

Valuing the Antigenicity of Sheep Milk Proteins after Fermentation at 40°C

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Abstract: The main objective of this work is to show a fermentation influence by some associations of two lactic acid bacteria and three bifidobacteria on proteolysis and the antigenicity of sheep milk proteins. This latter freshly collected is skimmed, sterilized and inoculated by a mixed culture. The mixture is homogenized and incubated at 40°C until it is a curd. On these fermented milks previously lyophilized, were measured the rate of lactic acid product, bacterial counts, the rate of total protein, α -NH₂ functions and the antigenicity of three proteins (β -Lg, α -La and SA). The obtained averages are compared with the "t" test of Student compared to sterile milk without ferment taken as control. The best proteolysis is achieved in the fermented milk by *Streptococcus thermophilus* (St) associated with *Lactobacillus plantarum* (Lp) with a high antigenic potential of β -Lg and the α -La probably due to the detection of unmasked antigenic sites.

Keywords: Sheep Milk, Bacteria, Fermentation, Proteolysis, Antigenicity, Awareness, Female rabbits

1. Introduction

Milk proteins are the main compounds capable of specific reactions with the immune system. They, generally, have a strong antigenicity power and a wide variety of epitopes to the immune system [1,2]. Under certain conditions, an increase in intestinal permeability to these proteins could be associated in allergies development, intolerance, gastrointestinal inflammation and diarrhea related to the degree of digestion of these latters [3-6,1,7].

The degree of protein digestion, therefore, determines the phenomena of tolerance, sensitivity and allergy to food proteins [8,4-7,1]. To prevent these symptoms, a full eviction of all sources of dairy protein is required, but this approach can lead to stunted growth [9].

The use of alternative products such as fermented sheep milks and even of other mammals, is an interesting alternative concerning proteolysis and preventing of manifestations of allergy. No study shows the superiority of a hydrolyzate type over another. [10] Different types of technological treatments on these proteins gave only inconclusive results. The lactic fermentation is a biological means for changing the allergenic character of these proteins [9-11,3].

Bacterial proteolysis is a complex biochemical phenomenon involving many enzymes. The preparation of fermented milk by associating lactic acid bacteria and bifidobacteria plays a key role since it is a process that is performed in a controlled manner on proteins mainly resistant to digestion such as β -lactoglobulin and α -lactalbumin; these are known to be allergenic, that's why it is important to evaluate this proteolysis against the allergic risk [10,3].

2. Materials & Methods

2.1. Preparation of Sheep Milk and Tested Bacterial Species

Sheep milk, freshly collected, is previously skimmed at 4°C by centrifugation at 3000 rev /min and sterilized at 105°C during 10 minutes to destroy the enzymes and the naturally occurring bacteria. It is inoculated with a mixed culture from two pure cultures at a concentration of 5% each. The mixture is homogenized and incubated at 40°C until it is a curd. The used bacteria, have allowed us to prepare the following fermented milk: *Streptococcus thermophilus* + *Bifidobacterium longum* (St + B long), *Streptococcus thermophilus* + *Bifidobacterium bifidum* (St + B bif), *Streptococcus thermophilus* + *Bifidobacterium infantis* (St +

B inf), *Streptococcus thermophilus* + *Lactobacillus plantarum* (St + Lp). On some of these fermented milks fermentation pattern and enumeration were measured. On the other previously lyophilized portion the levels of total protein, α -NH₂ functions released and the antigenicity of the main 3 proteins (α -La, β -Lg and SA) were measured, they are most implicated in the phenomena of allergy and their degradation products by ELISA.

2.2. Enumeration of Bacteria

Bacteria counting (cfu/ml) was performed on samples of fermented milk [12]. *Streptococcus thermophilus*, *Lactobacillus plantarum* species and bifidobacteria are counted respectively specific culture media: M₁₇ [13], Man Regosa Scharpe (MRS) [14] and Trypticase-Phytone-Yeast (TPY) [15].

2.3. Measurement of the Produced Acidity

The amount of produced acid is expressed in degrees Dornic / liter of sheep's milk (°D/ l) [16].

2.4. Measurement of the pH Change

pH, index of acidity developed in sheep milk during the fermentation, is measured as a function of time using a digital pH meter (inoLab).

2.5. Proteolytic Activity of Bacteria

2.5.1. Total Protein

The determination of the total protein content (g/mg of lyophilisate) in the samples of fermented milk, is carried out by the technique of Lowry *et al.*, [17].

2.5.2. Determination of α -NH₂ Released Functions

Bacterial proteolysis is assessed by measurement of α -NH₂ functions released (μ M/mg of lyophilized) in samples of fermented milk by the method of Doi *et al.*, [18].

2.6. Measuring the Antigenicity of Fermented Milk Proteins

Measuring the antigenicity of proteins (β -Lg, α -La and SA) is performed by ELISA according Engvall & Perlmann, [19]. It is expressed as μ g/mg of freeze-dried fermented sheep milk, with the corresponding serum antibodies produced by female rabbits of New Zealand which underwent parenterally a sensibilisation, followed by a collection of blood from the marginal ear vein.

Permission to use rabbits was obtained by the ethics committee of the Liabes Djillali University of Sidi Bel-Abbès. The general rules for health and use of laboratory animals recommended by the Council of the European Community [20] have been followed.

2.7. Statistical Analysis

For the statistical analysis, each operation has been repeated 5 times. Results are expressed as mean \pm standard error (X \pm SE). The mean values were compared using the "t"

test of Student relative to that of the sterile sheep milk without ferment taken in the same experimental conditions (control). The difference between the two means has been usually considered significant when $p < 0.05$ and non-significant in the other cases.

3. Results

3.1. Morphological Characterization of Ferments

The realized tests showed that all the bacteria are Gram positive, non-motile, non spore and are negative catalase and oxidase. Their growth is favored in anaerobic.

3.2. pH Variations Sheep Milk During Fermentation

The fermentation of sheep milk at 40°C showed a progressive decrease in pH which explains a metabolic activity of the bacterial species taken in combination. Our results show that lower pH is obtained in fermented milk by the association of (St + Lp) (4.55 ± 0.02); this pH is significantly lower than that of the sterile milk without ferment taken as a control (6.68 ± 0.01) ($p < 0.001$).

3.3. Measurement of the Acidity Produced by the Bacteria Used in Combination

Tested bacterial associations produce acid during the fermentation by degrading the sugars from sheep milk. Strongest acidification is obtained by the mixed culture (St + Lp) (71.00 ± 0.71 °D) compared to sterile milk without ferment taken as control (22.80 ± 0.58 °D) ($p < 0.001$); it corresponds to a low pH (4.55 ± 0.02) ($p < 0.001$), confirming the role of acidifying agent *Streptococcus thermophilus*.

3.4. Enumeration (log cfu/ml), on Appropriate Culture Media, Bacteria Put Together

Bacterial counting on appropriate selective media, shows that there is bacterial growth in all fermented milks and that all species have a symbiotic nature when they are put together.

The bacterial growth is of great variability and higher is St ($29.2.10^7$ cfu/ml) obtained with a parallel increase of Lp ($26.8.10^7$ cfu/ml).

3.5. Proteolytic Activity of the Bacteria During the Fermentation

3.5.1. Total Protein Content of Fermented Milk

The results show that the tested bacterial associations differently degrade sheep milk protein and it is the combination of (St + Lp) that gives the lowest profile (288.41 ± 79.41 μ g/mg of lyophilisate) compared to milk control (498.16 ± 2.88 μ g/mg of lyophilisate) a decrease of 42% ($p < 0.05$) (Figure 1).

3.5.2. α -NH₂ Functions Liberated in Fermented Milks

The best proteolysis of sheep milk protein is obtained by (St + B long) with a functions value of α -NH₂ free of (84,28

$\pm 8,60 \mu\text{M}/\text{mg}$ of lyophilisate) against ($16,62 \pm 0,71 \mu\text{M}/\text{mg}$ of lyophilisate) to the sterile milk (control) ($p < 0.001$) (Figure 2).

3.6. Measuring the Antigenicity of Proteins ($\beta\text{-Lg}$, $\alpha\text{-La}$, SA) of Fermented Milks

The results (Figure 3) show that the $\beta\text{-Lg}$ is detected in all fermented milks but with a significantly higher rate by associating of (St + B long) ($2,61 \pm 0,15 \mu\text{g}/\text{mg}$ of lyophilisate) compared to the milk control ($0,43 \pm 0,08 \mu\text{g}/\text{mg}$ of lyophilisate) ($p < 0.001$). The lowest rate for the antigenicity $\beta\text{-Lg}$ is obtained by combining (St + Lp) ($0,53 \pm 0,06 \mu\text{g}/\text{mg}$ of lyophilisate).

The results show that the $\alpha\text{-La}$ is present in all fermented milks but with a significantly higher rate by associating (St + B long) ($4,25 \pm 0,24 \mu\text{g}/\text{mg}$ of lyophilisate) compared to milk control ($2,48 \pm 0,25 \mu\text{g}/\text{mg}$ of lyophilisate) ($p < 0.001$). A significantly decreased rate ($0,66 \pm 0,17 \mu\text{g}/\text{mg}$ of lyophilisate) is obtained by association (St + Lp) compared to that of reference milk ($2,48 \pm 0,25 \mu\text{g}/\text{mg}$ of lyophilisate) ($p < 0.02$) (Figure 4).

The antigenic activity of SA is present in all fermented milks. The results (Figure 5) show that the antigenicity of the SA indicates that no significant difference was observed in the fermented milk by (St) associated with (Lp) and bifidobacteria. The highest antigenicity rate is obtained by combining (St + B bif) ($0,41 \pm 0,04 \mu\text{g}/\text{mg}$ of lyophilisate) compared to the milk control ($0,40 \pm 0,06 \mu\text{g}/\text{mg}$ of lyophilisate). The lowest rate is obtained by combining (St + B long) ($0,36 \pm 0,01 \mu\text{g}/\text{mg}$ of lyophilisate).

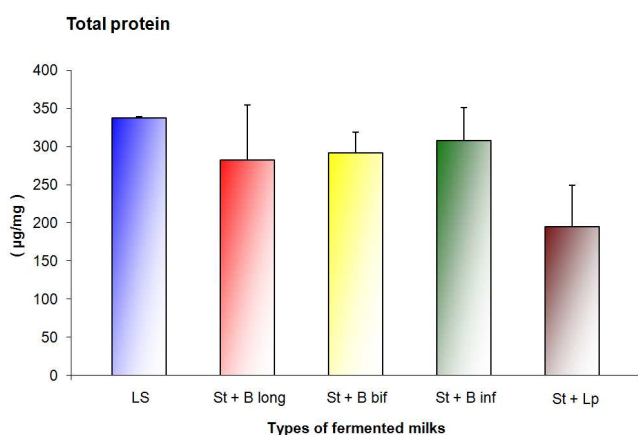


Figure 1. The amount of total protein ($\mu\text{g}/\text{mg}$ of lyophilisate) fermented milks at 40°C by *Streptococcus salivarius* subsp. *thermophilus* (St) associated with *Bifidobacterium longum* (St + B long); *Bifidobacterium bifidum* (St + B bif); *Bifidobacterium infantis* (St + B inf); *Lactobacillus plantarum* (St + Lp).

LS: sterile milk without ferment (control).

The values shown are mean \pm standard error ($X \pm \text{SE}$) ($n = 5$).

The averages were compared using the "t" test of Student between :

The sterile milk (LS) and fermented milks (LF)* ; Fermented Milks (LF)*.

There is no significant difference between fermented milks and the witness.

* $p < 0.05$ established only difference (St + Lp) relative to the sterile milk without closing.

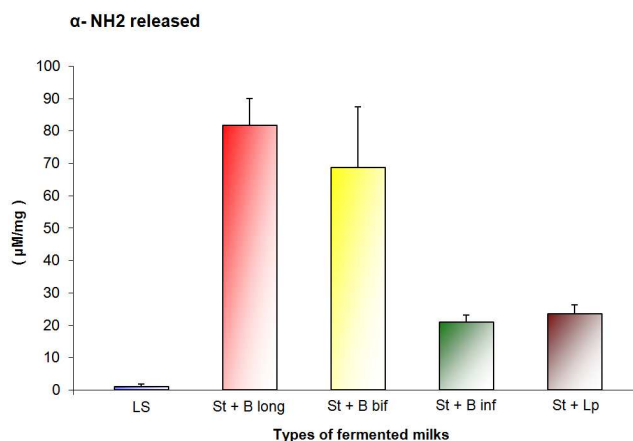


Figure 2. $\alpha\text{-NH}_2$ functions released in micromoles/milligrams ($\mu\text{M}/\text{mg}$ of lyophilisate) fermented sheep's milk at 40°C by *Streptococcus salivarius* subsp. *thermophilus* (St) associated with *Bifidobacterium longum* (St + B long); *Bifidobacterium bifidum* (St + B bif); *Bifidobacterium infantis* (St + B inf); *Lactobacillus plantarum* (St + Lp).

LS: sterile milk without ferment (control).

The values shown are mean \pm standard error ($X \pm \text{SE}$) ($n = 5$).

The averages were compared using the "t" test of Student between :

The sterile milk (LS) and fermented milks (LF)* ; Fermented Milks (LF)*.

*** $p < 0.001$ ** $p < 0.01$ * $p < 0.05$ established differences respectively (St + B long); (St + B bif); (St + B inf); (St + Lp) relative to the sterile milk without closing.

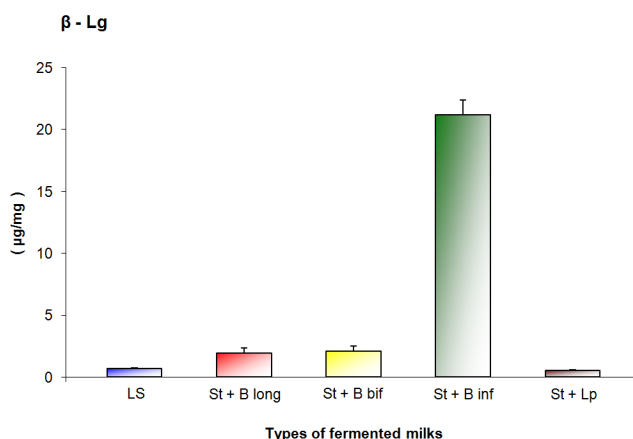


Figure 3. Measurement of residual antigenicity of β -lactoglobulin ($\beta\text{-Lg}$) ($\mu\text{g}/\text{mg}$ of lyophilisate) in fermented milks at 40°C by *Streptococcus salivarius* subsp. *thermophilus* (St) associated with *Bifidobacterium longum* (St + B long); *Bifidobacterium bifidum* (St + B bif); *Bifidobacterium infantis* (St + B inf); *Lactobacillus plantarum* (St + Lp).

LS: sterile milk ferment without (control).

The values shown are mean \pm standard error ($X \pm \text{SE}$) ($n = 5$).

The averages were compared using the "t" test of Student between :

The sterile milk (LS) and fermented milks (LF)* ; Fermented Milks (LF)*.

*** $p < 0.001$ only difference established (St + B long) compared to sterile milk without closing. *** $p < 0.001$ ** $p < 0.01$ respectively differences established (St + B long; St + B bif); (St + B bif; St + B inf) compared to associations (St + Lp) and (St + B long). ** $p < 0.02$ difference established (St + B inf) relative to the association (St + B bif).

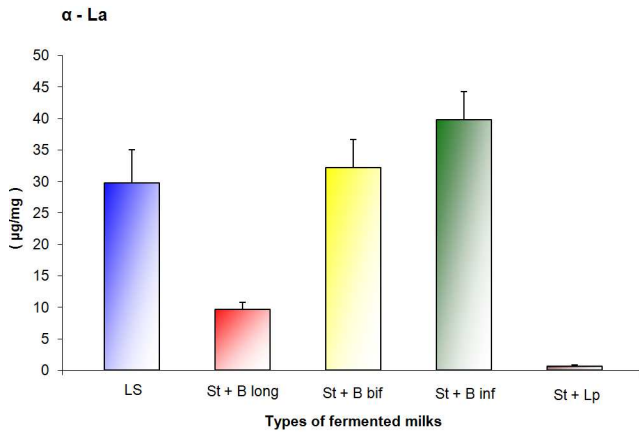


Figure 4. Measurement of residual antigenicity of α -lactalbumin (α -La) ($\mu\text{g}/\text{mg}$ of lyophilisate) in fermented milks at 40°C by *Streptococcus salivarius* subsp. *thermophilus* (St) associated with *Bifidobacterium longum* (St + B long) ; *Bifidobacterium bifidum* (St + B bif) ; *Bifidobacterium infantis* (St + B inf) ; *Lactobacillus plantarum* (St + Lp).

LS: sterile milk ferment without (control).

The values shown are mean \pm standard error ($X \pm \text{SE}$) ($n=5$).

The averages were compared using the "t" test of Student between :

The sterile milk (LS) and fermented milks (LF)* ; Fermented Milks (LF)*.

*** $p < 0.001$ ** $p < 0.02$ established differences respectively (St + B long) and (St + Lp) relative to the sterile milk without closing..

*** $p < 0.001$ * $p < 0.05$ established differences respectively (St + Lp) and (St + B inf) relative to the association (St + B long).

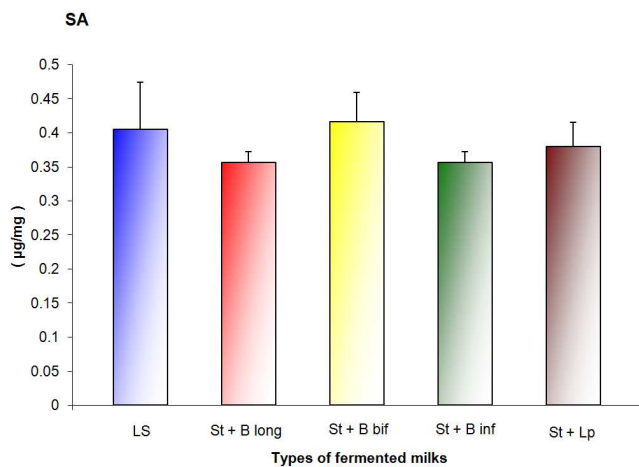


Figure 5. Measurement of residual antigenicity of serum albumin (SA) ($\mu\text{g}/\text{mg}$ of lyophilisate) in fermented milks at 40°C by *Streptococcus salivarius* subsp. *thermophilus* (St) associated with *Bifidobacterium longum* (St + B long) ; *Bifidobacterium bifidum* (St + B bif) *Bifidobacterium infantis* (St + B inf) ; *Lactobacillus plantarum* (St + Lp).

LS: sterile milk ferment without (control).

The values shown are mean \pm standard error ($X \pm \text{SE}$) ($n=5$).

The averages were compared using the "t" test of Student between :

The sterile milk (LS) and fermented milks (LF)* ; Fermented Milks (LF)*.

There is no significant difference between fermented milks and milk without closing the sterile and fermented milks them.

4. Discussion

This work's enterprise has been done in order to ferment sheep milk by various associations of *Streptococcus thermophilus*, *Lactobacillus plantarum* and bifidobacteria to

alter the antigenicity of three major proteins from sheep milk (α -La; β -Lg and SA) most implicated in allergy phenomena and their degradation products.

The bacteria used in this experiment are 5 ; they have been used in combination with a rate close to 5% for each of the tested bacteria [21]. This rate allows rapid sheep milk coagulation avoiding the proliferation of unwanted bacteria over long fermentation periods [14,22].

The results comparison concerning the production of acid shows that the bacterial associations have a greater acidifying power. Our results agree with those obtained by [22-23].

Our results also show that during the fermentation of the sheep milk at 40°C, there is a significant decrease in the pH of the fermented milk compared to the milk control; this decrease in pH reflects the metabolic activity of the tested species. Our results agree with those obtained by [24].

The results of counting bacteria show that the slower growth of one or the other bacteria which constitute the association probably partially caused by products such as lactic acid and acetic acid which reduce the milk pH during fermentation [25-26].

The bacterial counts on appropriate specific areas, in prepared fermented milk, reveals that the used bacterial species are equipped with a proteolytic and acidifying activity [24,27]. The strongest and fastest bacterial growth in mixed culture is that of (St) obtained with a parallel growth of (Lp). Our results agree with those obtained by [24], which explains the presence of a synergy between bacteria in mixed culture.

The mixture of bacterial species is clearly a more active example because each one benefits from the other making a symbiotic character. The results concerning the enumeration agree with those of [24,28-33] which have shown that certain bacterial strains stimulate growth of the other strains by producing nitrogen nutrients.

Sterilization at 105°C sheep's milk does not reduce the total protein rate. These results are in agreement with those of [34-36].

Concerning the bacterial proteolysis, our results showed that the tested mixed cultures degrade significantly the milk protein compared to the sterile sheep milk without leaven taken as control.

During the fermentation, the protein degradation by bacteria releases characteristic functions of proteolysis. The evaluation of these functions shows that all associations degrade significantly sheep milk proteins and the best proteolysis is obtained by the mixed culture : *Streptococcus thermophilus* and *Lactobacillus plantarum*. The protein hydrolysis by enzymes of bacteria can be explained by the existence of a protooperation between the two germs on nitrogenous matter, so to boost their fermentation performance. Our results are in agreement with those of [14,25-26,37-38].

The results of determination of α -NH₂ functions released shows that mixed cultures have a variable proteolytic power depending on the type of the association and have an affinity to degrade a particular sheep's milk protein [23,25,38-39].

The age of the bacteria, the external pH, incubation temperature and the pairing mode has an effect on the growth and proteolytic activity [24,28,32,40].

The antigenicity of proteins in the samples of fermented sheep's milk (β -Lg, α -La and SA) and their degradation products, studied in vitro, by ELISA allowed us to quantify the reactivity with IgG specific anti- β -Lg, anti- α -La and anti-SA [41].

Antigenic amounts of β -Lg, of α -La and SA, detected in samples of fermented milks, increased during the lactic fermentation at 40°C, and significantly for β -Lg and α -La compared to those found in the sterile sheep milk without ferment taken as reference control. This is probably due to the fact that sheep's milk contains high levels of total protein and bacterial proteolysis has unmasked the hidden antigenic sites in the protein and degradation products. Thus, our results are also confirmed by the increase in α -NH₂ functions released by proteolysis and whose values depend on the type of association used, allowing thus make a selection of species for performing proteolytic activity.

The increase of the proteins antigenicity in the prepared fermented milk may be explained by the non-exposure of some epitopes on the action of certain enzymes on the one hand, or to the release of new antigens [34,42]. The values obtained have allowed us to better understand the true incidence of bacterial proteolysis. Knowledge of proteolytic enzymes of the latter, really active and their properties, can be of fundamental importance in the selection of starters [26,30,43].

5. Conclusion

Following this study, the lactic acid fermentation is a process that revealed new antigenic sites in the degradation of sheep milk proteins by enzymes with an unmasking of antigenic epitopes initially hidden under the native form of the protein, but there are significant differences between the tested bacterial associations. Having said that our results will allow us to make the selection of the most efficient bacterial combination giving better proteolytic activity.

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