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FOOD ANALYSIS

Physicochemical, microbiological characterization and proteolysis of Algerian traditional *Bouhezza* cheese prepared from goat's raw milk

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ABSTRACT

Bouhezza is an Algerian traditional cheese made using different types of milk (goat, sheep and cow), ripened in a goat-skin or sheep-skin bag called *Chekoua* or *Djeld* of *Bouhezza*. The objective of the study was the characterization of *Bouhezza* cheese through ten cheese preparation trials using goat raw milk. The chemical and microbiological composition and proteolysis were monitored on ripened samples. The physicochemical characteristics showed that *Bouhezza* is acidic with a pH value of 3.95 and 2.36% for titratable acidity as lactic acid, 13.20% for protein and 12.74% for fat. At the end of ripening, *Bouhezza* was classified to be a ripened, soft and mid-fat cheese. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of *Bouhezza* samples showed low proteolysis after 30 days of ripening. Microbiological results showed a high content of lactic acid bacteria (LAB), 8 to 9 log cfu/ g, an absence of pathogenic bacteria, and a low level of contamination flora. This makes the cheese a healthy product and of an acceptable hygienic quality. Four proteolytic lactic acid bacteria were identified by 16S ribosomal deoxyribonucleic acid (rDNA) sequencing: two strains of *Enterococcus faecalis*, one *E. faecium*, and one *Lactobacillus paracasei* ssp. *paracasei*. Reversed phase-high performance liquid chromatography (RP–HPLC) of the soluble fraction at pH 4.4 showed changes occurred during ripening. This work highlights, characterizes, and improves the knowledge of cheese *Bouhezza* made with raw goat's milk. This product is rich in nutrients, such as proteins and minerals, that are useful to the consumer.

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Introduction

In Algeria, local products have a tendency to disappear due to the industrialization of most traditional preparation methods for many regional products. In addition, the rural exodus caused the abandonment of rural practices such as traditional cheese making.

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In countries where the production and variety of cheeses produced are limited, consumers remain skeptical about the sensory quality of the product and this is less relevant to the hygienic quality of the cheese.

Several studies have shown that the enzyme system of the indigenous microflora in raw milk is much more complex than bacteria used in cheese manufacture and, therefore, the indigenous microflora has an important influence on cheese proteolysis (Dagdemir and Ozdemir 2008). One of the most important biochemical processes involved in the manufacturing of many fermented dairy products is proteolysis (Fox 1989). El-Ghaish et al. (2010) reported that proteolytic or peptidolytic enzymes of lactic acid bacteria (LAB) contribute to organoleptic properties of the final milk products. More extensive is the proteolysis and acidification affected by microflora present in cheeses obtained from raw milk, and this contribute to cheese flavor (Beuvier et al. 1997).

Bouhezza is a traditionally made farmhouse cheese for a long time in the *Chaouia* region in eastern Algeria, originally prepared from raw goat milk. This cheese is prepared without the addition of coagulating enzymes, in a permeable container, prepared from goat or sheep's skin called *Chekoua* or *Djeld* of *Bouhezza*. During the cheese preparation, *Bouhezza* is subjected to ripening in the *Chekoua*, in which draining is occurred through its pores. During manufacture, there is no heat treatment of *Bouhezza*. The main factors involved in the transformation process are the salt and acidity of the cheese. Thus, they contribute to protect the cheese against the microbial contaminations.

The aim of this work was to characterize *Bouhezza* cheese through productions using goat's milk. Microbiological and physicochemical characterization have been carried out. Proteolysis including peptide profile was studied during the ripening process, as well as the isolation and identification of proteolytic LAB from *Bouhezza* cheese. This work is one of the first studies on *Bouhezza* cheese using raw goat's milk; it contributes to improve the knowledge on the product's characteristics.

Materials and methods

Processing: Raw materials and preparation of Bouhezza cheese

Bouhezza manufacturing begins with the *lben* that filled half or the entire volume of the *Chekoua* and followed by several additions comprise salted *lben* and finally raw milk. The stages of *Bouhezza* cheese manufacture are the same as reported by Medjoudj et al. (2017).

The *lben* used for *Bouhezza* manufacturing, a fermented milk, was obtained according to well-defined steps. They start with a spontaneous fermentation of raw milk (6 liters) and coagulation processes during 24 to 48 h at ambient temperature (25–30 °C). Then, the coagulated milk named "Rayeb" was churned for 30–45 min and 0.5 L of warm (20–25 °C) water were added to this mixture. Water was added to maintain the temperature at 20–25 °C, which promotes gathering of butter grains, and so facilitates the skimming operation.

After a partial skimming, the quantity of recovered *lben* was employed in manufacturing of *Bouhezza* in one or two containers. The twice *Chekouates* (F = cheese making) prepared simultaneously (F4/F5), (F6/F7) and (F8/F9), received the same quantity of *lben* (3 liters per *Chekoua*) at the same time, since the collected raw material was from the same farm. In this assay, the fermentation of the raw milk during *lben* preparation was achieved without the addition of a starter.

The cheese-making process was started using salted *lben* (25 g/L of raw material), followed by continuous *lben* addition for six weeks (1 volume to start at 1/2 volume on the first day, and then 1/4 volume every three days until the end of production) as reported by Medjoudj et al. (2017). The *Chekoua* was suspended in a ventilated area or in the shade. During cheese manufacturing, the outside of the *Chekoua* was cleaned daily with water. Milk was added at the end of cheese making process to stabilize the level of acidity and salt and also to increase the level of fat and to complete the final volume of cheese.

The cheese samples were collected and analyzed for chemical and microbiological analyses at end of ripening (unspicy and spicy with red hot pepper). The hot pepper in a powder form was mixed with raw milk in the final stage. The same stages of preparation and production of cheese *Bouhezza* were reported by Medjoudj et al. (2017); Medjoudj et al. (2018).

Manufacture of bouhezza *cheese with goat's milk*

Bouhezza cheese was produced according to the diagram obtained from surveys (data not shown) in different areas of *Chaouia* and according to Medjoudj et al. (2017). Thus, ten cheese-making trials (F1 to F10) were conducted between 2010 and 2015 from goat's milk in order to characterize the chemistry, biochemistry and microbiology of this cheese.

All cheese-makings were carried out as follows: two (F1, F2) in the laboratory of nutrition and food technology (LNTA), Institute of Nutrition, Food and Agro-food Technologies (INATAA), University of Constantine 1, Constantine; three in Tebessa [one (F3) in a rural area in Hammamet and two (F4, F5) in urban area of Boukhadra], five in rural areas in the province of Oum El-Bouaghi [two (F6, F7) at Ain Fakroun and three (F8, F9, F10) in Ain Beida]. *Bouhezza* cheese was made from goat milk in a *Chekoua*. *Lben* and raw milk were used during a period of production of about 50, 60 and 64 days for the ten batches. The cheese samples were taken directly from the *Chekoua* using a sterile stainless-steel spoon and put in sterile container in order to characterize parameters of ripened cheese.

The results of final products, whether spicy or unspicy, are presented in this study. Samples of raw milk (n = 5) and *lben* (n = 6) have been characterized before their introduction into the *Chekoua*. The results showed in this study are of the final products (cheese samples). For unspicy cheese (n = 10) between 49 and 56 days and the spicy product (n = 10); F1, F2 and F9 for 50 days; F3, F4, F5, F6, F7, F8 and F10 for 60 and 64 days. Samples F4 and F5 were not subjected to the microbiological analysis. In whole, twenty samples of Bouhezza were collected and analyzed in triplicate. While in case of SDS-PAGE, samples analyzed were levies from F1, F2 and F3 where sampling was from first day at each eight-day interval during ripening until the end of the manufacturing process.

Chemical and microbiological analysis

The raw material for manufacturing *Bouhezza* was *lben*. Each sample was thoroughly mixed in a sterile jar and analyzed. The physico–chemical characteristics were determined as follows. The pH was measured with a pH meter (Hanna Microprocessor pH Meter 8521.model instrument 8571n, Singapore) (Afnor 1985).

Table 1. Characteristics of the microbiological analysis.

Microorganisms	Media culture	Time and incubation temperature
Lactococcus	M 17 glucose agar	30 °C / 48 h
Lactobacillus	Man Rogosa & Sharp	37 °C / 48 h
Total aerobic mesophilic bacteria's	Plat Count Agar	30 °C / 72 h.
Total coliform	Lactose bile broth Brilliant Green)	37 °C / 24 to 48 h.
Fecal coliform	Lactose bile broth brilliant green and Peptone-free water indole	44 °C / 24 h
Fecal streptococci	Broth of Rothe	37 °C / 24 to 48 h.
	Broth of Eva Litsky	37 °C / 48 h
Sulphite-reducing Clostridium	Meat-Liver	37 °C / 24 to 72 h
Yeasts and molds	Oxytetracycline Glucose Agar	20 – 25 °C / 5 days
Salmonella	Sodium Selenite Broth	37 °C / 24 h.
	Salmonella deoxycholate citrate agar (Salmonella-Shigella)	37 °C / 24 h
Staphylococcus aureus	Broth of Giolitti-Cantoni	37 °C / 48 h
	Chapman agar	37 °C / 24 to 48 h

The titratable acidity was determined according to Afnor (1993) and the dry matter was performed in an oven (Memmert) set at 103° C ± 2° C and left for 3 h. Ash incineration was performed at a temperature of 500° C in a muffle furnace (Linn HighTherm) in a slow stream of air for 5 hours. For fat, 3 g of the crushed cheese sample (11 mL of milk or Lben) were introduced into the Gerber butyrometer or Van Gulik cup. (Afnor 1993). The proteins were measured by reading the optical density at 750 nm using a spectrophotometer (Cecil, CE 2041, Prim, Secomam, France) (Lowry et al. 1951).

Total chlorides were determined using the Vollard method (FAO 1997). All of these measurements were performed in triplicate. Microbiological analysis was performed for goat milk, *lben* and cheese at the end of production after 7 to 8 weeks (Guiraud 2003). Table 1 presents the details of microbiological analysis, which were carried out in triplicate.

The assay of protein fractions was performed on 5 mL by the Kjeldahl method. Total nitrogen was determined using a digestion apparatus (Buchi Digestion type K-424/ Gerhardt, Vapodest type VAP10), with a Buchi distillation apparatus unit type 339.

Microbiological characterization

Isolation, purification, and pre-identification of lactic acid bacteria cultures

Lactic acid bacteria (LAB) were isolated and pre-identified by morphological and physiological tests as described by El-Ghaish et al. (2010). The lactic acid bacteria morphological characteristics as the form and size of cells were checking in the preliminary tests (Gram staining and catalase negative). For purification, the cultures were streaked on Man Rogosa and Sharp (MRS) for lactobacilli and growth medium M17 glucose agar (M17) for streptococci media. The cultures were classified as Gram-positive catalase-negative in rods and cocci. The purified strains were reconstituted in sterilized skim milk (12.5%, w/v) supplemented with 30% (w/v) glycerol and stored at −20 °C.

Bacterial proteolytic activity on sterile skim milk and cheese

Thirty-five isolates have been characterized including 15 cocci and 20 rods. The 35 isolates were inoculated into skim milk and prepared for electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE).

The lactic acid bacteria isolates were reactivated twice by inoculating 50 µL of the previously prepared pre-culture into 950 µL of sterile Délisse skim milk and incubated at 37 °C overnight. The mixture was diluted 1/10 (v/v) in sample buffer and heated at 100 °C for 3 min and allowed to cool. Finally, all isolates were analyzed for their proteolysis level by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by El-Ghaish et al. (2010).

Molecular identification of some selected LAB isolates (amplification and sequencing of 16S ribosomal deoxyribonucleic acid (rDNA))

Deoxyribonucleic acid (DNA) was extracted from isolates according to described method by Delley et al. (1990), and used as a template for 16S ribosomal ribonucleic acid (rRNA) gene amplification (El-Ghaish et al. 2010). Universal primers fD1 (5′-AGA GTTTGATCCTGGCTCAG-3′) and rD1 (5′-TAAGGAGGTGATCCAGGC-3′) Qiagen GmbH, Hilden, Germany were used (Weisburg et al. 1991).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The samples intended for electrophoresis analysis were prepared by weighing 0.05 g of *Bouhezza* cheese and were diluted in 450 µL (1:9 w/v) of sample buffer already prepared to obtain a dilution of 1/10, mixed, and vortex mixed for 1 min for homogenization. Subsequently, the mixture was placed in a boiling water bath at 100° C for 3 min. After cooling, the injected volume per well of the prepared SDS-PAGE gels was 5 µL using a Protean II XI vertical gel unit (Bio-Rad Laboratories Ltd., Watford, UK).

The diluted prepared cheese samples were applied to a 12% polyacrylamide slab gel. The separating buffer contained 50 mM Tris, 0.384 M glycine and 0.1% SDS. After running at 10 mA on the stacking gel and 20 mA on the running gel, staining was performed with Coomassie brilliant blue G250 followed by a convenient destaining. The analysis was performed using the Film Fuji Image Gauge V3.0 software (Fuji Photo Film Company, Japan).

The gels were scanned with Image scanner III (Ge healthcare, USA) and the intensity of the bands was quantified by Image software (Ong et al. 2006). The protein bands were identified by comparison with Broad Range Prestained Molecular Weight Marker SDS-PAGE Standards from BioRad.

Reversed phase-high performance liquid chromatography (RP-HPLC)

The pH 4.4-soluble fraction of the cheeses was extracted according to the protocol proposed by Fallico et al. (2004). A mass of 0.75 g of cheese was homogenized with 1.5 mL of distilled water and mixed for 10 min. The pH was adjusted with 0.1 M HCl solution to 4.4 and 30 min were allowed stabilization. The samples were heated in a water bath at 40 °C for 1 h and centrifuged at 10,000 g for 20 min at 4 °C. The filtration of the supernatant was performed using Whatman filter paper Number 113.

The samples were stored at −20 °C until analyzed by RP-HPLC. The working conditions were as follows. The samples were separated on a Symmetry, 300 Å C18 column, 5 μm (2.1 mm x 150 mm) waters HPLC using a Waters Alliance system (Milford, MA,

USA) with a flow rate of 0.2 mL/min. Solvent A was trifluoroacetic acid (TFA) 0.055% (v/v) in water (sequencing class Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany) HPLC grade (Merck KGaA, Darmstadt, Germany). Solvent B was acetonitrile water/TFA, 80/20/0.045 (v/v/v) (Sartorius GmbH, Gottingen, Allemagne). The instrument was a Shimadzu LC 20 AD Prominence HPLC system (Shimadzu, Kyoto, Japan).

The elution was performed with a linear gradient of solvent B from 0% to 50% with a flow rate of 0.25 mL/min (45 min) and 50 to 100% from 45 to 52 min. Absorbance was measured at 216 nm (arbitrary units). The analysis time was 60 min per sample.

Statistical analysis

Physicochemical and microbiological data were expressed as the average ± standard deviation (sd). An average comparison test for paired series between the physicochemical parameters of the different productions at the 5% confidence level was done by the Xlstat 2009 version (Xlstat 2009).

A one-way analysis of variance (ANOVA) was performed on the RP-HPLC measurements using three chromatograms followed by Duncan's multiple-range test using the IBM SPSS Statistics (v 17.0) statistical software program version 2009.1.02 (Copyright Addinsoft program 1995–2009). The differences between the retention times of peaks were compared at the 5% ($P < 0.05$) level of significance by use of Fisher's least significant difference (LSD) test (Forthofer et al. 2007).

Results and discussion

Physicochemical characteristics of bouhezza *and its raw material*

Table 2 shows the main physicochemical characteristics (pH, titratable acidity, and dry matter, fat, proteins and ash) of *Bouhezza* cheese, raw milk and *lben*.

The average pH value of goat milk was 6.30. The dry matter content was 13.21%; higher than those of Masle and Morgan (2001), Greppi et al. (2008), and Bontinis et al. (2012). The fat content of goat milk was 2.74%, these results was close to those obtained by Masle and Morgan (2001) from 2.63 to 2.86%, but lower than reported by Bontinis et al. (2012).

The protein content was 4.61%, comparable to the value reported by Temizkan et al. (2014); however, higher than the results of Masle and Morgan (2001) and Bontinis et al. (2012). The pH of *lben* used as a raw material was 4.39 (n = 8). However, the *lben* used

Table 2. Physicochemical characteristics of milk, lben (acidity in g/100 mL), and Bouhezza (acidity in g/100 g) unspicy and spicy goat cheese.

Sample				
Parameter	Goat milk[a] (n = 7)	Goat *lben*[a] (n = 8)	Unspicy cheese[a] (n = 10)	Spicy cheese[a] (n = 10)
pH	6.30 ± 0.11	4.39 ± 0.67	3.91 ± 0.47	3.99 ± 0.49
Titratable acidity %	0.19 ± 0.02	0.76 ± 0.22	2.27 ± 1.24	2.45 ± 1.41
Dry matter %	13.20 ± 0.10	10.33 ± 4.00	41.30 ± 8.30	40.90 ± 9.00
Fat/dry matter %	20.76 ± 0.91	12.10 ± 0.83	29.64 ± 6.76	32.84 ± 6.46
Protein/dry matter %	34.92 ± 0.53	49.66 ± 1.05	10.46 ± 0.66	10.59 ± 0.68
Ash %	0.35 ± 0.06	2.53 ± 0.87	3.67 ± 1.91	3.39 ± 2.17

[a]Average and standard deviation of seven samples of raw goat milk, eight samples of goat *lben* and 10 samples of cheese as a mixture of 2 samples each during the manufacturing process.

by Aissaoui Zitoun et al. (2012) had a pH value of 5.05. The result was in accordance with that of Ouadghiri et al. (2009) which was close to the traditional *lben* (4.45), and experimental value (4.40) reported by Samet Bali et al. (2010).

The average acidity of *lben* was 0.76%. This value was close to that of Samet-Bali et al. (2010), Ouadghiri et al. (2008), and Aissaoui Zitoun et al. (2011; 2012). The dry matter of *lben* was 10.33% (Table 2), slightly higher than that presented by Aissaoui Zitoun et al. (2012) (8.97%) and those reported by Samet Bali et al. (2010) for different samples of *lben* (from 7.1 to 7.4%).

The average values of pH of unspicy and spicy *Bouhezza* cheese were similar, 3.91 ± 0.47 and 3.99 ± 0.49, respectively. Aissaoui Zitoun et al. (2011; 2012) also reported the same pH values were obtained with unspicy and spicy *Bouhezza* cheese after 70 days of ripening. Our results were comparable with those obtained with Xinotyri cheese (Bontinis et al. 2008), but lower than Darfiyeh (Serhan et al. 2010), Tulum (Hayaloglu et al. 2007a) and Anevato (Xanthopoulos et al. 2000) cheeses.

The values for the titratable acidity of unspicy and spicy *Bouhezza* were 2.27% and 2.45%, respectively. These values were higher than those provided by Hayaloglu et al. (2007a) at 60 and 90 days of maturation of Tulum and Xinotyri cheeses aged for 90 days (Bontinis et al. 2008), respectively. The average dry matter for unspicy and spicy cheese content was 41.27% to 40.90%, respectively. This average was higher than the value obtained in *Bouhezza* cow's milk reported by Aissaoui Zitoun et al. (2012), and Anevato cheese made from goat milk, 37.66% (Xanthopoulos et al. 2000). However, these values of dry matter of *Bouhezza* cheese were lower than for Tulum cheese ripened in goat-skin obtained after 60 and 90 days by Hayaloglu et al. (2007a) and than Darfiyeh at 60 days of ripening (Serhan et al. 2010).

The ash content of unspicy and spicy *Bouhezza* was 3.67% and 3.39% ($n = 8$), respectively. These were higher than obtained with Xinotyri goat cheese at 60 and 90 days of ripening (2.93 and 2.75%) (Bontinis et al. 2008). The fat content of the *Bouhezza* samples ranged from 12.24% to 13.43% ($n = 8$). These values were close to those reported by Aissaoui Zitoun et al. (2012) between 56 and 70 days old in *Bouhezza* cheese with cow's milk, unspicy and spicy. However, the value was lower than of Anevato cheese with 46.57% dry matter (Xanthopoulos et al. 2000).

The average levels of proteins were 12.68% and 13.63%, slightly less than reported in the literature. These values were lower than those for goat's milk cheeses, such as Anevato with 33.34% dry matter (Xanthopoulos et al. 2000); Darfiyeh at 60 days, between 31.21 and 40.91% (Serhan et al. 2010), and Tulum at 60 and 90 days of maturation with 18.06 and 19.97% (Hayaloglu et al. 2007a) .

Lastly, the chloride content was stable for the end of ripening samples with the value of 1.26 and 1.25% ($n = 4$) cheese. The values were lower than those of *Bouhezza* cow's milk cheese by Aissaoui Zitoun et al. (2012), and of Darfiyeh goat cheese at 60 days of ripening (Serhan et al., 2010). All differences, between *Bouhezza* goat's milk cheese and other cheeses, reside probably in the cheese making method. The differences in these results also may be due to the geography, feed of animals, type of goats, milking season, and techniques used in the characterization of the cheeses.

Nevertheless, compared to *Bouhezza* cheese from cow's milk, it may be due to type and composition of the milk, and the nature of the skin and their different porosity.

Most of our products are manufactured in newly prepared *Chekouates*; therefore draining was most important with the exception of the two experimental cheeses manufactured in the laboratory, which were used for the second time. The nature of the raw material and the manufacturing conditions played an important role during cheese ripening. For the other cheeses, the goat-skin was used only for ripening, unlike *Bouhezza*.

The *t*-test for two paired samples (bilateral test at 5% probability) was done between the values of pH, titratable acidity, moisture content, total dry matter, ash, fat, protein and chloride contents. The statistical analysis showed no significant differences between the values of different productions of unspicy ($P > 0.05$) and spicy *Bouhezza* ($P > 0.05$).

According to the *Codex alimentarius* (1978) and classification presented by St-Gelais and Tirard-Collet (2002), *Bouhezza* was classified as a ripened soft cheese; the average of moisture in nonfat substance was 69.67%, and mid-fat according to its fat-in-dry matter which is 34.44%, for unspicy and spicy cheese. Aissaoui Zitoun et al. (2012) reported the same classification for *Bouhezza* cow's milk, which was classified also as one of ripened soft cheese.

Proteolysis

Protein fractions

The results of the protein fraction quantification in traditional Algerian *Bouhezza* cheese made from goat's raw milk are shown in Table 3. The total nitrogen (TN) level was highest in the cheese-making 5 (F5) followed by that of the production 4 (F4) and the lowest was that of manufacture 2 (F2).

The proteolysis rate is expressed by ratio of water-soluble nitrogen (WSN) at pH 4.4 as the percentage of total nitrogen (TN). The results showed that the rate was higher in F2 with 14.8% and the lowest was of F3 with is 7.93%. These results were lower than those obtained by Aissaoui Zitoun et al. (2011) in *Bouhezza* made from cow's milk and with cheeses ripened in goat-skin bag as Darfiyeh (Serhan et al. 2010) and Tulum cheese (Hayaloglu et al. 2007 b).

However, our results were close to those reported in 2012 on *Bouhezza* prepared with cow's milk by Aissaoui Zitoun et al., and higher than the proteolysis rate in Xinotyri cheese from goat's milk obtained at 45, 60 and 90 days of ripening (Bontinis et al. 2012). The proteolysis rate of goat's milk cheese *Bouhezza* had an average level for five productions of approximately 11.00%. The variation that exists between the different fabrications was probably be due to the number of lactic bacteria and their enzymatic

Table 3. Protein fractions (average ± standard deviation) in the final product of ripening Bouhezza cheese made with raw goat's milk expressed in g/100 g of cheese.

Sample					
Fractions in %	Cheese prepared by F1 process (50 d)	Cheese prepared by F2 process (50 d)	Cheese prepared by F3 process (64 d)	Cheese prepared by F4 process (60 d)	Cheese prepared by F5 process (60 d)
Total nitrogen	2.50 ± 0.06	1.35 ± 0.06	2.27 ± 0.05	2.68 ± 0.21	2.72 ± 0.22
Water-soluble nitrogen/ total nitrogen	13.20 ± 0.16	14.80 ± 0.06	7.93 ± 0.04	11.90 ± 0.16	8.46 ± 0.10

activity by their proteases and peptidases as well as that of the fungal flora. *Bouhezza*, which is prepared from raw milk, the origin as well as the content of microbial flora in the latter may vary from one sample to another.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The results of electrophoresis analysis of the cheese samples per interval of eight days are presented in Figure 1. The gels show the main bands of protein fractions with low electrophoretic mobility, which are αs1-casein, followed by αs2-casein and β-casein as native fractions of milk, found intact in first days of cheese-production. Nevertheless, new small protein fractions were observed which were derived under proteolysis action that the native fractions undergoes, which shows that is a ripening process occurred during the cheese manufacture.

These new fractions are visible in Figure 1 from one month from sample 4 until 9 of fabrication 2 of the cheese ripening. The cleavages occurring in the *Bouhezza* cheese were probably due to the indigenous enzymes like plasmin and/or cathepsine with proteinases activity of native microflora of the milk and by nonstarter lactic acid bacteria. The important influence of indigenous microflora on cheese proteolysis has been reported by Dagdemir and Ozdemir (2008).

Proteinases and peptidases of indigenous flora contribute to release in cheese, short-chain peptides and free amino acids as been reported by Medjoudj et al. (2017 ; 201). Aissaoui Zitoun et al. (2011; 2012) have reported those allegations for 75 day-old *Bouhezza* prepared with cow milk. The hydrolysis has occurred both on the αs and β-caseins but not with the same levels. The β-caseins was much more hydrolyzed than αs-caseins, where the γ-casein fractions on the gels were probably from cleavage of β-casein under the action of the plasmin as reported by Medjoudj et al. (2017 ; 201) by the urea-PAGE electrophoresis analysis of the insoluble fractions proteins of goat's milk *Bouhezza* cheese.

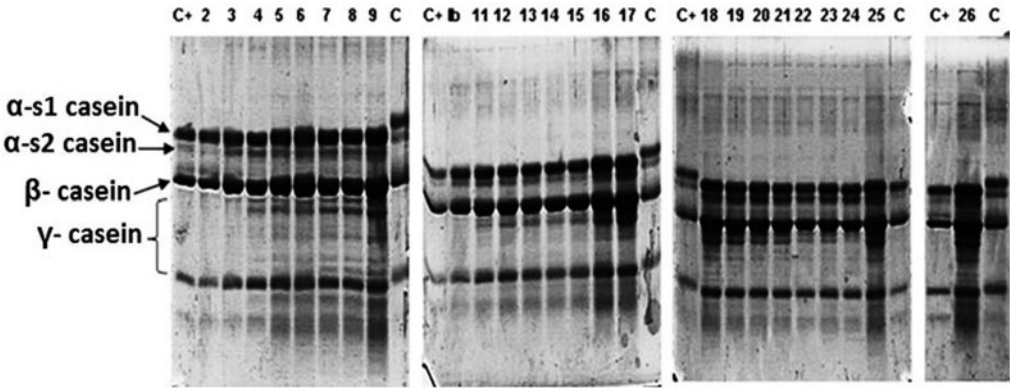

Figure 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE at 12%) of the Bouhezza cheese samples, manufactured from raw goat's milk, showing the emergence of new small fractions (Casein-γ) due to proteolysis from one month of ripening until the end-production in three cheese preparations. C+: goat milk; lb: *Iben*; C: cow's milk. Wells 2 to 9 are samples from preparation 2. Wells 11 to 19 are samples of preparation 1. Wells 20 to 26: samples from preparation 3.

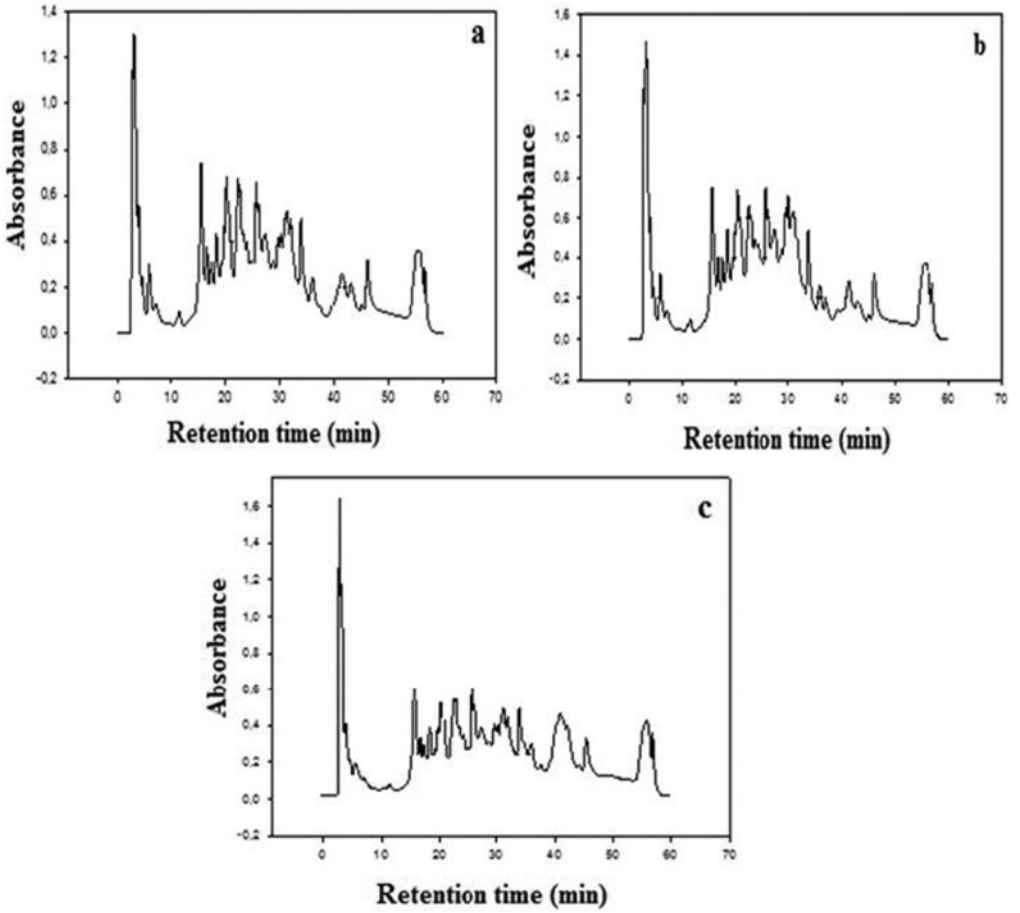

Figure 2. Reversed phase high performance liquid chromatography (RP HPLC) of the spicy Bouhezza cheese samples. Chromatographs (a), (b), and (c) show the retention times of the soluble fractions at pH 4.4 as a function of the absorbance at 216 nm for the three cheese preparations. (a) and (b) are from manufacturing process 1 and 2; the samples were analyzed after 50 days of ripening from the batches in the laboratory; (c) shows the chromatogram for manufacturing process 3 after 72 days of cheese ripening at the farm.

Reversed phase-high performance liquid chromatography (RP-HPLC) analysis

The results of RP-HPLC analysis on the soluble fractions at pH 4.4 showed the emergence of important peaks at retention times between 15 and 35 min (Figure 2). This demonstrated that a proteolysis and production of some new peptides were visible, which could undergo an enzymatic hydrolysis and a release of amino acids available and digestible for the consumer.

The statistical analysis showed no significant difference between samples analyzed for the first (F1) and the second cheese preparation (F2) that were both ripened after 50 days. Nevertheless, there was a significant difference between samples analyzed in the first (F1) and the second manufacture (F2), with sample of 72 days of ripening, of the third manufacture (F3) at the level of 5%.

Table 4. Counting of microbial flora in the raw material in log cfu/mL (milk, *lben*) and cheese Bouhezza goat's milk in log cfu/g.

Sample				
Flora sought	Raw milk (n = 7)	*Lben* (n = 8)	Unspicy cheese of 49 d (n = 8)	Spicy cheese of 60/64 d (n = 8)
Total aerobic mesophilic bacterias	9.12 ± 2.58	9.65 ± 1.88	9.52 ± 0.43	8.03 ± 0.93
Lactococcus	7.95 ± 1.01	9.52 ± 1.64	8.77 ± 0.90	8.91 ± 2.23
Lactobacillus	6.51 ± 0.00	8.94 ± 0.74	9.71 ± 2.70	9.30 ± 2.48
Total coliforms	2.40 ± 0.32	2.62 ± 0.20	2.67 ± 0.88	2.46 ± 2.59
Fecal coliforms	0.70 ± 0.00	1.44 ± 1.35	1.63 ± 1.04	0.94 ± 1.21
Yeast Molds	6.24 ± 1.63	7.61 ± 0.55	4.98 ± 1.26	2.16 ± 2.02
Halotolerant bacteria	4.87 ± 1.73	3.40 ± 3.33	3.20 ± 3.07	2.30 ± 2.06

The amount of soluble nitrogen in cheese water or buffers at pH 4.6 is widely used as an index of proteolysis. These extracts contain numerous of small and medium-sized peptides, amino acids and their degradation products (organic acids and their salts NaCl). Extraction with water efficiently separates the small peptides in cheese from proteins and large peptides (Fox et al. 2000).

According to De Llano et al. (1995), Laborda et al. (1999), Hayaloglu et al. (2004), and Fallico et al. (2005), RP-HPLC chromatograms of soluble fractions at pH 4.6 can be divided into three sections; most free amino acids were eluted from 0 to 10 min, the hydrophilic peptides in the range from 10 min to 35 min, and the hydrophobic peptides were eluted between 35 and 65 min.

As shown in Figure 2, the peptides of the hydrophilic area were greater in quantity and retention time. The peaks (or areas) for those peptides were higher in arbitrary units (between 0.6 and 0.8) for chromatogram (a) of F1 and chromatogram (b) of F2. However, for chromatogram (c) of manufacture 3 (F3), the values were less than 0.6 AU. This result was probably due to the cheese making method where salting was more important at the beginning of traditional F3 production, and may have negatively influenced the level of proteolysis.

Microbiological characterization of milk, lben *and cheese*

The results of microbiological analysis of *Bouhezza* cheese manufactured are summarized in Table 4 as logarithmic values.

The total aerobic mesophilic bacteria in milk and *lben* accounted for 9 log cfu/mL which decreased in cheese after the manufacture (60 and 64 days) (Table 4). These results were higher than those found by Masle and Morgan (2001) in goat milk ranged between 4.8 and 5.3 log cfu/mL; and similar to those found by Aissaoui Zitoun et al. (2012) in milk and *lben* between 10.94 and 8.81 log cfu/mL, respectively.

Bouhezza microflora was composed essentially of total aerobic mesophilic bacteria with an average of the order of 8 log cfu/g. This level was close to those found in cow's milk *Bouhezza* at 8 and 10 weeks of ripening, 8.02 and 8.03 log cfu/g (Aissaoui Zitoun et al. 2012), and in Anevato cheese made with raw goat milk, 8.14 log cfu/g (Xanthopoulos et al. 2000); but higher than those obtained with Tulum cheese after 60 days with 6.30 and 6.20 log cfu/g (Hayaloglu et al. 2007a) .

The counts for lactic acid bacteria were of 7.95 and 6.51 log cfu/mL in milk for lactococci and lactobacilli, respectively, which increased after the processing of milk into *lben*. Levels of 9.52 and 8.94 log cfu/mL were obtained for the two types, respectively. Ouadghiri et al. (2008) observed the same numbers for lactic acid bacteria with values of 7 log cfu/mL in raw milk.

In contrast, Aissaoui Zitoun et al. (2012) reported a decrease of the lactic acid bacteria counts. In milk, the values were 9.1 and 10.09 log cfu/mL for the lactobacilli and lactococci. In *lben,* the values were 7.49 and 6.74 log cfu/mL respectively. Samet-Bali et al. (2010) found comparable values in the traditional Tunisian *lben* (8 log cfu/mL).

Lactic acid bacteria in *Bouhezza* goat's cheese milk reached 9 log cfu/g lactobacilli and 8 log cfu/g lactococci. These levels were higher than those reported by Aissaoui Zitoun et al. (2011) in *Bouhezza* cheese with cow's milk, 8.14 and 7.56 log cfu/g of lactobacilli and 7.38 and 7.64 log cfu/g for lactococci at 8 and 10 weeks old, respectively. These measurements were also higher than the results of Aissaoui Zitoun et al. (2012). The values for unspicy and spicy *Bouhezza* at 70 days were 7.83 log cfu/g for lactobacilli and 7.53 log cfu/g for lactococci. They were also higher than in Anevato cheese with 7.70 and 7.93 log cfu/g, respectively, for the two types (Xanthopoulos et al. 2000).

Total coliforms were present in milk and lben at 2.40 and 2.62 log cfu/mL, respectively, and were higher in the unspicy cheese at 2.67 log cfu/g. This value decreased to an average of 2.46 log cfu/g at the end of production. The fecal coliforms were quite low with counts of 0.7 log cfu/mL in milk and 1.44 log cfu/mL in the *lben* (Table 4). This level increased to 1.63 log cfu/g in unspicy cheese and decreased to 0.94 log cfu/g in the spicy product.

The total coliform and fecal coliform numbers in *lben* of cow and in cheese found by Aissaoui Zitoun et al. (2012) were higher at 6.23 and 2.99 log cfu/mL. For total coliform, the values decreased at the end of ripening to 5.22 and 5.13 log cfu/g, respectively, in unspicy and spicy cheese at 70 days. While for fecal coliform, the counts were 0.88 log cfu/g at 70 days in unspicy cheese and absent in the spicy product. The coliform number in Tulum cheese from sheep's milk was between 3.59 and 4.30 log cfu/g at 60 and 90 days of age and disappeared in the following ripening stages (Hayaloglu et al. 2007a) .

Yeasts and molds accounted for 6.24 and 7.61 log cfu/mL in milk and *lben*, respectively. In *Bouhezza* cheese from goat's milk, the fungal flora was 4.98 log cfu/g in unspicy cheese at the seventh week and decreased at the end of ripening in the last spicy sample at eight weeks to 2.16 log cfu/g. Aissaoui Zitoun et al. (2011) found a load of 4.76 and 4.81 log cfu/mL in milk and *lben*, respectively.

The load in cow's milk *Bouhezza* cheese was 5.9 and 4.30 log cfu/g after 8 and 10 weeks of ripening. Levels of 5.44 and 5.75 log cfu/g in Tulum at 60 days of age increased to 5.79 and 6.95 log cfu/g after 90 days of aging (Hayaloglu et al. 2007a) . Pathogenic germs *Salmonella* and sulfite-reducing *Clostridium* were absent in the raw material (milk and *lben*) except for *Staphylococcus aureus*, which was present (1.67 log cfu/mL) in the *lben*, but disappeared during the following analyses in unspicy and spicy *Bouhezza* cheese. These last analyzes carried out showed that the studied samples of *Bouhezza* cheese were of good hygienic quality.

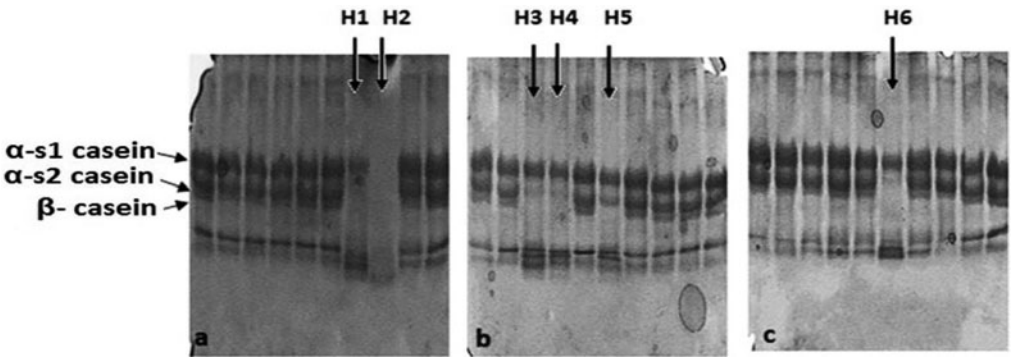

Figure 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE at 12%) of six isolates numbered from H1 to H6, identified as lactic acid bacteria, showing proteolysis zones, derived from the three cheese preparations (1, 2 and 3) of Bouhezza cheese goat's milk ripened for 50 days and longer. (a) H2 shows more extensive proteolysis than H1 issued from the two experimental batches. (b) Three isolates are identified where H4 shows more proteolysis on the protein fractions than H3 and H5. (c) H6 shows also extensive proteolysis on caseins such as H3, which is the same identified strain. The last well was the cow's milk. Isolates: H1 is unidentified and isolated from sample F1 at 50 d; H2 is unidentified from sample of F2 at 50 d; H3 is Enterococcus faecalis from sample of F2 at 50 d; H4 is Enterococcus faecium from sample of F3 at 56 d; H5 is Lactobacillus paracasei subsp. Paracasei from sample of F3 at 72 d; and H6 is a copy of Enterococcus faecalis from sample of F2 at 50 d.

Lactic acid bacteria strains with proteolytic activity

Microbiological analyzes have focused on the detection and enumeration of lactic acid bacteria (LAB) in appropriate media for lactic streptococci and lactobacilli and to test the proteolytic activity of those with consistent characteristics. Samples of each fabrications of approximately 30 days old were chosen to evaluate their lactic acid bacteria content and to test the isolates found in these samples. This choice was made according to the results of SDS PAGE electrophoresis that showed proteolytic activity within a month of ripening.

The enumerations of streptococci and lactobacilli were higher in raw milk and *lben*. They were also in *Bouhezza* cheese at 49 and 64 days, respectively, as the mean of eight samples, near to 9 log cfu/g for streptococci, and higher than 9 log cfu/g for lactobacilli in unspicy and spicy cheese, respectively.

These levels of LABs were high enough to contribute to the production of an appreciable acidity, which was able to limit the development of pathogenic bacteria. These numbers of LABs also make it possible to provide the cheese matrix with enzymes contributing to proteolysis during the ripening of *Bouhezza*.

The results showed a proteolytic activity in the case of six isolates after 48 h of the incubation of the samples. As shown in Figure 3, on gel (a) in the wells 7 and 8 were isolates H1 and H2; on the gel (b) in the wells 3, 4 and 6 were H3, H4 and H5; and on the gel (c) in well 6 was the isolate H6. The isolates giving a proteolysis have been identified by polymerase chain reaction (PCR). These isolates were identified as strains; H3 and H6 as *Enterococcus faecalis*, H4 as *Enterococcus faecium* and H5 as *Lactobacillus paracasei subsp. paracasei*. The others, H1 and H2, were not identified due to a technical error.

Previous studies on technological characterization of LABs in a natural artisanal marinated White Turkish cheese (Dagdemir and Ozdemir, 2008) showed that the proteolytic activity of strains *Enterococcus faecalis* was higher than for other species of enterococci. This confirms the previously published results indicating that *Enterococcus faecalis* is usually more active than *Enterococcus faecium* in respect of the proteolytic activity (Suzzi et al. 2000; Morandi et al. 2006). Those allegations confirm what appears in our results in comparing the two strains identified as *Enterococcus faecalis* (H6) and *Enterococcus faecium* (H4), where proteolysis was more extensive in H6 that in H4.

Bouhezza is a soft cheese refined for at least 50 days to two months and more. During its preparation, the additions of *lben*, which is an acid product rich in lactic acid bacteria, contribute widely. The results have demonstrated the biosafety of the products as the acidity resulting in decreasing pH. *Bouhezza* has an average pH of 4 and lower during the period of its manufacture. The acidity produced by to lactic acid helps to inhibit the pathogenic bacteria.

Concerning the presence of enterococci, it is true that this group was in the past considered as an indicator of fecal contamination, the presence of which may be accompanied by pathogens. Nevertheless, they are not necessarily pathogenic by themselves. Several studies have shown that this group of enterococci was very often present in products made and processed from raw milk.

On the contrary, as we have shown, these species contributes greatly to proteolysis during ripening, which is one of the important processes in cheese maturation. They contribute by their proteases and peptidases to provide the cheese, using different metabolic pathway of proteins and amino acids, in low molecular substances as aromatic components which they give a good flavor to traditional cheese made with raw milk and specially from goats that gives a specific caprin aroma.

Conclusion

Bouhezza is a ripened soft cheese exclusively manufactured in the *Chaouia* region in northeastern Algeria according to a traditional method. The manufacture of *Bouhezza* cheese as a traditional product was carried out in good hygienic conditions which was confirmed with the microbiological quality and the absence of the pathogenic flora in cheese. The physicochemical characterization allowed classifying *Bouhezza* as a soft cheese with mid-levels of fat. The results of this study have led to a better knowledge of the characteristics of this traditional cheese.

The proteolysis during ripening was highlighted by SDS-PAGE, where after one month of cheese ripening, the hydrolysis of proteins was detected by the appearance of new bands in the gel corresponding to new protein fractions. The presence of proteolytic activity on bovine and caprine milk has been revealed among six of the isolated strains from *Bouhezza* cheese. Four of the strains were identified as lactic acid bacteria. In addition, proteolysis was confirmed by RP-HPLC of the soluble fraction in pH 4.4 buffer.

Other studies must be conducted to determine and identify the strains involved in the ripening. Protein and flavor profiles must be evaluated in order to reveal the organoleptic properties of the *Bouhezza* cheese made using raw goat's milk.

Disclosure statement

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