat globule; cm: casein matrix; arrow: fat globule membrane.

Figure 3. Microstructure des fromages NL après une semaine d'affinage. La barre représente 2 µm. fg : globule de matière grasse ; cm : matrice caséique ; flèche : membrane de globule de matière grasse.

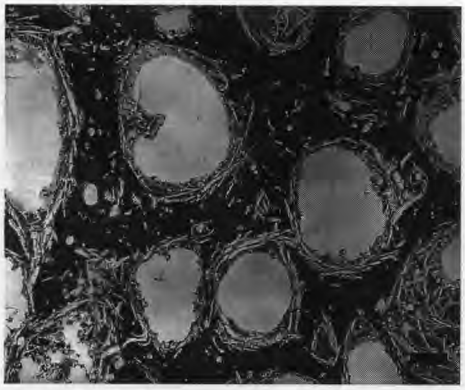

Figure 4. Microstructure of HL cheeses after one week of ripening. Bar represents 2 µm.

Figure 4. Microstructure des fromages HL après une semaine d'affinage. La barre représente 2 µm.

Figure 5. Microstructure of casein matrix in HL cheeses after one day of ripening. Bar represents 200 nm; arrows point to liposomes.

Figure 5. Microstructure de la matrice caséique de fromages HL après une journée d'affinage. La barre représente 200 nm ; les flèches désignent les liposomes.

liposomes in the cheeses showed that after one day of ripening, liposomes appeared to be either grouped together in the casein matrix or dispersed around the fat globule membrane (*figures 5* and *6*, respectively). In *figure 5*, a cluster of small vesicles (about 50–150 nm) appears to be surrounded by a bilayer membrane. The inside of the vesicle was slightly grey, which suggests that these small vesicles still retain part of their protein content, which means that CFE was still not completely released. *Figure 6* corresponds to the periphery of fat globules, where vesicles were generally larger than those observed in the casein matrix and where only a few vesicles were undamaged. Most of the bilayer membranes appeared to have fused with one another to give either larger empty vesicles or large sheets or cylinders. This observation and the phenomenon of black spots in the fat globule membranes suggest that liposomes were greatly destabilized in the periphery of fat globules as soon as the first day of ripening. The affinity of liposomes for the periphery of fat globules has been observed previously [12]. In the latter study, liposomes that were confined to areas between the fat globules and the casein matrix were completely excluded from regions of the casein matrix. These authors also reported extensive destabilization of liposomes composed of phosphatidylcholine, suggesting some type of interaction between liposomes and milk fat globule membranes. The results presented in this work tend to confirm this hypothesis. The whey pockets around fat globules would constitute a favored environment for the retention of liposomes. This observation supports the results of a recent study that determined the location of starter bacteria in cheese curd [17]. In the latter study, bacteria were shown to be located essentially in whey pockets around fat globules.

This work is the first to report the presence of liposomes within the casein matrix (*figure 5*). This original observation could be explained by the fact that Pro-Lipo® S contained 80 % of phosphatidylcholine and more than 15 % of polar lipids. The vesicles were slightly negatively charged and could therefore interact with ionic groups that were available in the casein matrix.

After two months of ripening, it was still possible to observe vesicles inside the casein matrix (*figure 7*). They were generally larger and less colored than those observed

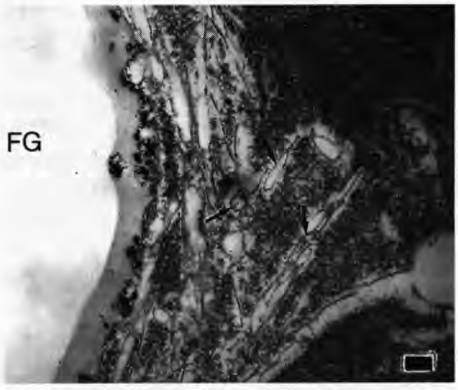

Figure 6. Liposomes (arrows) in the periphery of fat globules (FG) after one day of ripening. Bar represents 200 nm.

Figure 6. Liposomes (flèches) à la périphérie des globules de matière grasse (FG) après une journée d'affinage. La barre représente 200 nm.

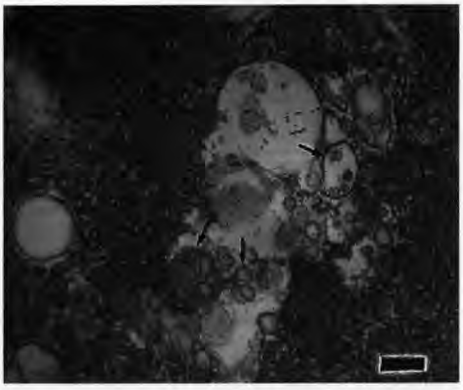

Figure 7. Liposomes (arrows) in the cheese curd after two months of ripening. Bar represents 200 nm.

Figure 7. Liposomes (flèches) dans le caillé fromager après deux mois d'affinage. La barre représente 200 nm.

after one week of ripening. These observations suggest that they released a part of their contents. The casein matrix surrounding the liposome clusters was generally less dense, suggesting a possible hydrolysis of the proteins and peptides by enzymes released from the liposomes. At the periphery of fat globules, very different features were observed (*figure 8*). The fusion of liposome membranes observed in *figure 6* appeared to be amplified, and fused membranes folded spirally to form cylinders in a casein matrix that was weakened by the proteolytic action of proteinases in the liposomes. This type of membrane deformation has been observed by Papahadjopoulos et al. [22]. These authors showed that the addition of Ca^{2+} to preparations containing small spheroidal vesicles led to the formation of large and apparently multilamellar structures, many of which were cylindrical in shape. They proposed the term 'cochleate' for these structures.

The spiral form of liposome membranes observed in the cheeses after two months of ripening suggests the following hypothesis. It is known that pockets of residual

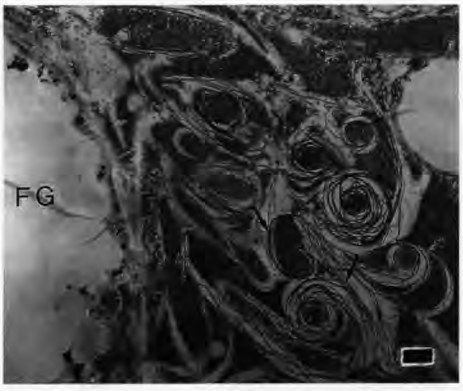

Figure 8. Liposome membranes (arrows) in the periphery of fat globules (FG) after two months of ripening. Bar represents 500 nm.

Figure 8. Membranes liposomales (flèches) à la périphérie des globules de matière grasse (FG) après deux mois d'affinage. La barre représente 500 nm.

whey are located between the fat and casein phases [3]. The same authors showed that crystalline inclusions appear in these regions during ripening, and these inclusions were identified as calcium lactate or calcium phosphate. The periphery of fat globules is

probably rich in calcium and the spiral form of liposomes could be the same cochleates that were observed in vitro [22]. Therefore, calcium would be a major destabilization factor of liposome structure in cheese curd during ripening.

3.7. Cheese rheology

Firmness, brittleness, cohesiveness and springiness of cheeses are presented in *figure 9*. All these parameters were generally influenced by the presence of liposomes and the concentration of encapsulated CFE ($P < 0.01$).

Firmness was found to be lower ($P < 0.05$) in L2 and L1 cheeses than in L0 and NL cheeses (*figure 9a*). During the first two weeks of ripening, the presence of CFE (L1 and L2 cheeses) was the major factor that influenced cheese firmness. However, this influence seemed to become weaker between the second and fourth weeks of ripening. After one month, the four cheeses were characterized by the same firmness, which then increased up to two months in NL and L0 cheeses. However, after two months, L0 cheeses were softer than NL cheeses ($P < 0.05$), showing that the presence of liposomes could affect the rheological properties of cheeses. In L1 and L2 cheeses, firmness decreased significantly ($P < 0.05$) between one and two months. It is possible that an extensive proteolytic activity by the CFE, originally immobilized in liposomes, could account for this loss in firmness. Picon et al. [24] observed a lower

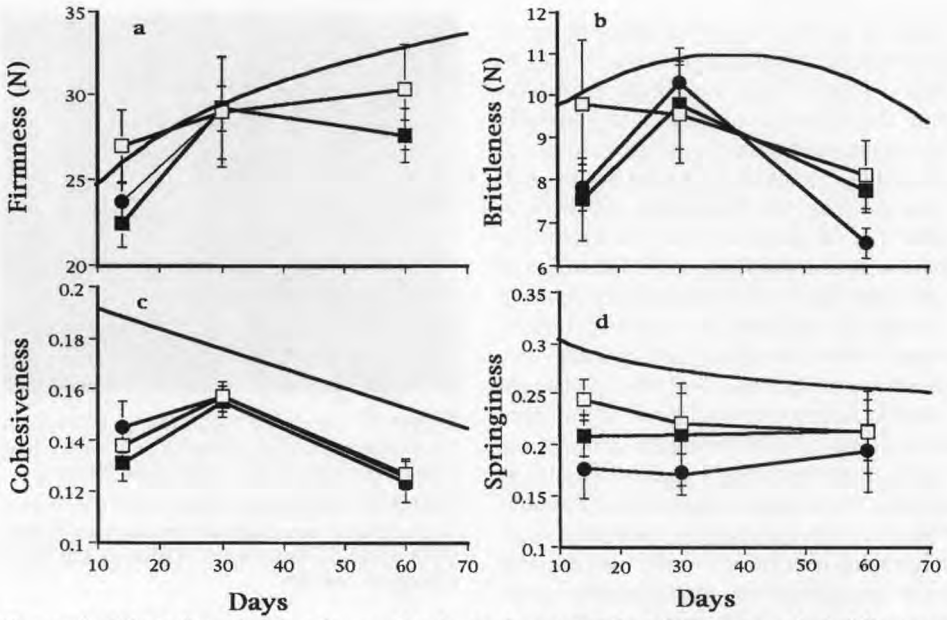

Figure 9. Effect of the ripening time on the rheological properties of NL cheeses (—); L0 cheeses (□); L1 cheeses (■); L2 cheeses (●). The response of the control cheeses (NL) were modelized with the best-fitted curve for firmness (r = 0.998); brittleness (r = 1.0); cohesiveness (r = 0.992); springiness (r = 0.994).

Figure 9. Effet de la durée d'affinage sur les propriétés rhéologiques des fromages NL (—) ; des fromages L0 (□) ; des fromages L1 (■) ; des fromages L2 (●). Les courbes des fromages témoins (NL) ont été construites à l'aide de modèles ayant le meilleur coefficient de corrélation pour la fermeté (r = 0,998), la friabilité (r = 1,0), la cohésion (r = 0,992) et l'élasticité (r = 0,994).

hardness in cheeses treated with liposome-encapsulated enzymes but did not obtain any decrease in hardness until two months of ripening.

The changes in brittleness (or fracturability) during ripening were similar to firmness (*figure 9b*). After two weeks of ripening, the presence of CFE was the major factor affecting this parameter ($P < 0.01$). After two months, the presence of liposomes appeared to be as important as the presence of CFE. The brittleness of L0, L1 and L2 cheeses was significantly different ($P < 0.001$) from that of NL cheeses. Both CFE and liposomes contributed to the decrease in the resistance of cheeses to external forces.

The data for cohesiveness confirm this phenomenon (*figure 9c*). The cohesiveness of cheeses was greatly affected by the introduction of liposomes. Between 2 and 8 weeks of ripening, liposome-treated cheeses were significantly less cohesive ($P < 0.001$) than control cheeses. This parameter was not affected by the introduction of CFE. Larivière et al. [18] also reported a decrease in cohesiveness and fracturability in cheeses containing liposomes.

Cheese springiness appeared to be influenced by the addition of either encapsulated CFE or empty liposomes ($P < 0.001$) (*figure 9d*), especially at the beginning of ripening. After two months of ripening, the most significant factor was again the presence of liposomes.

The presence of liposomes (whether they contained CFE or not) has been shown to influence the rheological parameters of cheese. These results could be related to the higher moisture content of liposome-treated cheese. In the range of moisture content of Cheddar cheese, an increase in moisture could favor the activity of endogeneous ripening enzymes. However, the rate of proteolysis in L0 cheeses was not significantly different from that of NL cheeses. However, Visser [31] found that increases in moisture led to a decrease in the compression modulus E (a measure of the stiffness) and in the fracture stress of Gouda cheeses. The increase in moisture observed in liposome-treated cheeses could then explain the difference in rheological properties observed in such cheeses compared with control (NL) cheeses. Moreover, the determination of liposome location in the cheese microstructure showed that the periphery of fat globules was weakened by the presence of liposome membranes, which lead to a less cohesive and a less elastic structure. Microstructural and rheological analyses were highly correlated, confirming that the presence of liposomes in the cheese microstructure can modify the textural properties of cheese.

4. CONCLUSION

The encapsulation of cell-free extracts in liposomes obtained from Pro-Lipo® was simple and efficient in terms of liposome retention in cheese curd.

Liposome stability in cheese curd was dependent on the interaction between liposomal bilayers and milkfat membranes. Vesicles that were located close to the milkfat globules were destabilized as early as the first day of ripening. After two months of ripening, liposomal bilayers showed a spiral form, probably due to an interaction with calcium.

The release of cell-free extracts from liposomes caused an increase in proteolysis, mainly peptidolysis. However, the presence of liposomes (even without any encapsulated material) resulted in some changes in rheological properties of cheeses.

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