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Full Length Research Paper

Development of fermented milk "Leben" made from spontaneous fermented cow's milk

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Formation and characterisation of Industrial Leben produced with Traditional Starters (ILTS) was investigated and compared with industrial Leben produced using commercial starters (ILCS). PH and titratable acidity determinations showed that both traditional and commercial starter cultures performed well in Leben fermentation. Physicochemical analysis showed a similarity between ILTS and ILCS with a slightly higher content of Na in ILTS. Textural analysis indicated that ILTS curd was firmer than ILCS. This textural difference was explained by microstructure observations. Indeed, scanning electron microscopy observation confirmed that ILTS gel was more compact than ILCS gel. Sensory evaluation showed that ILTS was more appreciated by panellists for taste, appearance and overall acceptance.

Key words: Fermentation, Leben, physicochemical, texture, microstructure, sensory evaluation.

INTRODUCTION

Lactic acid fermentation is the most widely used acidification process to coagulate bovine milk during the manufacture of cultured dairy products. Lactic acid bacteria are responsible for this bioprocess (Belkaaloul et al., 2010; Liu et al., 2011). The pH decrease caused lactic acid fermentation alters aggregation of casein micelles. These modifications lead to the formation of a coagulum (Attia et al., 2001; McCann et al., 2011). Physicochemical changes induced by lactic acid fermentation exert a major influence on the macrstructure and on the sensory properties of fermented dairy products. Final characteristics of fermented milks depend on milk composition, heat treatment of milk, fermentation conditions and the composition of the starter's cultures (Chammas et al., 2006).

The interest of consumers was increased to traditional fermented dairy products due to their nutritional value and tastiness. Leben is the most popular traditional product known in the North Africa and the Middle East. Chemical and microbial properties of traditional Leben were investigated in an effort to contribute to the standardization of the product (Tantaoui-Elaraki et al., 1983; Guizani et al., 2001; Benkerroum and Tamine, 2004).

In Tunisia, traditional Leben (TL) is produced by spontaneous fermentation of cows' milk with natural microflora. The fermentation is allowed to proceed for a period of up to 18 h, after which the fermented milk obtained called "Rayeb", was churned. Traditionally, churning takes place in a goat leather bag called a "Checoua" (Figure 1). The churning is achieved by hanging the "Checoua" filled with "Rayeb" and vigorously shaking it back and forth till the coalescence of the fat globules. To avoid the unpleasant appearance of smells, the "Checoua" was salted between two successive churning operations. Consumers prefer TL due to its organoleptic quality (fresh and sour taste and characteristics aroma). Scientific data on the traditional fermentation process and characteristics of TL were carried out by Samet-Bali et al. (2009): TL was rich in proteins, mesophilic lactis acid bacteria (LAB) were the predominant microflora, mostly of the genus *Lactococcus* (Ziadi et al., 2005), and several yeasts species contributed to the spontaneous fermentation process. The

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Abbreviations: ILTS, Industrial Leben produced with traditional starters; **ILCS,** industrial Leben produced using commercial starters.

Figure 1. Photo of the traditional churning process in Tunisia.

growth of these micro-organisms leads to production of many desirable aroma and flavour compounds in the TL.

To standardize product quality and increase safety of TL, selected bacterial cultures composed of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *diacetylactis* and *Lactococcus lactis* subsp. *cremoris* were used to produce an industrial Leben in Tunisia. In spite of the effort deployed to imitate TL, consumers prefer TL since artisanal starters give the product more typical flavours and texture (Wouters et al., 2002).

Therefore, the aim of this study was to produce Leben according to the industrial method, using the TL as a starter culture in the scope of the transfer of the products' technology to small industrial scale. ILTS (Industrial Leben produced with Traditional Starters) was compared to Industrial Leben produced using the Commercial Starters (ILCS).

MATERIALS AND METHODS

Milk sample

Cows' milk (Holstein breed) samples were obtained from a local farm in the southern part of Tunisia. Samples of cows' milk were collected, kept refrigerated (4°C) and transported to our laboratory within 6 h. Upon arrival, samples were skimmed, at 2000 ×g and 10°C for 15 min. Each sample was a pooled skim milk taken from 20 to 25 animals.

Used starters

For the production of ILCS samples, commercial starters were used containing a mixed culture of mesophilic acidified and aromatic lactic bacteria: *L. lactis* subsp. *lactis*, *L. lactis* subsp. *diacetylactis* and *L. lactis* subsp. *cremoris* (Danisco, Rhodia Food, France).

For ILTS samples, used starters were produced from traditional Tunisian spontaneous fermented milk (TL). TL was produced using the following steps: five liters of raw milk was left spontaneously at 25 ± 2°C in a jar until coagulation occurred (~ 18 h). On gelation, the product is called "Rayeb". By churning during 40 min, the "Rayeb" is separated into an aqueous fraction containing (TL) and a fat-rich fraction (traditional raw butter). A portion of TL was used as starters for the production of ILTS samples. TL contained a mixed culture of mesophilic LAB (~1.17 10^8 UFC/ml) especially the genus *Lactococcus* and a considerable numbers of yeasts (~1.99 10^7 UFC/ml) (Samet-Bali et al., 2009).

Fermentation process

Following to the industrial condition, 0.2% (w/v) of commercial starters was used to obtain ILCS after 12 h fermentation time at 27°C. To be able to compare ILTS and ILCS samples, level of added traditional starter must be optimized follow the industrial

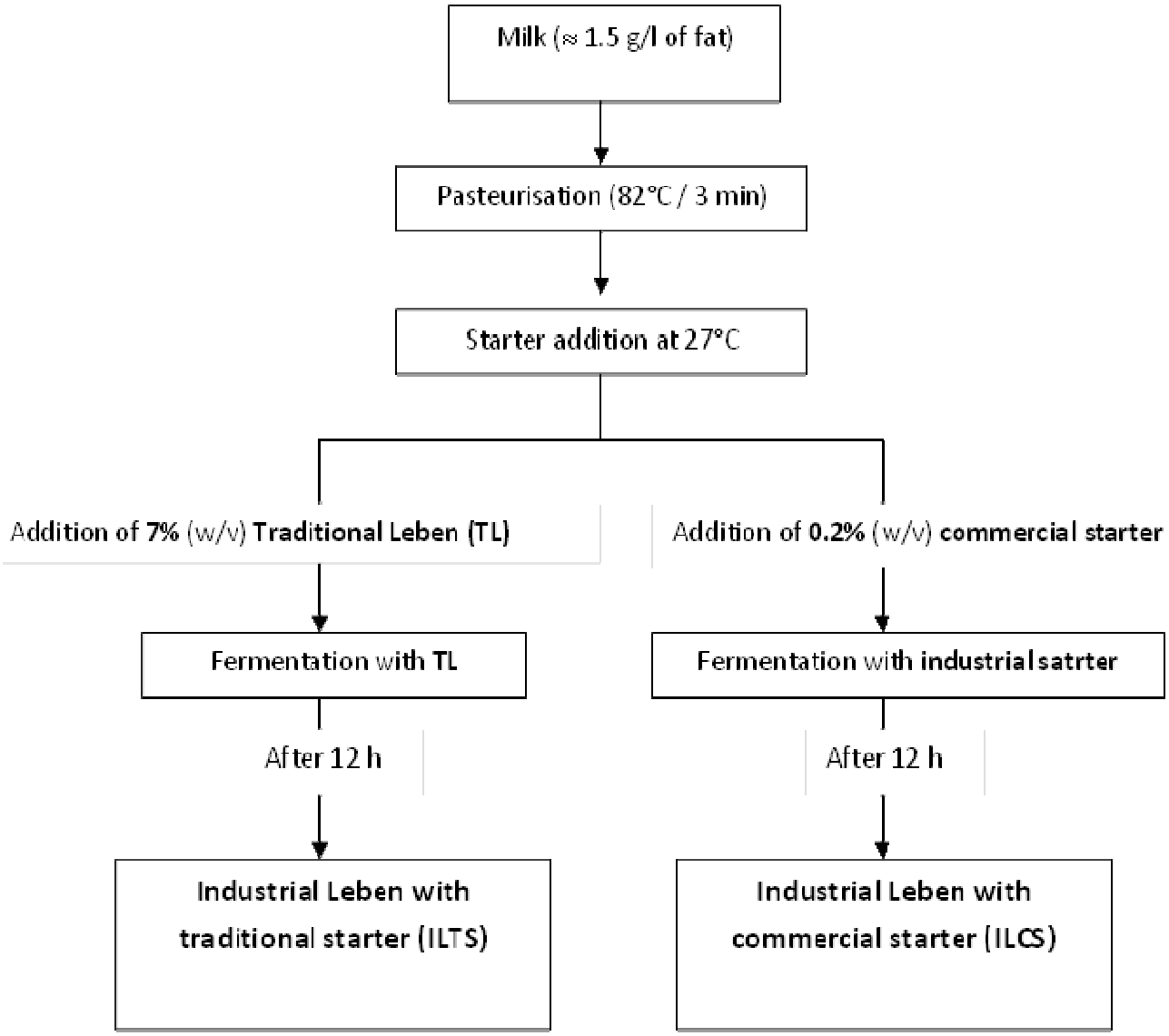

Figure 2. Schematic diagram of Industrial Leben produced with Traditional Starters (ILTS) and Industrial Leben produced using Commercial Starters (ILCS).

procedure (12 h fermentation times at 27°C) to obtain comparable products. Thus, a preliminary study was carried out to optimize the level of TL incorporated during fermentation of ILTS samples (comparable acidity and pH of final ILTS and ILCS samples). Several TL (traditional starters) levels were tested (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10% (w/v)). Obtained results (of this preliminary study) showed that 7% of TL give, after 12 h fermentation time at 27°C, an ILTS with comparable titratable acidity and pH values than ILCS.

ILTS and ILCS samples were produced according to diagram presented in Figure 2. After pasteurisation (82 °C for 3 min) of five liters of milk samples, incubation temperature was maintained at 27 ± 0.1°C in circulating water bath (Huber, Kältemashinenbau, Offenburg, Germany).

After production, samples were stored at 4°C and packed in glass. The fermentation process was carried out in triplicate for each sample. During fermentation process, pH value, titratable acidity and viable bacteria number were recorded. The pH was monitored during the fermentation by using a pH meter (METTLER TOLEDO MP 220 pH meter). 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 N/9 sodium hydroxide to pink endpoint using phenolphthalein as indicator (AFNOR, 1993). The number of viable bacteria was estimated in ILTS and ILCS, during fermentation process, by using serial decimal dilutions according to the International Dairy Federation (IDF, 1996). MRS (Difco) medium was used for counting mesophilic (LAB). Plates were incubated for 48 h at 30°C (Garrote et al., 2001). Yeasts were enumerated on sabouraud dextrose agar after incubation at 30°C for 3 days (Samet-bali et al., 2009).

Chemical analysis

Total nitrogen (TN), non protein nitrogen (NPN) and non casein nitrogen (NCN) contents of samples were determined by the Kjeldahl method (AFNOR, 1993) using a Büchi 325 apparatus

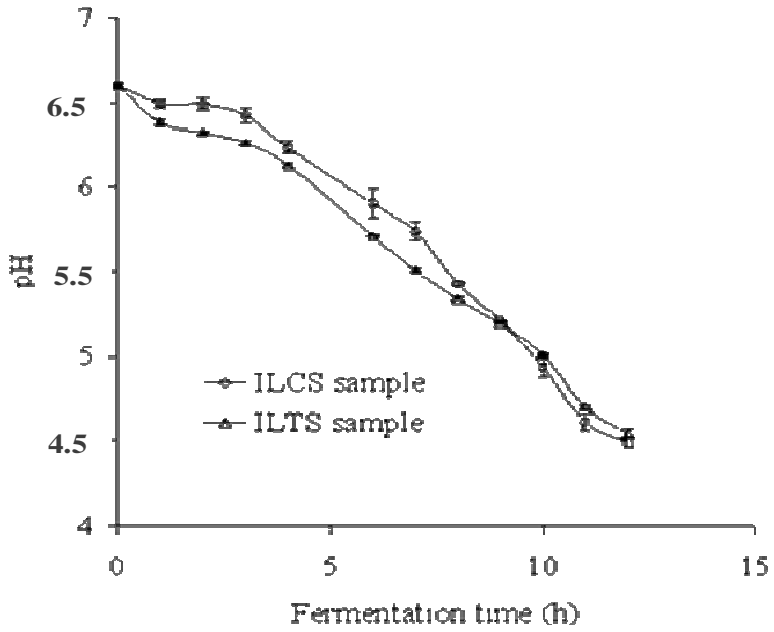

Figure 3. pH changes during lactic fermentation at 27°C.

(Büchi, Flawil, Switzerland). The total casein content was calculated by difference between TN and NCN after separation according to Rowland (1938). Total solids, ash, lactose and fat contents were determined according to the methods described by AFNOR (1993).

Calcium (Ca), magnesium (Mg), potassium (K) and sodium (Na) were measured by atomic absorption spectrometer model Hitachi Z-6100 (Hitachi Instruments Engineering Co., Ibaraki-ken, Japan) in the presence of lanthanum oxide (Sigma, St. Louis, MO, USA) for Ca and Mg and in the presence of cesium chloride for K and Na. The concentration of phosphorus (P) was determined by a colorimetric method with ammonium molybdate (Pien, 1969).

Texture evaluation

All instrumental texture analyses were done on ILTS and ILCS samples stored at least for 24 h at 4°C. For every sample three repeated measurements were taken for each replicate and mean values were reported. Texture profile of ILTS and ILCS were performed using a Texturometer (LLOYD instruments, England) by applying hardness procedure (40/0613). A cylindrical probe (1.5 cm diameter) was used at a speed of 40 mm/min with a detection value of 0.05 N and a first limit of 1mm and a second limit of 2 cm. After each test the mechanical power dissipated in the sample (dissipated work) and the hardness of the gel were calculated.

Scanning electron microscopy (SEM)

Samples of ILTS and ILCS 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).

Sensory analysis

Sensory analysis were conducted by 36 panellists, who were experienced in sensory evaluation of foods, but received no specific training relevant to these products. Panellists were asked to indicate how much they liked or disliked each product on a 7-point hedonic scale (7 = like extremely; 1 = dislike extremely) according to appearance, consistency, taste and overall acceptability. ILTS and ILCS samples were distributed at 4°C (after storage of 24 h at 4°C) in white polystyrene glasses. Tap water was supplied to the panellist for rinsing between samples. Experiments were conducted in an appropriately designed and lighted room and a mean value was calculated for each product.

Statistical analysis

Fermentation process was carried out in triplicate and duplicate analyses were performed on each replicate. Values of different tests were expressed as the mean ± standard deviation (X ± SD). The SPSS packet program for Windows (SPSS, version 11, Chicago, IL, USA) was used for the statistical analysis. Significant differences between mean (P<0.05) were determined by using a one-way ANOVA (Duncan's test).

RESULTS AND DISCUSSION

Lactic fermentation ability

Figure 3 presents the evolution of pH during fermentation of ILCS and ILTS samples. This figure shows that for both sample an induction period of ~ 4 h was observed. Indeed, after 4 h post-inoculation, pH of ILCS varied from 6.6 to 6.23 whereas pH of ILTS varied from 6.6 to 6.13. After this induction period, pH value declined considerably during lactic fermentation to reach a value of 4.48 for ILCS and 4.5 for ILTS. These pH changes could be attributed to the number and/or metabolic activity of acid

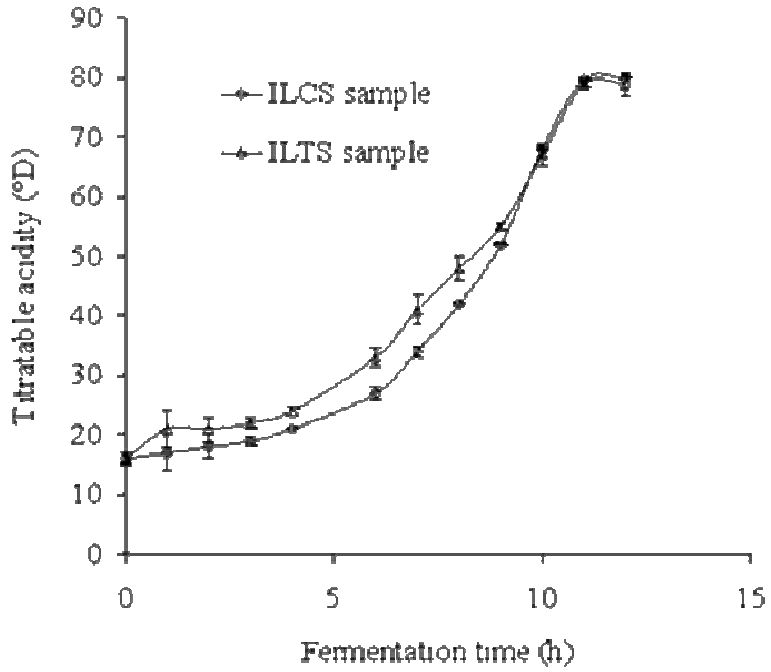

Figure 4. Lactic acid production during lactic fermentation at 27°C.

producing micro-organisms. As Leben starters grow, they produce acid which causes a decrease in pH. Gassem and Abu-Tarboush (2000) reported that low pH had an effect on cell growth, lactose utilization and lactic acid production. The titratable acidity increased with increasing fermentation time (Figure 4) to reach final values of 79°D and 80°D for ILTS and ILCS, respectively. These results are in agreement with those previously reported for other "Lebens" (Boubekri et al., 1984; Benkerrom and Tamine, 2004). Figure 5 shows LAB evolution during fermentation process for both ILCS and ILTS samples. In both cases, LAB number increases versus fermentation time. This LAB growth was slightly more important (P<0.05) in ILTS than in ILCS. This richness on LAB could develop more aroma and nutritional compounds. This confirms many studies which reported that fermentation by the microbial starter cultures preserves the product through the production of lactic acid from lactose and contributes to the development of characteristic flavour compounds; and that fermented dairy products are now recognized for their nutritional benefits (Boylston et al., 2004; Hilali et al., 2011).

Composition of fermented milks

Table 1 indicates average composition of raw milk, ILTS and ILCS samples. Chemical characteristics of fresh raw milk show suitable technological properties. For ILTS and ILCS chemical composition, no significant (P<0.05) differences were observed except for ash contents where ILTS is richer than ILCS. Table 1 shows too that NPN has increased to 2.34 and 2.31 g/Kg in ILTS and ILCS, respectively versus 1.67 g/Kg in bovine milk. The increase of NPN could be attributed to the proteolytic activity of LAB during lactic fermentation (Attia et al., 2001). Protein contents in ILTS and ILCS were almost similar compared to cow's milk. Lactose contents decrease from 48.7 g/Kg in raw milk to 36.7 and 36.5 g/kg in ILCS and ILTS, respectively. The decrease in lactose content was due to lactic acid formation. Obtained results (LAB numbers, pH, acidity and lactose consumption) show that with the same lactose quantity (carbon source), LAB number in the final product were slightly more important in ILTS (~3 10^8 UFC/ml) than ILCS (~2.4 10^8 UFC/ml) with closed values of pH and acidity. The presence of a relatively great number of LAB in ILTS sample could be explained by the presence of other micro-organisms such as yeasts in the TL (~1.99 10^7 UFC/ml) according to Samet-Bali et al. (2009). Indeed, yeasts count were more important in ILTS (~1.5 10^6 UFC/ml) compared to ILCS (~1.4 10^5 UFC/ml). Tamime et al. (2011) reported that lactic acid bacteria (LAB), yeasts and moulds are employed in the manufacture of fermented milk products. Yeasts could interact with LAB according to De Vuyst and Neysens (2005) which reported that for fermented dairy products microflora is composed of stable association of LAB and yeasts, in particular due to metabolic interactions. According to Gobbetti et al. (1994), a symbiosis between yeasts and LAB has been suggested: Whereby the bacteria provide the acidic condition favourable for the growth of yeasts. The latter provides vitamins and other

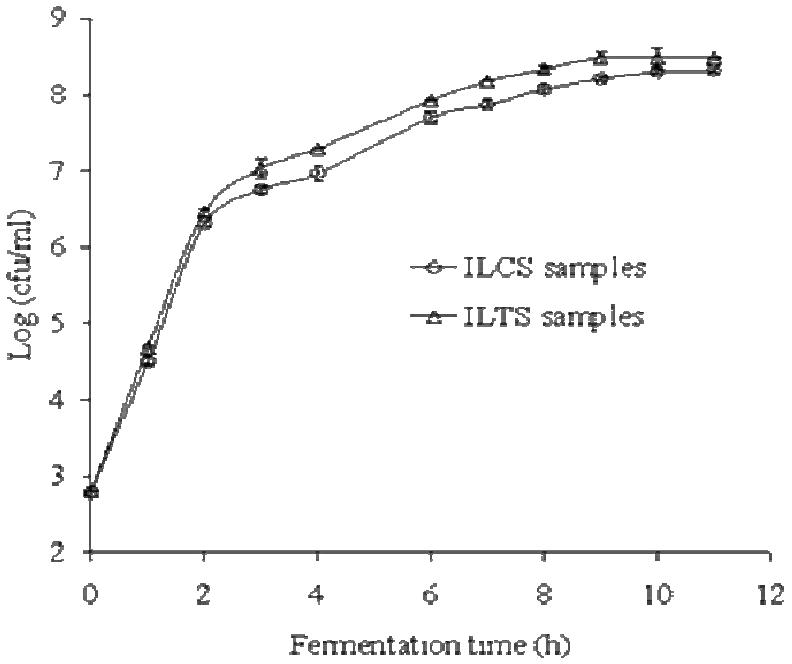

Figure 5. Bacterial growth during lactic fermentation at 27°C.

Table 1. Average composition (g/Kg; mean [a] ± SD) of raw skim milk, ILCS and ILTS.

Composition	Skim milk	ILCS	ILTS
Total solids	92.02 ± 1.82[a]	82.19 ± 1.40[b]	82.58 ± 1.30[b]
Protein	32.91 ± 0.61[a]	31.61 ± 0.28[b]	31.41± 0.23[b]
Caseins	26.60 ± 0.41[a]	26.30 ± 0.30[b]	26.27 ± 0.31[b]
NPN	1.67 ± 0.05[a]	2.31 ± 0.03[b]	2.34 ± 0.04[b]
Fat	1.40 ± 0.15[a]	1.35 ± 0.10[b]	1.35 ± 0.10[b]
Lactose	48.70 ± 1.04[a]	36.7 ± 0.30[b]	36.5 ± 0.27[b]
Ash	8.26 ± 0.10[a]	7.47 ± 0.03[b]	8.58 ± 0.06[c]

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

Table 2. Mineral composition (g/Kg; mean [a] ± SD) of ILTS and ILCS.

Parameter	Calcium	Magnesium	Sodium	Phosphorus	Chlorides	Potassium
Skim milk	1.20 ± 0.02[a]	0.10 ± 0.02[a]	0.40 ± 0.02[a]	1.15 ± 0.02[a]	1.54 ± 0.02[a]	1.51 ± 0.01[a]
ILTS	1.20 ± 0.04[a]	0.11 ± 0.03[a]	0.64 ± 0.04[b]	1.14 ± 0.03[a]	1.80 ± 0.01[b]	1.51 ± 0.01[a]
ILCS	1.18 ± 0.04[a]	0.10 ± 0.01[a]	0.36 ± 0.01[a]	1.12 ± 0.04[a]	1.52 ± 0.05[a]	1.50 ± 0.02[a]

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

growth factors to the bacteria. However, according to Samet-Bali et al. (2009), a symbiosis between yeast and LAB has been suggested for the production of aroma compound in TL. This leads us to suspect that ILTS will have a great nutritional value and will be more appreciated by the consumers. In the same trend, Erbaş et al. (2006) reported that association of LAB and yeast during fermentation may contribute to the production of metabolites, which could impart pleasant tastes and flavours to foods.

Table 2 shows mineral composition of ILTS and ILCS samples. Mineral contents show that globally ILTS and ILCS contain comparable values. ILTS samples show a higher content (P<0.05) of sodium (Na) and chloride (Cl) compared to ILCS samples. This difference could be attributed to the higher content of Na and Cl in the

Table 3. Textural properties (mean [a] ± SD) of ILTS and ILCS.

Parameter	Hardness (N)	Dissipated work (N/mm)
ILTS	0.00977 ± 0.00005[a]	0.13545 ± 0.01100[a]
ILCS	0.00853 ± 0.00016[b]	0.026185 ± 0.00045[b]

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

traditional starters (TL which is utilised to coagulate cow's milk). In fact, the salting of "Checoua" between two successive churning operations (to avoid the unpleasant appearance of smells) was responsible for the passage of sodium chloride (NaCl) hexogen to TL during manufacture. The presence of this excess Na could have an impact on the gelling properties of ILTS. Indeed, Le Great and Brulé (1993) reported that ionic strength increase via addition of sodium chloride leads to the disorganisation of micellar structure and then to the modification in the textural of dairy products gels.

Textural properties

At the end of the incubation time, there was a real curd in both fermented milks. It was a homogeneous and smooth mass, which entirely occupied the initial volume of milk. A better understanding of the fermented dairy products properties, necessary for controlling product quality, requires further studies of gel formation due to bacterial acidification (Aichinger et al., 2003). An important criterion for quality assessment of set-style cultured milk products is the texture of the gel. Table 3 shows results of textural characteristics of ILTS and ILCS samples after the fermentation process. Textural parameters (hardness and dissipated work) show that ILTS gel is firmer than ILCS one. Oliveira et al. (2001) reported that gel fermented milk firmness depends on the total solids as well as on protein contents and type. Although, total solids and protein contents for both fermented products (ILTS and ILCS) were similar (Table 1), firmness of ILTS was higher than that of ILCS. This result could be explained by the difference in Na content (NaCl) as previously shown and by the arrangement of casein micelles into more compact aggregates. In this sense, Zoon et al. (1989) reported that the variation on textural properties and then the disorganisation in the micellar structure caused probably by the difference in mineral contents. Indeed, by increasing of NaCl, gel firmness is increased and brittleness is reduced. Similarly, Salaün et al. (2005) reported that during production of dairy products, significant hydration changes occur in curd and an increase in ionic strength were observed by salt addition. Curd was observed to be more stable as concentration of NaCl and ionic strength increased. Moreover, Schkoda et al. (1999) reported that the tendency of casein micelles to aggregate in the presence of NaCl was also proved by electron microscopy.

Coagulum microstructure

Fermented dairy products are of great economic importance for the food industry. 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 slow acidification through LAB. SEM micrographs of the fermented bovine gels proved to be helpful in describing the network structure of gels (Aichinger et al., 2003). The SEM micrographs shown in Figure 6a and b demonstrates that the casein micelles in a gel have aggregated to form clusters and chains resulting in a three-dimensional network with the nodal points being connected by strands of aggregated casein micelles. These SEM observations also demonstrated that a casein gel network has a pores structure. Thus, fermented milks microstructure consisted of a protein network composed by chains and aggregates of fused casein micelles, whose globular shape is still discernible, interspaced by spaces and where the lactic bacteria are easily distinguished.

In ILTS, the casein micelles were predominantly linked by particle to particle attachment in chains with comparatively small interspaced voids (Figure 6c), rather than by particle fusion into aggregates (Figure 6d). However, for ILCS samples a protein structure characterized by short casein micelles chains and no appreciable casein micelle fusion were observed. ILCS exhibited a more open, loose and less dense protein network than ILTS. These SEM observations reinforce conclusion already drawn concerning hardness difference between ILTS and ILCS gels.

Sensory evaluation

Results of the sensory evaluation of fermented milk samples on a scale from 1 (dislike extremely) to 7 (like extremely) are shown in Table 4. ILTS had a high grading for appearance due to wheying off (syneresis) appeared in the fermented milk surface of ILCS. In relation to taste, there were significant (P<0.05) differences between samples. Indeed, ILTS presents a taste nearly similar to TL. For the overall acceptability and the consistency, panellists prefer ILTS than ILCS.

From the present study, it can be concluded that TL

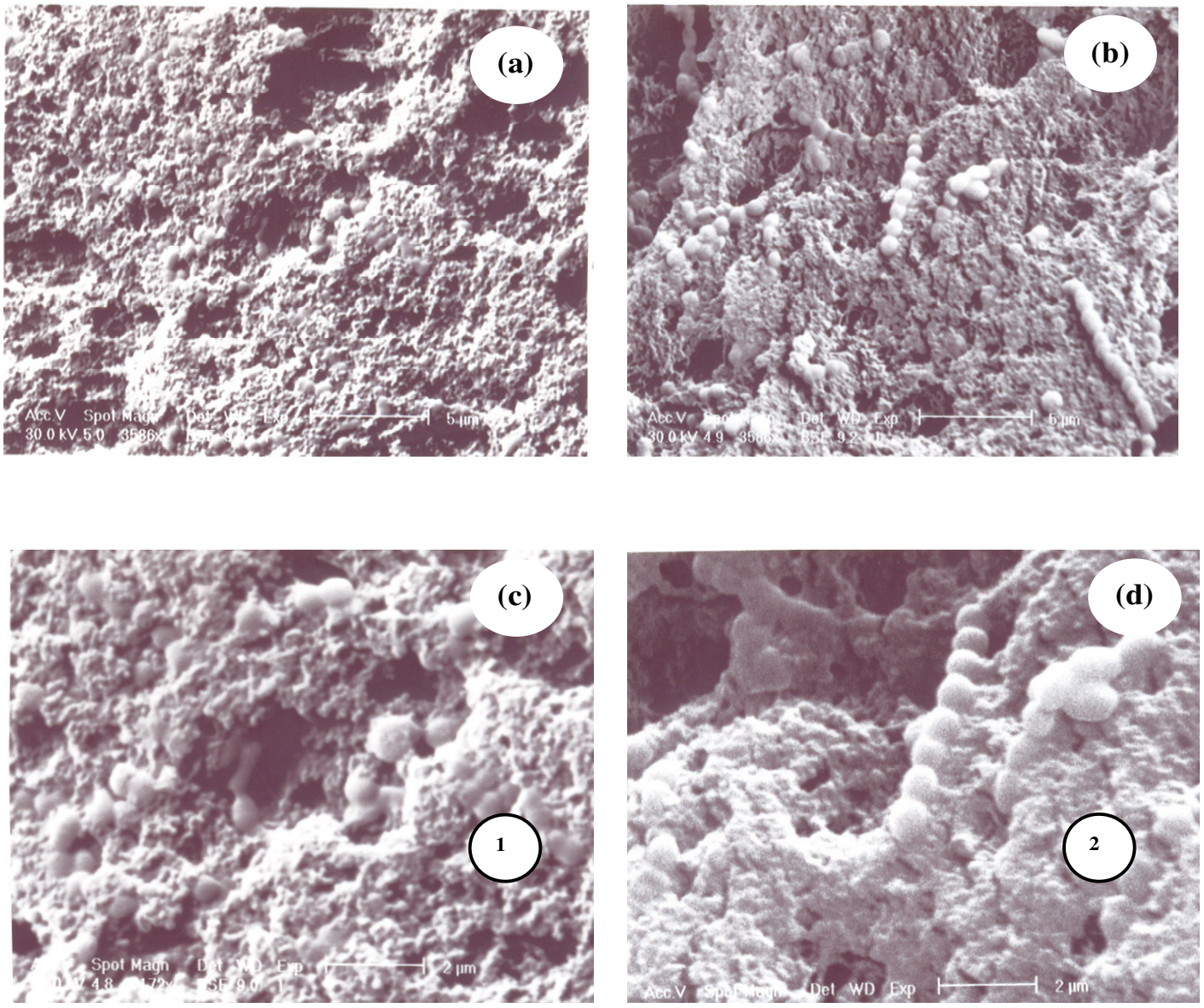

Figure 6. SEM micrographs of ILTS: **(a)** scale bar = 5 µm; **(c)** scale bar = 2 µm and ILCS: **(b)** scale bar = 5 µm; **(d)** scale bar = 2 µm. **(1)**: protein network formed by casein micelles chains, **(2)**: protein network formed by microparticulate protein and few fused casein micelles aggregates.

Table 4 Sensory analysis (mean [a] ± SD) of ILTS and ILCS.

Fermented milk	Appearance	Consistency	Taste	Acceptability
ILTS	6.40 ± 0.14[a]	6.53 ± 0.20[a]	6.33 ± 0.12[a]	6.5 ± 0.20[a]
ILCS	5.43 ± 0.16[b]	5.40 ± 0.21[b]	4.56 ± 0.20[b]	4.5 ± 0.40[b]

Scores vary between 1 (dislike extremely) and 7 (like extremely).
[a] Means are average from two independent trials. Different letters indicate significant differences ($P < 0.05$) between samples.

would be an adequate starter for the production of Leben or similar indigenous fermented milks on a small industrial scale. Indeed, the use of a sample from the TL as starters to produce Leben using the industrial procedure leads to industrial products (standard quality of the products and a high level of reproducibility of the process) with

traditional characteristics such as richness on LAB and aroma and an appreciated texture. Based on these experimental results, the possibility to use artisanal strains, isolated from TL, as starters for the development of fermented milks at an industrial scale is very promising.

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