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RESEARCH ARTICLE

Microbiological and physicochemical changes during ripening of Camembert cheeses made from raw and pasteurized cow milk produced in Tizi-Ouzou (north of Algeria)

Hillal Sebbane[1], Dalila Almi[1], Sonia Hadouchi[2], Louiza Hedjel[2], Noria Smail-Saadoun[3] and Abderrahmane Mati[1]

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Abstract: This study was carried out on two types of Camembert produced in the region of Tizi-Ouzou (northern Algeria), one artisanal (AC) made from raw milk and the other industrial (IC) made from pasteurized milk. This work shows the effect of milk quality and cheese making processes on the progress of physicochemical and microbiological parameters throughout a maturation period of 12 days. The result shows that the repining microflora and undesirable microbial populations were significantly higher ($P < 0.05$) in the artisanal Camembert (AC) than in the industrial Camembert (IC). The physicochemical and microbiological parameters, during the ripening of the cheeses developed in a similar way with significant differences according to the type and the stage of maturation. During the cheese repining period, AC showed more extensive ($P < 0.05$) lipolysis and proteolysis than IC. SDS-PAGE of water-soluble proteins (WSP) and insoluble fractions showed more extensive degradation of αs-casein (αs-CN) than β-casein (β-CN). The WSP profile, analyzed by Reversed-Phase High Performance Liquid Chromatography (RP-HPLC), was highest in AC than IC. The highest WSP profile was recorded at the 12th day of repining. The pathogenic flora decreased during the maturation process in AC. This development was confirmed by the results of the antibacterial effect of WSP, performed by the disc diffusion technique on *Escherichia coli* and *Staphylococcus aureus* strains. From this study, it can be concluded that AC has a better organoleptic quality, safe for the Algerian consumer and more profitable for the Algerian cheesemakers

Keywords: Algeria, Camembert, Cheese ripening, Lipolysis, Proteolysis, Raw and pasteurized milks

Introduction

Since the early 1990s, Algeria has implemented a dairy policy in order to enhance the value of local production from cattle breeding (Mamine et al. 2011). With the launch of the National Agricultural Development Plan (NADP) in 2000 and its extension to rural areas, total milk production increased from 1.9 billion liters in 2004 to 3.3 billion in 2018, covering about 60% of national milk needs. This production, even if it faces an insufficient collection circuit (Kali et al. 2011), has enabled some artisanal dairy farmers to process milk into raw milk cheese production. Nevertheless, this approach is quite innovative in Algeria and the microbiological and physicochemical characterization of raw milk cheeses is attracting the interest of public authorities and researchers (Aissaoui et al. 2011; Meribai et al. 2017). Indeed, hygiene requirements have become an important consideration in the production of artisanal cheeses, particularly those with a protected designation of origin (PDO), which are produced with raw milk, such as Camembert de Normandie (France), often produced in mountain areas (Gérard, 2015).

Traditional cheese making, especially for the Camembert, is distinguished from the industrial production by various aspects, and the better organoleptic qualities are obtained with the raw milk (Richard and Zadi 1983). Organoleptic properties are mainly influenced by the growth of lactic acid bacteria and the combined effect of proteolysis and lipolysis on the cheese matrix during the ripening process (Gebreyowhans et al. 2020). The release of bacteriocins and bioactive peptide fractions, endowed with antibacterial activities, inhibit bacterial pathogens (Mane and McSweeney, 2019), such as *Listeria ssp.* in the case of Camembert (Wan et al. 1997).This is how we set out to evaluate the quality of two soft cheeses, one made from raw milk and the other from pasteurized milk. We particularly explored the physicochemical

[1]Laboratory of Analytical Biochemistry and Biotechnology (LABAB), Mouloud Mammeri University, Tizi Ouzou, Algeria

[2] Faculty of Biological Sciences and Agronomic Sciences, Mouloud Mammeri University, Tizi Ouzou, Algeria

[3]Natural Resources Laboratory, Mouloud Mammeri University, Tizi Ouzou, Algeria

Hillal Sebbane (✉)
Laboratory of Analytical Biochemistry and Biotechnology (LABAB), Mouloud Mammeri University, Tizi Ouzou, Algeria
Email: hillalmicrobio@yahoo.com

and microbiological characteristics of these cheeses at different stages of the ripening process and demonstrate the safety of raw milk cheeses, especially Camembert, in Algeria. To the best of our knowledge, this is the first study to assess the antimicrobial effect of water-soluble peptide fractions extracted from Camembert.

Materials and Methods

Sampling procedure and cheesemaking

The samples of milk used for the production of Camembert, came from two farms in the region of Tizi-Ouzou. The farms are made up of cows of dairy cattle breeds (Holstein and Montbéliard), with an average age of 4 to 8 years and they are at the same stage of lactation. The maturing period is set at 12 days, with reference to Algerian industrial practices. Two types of Camembert, one artisanal, the other industrial, produced in the region of Tizi-Ouzou (northern Algeria), were selected within the framework of this study. The analyses were based on 108 samples from each Camembert type and on 27 samples from each kind of milk (raw and pasteurized). The sampling of the products were carried from March to June during the period 2014-2016, in order to avoid the seasonality effect on the physicochemical and microbiological composition of the milk. Three samples per month were collected from each milks (raw and pasteurized milk) and cheeses at four ripening stages: first, fourth, sixth and twelfth days (Table 1). Placed in a cooler, the samples were directly taken to the laboratory and the analyses were performed during the following 24 hours.

The moulding of the cheeses (AC and IC) was performed into polyurethane perforated moulds (cylinders 10 to 11 cm in diameter and 13 to 14 cm in height). Demoulding of cheeses (AC and IC) is carried out when the lactic acidity of the curd reaches values of 9.5 to 11% (w/w) and a pH of 4.5 to 4.9.

The artisanal Camembert (AC), made from raw milk, was produced in the locality of Ouacif (Tizi-Ouzou, Algeria) by an artisan cheesemaker breeding dairy cattle under extensive holding conditions and transforms milk to Camembert according to the guidelines of the French legislation related to cheeses and cheese specialties (Art. 9 and 14 of the decree n° 2013-1010 of the 12th of November 2013). Milk was heated up to 35 °C till it reaches an acidity of 22 to 25 °D (2.2 to 2.5 g/l of lactic acid) or at pH 6.30 to 6.35, before a liquid rennet (standard strength of 1:10000, Halal calf Rennet, Chr. Hansen Inc., Danemark) was added (15-20 ml/ 100 L of milk). After coagulation (1h10 min-1h30 min), the moulding of the cheese was performed directly with a ladle into polyurethane perforated moulds, without stirring the curd, at a rate of 5 ladles per mould, with a rest period of 15 to 20 minutes between each ladle, without stirring the coagulum. Draining was done on stainless drains (24 to 48 h, 25-28 °C) at a relative humidity (RH) of 95%, turning the cheese every five hours, before it was worked with sprinkling of fine salt. Then, the cheese (AC) was sprayed with a solution of *Penicillium camemberti* spores ($2x10^4$ spore/ml) and ripened during 12 days at a temperature of 10 to 12 °C and a RH of 90 to 95%, with a rotation every two to three days.

The industrial Camembert (IC), made from pasteurized milk, was produced in a cheese dairy located in Draa Ben Khedda (Tizi-Ouzou), which was supplied with milk from a close locality (Freha, Tizi-Ouzou). Without undergoing any standardization of fat and protein contents, milk is first pasteurized at 90 °C for 20 seconds and then cooled to 36 °C. After adding $CaCl_2$ (15-20 g/100 L of milk), milk was seeded with mixed lactic ferments (Mesophilous: DI-PROX M 229, BioProx, France/Thermophilous: DI-PROX TPM 2, BioProx, France) with 0.03% of fungal flora (Chr. Hansen Inc., Danemark) of *Penicillium camemberti* and *Geotricum candidum* in order to reach the final concentrations of 10^4 spores/ml and $2x10^4$ spores/ml, respectively. After the fermentation period of milk (20-25 minutes at 36°C), the pH reached a value of 6.35, commercial rennet fungal powder Marzyme™ (standard strength of: 150000, Danisco. France) was added (1.5 g/50 Kg of milk), then the coagulum is divided into small cubes (Ø = 30 mm). To accelerate draining, the coagulum was stirred twice during 15 minutes with an interval of five minutes. The whey was evacuated by pumping it out, then the moulding was carried out by

Table 1 Distribution of the samples according to the kind of the milk and the ripening stage

Sample	Ripening days	Sampling plan									Total number of samples
		1st year			2nd year			3rd year			
		1st month	2nd month	3rd month	1st month	2nd month	3rd month	1st month	2nd month	3rd month	
Artisanal or industrial camembert	1	3	3	3	3	3	3	3	3	3	27
	4	3	3	3	3	3	3	3	3	3	27
	6	3	3	3	3	3	3	3	3	3	27
	12	3	3	3	3	3	3	3	3	3	27
Total samples of Camembert at all stages of cheese repining											108
Raw or pasteurized milk		3	3	3	3	3	3	3	3	3	27

mechanically filling the moulds. The coagulum is drained in polyurethane molds placed on stainless drains during 15 to 20 hours at RH of 90 to 95% (27 °C-5 h and 20 °C-15 h), with rotations every five hours. After it was removed from the mold, the cheese was salted by brining (NaCl = 40%, pH = 4.5) during 30 to 60 minutes at 13 °C. The cheese surface was dried in a draining room during 15 hours (14 °C, RH = 85 %) with two rotations. Then, it was sprinkled with a suspension of *Penicillium camemberti* spores (2.10^9 Spore/ml) (Chr. Hansen Inc., Danemark) and ripened during 12 days (12-13 °C, RH = 90-95 %) with a rotation every two to three days.

Physicochemical analysis

Physicochemical parameters during cheese ripening were analysed after removing the cheese crust. Samples of milk were analyzed with Lacto-scan SP (Milkotronic LTD, Bulgaria) for density, fat and proteins). The pH was measured by immersing the pH-meter glass electrode (Hanna-instrument, Italy) directly in the products (cheeses and milks) (Bouton et al. 1994).The titratable acidity (TA) of the milk was expressed in Dornic degree (°D) (AFNOR, 1980) and as % of lactic acid for the cheese (Sulieman et al. 2012) The dry-matter (DM) was determined using an infrared desiccator IR35 (Denver instrument, Germany) by evaporation (105°C/20 min) of 3 g of milk (AFNOR, 1980) or 5 g of cheese (Randoin and Jourdan ,1952). The Fat content of cheese was determined by Van-Gulik butyrometer (Funkgerber Instrument, Germany) (JORADP, 2014).

Protein fractionation

Protein concentration was determined using the Lowry protein procedure at 750 nm with Folin-Ciocalteu's phenol reagent (Lowry et al. 1951). Bovine Serum Albumin was used as a standard. From slurry prepared from by mixing 20 g of cheese (crust removed and crushed) in 40 ml of sodium citrate buffer (0.5 M, pH 7), three protein fractions, which are the total protein content (TP), the acid soluble protein (ASP) and the non-protein nitrogen (NPN), were obtained by combining the methods reported by Gripon et al. (1975) and Guerra-Martinez et al. (2012).

Free amino acids

Total levels of free amino acids (FAA) in the pH4.6-Soluble fractions of the cheese were determined by the 2, 4, 6, trinitrobenzene-1-sulfonic acid (TNBS) method described by Polychroniadou (1988) modified by Bouton et al. (1993). Cheese extracts were prepared, as mentioned above. The use of a range of glycine standard (0.01-0.5 mM) allowed expressing the measurements in mEq glycine/g of protein.

SDS–polyacrylamide gel electrophoresis (SDS-PAGE)

Proteolysis during cheese ripening from the water-soluble proteins (WSP) and insoluble protein fractions at pH 4.6 was evaluated by SDS-PAGE (Barac et al. 2016). The fractions were prepared according to the techniques reported by Dupas et al. (2009).The PAGE-SDS was performed according the method described by Laemmli (1970). A marker kit was for bovine serum albumin (67 kDa), ovalbumin (45 kDa), β-Lactoglobulin (18 kDa) and α-Lactalbumin (14 kDa).

Reversed-phase high performance liquid chromatography (RP-HPLC) of the cheese solutions water-soluble fractions

The proteolysis during cheese repining was also assessed by RP-HPLC. Samples of WSP (3 mg/ml) were prepared in a Bis-Tris

Table 2 Milk and cheese microbial flora enumerated during ripening

Microflora	Culture medium	Incubation
Aerobic mesophilic flora (AMF)	PCA-Agar	30°C/24-72 h
Total coliforms (TC)	VRBL agar	30°C/24-48 h
Faecal coliforms (FC)		44°C/24-48 h
Faecal streptococci (FS)	BEA (bile esculineazide) agar followed by catalase and Gram reactions, growth at 37° C and at 6.5% NaCl in BHI	37° C/24-48 h
Mesophilic lactobacilli (MLAB)	MRS agar followed by a catalase test and a Gram stain	30°C/24-48 h
Mesophilic lactic *Streptococcus* (MLS)	M17 agar followed by a catalase test and a Gram stain	30°C/24-48 h
Yeast and molds (Y-L)	OGA agar	22°C/3-7 d
Staphylococcus aureus	Presumtive testing: Giolitti-Cantoni broth Confirmatory test: Baird Parker agar with the coagulase test	37°C/48 h
Salmonella spp. (samples:25 ml for milk or 25 g for cheese)	Enrichment on SFB broth and isolation on Hektoen agar	37 °C/48 h

buffer with a pH 8 and filtred through 0.45 µm nylon filters (Sartorius, Germany). Twenty µl of filtrate was injected in a Lichrosorb C8 column (5 µm, 125x4, 1mm). The mobile phase was constituted by a solvent A (10% acetonitrile, 0.1% trifluoroacetique acid: TFA) and a solvent B (60% acetonitrile, 0.02 % TFA). The elution was performed using a 0-50% binary gradient of the solvent B during 50 minutes with a flow rate of 0.2 ml/min at 35 °C. The detection was performed under UV at 214 nm.

Lipolysis

The lipolysis was assessed by measuring the amount of free fatty acids (FFA_s) according to the method described by Deeth et al. (1975). The FFAs were expressed in equivalent of oleic acid per 100 g of fat, referring to a standard curve of oleic acid (0.10-10 mg/ml).

Microbiological analysis

Milk and cheese microflora during ripening were determined by the conventional techniques for which the incubation conditions and the medium used are reported in Table 2. Cheese samples were collected at 1, 6 and 12 days of repining. The dilutions were prepared either directly from milk or from cheese prepared according the method described by Lenoir (1963) after some modifications. After removing the crust, 10 g samples of cheese were homogenized in a Stomacher 400 (Seward Medical, Londres, UK) with 90 ml of sterile sodium citrate (2%) at 40 °C. Decimal dilutions of milk and cheese solution were prepared with Ringer's solution. Germ counts were expressed according to the formula of Joffin and Joffin (1999).

Anti-microbial activity of water-soluble extract

The raw water-soluble extracts (WSE) were extracted at the 12th day of cheese ripening, as mentioned above (fractionation of WSP), without acidification. In order to eliminate the antagonistic effects of the pH and H_2O_2 , the WSE was neutralized to pH 6.8 with NaOH (2N) and after addition of few drops of catalase (1 mg/ml),it was incubated for one hour at 30 °C (Sahraoui et al. 2015). The raw WSE and the neutralized WSE were filtered through 0.22 µm filters .The agar-disc diffusion assay was performed according to protocol reported by Motta and Brandelli (2002). The antibacterial activity was tested against *Escherichia coli* ATTCC 25922 and *Staphylococcus aureus* ATTCC 25923. All the strains were standardized by spectrometry (620 nm) at an optical density (OD) of 0.08-01, according to the Antibiogram Committee CASFM/EUCAST (2019).

Aliquots of 20 µl were applied on five discs (6 mm): two discs of the raw WSE, two discs of neutralized WSE and one disc with broth (as negative control). The Petri dishes were placed in a refrigerator at 4 °C for two hours. The antimicrobial effect of WSE (expressed in mm) was examined after incubation for 24 hours under appropriate culture conditions. Three replicates were performed on different dates.

Statistical analysis

The physicochemical composition and microbiological counts, between raw milk and pasteurized milk, were assessed by the Student's t-test (5% level of significance). An Analysis of variance (ANOVA), using the Tukey test for pair wise comparison of means, was performed (significant level of 5 %), to follow the changes in the physicochemical and microbiological parameters during cheese ripening. A principal component analysis (PCA) was run in order to analyze the effect of the ripening stage on the physicochemical and microbiological interactions during cheese maturation. The statistical analyses were performed using STATbox software ver. 6.4.

Results and Discussion

Physicochemical characteristics of milk

The milk physicochemical characteristics are reported in Table 3. Among the analyzed parameters, significant difference was only found between the DM and milk TP ($P < 0.05$). The sampling season can affect the physico-chemical and microbiological quality of milk (Spike and Freeman, 1967; Nalepa et al. 2018). The effect of seasonality was discarded in this study, as sampling occurred during the same periods of the year.

Changes in physicochemical parameters during ripening

The statistical analyses of the physicochemical parameters (Table 4) showed significant differences ($P < 0.05$) depending on the stage of ripening and the type of cheese.

Table 3 Physicochemical compositions of the milk

Composition	Raw milk	Pasteurized milk
pH	$6.69^{a} \pm 0.02$	$6.66^{a} \pm 0.06$
TA (°D)	$17^{a} \pm 0.93$	$15.66^{a} \pm 0.41$
Densité	$1.03^{a} \pm 0.01$	$1.027^{a} \pm 0.7$
DM (g/l)	$122.1^{b} \pm 0.3082$	$108.13^{a} \pm 2.31$
FAT (g/l)	$33.66^{a} \pm 2.16$	$36^{a} \pm 0.61$
TP (g/l)	$29.45^{b} \pm 0.09$	$26.66^{a} \pm 0.41$

Results in the same column for the same parameter with different superscripts are significantly different at $P < 0.05$.

The pH is negatively correlated with titratable acidity between the different cheese types and the ripening stages. From the first to the twelfth day of ripening, the pH values increased from 4.59 and 4.67 (IC) to 5.13 and 5.36, respectively. This is due to the deacidification of the cheese, which is better assessed through measuring the titratable acidity. Indeed, cheese acidity decreased between the first day (9.59% for the artisanal cheese and 9.11% for the industrial) and the last dayof ripening (5.83% artisanal cheese and 6.17% for the industrial**)**

In the case of Camembert the neutralization and deacidification of cheese is due to the assimilation of lactic acid and the releasing of NH_3 by the fungal flora, especially *Penicillium camemberti* and *Geotichum candidum,* which promoted the action of proteolytic enzymes and increased the proteolysis index (ASP%, NPN%, and FAA) (Leclercq-Perlat et al. 2004).

Dry Matter increased during ripening with significant differences between the stages and the cheese types. The values range from 39.40% (AC) and 40.89% (IC) at the first day, to 50.09% (AC) and 49.51% (IC) at the 12th day of ripening. These findings may be attributed to the dehydration during cheese repining. This phenomenon is caused by water loss and exchanges in volatile compounds (ammoniac, fat volatile fatty acids, etc.) between the cheese surface and the ripening room environment (Bertolino et al. 2011). The dehydration of cheese affects the contents of DM, fat and protein during ripening process. Indeed, at the last ripening day, the fat content reached at 25.99% for the AC and 28.75% for the IC and the protein contents attained 15.25% for the AC and 17.20% for the IC.

Progress of proteolysis and lipolysis during ripening

The proteolysis index, ASP/TP (ASP %) and NPN/TP (NPN %) ratios, have been used by many authors as cheese ripening indicators in order to estimate the degree of the proteolysis (Leclercq-Perlat, 2000). Statistical analysis showed significant differences between the types of cheeses and stages of ripening. ASP% increased from the first to the twelfth day of ripening, with the following values respectively: 5.22% to 22.24% for AC and from 4.69% to 17.44% for IC. The non-protein nitrogen values also increased from 0.310% to 0.44% for AC and from 0.22% to 0.28% for IC. The results of the free amino acids contents during cheese repining, revealed significant differences ($P<0.05$) between the cheeses. Free fatty acids increased from 0.12 (first day) to 0.25 (12th day) for AC and from 0.09 (first day) to 0.23(12th day) for IC. The combined effect of the microflora and indigenous enzymes of the raw milk explains the high degree of the proteolysis in AC. The action of intracellular enzymes, released during cell lysis, on the proteolysis of the cheese caseins has been reported by Saboya et al. (2001).The heat treatment of milk slows down cheese proteolysis by modifying the total flora and the proteolysis system during cheese ripening, such as the Camembert (Samelis et al. 2009). The proteolysis activity of the plasmin in ripened cheeses depends on the technology applied in milk processing. The effect of plasmin on caseins of cheese made from pasteurized

Table 4 Changes in physicochemical parameters during ripening of artisanal and industrial cheeses

Physico-chemical parameters	Cheeses	Ripening period (days)			
		1	4	6	12
pH	Artisanal ^{A}	$4.59^{a} \pm 0.38$	$4.60^{a} \pm 0.49$	$4.90^{ab} \pm 0.35$	$5.13^{ab} \pm 0.38$
	Industrial^{B}	$4.67^{ab} \pm 0.53$	$4.94^{ab} \pm 0.42$	$5.28^{ab} \pm 0.4$	$5.36^{b} \pm 0.46$
TA (g /100g of cheese)	Artisanal ^{B}	$9.59^{f} \pm 0.18$	$8.70^{de} \pm 0.43$	$8.06^{cd} \pm 0.42$	$5.83^{a} \pm 0.23$
	Industrial ^{A}	$9.11^{e} \pm 0.64$	$7.90^{c} \pm 0.39$	$7.04^{b} \pm 0.52$	$6.17^{a} \pm 0.22$
DM (g/100 g of cheese)	Artisanal ^{A}	$39.40^{a} \pm 2,49$	$41.84^{a} \pm 4.46$	$48.11^{b} \pm 4.48$	$50.09^{c} \pm 2.57$
	Industrial ^{A}	$40.89^{b} \pm 4.61$	$42.61^{b} \pm 3.41$	$47.11^{cd} \pm 2.59$	$49.51^{d} \pm 2.98$
FAT (g/100 g of cheese)	Artisanal ^{A}	$18.76^{a} \pm 0.82$	$20.15^{a} \pm 0.68$	$23.71^{b} \pm 0.77$	$25.99^{c} \pm 0.44$
	Industrial ^{B}	$22.95^{b} \pm 1.53$	$23.61^{b} \pm 1.92$	$27.11^{cd} \pm 1.59$	$28.75^{d} \pm 1.64$
TP (g/100g of cheese)	Artisanal ^{A}	$10.01^{a} \pm 1,34$	$12.07^{b} \pm 1.55$	$14.19^{c} \pm 0.63$	$15.25^{c} \pm 0.67$
	Industrial ^{B}	$12.17^{b} \pm 0.32$	$14.27^{c} \pm 0.77$	$15.29^{c} \pm 0.85$	$17.20^{d} \pm 0.78$
APS-PT (g/100g of TP)	Artisanal ^{B}	$5.22^{a} \pm 0.55$	$5.52^{a} \pm 0.53$	$12.49^{b} \pm 0.59$	$22.24^{c} \pm 2.87$
	Industrial ^{A}	$4.69^{a} \pm 0.27$	$4.63^{a} \pm 0.33$	$6.97^{a} \pm 0.42$	$17.44^{c} \pm 2.85$
NPN-PT (g/100 g of TP) %	Artisanal ^{B}	$0.31^{b} \pm 0.07$	$0.34^{b} \pm 0.06$	$0.42^{c} \pm 0.03$	$0.44^{c} \pm 0.04$
	Industrial ^{A}	$0.22^{a} \pm 0.03$	$0.22^{a} \pm 0.03$	$0.21^{a} \pm 0.03$	$0.28^{ab} \pm 0.05$
FAA(mEq glyc/g TP)	Artisanal ^{A}	$0.12^{ab} \pm 0.03$	$0.14^{bc} \pm 0,01$	$0.18^{cd} \pm 0.02$	$0.25^{e} \pm 0.02$
	Industrial ^{B}	$0.09^{a} \pm 0.004$	$0.13^{ab} \pm 0.003$	$0.21^{cd} \pm 0.03$	$0.23^{d} \pm 0.014$
FFA (Eq Oleic acid /100g of FAT)	Artisanal ^{B}	$0.90^{a} \pm 0.31$	$2.04^{c} \pm 0.006$	$2.61^{d} \pm 0.38$	$3.19^{e} \pm 0.18$
	Industrial ^{A}	$0.99^{a} \pm 0.11$	$1.22^{ab} \pm 0.14$	$1.28^{ab} \pm 0.007$	$1.40^{b} \pm 0.23$

Results in the same row or column for the same parameter with different superscripts (in upper case or lower case) are significantly different at $P < 0.05$

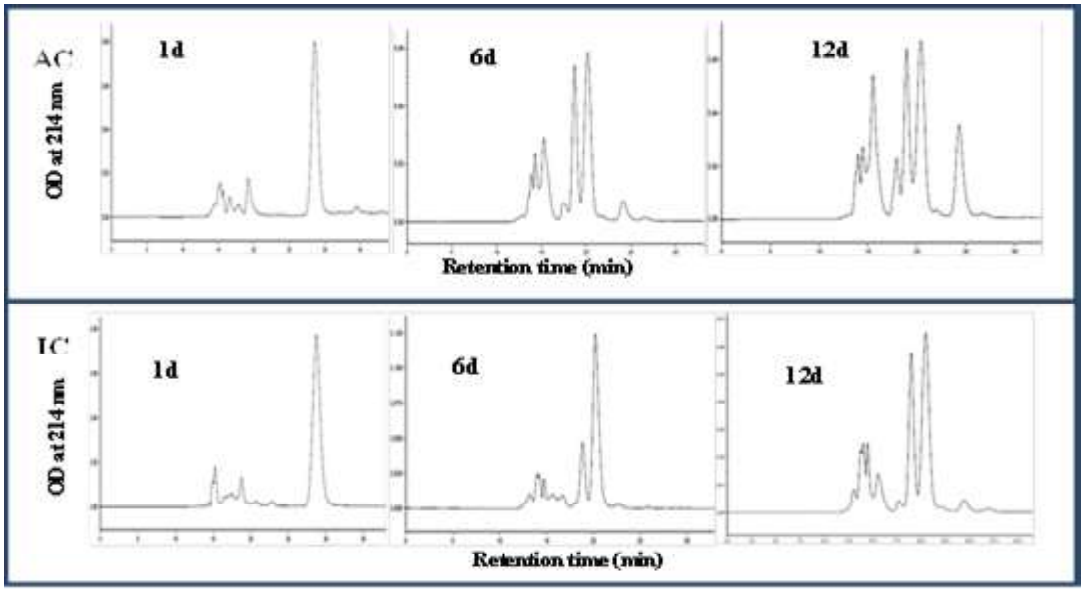


Fig. 1 RP-HPLC water soluble peptide (WSP) fractions from AC and IC at different periods of cheese repining (1, 6 d and 12 days)

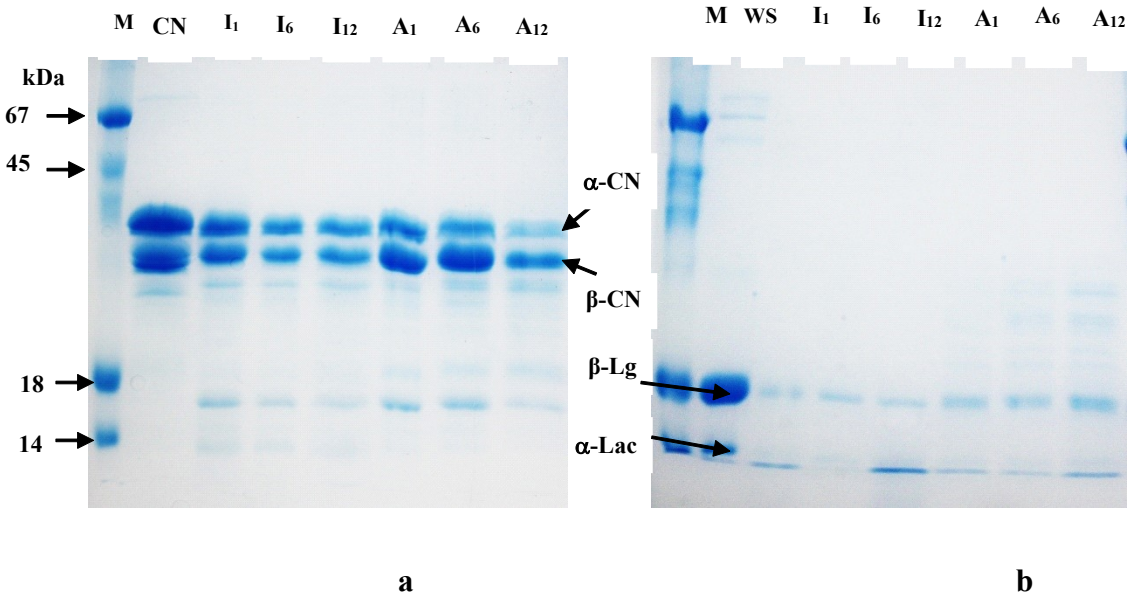


Fig. 2 SDS-PAGE electrophoretic profiles of the insoluble (a) and WSP (b) fractions at pH 4.6 of AC and IC at different days of repining (1, 6 and 12 days)

milk, decreases significantly with increasing temperatures (Benfeldt et al. 1997; Buffa et al. 2000). The action of the rennet, the plasmin, the proteases and the microbial peptidases on the caseins throughout ripening releases peptides and amino acids, which increases the ripening index: ASP%, NPN% and FAA % (Gonzalez et al. 2003; Upreti et al. 2006; Orlyukand Stepanishchev, 2014).

Free fatty acids during cheese ripening, increase as does the proteolysis and the fat content. Similar results have been reported by Batool et al. (2018). FFA presents significant differences between the stages and the types of cheeses. The high level of FFA in the AC can be explained by the micro floral composition of raw milk and the lipoprotein lipase activities. Indeed, the negative effect of pasteurization on LPL activities and the highest of lipolysis degrees in cheeses made from raw milk compared to those made from pasteurized milk have been reported by several authors (McSweeney et al. 1993; Franco et al. 2001).

RP-HPLC profiles

The RP-HPLC profiles of WSP of the two cheeses, showed an increase in the number of peaks during the ripening process (Figure 1). These peaks were more frequent in AC than in IC. This is due to the high level of proteolysis in AC, the proteolysis activity of the indigenous milk enzymes, the microbial enzymes

as well as the activity of the residual rennet. The heat treatment has a negative effect on the proteolysis process during cheese repining. Benfeldt and Sorensen (2001) showed an inverse relationship, during cheese ripening, between the temperature of heat treatment of milk and the proteolysis as followed by an RP-HPLC. According to Trujillo et al. (1997) and Van Hekken et al. (2007), most of the peptides which are released during ripening, are generated from the caseins hydrolysis by rennet and plasmin with a molecular weight ranging from 10 to 20 kDa.

Electrophoresis of WSP and insoluble proteins fractions

The figure 2_a, shows that the indigenous casein migrates as two main bands corresponding to αs-CN and β-CN. The band intensity decreased throughout ripening, leaving two news bands of low molecular weight (Figure 2_b).

This trend was most pronounced for AC at the 12th day of ripening. These profiles revealed that the caseins proteolysis changes over time and with the type of the cheese, with higher αs-CN proteolytic rates than with β-CN. Indeed, new bands with lower molecular weight and the increase in peaks of WSP obtained with RP-HPLC, explain the higher proteolysis index and the free amino acids rates observed in AC than in IC. Our findings are consistent with those reported by Gobbetti et al. (2002), who noted that chymosin hydrolyzes easily αs_1-CN than β-CN, releasing a peptide identified as α_{s1}-I-casein f(24-199). Chymosin is also able to hydrolyze β-CN (Addeo et al. 1980). The main cleavage site of chymosin in β-CN is Leu^{192}-Tyr^{193}. However, in pure solution, it can hydrolyze β-CN at seven sites, generating f1-192, f1-189, f1-163/4/5 et f1-139 peptides, which are called -β-I^{I}, -β-I^{II}, -β-II and -β-III respectively (Visser, 1993). Orlyuk and Stepanishvhev (2014) reported that between the first and the 21st days of the Camembert ripening, the content of αs-CN and β-CN decreased respectively, by 53% and 25%. Proteolysis during the Camembert ripening is mainly due to the action of five proteinases: rennet (chymosin and pepsin), plasmin, aspartyl and methaloprotease of *Penicillium caseicolum.* However, the rennet seems to be inactive on β-CN, but acts early on αs_1-CN with a similar action to that of aspartyl-proteinase on αs_1-CN. On the contrary, the action of the plasmine on β-CN is observed at the 21st day, while the proteolysis of β-CN by the aspartyl and by the methaloproteases is detectable after seven and ten days of ripening respectively (Mane and McSweeney, 2019).

Growth of microflora during ripening

The results of milk microorganism counts are reported in table 5 .The samples comply with the standards described in the Official Journal of the People's Democratic Republic of Algeria n° 39 of July 2017 (JORADP, 2017).The tests to detect pathogens in the milks and the cheeses, especially for *Staphylococcus aureus* and *Salmonella*, were negative. The number of microorganisms is significantly higher in the raw milk than in the pasteurized. This reflects the lethal effect of the pasteurization on milk microflora.

The results of the microflora evolution during ripening show an inverse relationship between two microbial groups (Figure 3). The group a, including faecal streptococci and faecal and total coliforms, decreased gradually during ripening. The group b, which consists of total aerobic mesophilic flora, streptococci, Lactobacillus, yeast and molds, increased during the ripening process. The trends were similar for both studied cheeses. The differences were rather concerned with variations in the number and in the distribution of the microflora. Indeed, the growth of the microflora during ripening of AC recorded significant differences between the first and the twelfth days for AMF (7.41±0.35 log cfu/g and 9.35±0.31 log cfu/g respectively), MLAB (4.41±0.23 log cfu/g and 5.98±0.49 log cfu/g respectively), FC (1.60 ±0.08log cfu/g and 0.33l±0.41og cfu/g respectively) and FS (1.33±0.2 log cfu/g and 0.58±0.15 log cfu/g respectively). However, IC recorded a slight contamination of TC from the beginning until the sixth day of ripening then it dropped drastically at the twelfth day. With regard to fecal coliform, it disappeared from the sixth day of ripening stage of IC.

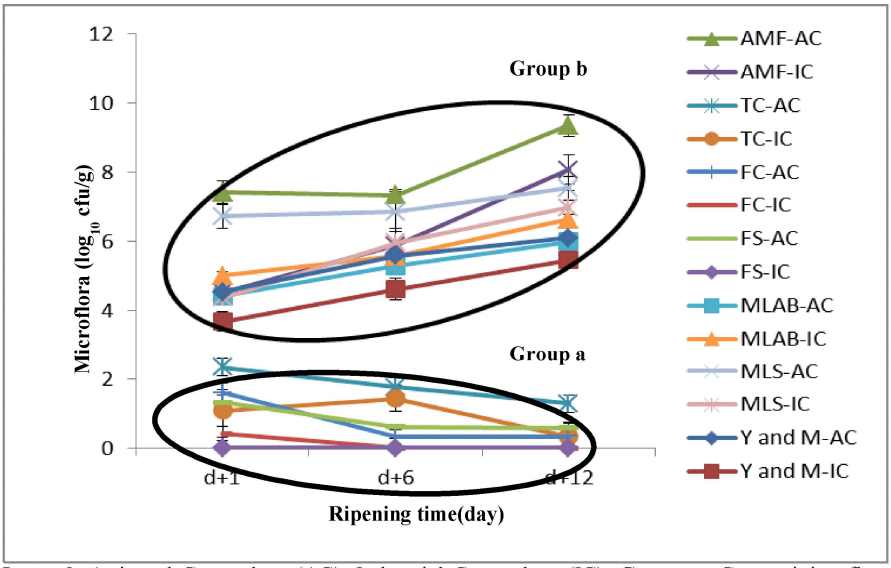
The decrease of contamination and the pathogen microflora, during the ripening process of cheese raw milk, is due to the increase in the ripening microflora, which modifies the physico-chemical parameters (a_w and pH) and releases peptides with antibacterial activity (Fontan et al. 2001; Arenas et al. 2004; Kirdar et al. 2018). However, some studies demonstrated that coliforms decrease during the first week and then increase till the end of the ripening process, with higher rates for the Camembert made

Table 5 Microbiological count in milk

Microflora (log_{10} CFU/mL)	Raw milk	Pasteurized milk
Aerobic mesophilic flora (AMF)	4.99^{b} ±0.306	3.71^{a} ±0.42
Total coliforms (TC)	1.66^{b} ±0.11	0.32^{a} ±0.24
Faecal coliforms (FC)	0.19^{b} ±0.17	0.0033^{a} ±0.004
Faecal streptococci (FS)	0	0
Mesophilic lactobacilli (MLAB)	3.63^{b} ±0.46	0.96^{a} ±0.178
Mesophilic lactic *streptococcus* (MLS)	2.49^{b} ±0.22	0.4^{a} ±0.14
Yeast and molds (Y and M)	1.57^{b} ±0.19	0.15^{a} ±0.19

Results in the same column for the same parameter with different letters are significantly different at $P < 0.05$

Fig. 3 Microflora evolution in the cheeses during ripening



Legend: Artisanal Camembert (AC), Industrial Camembert (IC). **Group a:** Contamining flora (Total coliforms (TC), Faecal coliforms (FC), Faecal streptococci (FS)). **Group b:** Ripening flora (Aerobic mesophilic flora (AMF), Mesophilic lactobacilli (MLAB), Mesophilic lactic streptococcus (MLS), Yeast and molds (Y and M)).

Table 6 Antibacterial activity statistics for the raw and neutralized supernatants

Strains	Supernatants	Cheeses type	Inhibition zone diameter (mm) Mean ± SD
Staphylococcus aureus ATCC 25923	Raw	IC	10.8 ± 0.97
		AC	10.5 ± 0.98
	Neutralized	IC	10 ± 1.94
		AC	8.8 ± 0.32
Escherichia coli ATCC 25922	Raw	IC	11.7 ± 0.21
		AC	11.3 ± 0.21
	Neutralized	IC	8.2 ± 0.64
		AC	7.9 ± 0.32

from raw milk than for those made from pasteurized milk (Mourgues et al. 1977; Rutzinski et al. 1979).

Antimicrobial activity analysis

The results of the analysis of the supernatant antimicrobial activity are reported in the Table 6. The presence of inhibition zones after neutralization of the supernatants suggests that the antibacterial effect could only be due to the presence of antibacterial substances, such as bacteriocins and bioactive peptides (Ortolani et al. 2010; Corrêa et al. 2011).

Cheese protection against pathogens proliferation can be performed by the indigenous antimicrobial agents of milk, such as lactoferrin (Farnaud and Evans, 2003), lactoperoxydase system (LPS) (Seifu et al. 2005) and lysozyme (Claeys et al. 2013). These agents are inactivated by the heat treatment (Conesa et al. 2010; Dumitraşcu et al. 2012; Claeys et al. 2013).These results explain in large part the decrease in the contaminating microflora during the ripening of AC and the role of the lactic microflora of the raw milk during the cheese ripening, thanks to their proteolytic activity and the production of bacteriocins.

Most of known varieties of cheeses contain a certain amount of kappacins (A and B) in form of caseinomacropeptide, with an antimicrobial activity, which are released as a result of the action of coagulant enzymes (rennet or other coagulant enzymes) on the κ-casein (Vajiheh, 2012). In the case of the Camembert, many studies demonstrated the effect of lactic bacteria and their products on the inhibition of *Listeria ssp* during cheese ripening (Wan et al. 1997). Lignitto et al. (2012) and Nguyen Thi et al. (2013) reported that inhibition of *Listeria monocytogenes* and *Listeria innocua* by water soluble extracts of cheese made from raw milk is attributed to peptides with MW < 1 kDa and to the

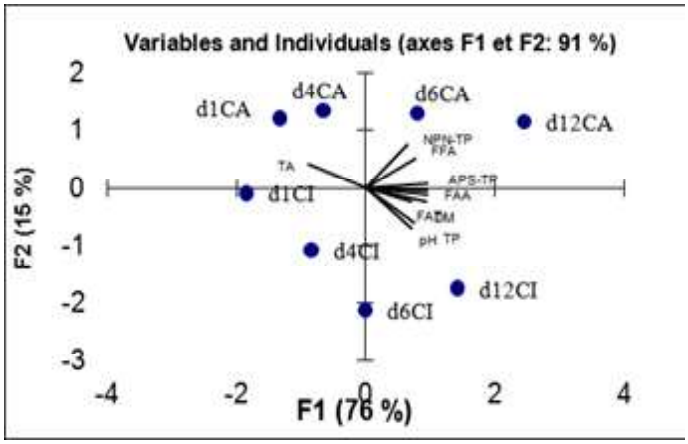


Fig. 4 Factorial plane 1-2 of the component analysis for the physicochemical changes during cheese repining

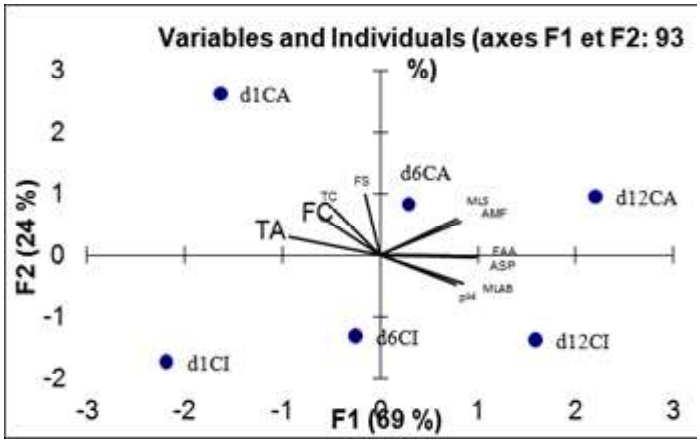


Fig. 5 Factorial plane 1-2 of the component analysis for the physicochemical and microbiological parameters interactions during ripening

antimicrobial peptides αs_1-CN f(1-23) et αs_2-CN f(183-207). The antimicrobial activity of the cheese water-soluble extracts could not only be attributed to the known intact bacteriocins with MW > 3 kDa, but also for the fraction with MW > 10 kDa (Drider et al. 2006; Pritchard et al. 2010). During the ripening process of the Camembert, 15 peaks-RPHPLC were identified as potentially bioactive, all stemming from β-CN with predominance of antibacterial fragments β-CN f(193-209) (Galli et al. 2019).

Interactions among the physicochemical and microbiological parameters and their progress during ripening

A PCA was run to analyze the interactions between the physicochemical and microbiological parameters and their evolution during ripening. The results related to physicochemical parameters during cheese repining are presented in Figure 4.

The first two axes explain 91% of the total variance. Axis F1 represents the maturity (temporal) gradient during the ripening process. F2 represents the type of the product. The projection of the means obtained from the three years of study from the same stage of ripening shows that F1 opposes the artisanal Camembert to the industrial. The small distance between d1AC and d4AC shows the high correlation (low changes) registered between the two first considered ripening stages of AC. In contrast, the temporal gradient revealed great changes (lower correlations) from the second stage of ripening, expressed here by an increase in the distance between successive stages (d4AC, d6AC, and d12AC). At the same stages, these differences were less pronounced during IC ripening, which registered an appreciable change only between the two last stages (J6IC and J12IC). The degree of maturation of AC is the result of the combined effects related to the production conditions and the physicochemical and microbiological proprieties of the raw milk. Consequently, IC is richer in DM, fat and proteins, but less matured than AC. Moreover, the factorial plane 1-2 highlights the concentration of the points representing fat, DM and proteins close to the last stage of ripening for IC, while the points representing the maturation index (ASP, NPN, FAA and FFA) are gathered towards this final ripening stage of AC.

The results of the component analysis for the physicochemical and microbiological parameters interactions during ripening are presented in Figure 5.

The axes F1 and F2 expressed 69 % and 24 % of the total variance respectively. The change in the physicochemical parameters is opposed to the contaminating microorganisms. F2 opposes the coliforms (faecal and total), the faecal streptococci to the mesophilic lactic bacteria (*Streptococci* and *Lactobacillus*), the MFAT, molds and yeasts. This axis also opposes contaminating germs to pH, FAA and ASP.

A scan of figures 4 and 5 explains the decrease of pathogenic organisms during ripening of the artisanal cheese. This could be due to the action of indigenous enzymes of the raw milk and increase in of lactic bacteria and the fungal flora, which lead to the release of bioactive peptides, with antibacterial effects by their proteolytic action, and production of bacteriocins by lactic bacteria. These results are confirmed by the correlation matrix obtained between the physicochemical and microbiological parameters, which revealed that molds and yeasts have an impact on the proteolysis, with significant ($P < 0,05$) positive correlations with the proteolysis products of ASP ($r = 0.88$) and FAA ($r = 0.90$). MLAB showed significant negative correlations with the pathogenic organisms TC ($r = -0.87$), FC ($r = -0.84$) and FS ($r = -0.86$). This explains the effect of MLAB on the decrease of these pathogenic germs during ripening of the cheeses made from raw milk and the role of lactic microflora in their bio-conservation.

Conclusions

The physicochemical parameters showed the same trends and recorded higher values in the Camembert made from raw milk. The inhibition of *Escherichia coli* and *Staphylococcus aureus* by the neutralized water-soluble fractions revealed the effect of the bioconservation of the camembert made from raw milk by an eventual presence, in the cheese, of bacteriocins and bioactive peptides with antibacterial effect. The current study opens up new perspectives within the framework of the dairy industry in Algeria, encouraging stockbreeders and farmers to transform the raw milk into healthy dairy products and provide steady and more active incomes than those generated from selling milk to collectors.

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