

Full Length Research Paper

# Growth, acidification and proteolysis performance of two co-cultures (*Lactobacillus plantarum*-*Bifidobacterium longum* and *Streptococcus thermophilus*-*Bifidobacterium longum*)

K. Belkaaloul<sup>1</sup>, A. Chekroun<sup>1</sup>, A. Ait-Abdessalam<sup>2</sup>, D. Saidi<sup>1</sup> and Kheroua O.<sup>1\*</sup>

<sup>1</sup>Laboratory of Physiology of Nutrition and Food Safety, Department of Biology, Faculty of science, University of Oran, Es-Senia Oran-31000, Algeria.

<sup>2</sup>Laboratory of Food and Industrial Microbiology, Department of Biology, Faculty of science, University of Oran, Es-Senia Oran-31000, Algeria.

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In this study, the fermentation and proteolysis of two co-cultures were investigated. Two fermented cow skim-milk lactoferrin 1 (LF1) and lactoferrin 2 (LF2) were prepared. LF1 was inoculated with *Bifidobacterium longum* (Bf I) and *Streptococcus thermophilus* (St I) while LF2 was inoculated with *Lactobacillus plantarum* (Lb O) and *B. longum* (Bf I). Incubation was at 42°C for 8 h. The enumeration revealed bacterial growth in all fermented milk. Maximum growth of (Lb O) and (Bf I) was observed when mixed together after 2 h of fermentation in comparison with Bf I and St I with values of 10<sup>9</sup> and 3.10<sup>8</sup> cfu/ml, respectively. The kinetics of acidification (pH and lactic acid production) gave significant values ( $p < 0.01$ ) for LF2 when compared to LF1 and sterile milk (LS). The proteolytic activity (functions  $\alpha$ -NH<sub>2</sub> released in  $\mu$ M/mg) and total proteins (in  $\mu$ g/mg) gave significant values ( $p < 0.05$ ) for LF2 when compared to LF1. Two mixed cultures (Lb O- Bf I) and (St I-Bf I) showed proteolysis of  $\beta$ -lactoglobulin ( $\beta$ -Lg) and  $\alpha$ -lactalbumin ( $\alpha$ -la).

**Key words:** *Bifidobacteria longum*, *Lactobacillus plantarum*, *Streptococcus thermophilus*, cow milk, fermented milk, milk's proteins, proteolysis.

## INTRODUCTION

Throughout history, humans have made use of lactic acid bacteria (LAB), which are distributed widely in nature. LAB has traditionally been employed to produce fermented milk products, including yogurt, leiben, dahi, kefir and koumiss (Miyazaki and Matsuzaki, 2008). Fermented milks products were significantly more digestible than the milk mixture from which it is made (Breslaw and Kleyn, 1973; Tamime and Robinson, 1999). Although twelve genera of LAB are now recognized (Axelsson, 2004), starter cultures of LAB belong to one of four genera, with the dairy LAB representing the largest group. This includes species of *Lactococcus*, *Streptococcus*, *Leuco-*

*nostoc* and *Lactobacillus* (Hutkins, 2006). In addition to lactic acid producers, other types of organisms may also be employed to impart therapeutic properties to fermented products, such as *Bifidobacterium* sp. (Leahy et al., 2005). One important result of the addition of the LAB necessary for fermentation is the resulting proteolytic activity. Although this activity is slight, resulting in a breakdown of only 1 to 2% of milk protein (Rasic and Kurmann 1978), it is essential for the release of small peptides and amino acids for the growth of the bacteria. The principal substrate for such proteolysis is casein, but limited degradation of whey proteins may also occur. (Chandan et al., 1982; El-Zahar et al., 2003; Khalid et al., 1991).

The aim of this work was to evaluate the growth, acidification and proteolytic activity of three strains (*Bifidobacteria longum*, *Lactobacillus plantarum* and

\*Corresponding author. E-mail: [omar\\_kheroua@hotmail.com](mailto:omar_kheroua@hotmail.com)  
.Tel/Fax: +213 41 513025.

*Streptococcus thermophilus*) in cow's skimmed milk.

## MATERIALS AND METHODS

### Bacterial strains and growth conditions

The bacterial strains used in this study were *B. longum* (Bf I) and *S. thermophilus* (St I) from feces of breast-fed new born and *L. plantarum* (Lb O) from Olives origins, belonging to the Culture Collection of the Laboratory of Physiology of the Nutrition and Food Safety, Department of Biology, University of Oran (Es-senia), Algeria.

The freeze-dried stocks are revived by sowing in liquid media. Medium MRS (De Man et al., 1960) was used for *L. plantarum* (Lb O), medium M17 (Terzaghi and Sandine, 1975) for *S. thermophilus* (St I) and medium MRS containing L-cysteine hydrochloride (0.05%, w/v) (Merck) for *B. longum* (Bf I). All sown media were incubated at 37°C for 2 nights. This was done aerobically for *S. thermophilus* (St I) and anaerobically (GENbox anaer, bioMérieux® sa, France) for *B. longum* (Bf I) and *L. plantarum* (Lb O).

### Cow's milk provenance and treatment

The raw cow's milk freshly collected in a breeding farm was skimmed by centrifugation (SIGMA 4 K 10) at 3500 rd/min for 20 min at 4°C and then sterilized at 105°C for 10 min.

### Experimental process of fermentation of skim-cow milk

#### Preparation of inocula

Each strain was first sub-cultured in its specific medium at 37°C for 24 h and then sub-cultured twice in sterile reconstituted skim-milk (10%, w/v) supplemented with (0.5%, w/v) yeast extract (Difco) and L-cysteine hydrochloride (0.05%, w/v) for culturing *B. longum* (Frank et al., 1993). This was followed by a final subculture in sterile skim-cow milk, in order to be better adapted to the cow's milk environment. All subcultures were done at 37°C for 18 h.

#### Inoculation of skim-cow milk

Two fermented milk, lactoferrin 1 (LF1) and lactoferrin 2 (LF2) obtained from cow-skim milk were inoculated with two mixed cultures of 2% (v/v) *B. longum* (BfI) ( $10^7$  ufc/ml) + 2% (v/v) *S. thermophilus* (St I) ( $10^8$  ufc/ml) for LF1 and 2% (v/v) *L. plantarum* (LbO) ( $10^7$  ufc/ml) + 2% (v/v) *B. longum* ( $10^7$  cfu/ml) for LF2. Incubation was at 42°C for 8 h.

#### Viable microorganism

Viable counts were determined in fermented milks samples (1 ml) after the fermentation process by using serial decimal dilutions prepared in ¼ strength Ringer's solution (supplemented with 0.3 g.l<sup>-1</sup> cysteine-HCl for bifidobacteria). One ml aliquot dilutions were poured onto plates of the various selective and differential agar in triplicate. Counts of *S. thermophilus* (St I) were enumerated on M-17 agar and incubated aerobically at 37°C for 48 h. For enumeration of *L. plantarum* (Lb O), appropriate dilutions were spread out on MRS agar pH 5.2 and plates were incubated anaerobically at 37°C for 72 h. Beerens medium was adapted from bifidobacteria selective media described in the literature by Bonaparte et al. (2001). Plates were incubated for 3 days at 37°C under anaerobic

conditions. Plates containing 30 - 300 colonies were counted and the results expressed as colony-forming units per ml (cfu ml<sup>-1</sup>) of sample.

#### Acidification kinetics

Lactic acid determination produced during the growth in skimmed fermented milk at 0, 2, 4, 6 and 8 h was measured by the method described by Accolas et al. (1977) using NaOH (N/9) in the presence of phenolphthalein (1%). The acidity developed in milk was also followed by a measurement of pH, using a digital pH meter (Kika Laborechnik, West Germany). The pH meter was calibrated using standard buffer solutions (Merck) at pH 4.0 and 7.0.

#### Proteolysis measurement

The measurement of total proteins was carried out by the method of Lowry et al. (1951) measuring absorbance at 750 nm. Bovine serum albumin was used as a standard. The measurement of the bacterial proteolysis activity was obtained by measurement of the released  $\alpha$ -NH<sub>2</sub> functions during fermentation according to the method of Doi et al. (1981). The absorbance of the solution was measured by a spectrophotometer (JASCO V-530, Indonesia) at 540 nm. The concentration of functions  $\alpha$ -NH<sub>2</sub> released per sample was calculated with leucine standard curve established with standards studied under the same conditions.

#### SDS-PAGE of proteins from cow's fermented milk

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of milk samples was performed under denaturing conditions as described by Lammeli (1970). Proteins were separated in an acrylamide gel (12.5%) and stained with Coomassie blue R-250. All material and instruments were purchased from Bio-Rad. laboratories (Richmond, Calif.).

#### Statistical analysis

Experiments were repeated six times and results were expressed as mean  $\pm$  standard errors. The obtained data were statistically analyzed using the student's t test as programmed by MSII.

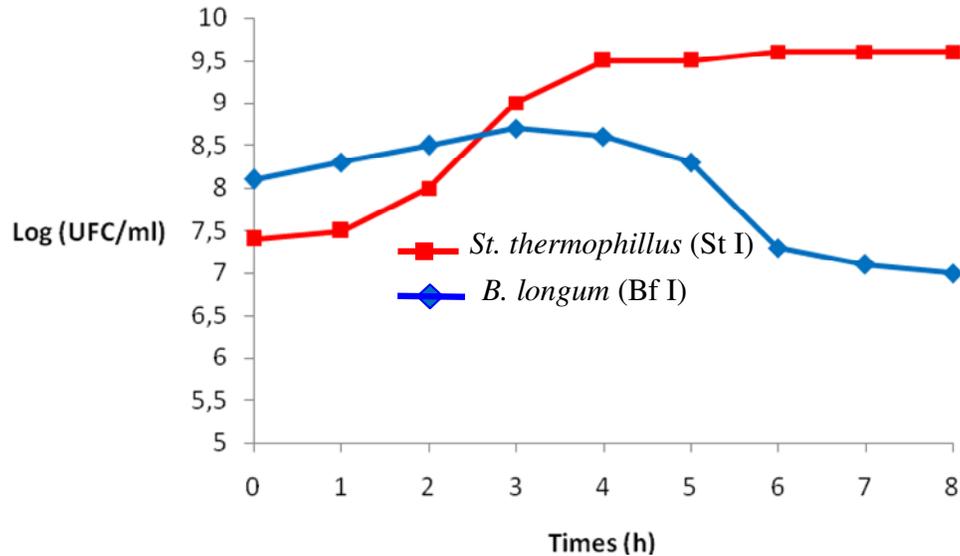
## RESULTS

### Viable microorganisms

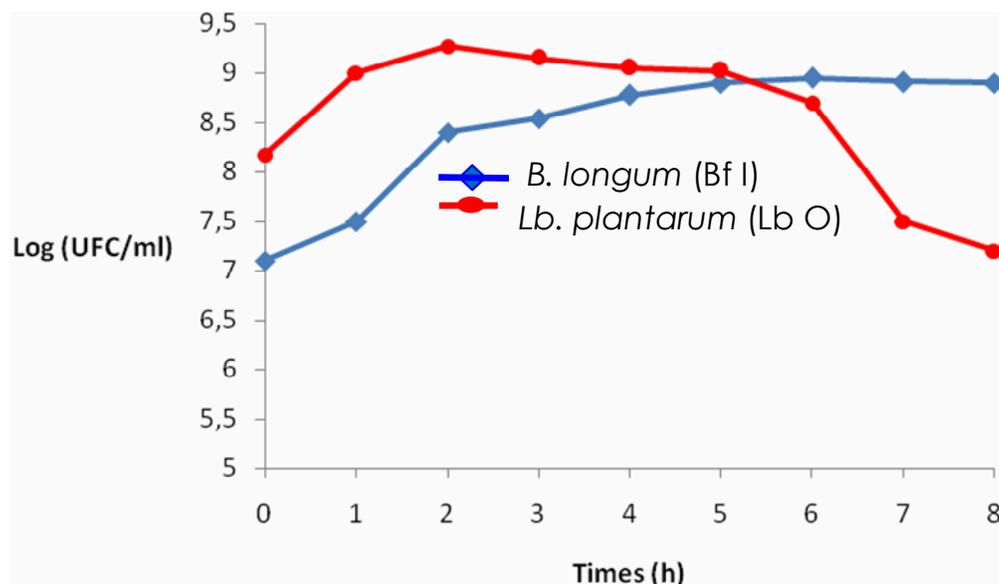
The enumeration revealed bacterial growth in all fermented milks. The maximum growth of *L. plantarum* (Lb O) and *B. longum* (Bf I) was observed when mixed together after 2 h of fermentation (Figure 2) compared to *B. longum* and *S. thermophilus* (St I) with values of  $10^9$  and  $3.10^8$  cfu/ ml, respectively. However, the maximum growth was after 3 h (Figure 1).

### Acidification kinetics

All bacterial associations tested produced lactic acid and progressive diminution of the pH during the fermentation.



**Figure 1.** Kinetics of growth in Log (cfu/ml) of *B. longum* (BfI) and *S. thermophilus* (St I) in cow-skim milk at 42°C.



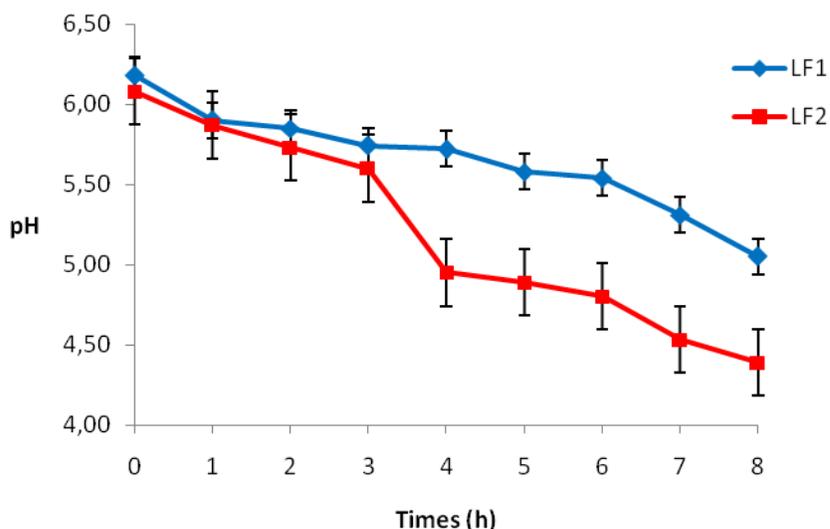
**Figure 2.** Kinetics of growth in Log (cfu/ml) of *B. longum* (BfI) and *L. plantarum* (LbO) in cow-skim milk at 42°C.

A significant difference of the pH and acidity between milk LF1 and LF2 was noted after coagulation ( $p < 0, 05$ ). It was noted that higher lactic acid production was obtained by the association between Lb O and Bf I (Figure 4). The values  $4.8 \pm 0.3$  g/l were significant with an acidification rate (Figure 4) and a minimum pH of  $4.6 \pm 0.1$  (Figure 3). The lowest lactic acid production was obtained by (St I + Bf I) association with  $4.19 \pm 0.04$  g/l and pH of  $5.55 \pm 0.1$  ( $p < 0.05$ ).

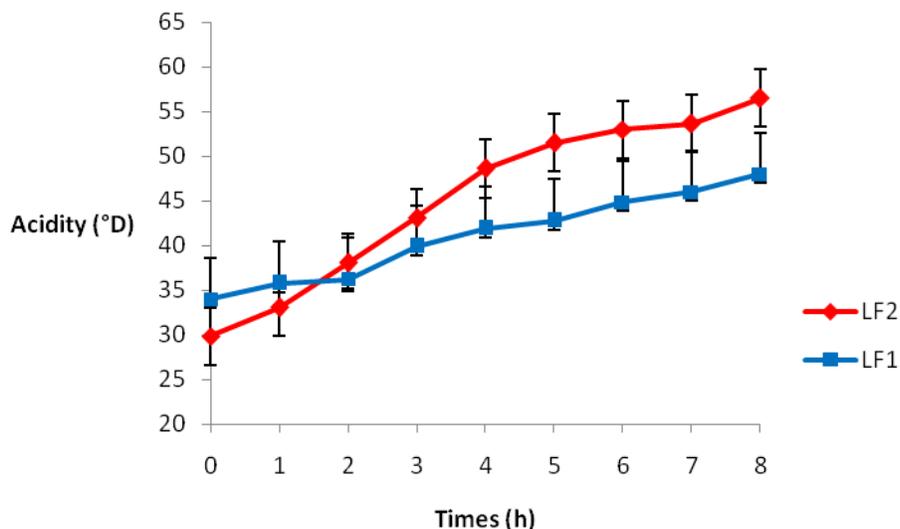
### Proteolysis activity

#### Measurement of total proteins in cow's fermented milk

The results showed that bacterial associations tested degrade proteins of cow's milk differently. The associations (Lb O + Bf I) give the best protein degradation of  $220 \pm 0.06$   $\mu\text{g}/\text{mg}$  compared to (Bf I + St I) that gave



**Figure 3.** Curve of pH evolution of milks LF1 (Bf I - St I) and LF2 (Bf I - Lb O) at 42°C (\*p < 0.01).



**Figure 4.** Acidification kinetics of (Bf I - St I) in LF1 and (Bf I - Lb O) in LF2 at 42°C. (\*p < 0.01).

235 ± 0.1 µg/mg (p < 0.05) (Figure 5).

**Measurement of the release of the functions α-NH<sub>2</sub> of cow’s fermented milk**

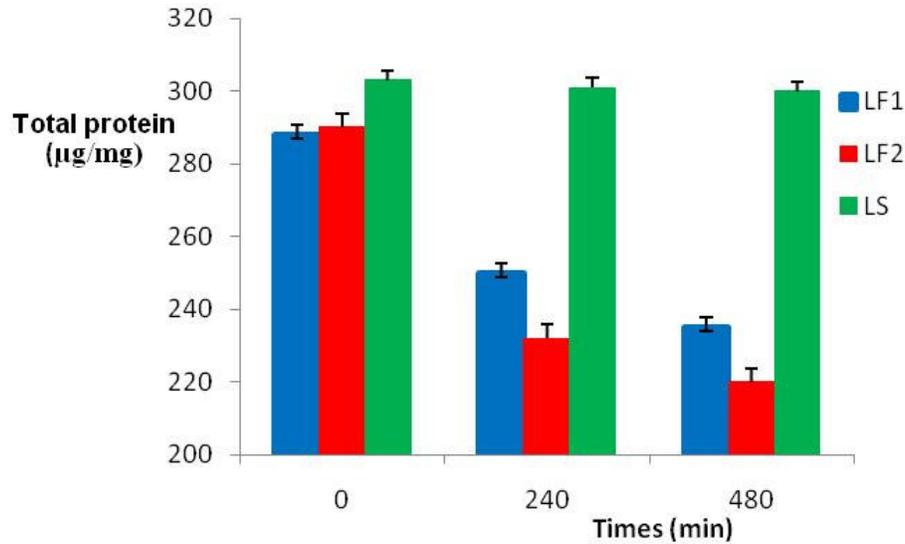
The results showed that all associations degraded proteins. Significant value of α-NH<sub>2</sub> functions (90±0.01) was obtained with the association (Lb O + Bf I) compared to (Bf I + St I) that gave 62 ± 0.1 µM/mg (p < 0.05) (Figure 6).

At the end of fermentation, a significant release of the

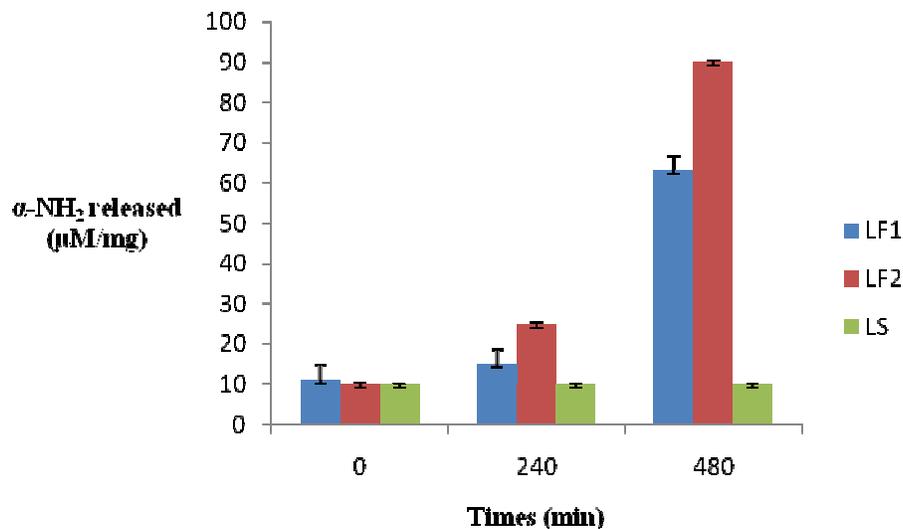
function α-NH<sub>2</sub> was observed for LF2 (90 ± 0.01) µM/ml compared to LF1 (63 ± 1.4) µM/ml (p < 0.05).

**Effect of fermentation on milk and electrophoresis profiles**

In sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, all proteins were easily identified by comparison with purified markers. The electrophoresis profiles clearly demonstrated the persistence of caseins in all milk. Significant difference in



**Figure 5.** Content of total proteins (in µg/mg) of LF1, LF2 and LS during 480 min of incubation at 42°C (\*p < 0, 05).



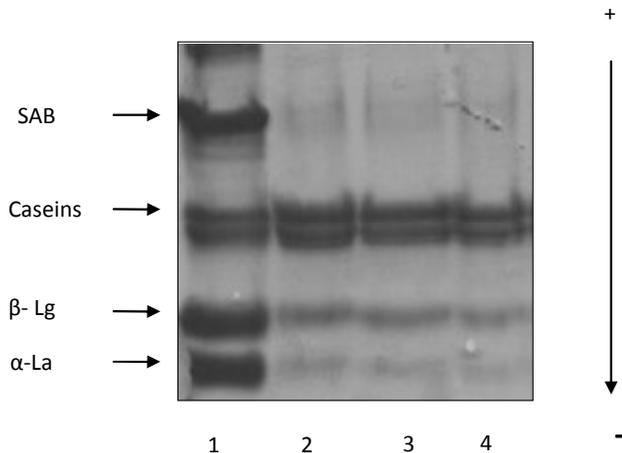
**Figure 6.** Content of Functions α-NH<sub>2</sub> released (in µM/mg) during 480 min of incubation at 42°C (\*p < 0.05).

band intensity was observed between sterile milk (LS) and LF1 (4) and LF2 (5) level of β-lactoglobulin (β-Ig) (Figure 7).

## DISCUSSION

This study showed the potentiality of two strains of LAB *S. thermophilus* (St I), *L. plantarum* (Lb O) and one strain of bifidobacteria *B. longum* (Bf I) to grow (co-cultures) in skim cow's milk and to degrade the major proteins, β-Ig and α-lactalbumin (α-la). Bacteria grew well in fermented

milk with different acidification rates. A good growth was observed for *B. longum* (Bf I) when mixed with *L. plantarum* (Lb O) in LF2 with a large viable concentration and a longer survival for *B. longum* and *L. plantarum*. According to Chekroun et al. (2006) and Shihata and Shah (2000), a better growth of bifidobacteria was observed when it associated with lactobacilli due to the acidifying activity of streptococci and the activity proteolysis of the lactobacilli. Also, Wang et al. (2005) affirmed that the maximum population of starter organism could be obtained during a shorter period of fermentation when mixed cultures were used (bifidobacteria with either



**Figure 7.** SDS- PAGE of proteins from cow's fermented milk. 1 = Reference markers; 2 = sterile milk (LS); 3 = LF1; 4 = LF2. The markers included molecular weight standard: bovine serum albumins (BSA = 68,000 Da), caseins (Cas = 24,000Da),  $\beta$ -lactoglobulin ( $\beta$ -lg = 18,000 Da) and  $\alpha$ -lactalbumin ( $\alpha$ -la = 14,000 Da).

*S. thermophilus* or *Lactobacillus acidophilus*). Indeed, it was mentioned that there has been an active synergy between the strains in mixed cultures (Ait Abdeslam et al., 2009; Altieri et al., 2008; Chekroun and Bensoltane, 2007; Garro et al., 2004). The pH and acidity also showed significant values ( $p < 0.01$ ) for LF2 with maximum rate when compared to LF1 after 8 h of fermentation. However, this rate of acidification has less performance than dairy product's industry. Generally, the acidification was better when *S. thermophilus* was associated with *Lactobacillus delbrueckii* subsp. *bulgaricus* as shown by Ait Abdeslam et al. (2009). These researchers showed that mixed culture (*S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus* and *Bifidobacterium animalis*) gave a fast acidification of ewe's and cow's milk and attained pH 4.6 – 4.5 after 4 h of fermentation. Proteolysis activity was observed by co-cultures (Lb O-Bf I) in LF2 with significant values ( $p < 0, 01$ ) compared to LF1 fermented by co-culture (St I- Bf I). It is interesting to mention that lactobacilli gave a good proteolysis when it associated with bifidobacteria. Meanwhile, it has being reported that lactobacilli are used in various fermentation processes and that milk contains too little free amino acids and small peptides for sufficient growth of these microorganisms (Sakellaris and Gikas, 1991). Electrophoresis profiles demonstrated the persistence of caseins in all milk and a significant degradation of  $\beta$ -lg was observed between LS, LF1 and LF2 according to Chekroun et al. (2007). Prioult et al. (2003) suggested that *Bifidobacterium lactis* NCC362 could be a potential probiotic for preventing cow's milk allergy through degradation of the allergenic portion of  $\beta$ -lg generated by trypsin/chymotrypsin hydrolysis. Also it has being reported by Bertrand-Harb et al. (2003) that the proteo-

lysis of  $\beta$ -lg and  $\alpha$ -la by LAB could increase their digestibility and hydrolyze allergenic peptides. In this work, it was demonstrated that these co-cultures could display high proteolytic properties toward  $\beta$ -lg and  $\alpha$ -la.

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