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Full Length Research Paper

Characterization of typical Tunisian fermented milk, rayeb

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Traditional Tunisian fermented milk, rayeb, was produced according to the traditional method. Physicochemical, microstructural, microbiological characteristics and major aromatic compounds evaluation were studied. The results show a decrease in lactose content and pH value and an increase in lactic acid during spontaneous fermentation. The microstructure of rayeb consisted of individualized particles that were coalesced in chains leading to relatively homogeneous sieve. Lactic acid bacteria (LAB) and yeasts present in rayeb were responsible for lactic acid fermentation and aroma development. Dynamic headspace (DHS) extraction procedure shows the existence of four major volatile compounds: acetaldehyde, ethanol, diacetyl and acetoin in the rayeb.

Key words: Rayeb, spontaneous fermentation, physicochemical composition, microstructure, microbiological, volatile compounds.

INTRODUCTION

Rayeb is a traditional Tunisian curdled dairy product that has been known and highly appreciated by consumers for centuries. It is produced by spontaneous fermentation of cow's milk. It can be consumed as a fresh beverage or accompanied with some foods such as bread and couscous. Rayeb can be churned to separate Leben from traditional butter (Samet-Bali et al., 2009). The spontaneous fermentation process results from the action of lactic acid bacteria (LAB) and yeasts. Indeed, LAB has traditionally been employed to produce fermented milk products (Belkaaloul et al., 2010). LAB was known to have a beneficial role in health and in the inhibition of undesirable bacteria (Benkerroum et al., 2002). During fermentation, LAB produced lactic acid: a natural organic acid widely used in food industry as an acidulant, preservative and flavour enhancer. Furthermore, LAB aids the development of many desirable aroma and flavour compounds in fermented milk (Benkerroum and Tamine, 2004) during degradation of proteins, fats and lactose (Tamine and Robinson, 1990). Yeasts also synthesises volatile compounds that contribute to the flavour of traditional fermented milks (Benkerroum and Tamine, 2004; Lore et al., 2005; Alvarez-Martin et al., 2008; Samet-Bali et al., 2010). Extraction of volatile compounds can be achieved using dynamic headspace (DHS) prior to gas chromatographic (GC) separation. During the preparation of fermented dairy products, an essential step in the manufacture of such products is the induction of gel formation, that is, destabilization of the colloidal system of dispersed casein micelles by acidification through LAB. Once destabilized, the casein micelles start to aggregate and finally form a three-dimensional network entrapping the serum phase (Aichinger et al., 2003). The structure of the gel can be investigated by scanning electron microscopy (SEM).

Recently, rayeb is produced in Tunisia using industrial manufacturing practice in order to obtain safe fermented milk and to provide the product with standard characteristics. Nevertheless, consumers prefer the traditional product due to its organoleptic quality (fresh taste and characteristics aroma). The objective of this study was to produce rayeb according to the traditional method and the determination of its physicochemical,

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Abbreviations: LAB, Lactic acid bacteria; **DHS,** dynamic headspace.

microstructural,microbiological and aroma characteristics.

MATERIALS AND METHODS

Milk sample

Cows' milk (Holstein breed) was obtained from a private farm in southern part of Tunisia. Samples of cows' milk were collected, kept refrigerated (4°C) and transported to our laboratory within 6 h. Each sample was taken from 20 to 25 animals.

Fermented milk preparation

Five liters of raw milk was left spontaneously at 25 ± 2°C for coagulation, requiring up to 18 h. After gelation, the product was called "rayeb". After production, samples were stored at 4°C and stored in glass wares. Fermentation process was triplicate for each sample.

Physicochemical analysis

Total nitrogen (TN) and non casein nitrogen (NCN) contents of the Leben were determined using the Kjeldahl method (AFNOR, 1993) using a Büchi 325 apparatus (Büchi, Flawil, Switzerland). The total casein content was calculated by difference between TN and NCN after separation according to Rowland (1938). Dry matter, ash, lactose and fat contents were determined according to standard methods (AFNOR, 1993). Titratable acidity, expressed in Dornic degrees (1°D = 0.1 g lactic acid/l of milk), was determined by titration of 10 ml of sample with M/9 sodium hydroxide to pink endpoint using phenolphthalein as indicator (AFNOR, 1993). The pH was determined using a pH meter (METTLER TOLEDO MP 220 pH meter) calibrated with standard buffer solutions at pH 4.0 and 7.0.

Scanning electron microscopy (SEM)

Samples of milk and rayeb were prepared according to Attia et al. (1991), and were observed under a scanning electron microscope Philips XL30 (Philips, France) after drying to CO_2 critical point using a Baltec CPD 030 apparatus and coating with gold using a Baltec MED 20 apparatus (Balzers Union, Balzers, Germany).

Enumeration of microorganisms

The number of viable mesophilic aerobic bacteria (AB), mesophilic LAB and yeasts were estimated in the milk and the rayeb. Sample preparation and decimal dilutions were made according to the International Dairy Federation (IDF) Standard method (IDF, 1992). The total counts of mesophilic (AB) were enumerated on plate count agar (PCA, Oxoid) after incubation at 30° C for 48 h (ICMSF, 1978). De Man, Rogosa and Sharpe (MRS) medium (Difco) was used for counting LAB. Plates were incubated at 30° C for 48 h (Garrote et al., 2001). Yeasts were enumerated in Sabouraud dextrose agar after incubation at 30°C for three days (Tantaoui-Elaraki et al., 1983).

Volatile compounds extraction and optimization

Volatile compounds extraction was performed by DHS procedure (Dhifi et al., 2005). 50 ml of rayeb sample were put into 120 ml Drechel gas washing bottle with a porous distributor. Volatiles were stripped with nitrogen (0.2 bars, 36°C) for 90 min, trapped on 50 mg of activated charcoal (0.5 to 0.85 mm, 20 to 35 mesh ASTM) from E. Merck (Schuchardt, Germany), at 36°C and eluted with 1 ml of diethyl ether. 10 µl of hexanol was used as internal standard and added for volatile compounds quantification.

Gas chromatography analysis

Volatile compounds were analyzed by GC, using a Hewlett-Packard 6890 gas chromatograph (Palo Alto, CA) equipped with a flame-ionization detector (FID) and an electronic pressure control (EPC) injector. A polyethylene glycol fused silica capillary column (HP-Innowax: 30 m x 0.25 mm ID, 0.25 µm film thickness) purchased from Agilent (Wilmington, DE) was used. The carrier gas flow (N_2) was 1.6 ml/min. The split ratio in the injector was 60:1. The detector and injector temperatures were held at 275 and 250°C, respectively. GC oven temperature was kept at 35°C for 10 min. Aroma compounds were identified by comparing their retention time with those of authentic standards analyzed under the same analytical conditions.

Gas chromatography-mass spectrometry (GC-MS) analysis

The volatiles were analysed also by GC-MS (HP 5890 (II) gas chromatograph). The compounds were separated by HP-5MS 5% phenyl methyl silicone and 95% dimethyl polysiloxane capillary column (30 m x 0.25 mm, 0.25 µm). The oven temperature was programmed to rise from holding times at 50 and 240°C at a rate of 5°C/min. The injection port was heated at 250°C. The carrier gas was He with a flow ratio of 1.2 ml/min; split ratio was 60:1. Detection was performed with the mass spectrometer detector (HP 5972, mass spectrometer) operating in the scan rate (3.81 scans /s) and the ionization energy set at 70 eV. The temperatures of the ion source and the quadrupole mass analyzer were held at 230 and 150°C, respectively. The identification of volatile compounds was made by their retention times and by comparison of their mass spectra with those in the Wiley Mass Spectral database (Wiley and Sons Inc., New York, USA).

Statistical analysis

Fermentation process was triplicate and duplicate analyses were performed on each replicate. Values of different tests were expressed as the mean ± standard deviation ($\bar{x} \pm SD$). SPSS packet program for Windows (SPSS, version 11, USA) was used for the statistical analysis. Significant differences between mean ($P < 0.05$) were determined by using a one-way analysis of variance (ANOVA-Duncan's test).

RESULTS AND DISCUSSION

Physicochemical analysis

The chemical analysis of rayeb (Table 1) showed similarities in nitrogen and casein composition comparatively to the milk. Rayeb can be considered as a product with important nutritional value since it constitutes a high source of digestible protein. There was also no significant difference ($P < 0.05$) in fat and ash contents between the milk and the fermented product. Rayeb was characterized by lower lactose content and pH value and was

Table 1. Physicochemical composition (g/Kg) of milk and rayeb (after 18 h fermentation) (mean[a] ± SD).

Parameter	Milk	Rayeb
Dry matter	117.13 ± 0.28[a]	115.28 ± 0.31[a]
Total nitrogen	33.41 ± 0.65[a]	32.81 ± 0.45[a]
Caseins	26.71 ± 0.53[a]	26.11 ± 0.61[a]
Fat	34.50 ± 0.54[a]	34.10 ± 0.66[a]
Lactose	41.37 ± 0.48[a]	30.60 ± 0.38[b]
Ash	8.25 ± 0.08[a]	7.87 ± 0.05[a]
pH	6.70 ± 0.03[a]	4.45 ± 0.04[b]
Lactic acid	1.62 ± 0.03[a]	6.44 ± 0.11[b]

[a]Mean are average from two independent trials. Different superscript alphabets indicate significant differences (P < 0.05) between samples.

Table 2. Evolution of lactose, pH and lactic acid at various sampling times during spontaneous fermentation of cow's milk in the manufacture of rayeb (mean[a] ± SD).

Parameter	Spontaneous fermentation time (h)					
	0	4	9	12	15	18
pH	6.70 ± 0.03[a]	6.55 ± 0.03[a]	5.94 ± 0.03[b]	5.20 ± 0.03[b]	4.60 ± 0.03[b]	4.45 ± 0.04[c]
Lactose (g/Kg)	41.37 ± 0.48[a]	40.61 ± 0.26[a]	37.80 ± 0.38[b]	34.65 ± 0.27[b]	32.10 ± 0.91[b]	30.60 ± 0.38[c]
Lactic acid (g/Kg)	1.62 ± 0.03[a]	1.80 ± 0.03[a]	2.74 ± 0.03[b]	3.78 ± 0.03[b]	4.92 ± 0.03[b]	6.44 ± 0.11[c]

[a]Mean are average from two independent trials. Different superscript alphabets indicate significant differences (P < 0.05) between samples.

more acidic than milk. This result is similar to those observed by authors for traditional fermented milks (Tantaoui-Elaraki et al., 1983; Beukes et al., 2001; Benkerroum and Tamine, 2004). Gassem and Abu-Tarboush (2000) reported that low pH had an effect on cell growth, lactose utilization and lactic acid production.

Regarding the mean changes in some physicochemical components during spontaneous fermentation (Table 2), the lactose content (41.37 ± 0.48 g/Kg) decreased slowly during the first 4 h (40.61 ± 0.26/kg), and then more significantly (P < 0.05) until the end of fermentation (30.60 ± 0.38g/kg). A very marked decrease in the pH was observed during spontaneous fermentation of cow's milk from an initial mean value of 6.70 ± 0.03 reaching a final mean value of 4.45 ± 0.04. The decrease in pH values coincides chronologically with the increase in the level of lactic acid. Indeed, the mean content of lactic acid (g/Kg) raised significantly (P < 0.05), from 1.62 ± 0.03 reaching a level of 6.44 ± 0.11 g/Kg after 18 h of fermentation. These physicochemical changes observed during spontaneous fermentation could be attributed to the number and / or metabolic activity of acid-producing microorganisms. Alvarez-Martin et al. (2008) reported that LAB species were mostly responsible for milk acidification. Attia et al. (2001) reported that lactic acid fermentation altered casein micelles that progressively lose their surface potential, minerals, caseins and salvation. The results of these modifications are the destruction of the micellar structure and the formation of a three-dimensional network or coagulum which can be visualized by SEM.

Microstructure of rayeb

Evolution of microscopic structure of milk to rayeb is presented in Figure 1. Microstructure of raw milk at native pH 6.7 (Figure 1a) shows the existence of individual micelles in a spherical shape (Attia et al., 2000). From pH 5.6 (Figure 1b), we observed a tendency for the micelles to combine with each other. Linear and irregular arrangements appeared, giving an original structure made up of chains. The whole formed a true network: "open" structure. Different development of the structure of the deposit was observed at pH 5 (Figure 1c). The chains previously observed appeared to stem from the "fusion" of micelles and which bore swellings that might be micellar residues or initial stage of new particles. At pH 4.45 (Figure 1d, rayeb), micelle aggregation became more extensive at the same time. Any individual micelles remained since the fall in their charge and the fact that they became closer in the deposit enhanced the formation of combinations. Thus, the microstructure of rayeb consisted of individualized particles that were coalesced in chains leading to relatively homogeneous sieve (Attia et al., 2001). It was noted that LAB contributed to the building up of biologically acidified milk. In addition, they delimit an empty space around them in

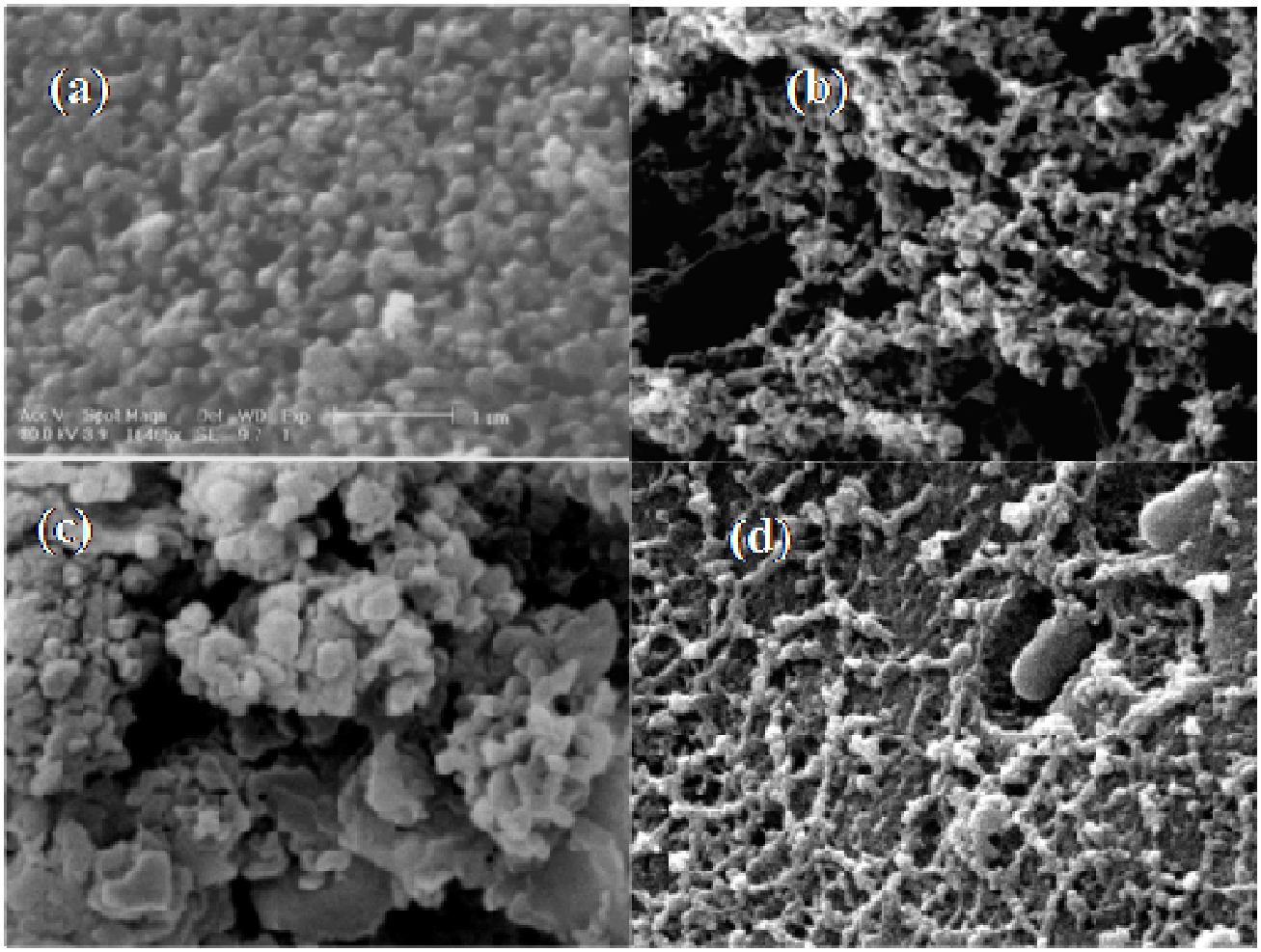

Figure 1. Scanning electron microscopy (SEM) photographs: (a) fresh raw milk (pH 6.7); during the spontaneous fermentation of cows' milk; (b) at pH 5.6; (c) at pH 5 and (d) rayeb. Scale bars = 1 µm.

the protein matrix, which is shown in rayeb (Figure 1d). This could be a result of physicochemical interactions between bacterial membranes and milk proteins (Attia et al., 1991).

Microbiological characteristics of rayeb

Table 3 summarises the microbial counts obtained from milk and rayeb. Aerobic mesophilic counts and mesophilic LAB counts were similar indicating that the micro flora responsible for the fermentation of rayeb was mesophilic and LAB were the dominating micro-organisms. The yeast counts in the rayeb were lower than the counts of LAB but were higher than those observed for other traditional fermented milks (Beukes et al., 2001; Benkerroum and Tamine, 2004). A symbiosis between yeasts and LAB has been suggested: whereby the bacteria provide the acidic condition favourable for the growth of yeasts. The latter provide vitamins and other growth factors to the bacteria (Gobbetti et al., 1994). Alvarez-Martin et al. (2008) reported that yeast growth can be essential to the development of the typical texture and aroma profiles of certain fermented milk products – the outcome of their strong proteolytic and lipolytic activity. Samet-Bali et al. (2010) reported that yeasts produce valuable nutriments (vitamins, essential amino-acids) and various aroma compounds such as diacetyl and ethanol in traditional fermented milk.

Aroma compounds of rayeb

Four major volatile compounds were found in the rayeb (Table 4): ethanol, acetaldehyde, diacetyl and acetoin. Ethanol was the first principal component present in the rayeb. Indeed, ethanol increased considerably during spontaneous fermentation of milk. It can be produced though lactose fermentation by LAB (Fox et al., 1995) and by yeasts (Fernendez-Garcia, 1996). O'Riordan and

Table 3. Counts of the different microbial groups (log UFC/ml) in milk and rayeb (after 18 h fermentation) (Mean[a] ± SD).

Microbial count	Milk	Rayeb
Aerobic mesophilic counts	5.97 ± 1.3[a]	14.85 ± 2.3[b]
Mesophilic LAB	5.11 ± 1.1[a]	13.44 ± 1.8[b]
Yeasts	4.30 ± 1.2[a]	11.64 ± 2.1[b]

[a]Mean are average from two independent trials. Different superscript alphabets indicate significant differences (P < 0.05) between samples.

Table 4. Evolution of the major volatile aroma compounds at various sampling times during spontaneous fermentation of cow's milk in the manufacture of rayeb (mean[a] ± SD)

Compound (mg/L)	A	B	C
Ethanol	+	50.76 ± 1.14[a]	866.87 ± 18.36[b]
Acetaldehyde	-	+	38.05 ± 3.35[a]
Diacetyl	-	20.86 ± 4.96[a]	43.62 ± 5.73[b]
Acetoin	+	20.35 ± 6.46[a]	762.40 ± 33.24[b]

A, Milk after 10 h fermentation time; B, milk after 15 h fermentation time; C, 18 h fermentation time (rayeb) +, compound detected as traces; -, compound was absent. [a]Means are average from two independent trials. Different letters indicate significant differences (P < 0.05) between samples.

Delahunty (2003) reported that ethanol can be produced also by enzymatic reduction of acetaldehyde by LAB. Acetaldehyde began to appear after 15 h of fermentation time and increased slightly until the end of fermentation. It was produced by LAB from lactose and threonine (Marshall and Tamine, 1997) and threonine aldolase can transform threonine to acetaldehyde. The amount of diacetyl and acetoin increased during spontaneous fermentation. Diacetyl was produced through lactose heterofermentation and citrate utilisation (Bourel et al., 2001). However, α-acetolactic acid oxidative decarboxylation is thought to be the dominant mechanism for the characteristic dairy flavour of diacetyl produced by LAB of the *Leuconostoc* and *Lactococcus* genus (Rondags et al., 1998). Acetoin can be derived from diacetyl metabolism (Rehman et al., 2000) or from pyruvate metabolism during the conversion of lactose to lactic acid (Chammas et al., 2006). Alvarez-Martin et al. (2008) reported that *Leuconostoc* species use citrate from which they produce acetoine and diacetyl, but other LAB species and yeasts also produce these compounds.

Conclusion

The production of rayeb is traced back to ancient times in Tunisia. It is a fermented and curdled dairy product characterized by its nutritional value and flavour. Rayeb microflora is composed of stable associations of LAB and yeasts, in particular due to metabolic interactions. These spontaneous starters were responsible of a typical taste (production of aroma compounds) appreciated by consumers. Further studies are required to isolate and characterize the microflora responsible for the fermentation of the traditional fermented cow milk. New isolates may attract the attention of the dairy industry for development of starter cultures as well as for new products and new tastes.

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