Probiotic effect of some local strains of the Lactobacillus genre against Escherichia coli responsible of infantile diarrheas (in vitro Study)

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Abstact

The aim of this study consist to isolate and identify stocks of the Lactobacillus genre starting from ewe , goat and cow's milk collected starting from the area of chlef, and the study of their antimicrobial effects against E.coli ATCC 25922 responsible of infantile diarrheas and the study of their survive in a media similar to the conditions of the digestive tract. 32 isolats of Lactobacillus among 80 were characterized, they are sticks gram positive, negative catalase and push in anaerobiosis and in a slightly acid media.

The interaction between the lactic strains and E.coli present a significant activity. The strains retained for their inhibiting effect are: Lb33, Lb31, Lb58, Lb63, Lb64 whose diameter of inhibition was between 7 mm and 17 mm. The study of the survive of these strains shows that five (Lb33, Lb31, Lb 58, Lb64, Lb63) have a good resistance with respect to extreme conditions of the digestive tract (gastric acidity and bile salts). the viable rate of bacteries is remarkable to deferent pH (2.5, 4.5 and 6.5) in absence and in presence of bile salts; they arrive at 10 9 UFC/ml after 6h and at 10 7 UFC/mL after 72h of incubation at 37°C. The biochemical identification shows that the strains Lb33 and Lb31 belong Lactobacillus plantarum, Lb58 is Lactobacillus rhamnosus , Lb63 and Lb64 are Lactobacillus casei. According to our study, we can deduce that our strainss can be regarded as the probiotic one for the prevention of the infantile diarrheas. In prospect we hope for acontinuity of this work by applying these results *in vivo.*

Key words: Infantile diarrheas, *E.coli, Lactobacillus*, antimicrobial activity, , digestive tract, probiotic

1. Introduction

The infantile diarrheas are a disease which threatens several children, especially less than 5 years, in the developing countries. Indeed, in spite of the progress achieved in the improvement of infantile health since the Eighties, the diarrheas accounts for today 17% of the deaths of children of less than 5 years [1]. It constitutes a state of water loss and electrolytes in the child with like consequences dehydration and the denutrition. Its treatment rests on the administration of oral rehydration solution (SRO) and the early refeeding and the medicamentous treatment. At present, the problems of resistance to antibiotics and the sensitivity of the patients associated with the incapacity to control this disease led in the continuous search of new treatments, at end to fight sometimes the microorganisms resistant to several antibiotics. For that many research directed themselves towards the sifting of new strains producing of antimicrobial substances.

A group of bacteria proves to be interesting; lactic acid bacteria have been used in the manufacture of food fermented for several centuries. In addition to the improvement of savour, fermentation makes it possible to increase the shelf life of the product. [2] showed that certain stocks of lactic acid bacteria can have an influence on many harmful bacteria which can cause acute diarrheas and which can be of bacterial origin (ex: *Escherichia coli*).

Among the lactic acid bacteria there is the lactobacilli which are used for their deferent properties. They have the capacity to produce at the time of their growth active compounds like organic acids, derived of the metabolism of oxygens (H2Ò2) and the natural substances of

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proteinic nature endowed with antagonistic activity against a great number of germs of deterioration [3]. According to that we were interesting to study, *in vitro*, the effect of some strains of Lactobacilli on certain pathogenic germs responsible for the infantile diarrheas.

Our study is articulated on three parts initially, isolation and identification of lactic strains belonging to *the lactobacillus* genre starting from believed milks of ewes, goats and cows collected in the area of Chlef; then the selection of the species of *the lactobacillus* genre who product antimicrobial products against *E.coli ATCC 25922* and the study of their survival under extreme conditions of the digestive tract (gastric acidity and bile salts), and finally biochemical dentification of the species of lactobacilli already insulated.

2. Materials and methods

2.1. Material

2.1.1. Biological material

- Milk

The samples of milk were collected of different areas of the wilaya of Chlef, eight samples are taken from ewe, goat and cow's milks.

- Pathogenic bacterias

Escherichia coli ATCC25922 provided by Pasteur institute of Algiers.

- Culture media

- MRS (de Man-Rogosa et Sharp) used for the culture of the lactobacilli,
- Mueller-Hinton used for the interaction and the antimicrobial activities
- Mak conkey for the culture of *E coli*.

2.2. Methods

2.2.1. Sampling

The samples were carried out asepticly, before the draft, we carried out the disinfection of the hands of farmer and the nipples of the animal by chlorinated water, the milk samples were collected in sterile bottles placed any meadows of the nipples to avoid any contamination. These samples are preserved in refrigerators during transport at the laboratory, and then preserved at 4°C before the beginning of experimentation.

2.2.2. Measure of acidity of milk

According to [4] using a pH meter, we took the measurement of the acidity of milk 24 hours after the collection, then the second measurement and the third measurement according to the ambient temperature . We also measured the assayable acidity of milk according to the method described by [5], this technique was used to control fermentation and to know the moment located to isolate the various species of lactic acid bacteria.

2.2.3. Preparation of the samples (decimal Dilutions)

According to [6], for to realize a bacteriological analysis, it is necessary o pass to decimals dilutions.

2.2.4. Technique of insulation and purification

2.2.4.1. Isolation

MRS media with pH=6,8 was used for the growth of the total lactic microflore, the lactobacilli are enumerated on MRS media acidified at pH=5, 4 [7].
The sterilization of the mediums is carried out by autoclave at 121°C during 20 min.

2.2.4.2. Purification of the isolated lactic acid bacteria

After growth of the colonies, we takes 10 colonies isolated from each limp on which will be carried out a colouring of Gram and a research of the catalase. The bacteria with Gram+, catalase - and not sporulate are retained and mended on MRS broth. The operation is renewed until obtaining a pure culture whose purity is estimated by microscopic observation after colouring of Gram [8, 9].

2.2.4.3. Priliminary identification

The purity of the strains will be controlled by macroscopic and microscopic observations, the colouring of Gram, the test of the catalase and are carried out before proceeding to the conservation.

2.2.4.4. Morphological study

The macroscopic study is for determination of the shapes of colonies, color, types of contour, pigmentation..... etc [4].
The microscopic study via the colouring of Gram, enabled us to examine the form, the mode of association and Gram of these bacteria.

2.2.5. Conservation of strains

The pure strains are s preserved at -20°C and 3 % of glycerol [10].

2.2.6. Probiotic characterization of the potential of the lactic aci bacteria

Two parameters were tested for the evaluation of the probiotic activity of the colonies isolated on MRS media.

- The antimicrobial activity of the isolated bacteria was tested on the growth of *Escherichia coli*.
- The survival of the bacteria in a media simulating digestif tract.

2.2.7. Characterization of the antibacterial activity

One studies the bacterial interaction between the pathogenic bacteria and the lactic acid bacteria by the method of the discs (carries germs) and the method of the wells:

2.2.7.1. Method of disc or carries germ

Described by [11]. The inhibition of the indicating strains results in the formation of clear zones around the discs whose diameter is measured starting from the center of the disc in mm.

2.2.7.2. Method of the wells

Described by [12], this method makes it possible to put in contact the supernatant of the of antimicrobial substance produced by lactic acid bacteria with the pathogenic bacteria.

2.2.8. The survive of the strains under the extreme conditions of the digestive tract

The aim of this study is to select lactic acid bacteria which can resist at the physiological barriers of the digestive tract (intestinal pH stomacal low, peristalsis and bacterial competition on the level of the large intestine). The method used is that of [13] which consists to have two fractions.

- **fraction 1:** without bile salts, divided into bottles and pressure-sealed with 120°C during 15 mn, then added with hydrochloric acid concentrated until obtaining the desired pH:
PH 2.5: represent the pH of the stomach with before eating.
PH 4.5: represent the pH of the stomach at the time or after the meal
PH 6.5: represent the pH on the level of the intestines.
- **fraction 2:** is added with 0,3 % of bile salts, after its pressure-sealing with 120 °C during 15 mn. pH is adjusted like previously described.

These fractions are then distributed in sterile tubes at a rate of 10 ml per tube.
These various fractions are added with 3 % of inoculum of a pure lactic acid stains. These fractions are then incubated to 30 °C or the growth of the leavens is controlled by enumerations on Petri dishes during several times: 0h, 3h, 6h, 24h, 48h, and 72h.

2.2.9. Identification by API 20 E gallery

The API gallery 20 E comprises 20 microtubes containing dehydrated substrates, these microtubes are inoculated with a bacterial suspension (to take only one isolated colony, and to homogenize in a tube containing 5 ml of physiological water).
The reactions produced for the incubation period result in spontaneous coloured turns or revealed by the addition of the reagents. The reading of these reactions is done using the table reading this gallery,

3. Results and discussion

3*.1. Isolation and purification of the lactic acid bacteria*

We have isolate 80 strains. The preliminary tests showed that these stocks are cocci or bacilli with positive Gram and negative catalase, these criteria being characteristic of the lactic acid bacteria [10].

3.2. Identification of the strains

The microscopic aspect of the strains after colouring of Gram revealed the two shapes of cells: hulls and Sticks. The hulls are laid out in pairs (diplocoques) or short chains. But Sticks present of the associated cells in pairs or short chains. Juste the strains in the forms of sticks are retained.
According to the macroscopic observation made on the strains developed on MRS media, we noticed that the colonies have an ireguler forme, eroded, acream color and 1 to 3 mm in diameter [14]. The results obtained following colouring of Gram and with the test of the catalase, show that 32 strains are sticks positive Gram and negative catalase not sporulated, the mode of association varies from one strain to another, these observations make it possible to initially classify the isolates according to the gram, their cellular morphologies and their mode of association [15]

3.3. Antimicrobial effect of Lactobacillus on E.coli

3.3.1. Method of disc or carries germ

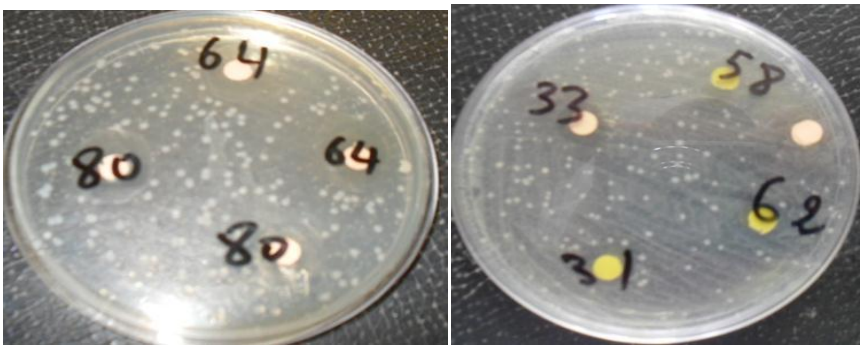


Figure 1: Inhibitions obtained by the method of the discs By the strains of *Lactobacillus* against *Escherichia coli*

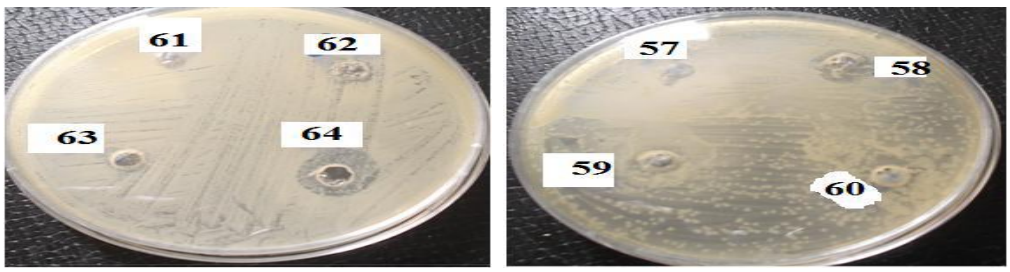
One of the interesting characteristics of the lactic acid bacteria is their capacity to slow down and inhibit the activity and the growth of the pathogenic bacteria by the production of inhibiting factors [16]. The inhibiting effect of the isolated colonies was tested on the growth of *Escherichia coli*.

Between the 32 strains used except five had an inhibiting activity against *E. coli.* According to the results obtained, their spectrum of inhibition is less significant because the diameter of the zones of variable inhibition is between 2 and 12 mm.

3.1.1.2. Method of Barefoot and kaenhammer

This method gave results different from the preceding method indeed the results of the interaction obtained,it reveal the presence of a clear zone to the turn of the wells with quite distinct edges, the diameter of inhibition is variable between 7 and 16 mm following the strain tested. The results of the interaction between the lactic stocks and the pathogenic bacterium *Escherichia coli* are showed in the figure 2

According to the results obtained, the majority of the pure strains of lactobacilli are at the origin of zones of significant inhibitions against *E.coli*, by way of examples: 13.21mm for Lb 64, 7.14mm for Lb58, 16.85mm for Lb 33, 15.64 mm for Lb 31, and 11mm for Lb 63. The other remaining strains do not have an inhibiting effect against *E.coli.* These results enabled us to note the presence of the bacteriocines, the organic acids or hydrogen peroxide produced by the strains [17]. The inhibition of *E. coli* by some strains of lactobacilli was already described by several work [18; 19].



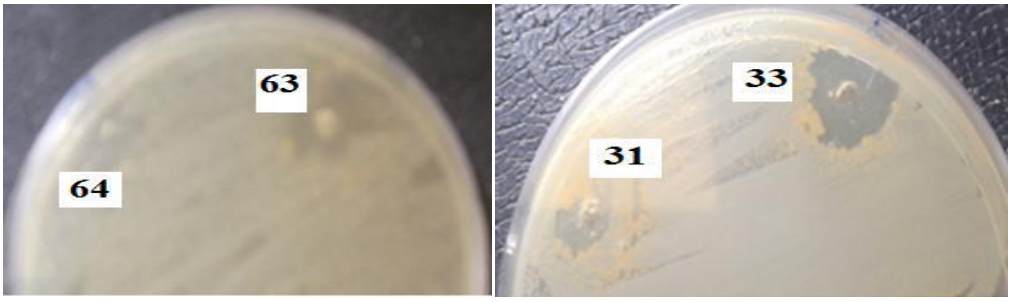


Figure 2: Inhibitions obtained by the method of Barefoot and kaenhammer by the strains of *Lactobacillus* against *Escherichia coli.*

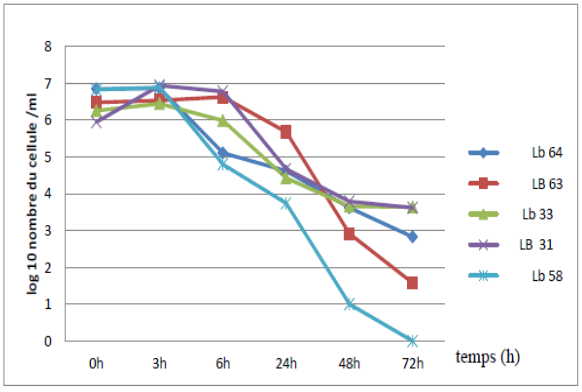
3.4. Survive of the lactic acid bacteria under the extreme conditions of the digestive tract

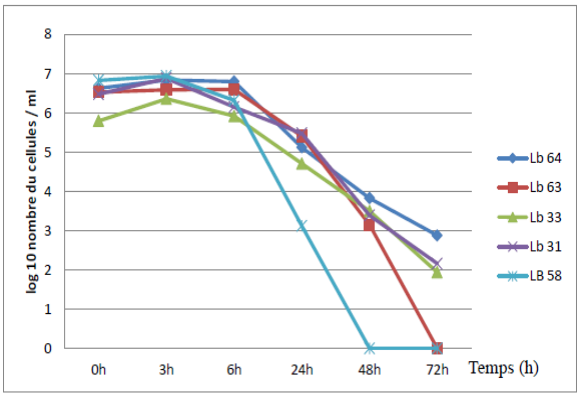
The current studies confirm that to exert an effect probiotic significant, the bacteria must arrive alive with a load equal or higher than 10^7 UFC/ml at the intestinal level [20]; but to reach this last compartment of the digestive tract, they must cross without irreversible damage the physiological barriers of its higher part which are represented by the acidity of the stomach, the intestinal peristalsis and bile secretions.
In order to test the growth of the five strains of the lactic acid bacteria according to times (0 , 3 , 6 , 24 , 48 and 72 h) with different pH (2.5, 4.5, 6.5) iin presence and in absence of 0.3 % of bile salts, the strains showed considerable variations of growth in these synthetic media. The obtained results are illustrated on figures 3, 4 and 5.

3.4.1 Survive at pH 2.5 in absence and in presence of bile salts

In absence of bile salts and at very acid pH (2.5), the tested strains of lactobacilli have a variable sensitivity, Lb 64, Lb 31, Lb 33, Lb 63, Lb 58 showed a significant resistance and persist with loads higher than 10^7 UFC/ml. After 6 h of incubation, this number falls to reach less than 10^6 UFC/ml for Lb 64, Lb 31,Lb 33 and 10^5 for Lb 63, Lb 58, the continuous reduction in the number of bacteria with time and remains in the surrounding of 10^3 UFC/ml for Lb 64, Lb 31,Lb 33 and 10^2 for Lb 63 UFC/ml and cancel after 72 h of incubation for Lb 58.

It is noted that Lb 58 and Lb 63 are sensitive at this pH and in the presence of 0.3 % of bile salts while the growth of Lb 64, Lb 31 and Lb 33 remains significant because the number of the recorded alive cells was higher than 10^6 UFC/ml after 3 h of incubation. This number falls to reach less than 10^2 UFC/ml for Lb 64, Lb 31 and Lb 33 after 72h , this number is cancelled only after 48 h for Lb 58 and 72 h for Lb 63.





(A) **(B)**

Figure 3: growth of Lb 63, Lb 64, Lb 33, Lb 31 and Lb 58 at pH 2.5 in absence (A) and presence (B) of bile salts according to time.

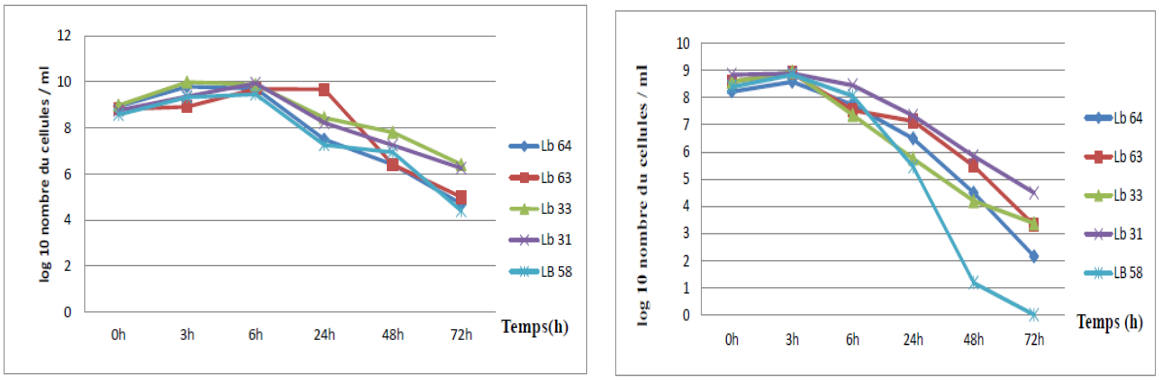
[21] showed that the lactic acid bacteria have the capacity to survive to pH too acid (up to 2.5) but this capacity varies much according to the kind and the species. In similar experiments [22] had recorded the aptitude for the growth of three strainss of lactic acid bacteria which, after to be incubated 2 hours at pH 3 were cultivated 12 hours in the presence of 0%, 0,5% and 1% of bile. The strength to the acid pH of the strains of lactobacilli shows that these last resist at pH = 2.5. This property supported our conclusion for the effect probiotic.

3.4.2. Survive at pH 4.5 in absence and in presence of bile salts

At pH 4.5, representing the pH of the stomach at the time or right after the meal, the strains showed a very good resistance, After 6 h of incubation, the bacterial loads reach figures higher than 10^9 UFC/ml for all the strains (figure 4). But after 24 h of incubation, this number falls to reach less than 10^7 UFC/ml. The reduction in the number of bacteria continuous with time and remains in the surrounding of 10^4 UFC/ml for Lb 63, Lb 64 and Lb 58 , and 10^6 UFC/ml for Lb 33 and Lb 31 after 72 h.

The addition of bile salt with pH 4,5 modified the behavior of the lactic acid bacteria (figure 4B). They showed a very good resistance, After 3 h of incubation, the bacterial loads are higher than 10^8 UFC/ml for all the strains.

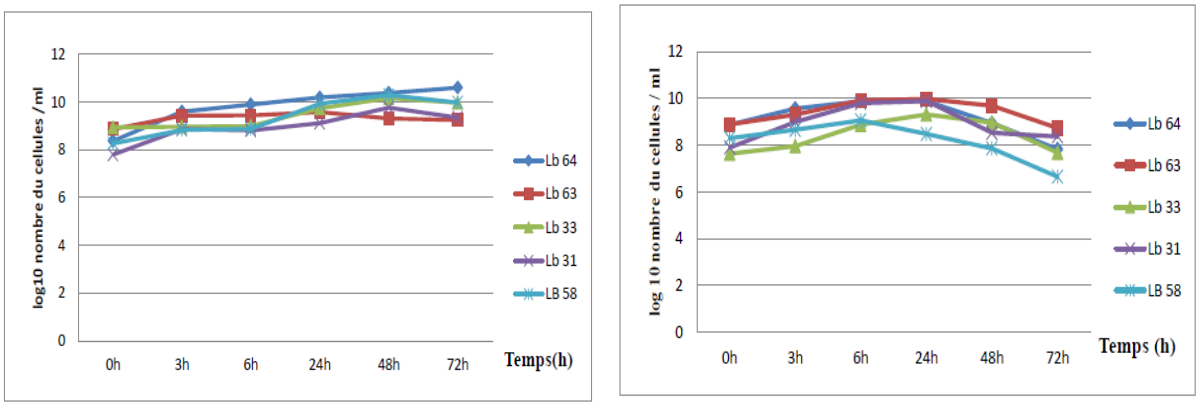
This number falls according to times but remains higher than 10^7 UFC/mL after 6h for the majority of the strains. The reduction in the number of bacteria continuous with time and remains in the surrounding of 10^4 UFC/ml for Lb 63, Lb 33, Lb 31 and 10^2 UFC/ml for Lb 64 and cancel after 72h of incubation for Lb 58. It is noticed that the majority of the strains have a very good growth at pH 4.5 and resist at this same pH in the presence of bile salts.These results are in analogy with those of [13] which shows that the lactic acid bacteria have variable behaviors with bile salts and pH 4.5. The same observations were made by [23] which finds that the pure mesophilic strains of *Lactobacillus paracasei* (3, 4, 6 and 7) have a good resistance and a better rate of survive at pH 4,3 in absence and in the presence of 0,3 % of bile salts.



(A) **(B)**

Figure 4: growth of Lb 63, Lb 64, Lb 33, Lb 31 and Lb 58 at pH 4.5 in absence (A) and presence (B) of bile salts according to time.

3.4.3. Survive pH 6.5 in absence and in presence of bile salts



(A) **(B)**

Figure 5 : growth of LB 63, LB 64, LB 33, LB 31 and LB 58 at pH 6.5 in absence (A) and presence (B) of bile salts according to time.

At pH 6.5, similar to the intestinal pH, we recorded a very significant growth of the lactobacilli, higher than 10^8 UFC/ml after 3h of incubation to 37 °C. The growth of the stains remains always high and it increases according to time to reach maximum values after 48 h of incubation: 2.32 $\times 10^{10}$ UFC/ml for Lb 64, 1,92. $\times 10^{10}$ UFC/ml for Lb 58 , 1.44$\times 10^{10}$ UFC/ml for Lb 33, 5,70 $\times$ 10^{10} UFC/ml for Lb 31 and 2$\times 10^9$ UFC/ml for Lb 63. A reduction is detected after 72 h of incubation.

When 0.3% of bile salts are added in the culture media the strains resist and continue to growth (figure 5B), their values loads exceed 10^8 UFC/ml after 48h of incubation for all the strains. These loads start to decrease after 72 h of incubation at 37C°. According to the results obtained we can conclude that the lactobacilli resist at pH (2.5, 4.5, 6.5) in absence and in presence of a high bile salts concentration (0.3%) with considerable differences in growth according to species', this is confirmed by [25] which showed that *lactobacillus plantarum* survive with its passage in the digestive tract, it finds that some strains of lactobacilli develop normally at pH 3 and 4 and resist even pH 2.

3.4. Results of identification by API 20 E gallery

The results of the fermentation of the carbohydrates on the API 20 E gallery, allowed the identification of the species, after the comparison between the results, and given of [14]. Strains are probably classified in the following species:

- Lb 31 and Lb33 are *Lactobacillus plantarum.*
- Lb 58 is *Lactobacillus rhamnosus.*
- Lb63 and Lb64 are *Lactobacillus casei.*

Conclusions

The lactobacilli are used for their deferent properties. They have the capacity to produce at the time of their growth active compounds. The results are as follows:

- 32 isolates of lactobacilli among 80 were characterized starting from cow, goat and ewe's milk collected starting from the area of chlef .They are sticks gram positive, negative catalase and push in anaerobiosis and in a slightly acid media.
- Lb33, Lb31, Lb64, Lb63, show a good inhibiting activity with respect to *E.coli* which arrive at 16,85 mm for Lb33 according to the method of well. The results are different according to the used method.

- Five lactobacilli showed a good survive under the conditions of the digestive tract, the viable rate of bacterias is remarkable with deferent pH (2.5,4.5,6.5) in absence and in presence of bile salts; they arrive at 10^7 UFC/ml after 6h and at 10^4 UFC / ml after 72h for Lb64 at pH 2,5 in the presence of bile salts, whereas they manage to survive 10^9 UFC/ml after 6h and higher than 10^4 UFC/ml after 72h for Lb33 at pH 4,5 but for the pH 6.5 which they manage to survive 10^8 UFC/ml after 48h and higher than 10^7 UFC/ml after 72h for Lb 63.

- The biochemical identification allowed us to deduce that Lb31 and Lb33 were probably identified as being *Lactobacilus plantarum*, Lb58 is *Lactobacillus Rhamnossus* , Lb63 and Lb6 were identified as being *Lactobacilus casei*. According to our study, we can deduce that our strains can be regarded as the probiotic one used for the prevention of the infantile diarrheas. In prospect we hope for a continuity with this work by applying these results " *in vivo* ".

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