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

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Abstract: This work shows the influence of fermentation by associations of lactic acid bacteria and bifidobacteria 3 proteolysis and protein antigenicity of sheep's milk. Freshly collected it is skimmed, sterilized and inoculated with a mixed culture. The mixture is homogenized and incubated at 40°C until a curd. These fermented milks previously lyophilized, are valued on the levels of total protein, α-NH_2 functions and antigenicity of three proteins (β-Lactoglobulin, α-Lactalbumin, Serum Albumin). The averages are compared to the test using the "t" of Student compared to the control milk. The proteolysis is best obtained in the fermented milk by *Lactobacillus plantarum Bifidobacterium bifidum* associated with a high antigenic potential β-Lg and the α-La probably due to the detection of antigenic sites exposed.

Key words: Sheep milk · Bacteria · Fermentation · Proteolysis · Antigenicity

INTRODUCTION

Milk proteins are the main compounds capable of specific reactions with the immune system. They, generally, have a strong antigenic 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 elements [1, 3-7].

To prevent these symptoms, a full eviction of all sources of dairy protein is required, but this approach can lead to stunted growth [8].

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 [3, 9, 10].

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 [3, 10].

This work shows the influence of fermentation by associations of lactic acid bacteria and bifidobacteria 3 proteolysis and protein antigenicity of sheep's milk.

MATERIALS AND METHODS

Preparation of Sheep Milk and Tested Bacterial Species: Sheep milk, freshly collected, is previously skimmed 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: *Lactobacillus plantarum* + *Bifidobacterium longum* (Lp + B long), *Lactobacillus plantarum* + *Bifidobacterium bifidum* (Lp + B bif), *Lactobacillus plantarum* + *Bifidobacterium infantis* (Lp + B inf).

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On some of these fermented milks were measured the fermentation pattern and enumeration. On the other previously lyophilized portion, were measured the levels of total protein, α-NH_2 functions released and the antigenicity of the main 3 proteins (α-La, β-Lg and SA) most implicated in the phenomena allergy and their degradation products by ELISA.

Enumeration of Bacteria: Bacteria counting (cfu/ml) was performed on samples of fermented milk [11]. *Lactobacillus plantarum* species and bifidobacteria are counted respectively specific culture media: Man Regosa Scharpe (MRS) [12] and Trypticase-Phytone-Yeast (TPY) [13].

Measurement of the Produced Acidity: The amount of produced acid is expressed in degrees Dornic / liter of sheep's milk (°D/ l) [14].

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).

Proteolytic Activity of Bacteria

Total Protein: The determination of the total protein content (µg/mg of lyophilisate) in the samples of fermented milk is carried out by the technique of Lowry *et al.* [15].

Determination of α-NH_2 Released Functions: Bacterial proteolysis is assessed by measurement of α-NH_2 functions released (iM/mg of lyophilized) in samples of fermented milk by the method of Doi *et al.* [16].

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 [17]. It is expressed as ìg/mg of freeze-dried fermented sheep milk, with the corresponding serum antibodies produced by female rabbits of New Zealandwhich 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-Abbes. The general rules for health and use of laboratory animals recommended by the Council of the European Community [18] have been followed.

Statistical Analysis: For the statistical analysis, each operation has been repeated 5 times. Results are expressed as mean±standard error (X±S.E). 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.

RESULTS

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.

pH Variations Sheep Milk During the 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 (Lp + B inf) (4,70±0,01) ; this pH is significantly lower than that of the sterile milk without ferment taken as a control (6,68±0,01) ($p<0.001$).

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 (Lp + B long) (60,40±0,93 °D) compared to sterile milk without ferment taken as control (22,80±0,58 °D) ($p<0.001$).

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 Lp ($2,5.10^7$ cfu/ml) obtained with a parallel increase of B inf ($2,4.\ 10^8$ ufc/ml).

Proteolytic Activity of the Bacteria during the Fermentation

Total Protein Content of Fermented Milk: The results show that the tested bacterial associations differently degrade sheep milk protein. The association (Lp + B long) gives the best protein degradation (271.56±52.58 µg / mg of lyophilisate) compared to the control milk (498.16±2.88 µg / mg of lyophilisate) ($p < 0.01$) (Figure 1).

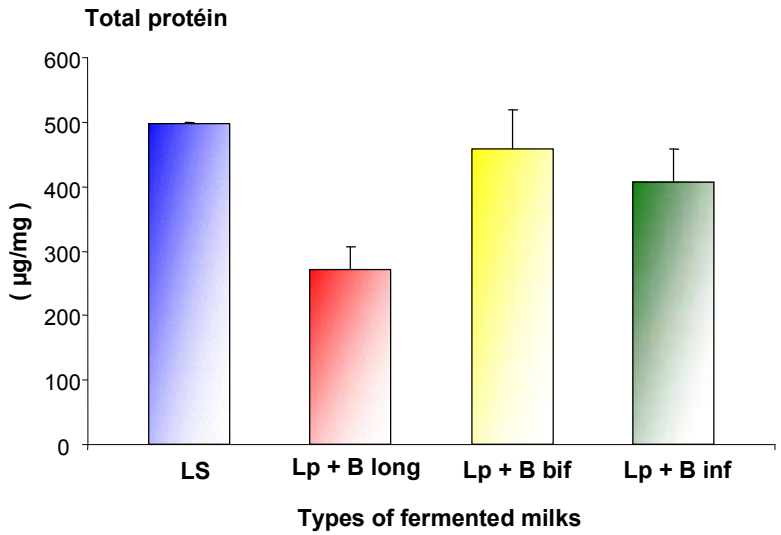

Fig. 1: The amount of total protein (µg/mg of lyophilisate) fermented milks at 40ºC by *Lactobacillus plantarum* (Lp) associated with *Bifidobacterium longum* (Lp + B long); *Bifidobacterium bifidum* (Lp + B bif); *Bifidobacterium infantis* (Lp + B inf).
LS: sterile milk ferment without (control).
The values shown are mean ± standard error (X ± SE) (n = 5).
The averages were compared using the "t" test of Student between :
The sterile milk (LS) and fermented milks (LF)*.
There is no significant difference between fermented milks and the witness.
**p<0,01 established only difference the (Lp + B long) relative to the sterile milk without closing.

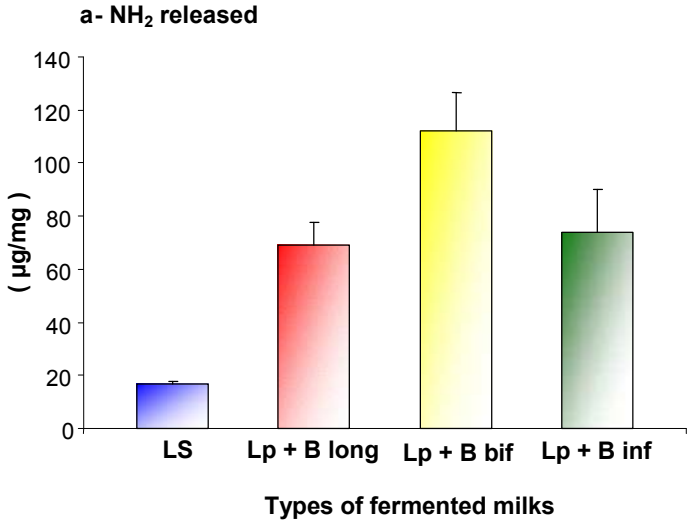

Fig. 2: a-NH2 functions released in micromoles/milligrams (µM/mg of lyophilisate) fermented sheep's milk at 40ºC by *Lactobacillus plantarum* (Lp) associated with *Bifidobacterium longum* (Lp + B long); *Bifidobacterium bifidum* (Lp + B bif); *Bifidobacterium infantis* (Lp + B inf).
LS: sterile milk ferment without (control).
The values shown are mean ± standard error (X ± SE) (n = 5).
The averages were compared using the "t" test of Student between :
The sterile milk (LS) and fermented milks (LF)* .
****p<0,0001 ***p<0,001 **p<0,01 established differences respectively of (Lp + B bif); (Lp + B long); (Lp + B inf) relative to the sterile milk without closing.

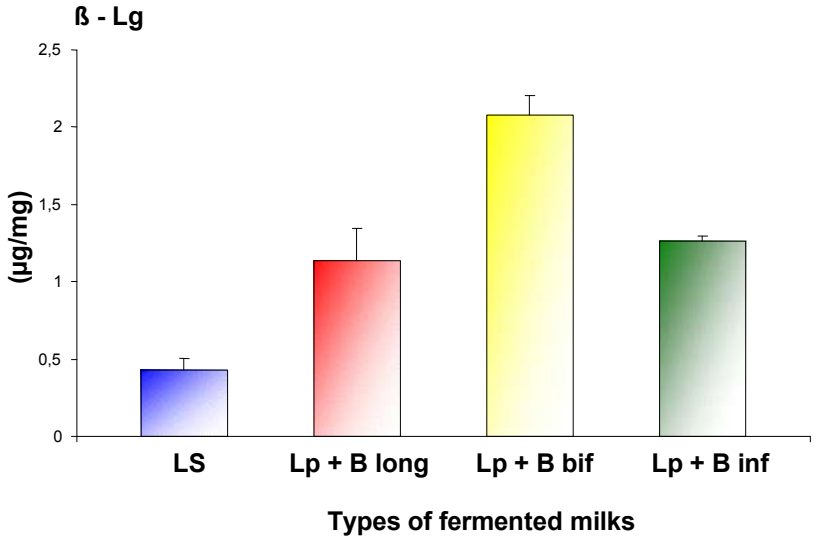

Fig. 3: Measurement of residual antigenicity of ß-lactoglobulin (ß-Lg) (µg/mg of lyophilisate) in fermented milks at 40ºC bay *Lactobacillus plantarum* (Lp) associated with *Bifidobacterium longum* (Lp + B long); *Bifidobacterium bifidum* (Lp + B bif); *Bifidobacterium infantis* (Lp + B inf).
LS: sterile milk ferment without (control).
The values shown are mean ± standard error (X ± SE) (n = 5).
The averages were compared using the "t" test of Student between :
The sterile milk (LS) and fermented milks (LF)*.
***p<0,001 only difference established of (Lp + B bif) compared to sterile milk without closing.

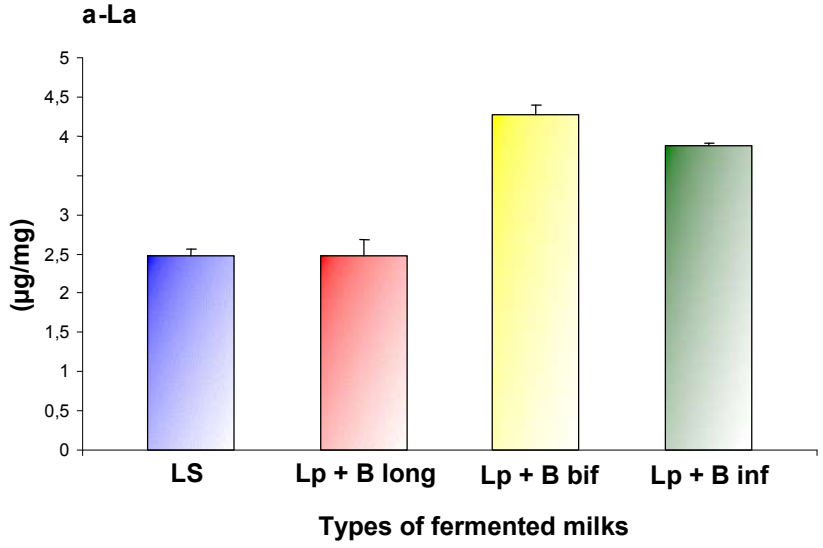

Fig. 4: Measurement of residual antigenicity of a-lactalbumin (a-La) (µg/mg of lyophilisate) in fermented milks at 40ºC by *Lactobacillus plantarum* (Lp) associated with *Bifidobacterium longum* (Lp + B long); *Bifidobacterium bifidum* (Lp + B bif); *Bifidobacterium infantis* (Lp + B inf).
LS: sterile milk ferment without (control).
The values shown are mean ± standard error (X ± SE) (n = 5).
The averages were compared using the "t" test of Student between :
The sterile milk (LS) and fermented milks (LF)*.
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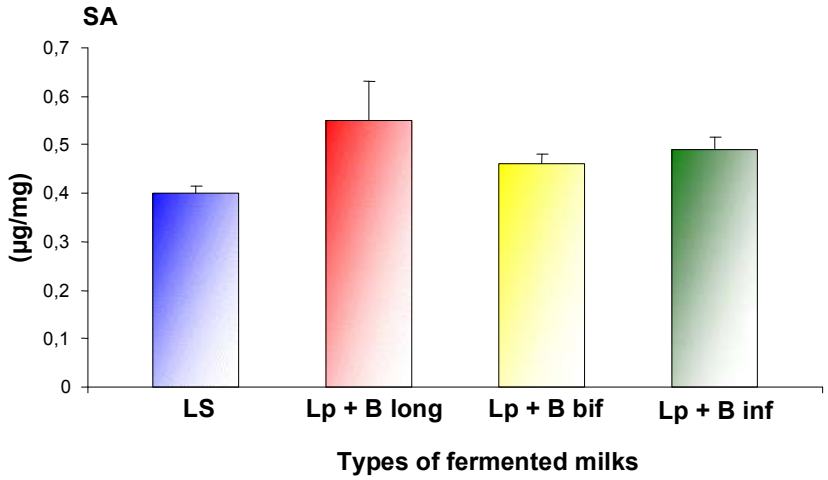

Fig. 5: Measurement of residual antigenicity of serum albumin (SA) (ug/mg of lyophilisate) in fermented milks at 40ºC by *Lactobacillus plantarum* (Lp) associated with *Bifidobacterium longum* (Lp + B long); *Bifidobacterium bifidum* (Lp + B bif); *Bifidobacterium infantis* (Lp + B inf).
LS: sterile milk ferment without (control).
The values shown are mean ± standard error (X ± SE) (n = 5).
The averages were compared using the "t" test of Student between :
The sterile milk (LS) and fermented milks (LF)*.
***p<0,001 established only difference of (Lp + B long) relative to the sterile milk without closing.

α-NH_2 Functions Liberated in Fermented Milks: The best proteolysis of sheep milk protein is obtained by (Lp + B bif) with a functions value of α-NH_2 free of (111,99±15,12 ìM/mg of lyophilisate) against (16,62±0,71 ìM/mg of lyophilisate) in the sterile milk without ferment taken as control (p <0.0001) (Figure 2).

Measuring the Antigenicity of Proteins (β-Lg, α-La, SA) of Fermented Milks: The results (Figure 3) show that the antigenic activity of the β-Lg is increased in all the fermented milk; the significantly higher antigenicity-rate is obtained by combining (Lp + B bif) (2.08±0.21 ìg/mg of lyophilisate) compared to the control milk (0.43±0.08 ìg/mg of lyophilisate) (p<0.001).

The antigenicity of the α-La the highest is obtained by the association (Lp + B bif) (4.28±0.19 ìg/mg of lyophilisate) compared to the control milk (2.48±0.25 ìg/mg of lyophilisate) (Figure 4).

The antigenicity of the SA (Figure 5) shows that there is only one significant difference in the fermented milk by the association (Lp + B long) (0.55±0.05 ìg/mg of lyophilisate) compared to the control milk (0.40±0.06 ìg/mg of lyophilisate) (p <0.001).

DISCUSSION

This work's enterprise has been done in order to ferment of the sheep milk by different associations of *Lactobacillus plantarum* and bifidobacteria to change 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 our experiment are 4 in number; they have been used in combination with a rate close to 5% for each of the tested bacteria [19]. This rate allows rapid of sheep milk coagulation and avoiding the proliferation of unwanted bacteria over long of fermentation periods [12, 20].

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 Benjamas *et al.,* [20] and Ayad *et al.,* [21].

Our results also show that during the fermentation of 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 Chekroun *et al.,* [22].

The results of counting bacteria show that the slower growth of one or the other bacteria, which constitute the association, may be probably partially caused by products such as lactic acid and acetic acid which lower of the milk pH during the fermentation [23, 24].

The bacterial count on appropriate specific areas, in prepared fermented milk, shows that the used bacterial species are equipped with a proteolytic and acidifying

activity [22, 25]. The strongest and fastest bacterial growth in mixed culture is that of (Lp) obtained with a parallel growth (B long). Our results agree with those obtained by Chekroun *et al.,* [22] which explains the presence of a synergy between bacteria in mixed culture.

The mixture of bacterial species is clearly a more active an example proves because each one benefits from the other making a symbiotic character. The results concerning the enumeration agree with those of Chekroun *et al.,* [22], Chopard *et al.,* [26], Requena *et al.,* [27], Bensoltane *et al.,* [28], Hunsinger [29], Chekroun *et al.,* [30] and Chekroun *et al.* [31] which have shown that certain bacterial strains stimulate the growth of other strains by producing of nitrogen nutrients.

Sterilization at 105°C sheep's milk does not reduce the total protein rate. These results are in agreement with those of Lorient, [32], Pougheon, [33] and French *et al.,* [34].

Concerning the bacterial proteolysis, our results showed that the tested mixed cultures degrade significantly the sheep milk protein compared to the sterile sheep milk without ferment taken as a 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: *Lactobacillus plantarum* and *Bifidobacterium longum*. The protein hydrolysis by enzymes of bacteria can be explained by the existence of a proto-cooperation between the two germs on the nitrogenous matter, so to boost their fermentation performance. Our results are in agreement with those of De Man *et al*., [12], Gomes *et al*., [23], Payne *et al*., [24], Bintsis *et al*., [35] and Mierau *et al*., [36].

The results of determination of α-NH_2 functions released shows that mixed cultures assay have a variable proteolytic power depending on the type of the association and have an affinity to degrade a particular sheep's milk protein from Ayad *et al*., [21], Gomes *et al*., [23], Mierau *et al*., [36] and Langella *et al*., [37].

The age of the bacteria, the external pH, incubation temperature and the pairing mode of association have an effect on the growth and proteolytic activity [22, 26, 30, 38].

The antigenicity of the proteins in the samples of sheep's milk fermented (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 [39].

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 the degradation products. Thus, our results are also confirmed by the increase in α-NH_2 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 [32, 40]. The values obtained have allowed us to better understand the real incidence of bacterial proteolysis. Knowledge of proteolytic enzymes of the latter, really active and their properties may be of fundamental importance in the selection of starters [24, 28, 41].

CONCLUSION

Following this study, the lactic acid bacteria and bifidobacteria, tested in combination, significantly increase the antigenicity of β-Lg and α-La in fermented sheep milk compared to the control of experimentation. This is probably due to proteolysis of the protein with an unmasking of antigenic sites within the molecule. To optimize our work, we must move towards a better understanding of the physiology of bacteria and mechanisms involved in the fermentation as the composition of milk, the fermentation temperature, pH control and ferments rates.

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