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Isolation and Identification of Lactic Acid Bacteria from Dhan, a Traditional Butter and Their Major Technological Traits

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Abstract: A total of 5 samples of traditional fermented milkbutter (Dhan) were collected from individual households. Lactic acid bacteria dominated the microflora of these samples, especially the genera *Leuconostoc*, *Lactococcus* and *Lactobacillus*. Other groups identified included pyogenic streptococci and enterococci. The dominant lactococci species was *Lactococcus lactis* subsp. lactis. Eighty-three percent of the *Leuconostoc* isolates were identified as *Leuconostoc mesenteroides* subsp. *dextranicum*. Other species identified included *Leuconostoc citreum*, *Leuconostoc lactis*, *Lactobacillus delbrueckii* subsp. *lactis* and *Lactobacillus plantarum*

Key words: Lactic acid bacteria · *Lactobacillus* · Dhan · Traditional fermented milkbutter · Households

INTRODUCTION

Lactic acid bacteria (LAB) have played along and important role in Food technology and have a long history of use by man for food production and food preservation. Isolation of wild-type strains from traditional product is a classical method to obtain starter cultures for food fermentations [1]. By using selected wild-type strains, the large-scale production of fermented foods can be developed without losing their unique flavour and particular characteristics [2].

The samples used in this study are Dhane, a traditional butter milk which made from unpasteurized cow milk. First, milk is left at room temperature until it tastes sour. A manual agitation is operated in a goatskin churn, during agitation a small amount of cool water is added. This cool water help to coagulate fat globules. This agitation lasts a few tens of minutes. the fat contents are collected and it represents butter. Butter thus obtained is salted, it should be noted that salting is to leave with the taste of sparing. the butter is preserved at room temperature.

Numerous researches demonstrated that the autochthonous microflora present in several traditional products, other than improving the flnal technological and sensory characteristics, possess inhibition activity towards spoilage and pathogenic bacteria and this has been reported for both dairy [3-6] and meat products [7,8].

The aims of this study were: i) to study the microbial ecology of lactic acid bacteria of Dhan (traditional butter milk) and ii) characterization of different groups of microflora, lipolytic, proteolytic and antimicrobial producer bacteria using classical methods.

MATERIAL AND METHODS

Collection of Samples: Five samples of traditionally butter milk (dhan) where purchased from local households in west Algeria. Samples were transported to the Laboratoire de Microbiologie Appliquée in room temperature.

Isolation and Identification of Lactic Acid Bacteria: Ten grams of butter was homogenized with 90 mL sterile NaCl solution (0.85%, w/v) to a homogenous suspension and then a tenfold serial dilution in NaCl solution (0.85%, w/v) was carried out. For isolation of lactic acid bacteria, acidified MRS pH 5,4 [9] incubated anaerobically for 72 h at 37°C was used for isolation of lactobacilli. MSE [10] incubated aerobically for 48 h at 35°C was used for isolation of leuconostocs. M17 agar [11] incubated aerobically for 48 h at 30°C for the isolation of lactococci.

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The colonies between 30 and 300 on each Petri dish were counted as total LAB. To isolate LABs, ten colonies were randomly picked from each countable plate. Attention was given to choose colonies with different macroscopic morphology. Isolates were reinoculated in MRS broth, incubated at 30 °C and checked for purity by streaking on MRS agar. Plates with pure cultures were used to test for cell morphology by phase contrast microscopy, Gram stain and catalase formation. Gram positive and catalase negative strains were selected. These isolates were maintained as frozen stocks in MRS broth supplemented with 10% (v/v) glycerol at 18°C during a month. Before experimental use, all LAB strains were recovered in MRS broth and were incubated at 30 °C.

Growth at different temperatures was observed in MRS broth after incubation for 5 days at 15°C and 45°C. Supplemented test was performed for bacterial cocci and resistance at 63,5°C for 30 min was done in order to discard enterococcus bacteria [12]. Hydrolysis of arginine was tested in M16BPC [13,14]. Growth in the presence of 4 and 6.5% NaCl was performed in MRS broth for 5 days. Utilization of citrate was realised in Kempler and Mc Kay [15] medium. Production of acetone from glucose was determined using Voges-Proskauer test [16]. Production of dextrane from sucrose was done in MRS agar [10] and CO_2 gas in Durham tube within MRS broth was checked from glucose.

The sugar profile of each strain was determined in mini preparation with ELISA microtiter plate [17], with MRS broth without lactose and meat extract and adjusted to pH 6.5 as fermentation medium and bromocresol purple (0.004%) as indicator (MRS-BCP). Individual sugars (Sigma Chemical Co., St. Louis, MO, USA) were sterilised by filtration and added to a final concentration of 2%. Microbial cells were washed and added in a sterile saline solution (8 g NaCl/L). Each ELISA microtiter plate well, receive 0,1 ml of MRS-BCP medium, 10 µl of microbial cell and 10 µl of sugar solution. To ensure anaerobical condition 0,1 ml of sterilized paraffin oil was added. the incubation was made at 30°C and the results read after 1 and 2 days of incubation.

Lipolytic Activity of Isolated Microorganisms: The culture medium was prepared by adding peptone 10.0g, NaCl 5.0g, CaCl 2 •2H 2 O 0.1g and agar 20.0 g in 1000ml water and autoclaved for 20 min; 10ml Tween-20 (Sigma) was separately sterilized and added into the autoclaved medium and the pH was adjusted to 6.0. About 20ml the medium was poured into each Petri dish and inoculated at the center using a pinpoint inoculum of the test fungus. Lipolytic activity was indicated by the appearance of a visible precipitate, resulting from the deposition of crystals of the calcium salt formed by the fatty acid liberated by the enzyme, or as a clearing of such a precipitate around a colony due to complete degradation of the salt of the fatty acid. At regular intervals of 24-h incubation, each plate was examined and measurements were taken to monitor lipolytic activity [18-20].

Proteolytic Activity of Isolated Microorganisms: Proteolytic activity was tested using Plat Count Agar PCA with 1% and 2 % (w/v) skim milk. The presence of clear zones around the colonies was recorder as positive activity. All strain with positive reaction in MRS with 1% skimed milk was considered as strains with slight activity [21].

Screening for Antagonistic Activity: Detection of antagonistic activity in LAB strains was initially screened by means of an agar well diffusion assay (AWDA) [22]. MRS agar was used for LAB strains while BHI agar was used for the rest of the indicator microorganisms (*Staphylococcus aureus, Escherichia coli* and *Listeria innocua*). Briefly, Petri dishes were overlaid with 15 ml of molten agar (1% agar), inoculated with 30 µl of an overnight culture of the indicator microorganism, in which wells were formed. Wells, of 5 mm in diameter and of 30 µl in capacity, were formed by carving the agar with a cork borer. Afterwards, 30 µl of an overnight culture of the putative inhibitor strain were placed in each well. The plates were then incubated aerobically for 24h at a temperature conductive to growth of the indicator microorganism and were subsequently examined for zones of inhibition. Inhibition was recorded as negative if no zone was observed around the agar well. Each antagonistic activity was related to the area (2 mm) of the inhibition zone displayed [23].

Antibiotic Susceptibility Testing: For the antibiogram of isolated lactic acid bacteria strains, the isolates were inoculated into MRS broth individually and incubated for 24 h. About 20 ml of Mueller-Hinton agar was seeded with the cultures of LAB isolates, mixed well, poured into sterile Petri plates and leave to solidify at room temperature. Antibiotic discs (OXOID) (Table 1) were placed up side down, pressed on the top of the agar plates. The plates were incubated at 37ºC over night. Resistance was defined as the absence of a growth inhibition zone around the discs.

Table 1: List of tested antibiotics on isolated lactic acid bacteria

Antibiotics	Concentration	Symbole	Antibiotics	Concentration	Symbole
Amikacin	30 µg	AN	Lincomycin	15 µg	L
Amoxycillin + Clavulanic Acid	20 µg + 10 µg	AMC	Nalidixic Acid	30 µg	NA
Ampicillin	10 µg	AM	Netilmicin	30 µg	NET
Bacitracin	0,02 à 0,04 IU	BAC	Nitrofurantoin	300µg	FT
Cefazolin	30 µg	CZ	Ofloxacin	5 µg	OFX
Cefotaxime	30 µg	CTX	Oxacillin	1 µg	OX1
Cefoxitin	30 µg	FOX	Pefloxacin	5 µg	PEF
Cefsulodin	30 µg	CFS	Penicillin	6 µg / 10 IU	P
Ceftazidime	30 µg	CAZ	Piperacillin	75 µg	PIP 75
Cephalothin	30 µg	CF	Pristinamycin	15 µg	PT
Ciprofloxacin	5 µg	CIP	Rifampin	30 µg	RA 30
Clindamycin	2 µg	CM	Spiramycin	100 µg	SP
Colistin	50 µg	CS 50	Tetracycline	30 µg	TE
Erythromycin	15 µg	E	Ticarcillin	75 µg	TIC
Fusidic Acid	10 µg	FA	Tobramycin	10 µg	TM
Imipenem	10 µg	IPM	Trimethoprim-Sulfamethoxazole (co-trimoxazole)	1.25 µg + 23.75 µg	SXT
Vancomycin	30 µg	VA			

RESULTS

Isolation and Identification of Lactic Acid Bacteria: The greater part of the total number of isolates was gram-positive and catalase-negative.

A total of 76 isolates from four samples could be identified and were divided into five genera:

Lactobacillus, *Lactococcus*, *Leuconostoc* and *Enterococcus*.Thirty five isolates belonged to the genus *Lactobacillus*, 8 isolates to *Leuconostoc* and 13 isolates to *Lactococcus*. Members of the genus *Lactobacillus* dominated in all Dhan samples. Figure 1 illustrated the percentage of distribution of the 76 bacteria identified from traditional butter.

All the lactococci isolates (a total of 8 isolates) belonged to *Lactococcus lactis* subsp. *Cremoris.* Four isolates from a total of 8 *Leuconostoc* isolates produced dextran from sucrose.

Four of the *Leuconostoc* isolates were non-dextran-producing isolates. Three of them belonged to*Leuconostoc mesenteroids* subsp. *mesenteroides* species and one to *Leuconostoc lactis*. 76.92% of *Lactobacillus* isolates belonged to *Lactobacillus plantarum* species. *Lactobacillus delbrueckii* reprisent 84,61% of the total of lactobacilli isolates. Three isolates are *Lactobacillus delbrueckii* subsp. *Lactis,* Four are *Lactobacillus delbrueckii subsp. delbrueckii* and 4 isolates are belonged to *bulgaricus* subspecies. Five isolates are belonging to *Lactobacillus casei.*

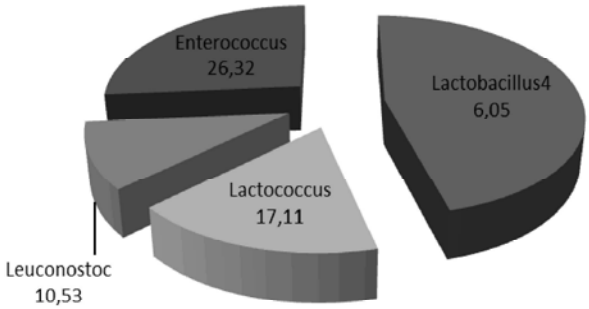

Fig. 1: Identity of 76 bacterial strains isolated from indigenous traditional butter (Dhan).

Eight isolates belonged to *Lactobacillus amylophilus* and finally one isolate belong to *Lactobacillus brevis* (Table 2 and 3).

Proteolytic and Lipolytic Activity of Isolates: From all tested isolates, only two isolates are lipolytic B11 (*Lactobacillus delbrueckii* subsp. *delbrueckii*) and A20 (*Lactobacillus delbrueckii* subsp. *bulgaricus*) (Figure 4). For proteolytic activity, the use 1% of skimed milk in PCA medium allow to detect slight proteolytic activity (Figure 2). We can list as an example 3 isolates belonging to *Lactococcus lactis* subsp. *cremoris* gave slight reaction with 1% and no activity was observed with 2% of added skimed milk in PCA medium. The isolate G4 (*Lactobacillus plantarum*) has no proteolytic activity. All result of proteolytic activity are listed in table 4.

Table 2: Physiological and biochemical characteristics of lactobacilli strains isolated from Dhan

Strains	1 G5, d4, S4	2 N4, H15, S1, N4	3 A24, d1, G1	4 H1, H11, H13, N3	5 S14, B5, N1, N11, d12, d14, H4, A2	6 S3, d3	7 s11	8 G4,H3,S12,N2, G2, A25,d11,S11, B3,B8
Gram	+	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	-	-
CO_2 from Glucose	-	-	+	-	-	-	+	-
A.D.H	-	-	-	-	-	-	-	-
Temperature								
15°C	-	-	+	-	+	+	+	+
45°C	+	+	-	+	-	+	-	-
Sugar proflle								
xylose	-	-	-	-	-	-	-	-
maltose	+	+	-	-	+	+	+	+
galactose	-	-	+	-	+	+	-	+
D-sorbitol	-	-	-	-	-	+	-	+
arabinose	-	-	-	-	-	-	+	-
mannitol	-	-	+	-	-	+	-	+
L-rhamnose	-	-	-	-	-	+	-	-
sucrose	-	+	-	-	+	-	-	+
lactose	+	+	-	+	-	+	+	+
D-fructose	-	+	+	+	+	+	+	+
glucose	+	+	+	+	+	+	+	+
Esculine	+	-	+	-	+	+	-	+

1:Lactobacillus delbrueckii subsp. Lactis; 2:Lactobacillus delbrueckii subsp. delbrueckii; 3:Lactobacillus casei subsp. casei; 4:Lactobacillus delbrueckii subsp. bulgaricus; 5:Lactobacillus amylophilus; 6:Lactobacillus casei subsp. rhamnosus; 7:Lactobacillus brevis; 8:Lactobacillus plantarum

Table 3: Physiological and biochemical characteristics of cocci strains isolated from Dhan

Souches	1 H10	2 B10, B11, M2, M11	3 M3, M5, M13	4 G6,G8,G10,G13, A7,A9, A20,H9,d9 ,d7,N9,d17,d18
Gram	+	+	+	+
Catalase	-	-	-	-
CO2 à partir du Glucose	+	+	+	-
A.D.H	-	-	-	-
Température				
15°C			+	+
45°C	-	-	-	-
Sugar proflle				
xylose	-	-	+	+
maltose	+	-	+	-
galactose	+	-	+	+
D-sorbitol	-	-	-	-
arabinose	-	-	-	-
mannitol	-	+	-	-
L-rhamnose	-	-	-	-
sucrose	-	-	+	-
lactose	+	+	+	+
D-fructose	+	+	+	+
glucose	+	+	+	+
Esculine	-	+	+	-

*1:Leuconostoc lactis; 2:Leuconostoc mesenteroides subsp. dextranicum; 3:Leuconostoc mesenteroides subsp. mesenteroides ;*4:*Lactococcus lactis subsp. cremoris*

Table 4: Proteolytic activity of isolated lactic acid bacteria

	Milieu PCA+Lait			Milieu PCA+Lait			Milieu PCA+Lait	
isolate	1%	2%	isolate	1%	2%	isolate	1%	2%
d17	+	+	H6	Nd	-	A9	+	+
N11	+	+	d9	+	+	B13	+	+
N15	+	+	N13	-	-	A25	+	+
N2	+	+	N16	-	+	M2	+	+
S'2	+	+	S15	+	+	A8	+	-
S10	+	+	G8	+	+	M13	+	+
N9	+	+	B7	+	+	B7	+	-
S8	+	+	S17	+	+	M11	+	+
d18	+	+	S14	+	+	M5	+	+
S7	+	+	B5	Nd	+	G16	+	+
N14	+	+	A24	Nd	+	B4	+	+
S18	+	+	M6	-	+	H5	-	+
N7	+	+	G1	+	-	B11	+	+
N5	-	+	G6	+	-	d14	+	+
N12	+	+	B10	+	-	H5	+	+
S11	+	+	G4	-	-	d5	+	+
S12	+	+	A7	+	+	S13	+	+
d10	+	+	A19	+	+	N10	+	+
M7	+	+	G5	+	+	A21	+	+
G13	+	-	G12	+	+	A18	+	+
B8	+	+	B15	+	+	d12	+	+
d18	+	-	M12	-	+	H9	Nd	Nd
G2	+	+	M1	+	-	B3	+	+
M10	-	+	A27	+	+	A5	+	+
S6	+	-	S9	Nd	-	A20	+	+
d4	+	-	G15	+	+	G10	+	+

Nd:not determined

Table 5: Antibiogram of representative isolate of lactic acid bacteria.

	isolates					
Antibiotics	N11	M5	A20	d18	d14	B11
OFX	S^+	S^+	S^+	S^+	S^+	R
AN	S^+	S^+	S^+	S^+	S^+	S^+
NET	S^+	S^+	S^+	S^+	S^+	S^+
CF	S^+	S^+	S^+	S^+	S^+	S^+
CIP	S^+	S^+	S^+	S^+	S^+	R
TIC	S^+	S^+	S^+	S^+	S^+	S^+
1OX	R	R	S^+	S^+	R	R
CS	S^+	S^+	S^+	S^+	S^+	R
AMC	S^+	S^+	S^+	S^+	S^+	S^+
TM	S^+	S^+	S^+	S^+	S^+	S^+
CTX	S^+	S^+	S^+	S^+	S^+	S^+
FT	S^+	S^+	S^+	S^+	S^+	S^+
XGHP	S^+	S^+	S^+	S^+	S^+	S^+
CAZ	R	S^+	S^+	S^+	S^+	S^+
PIP	S^+	nd	S^+	S^+	S^+	S^+
FOX	S^+	S^+	S^+	S^+	S^+	S^+
TE	S^+	S^+	S^+	S^+	S^+	S^+
VA_{30}	R	R	S^+	R	S^+	R
L	S^+	S^+	S^+	S^+	S^+	S^+
RA	S^+	S^+	S^+	S^+	S^+	S^+

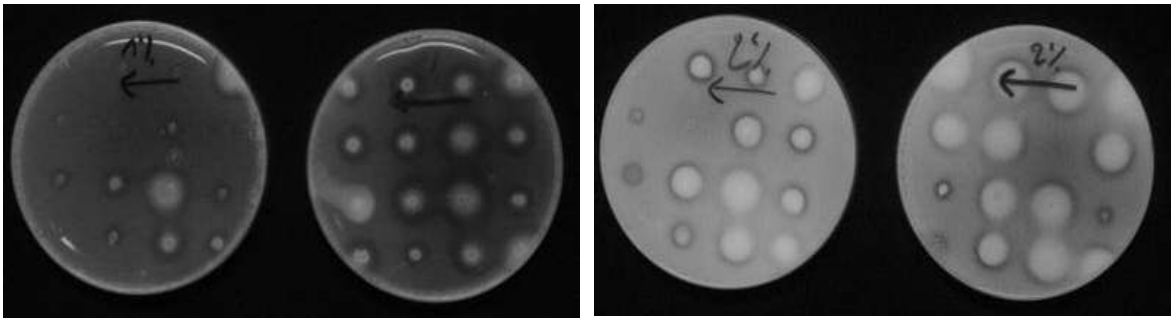

Fig. 2: Proteolytic activity of lactic acid bacteria in PCA medium additionned with 1% and 2% of skimmed milk

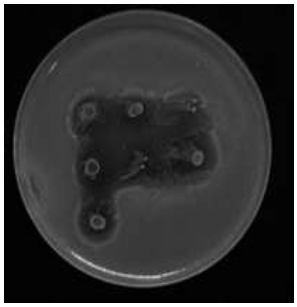

Fig. 3: antimicrobial activity of isolated lactic acid bacteria

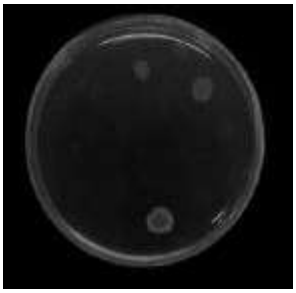

Fig. 4: Lipolytic activity of lactic acid bacteria strain

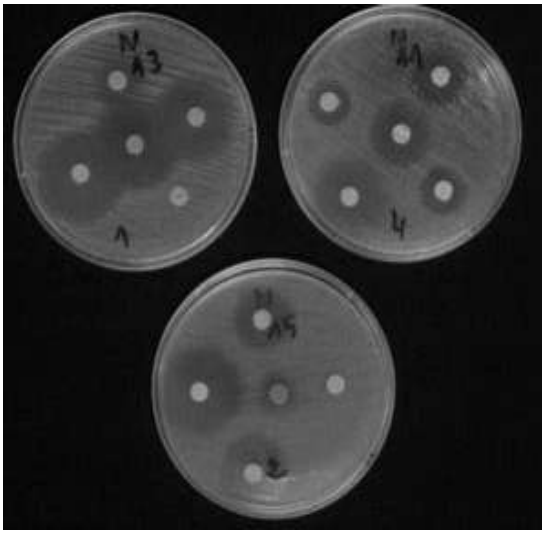

Fig. 5: Antibiogram of certain lactic acid bacteria strain

Antimicrobial Activity: From candidate isolates belonging to different species, some isolates gave positive inhibition toward pathogenic bacteria (Figure 3). The supernatant of Lactococcus lactis subsp. cremoris, Lactobacillus plantarum and Lactobacillus delbrueckii subsp. delbrueckii inhibit growth of *Escherichia coli, Staphylococcus aureus* and *Listeria innocua* respectively.

Antibiogram of Isolates: The behaviour of each isolate to different antibiotics in terms of sensivity and resistance has been shown in table 3. All isolates were found sensitive to most of the broad-spectrum antibiotics and resistant to antibiotics active on Gram-negative bacteria (Table 5 and Figure 5).

DISCUSSION

Lactic acid bacteria predominated the total microflora; Lactobacillus dominates the totality of these flora. This dominance is initialized by the pre-acidification of milk prior to its transformation to butter. According to many studies, *Lactobacillus* dominates all traditional fermented dairy products. *Lactobacillus plantarum, Lactococcus lactis ssp. lactis, Lactobacillus delbrueckii subsp lactis, Leuconostoc lactis* and *Leuconostoc citreum* were identified in South African traditional fermented milks [24]. In this study, Lactobacillus are dominant flore with 23% from total lactic acid bacteria. Missotten., [25] found that 131, strains isolated from fermented liquid pig feed, out of 145 lactic acid bacteria are *Lactobacillus.* Khedid *et al*., [26], found that *Lactobacillus* isolated from camel milk representing 37,5% from LABs flora. This work shows also, that the initial flora of raw milk contributes to that residual in the finished product, the method of traditional transformation and no pasteurization is carried out in these methods. The lactobacilles accounts are for 54.4% of the lactic flora in traditional fermented product

containing manioc [27]. The most dominant species for this group is *Lactobacillus plantarum*. This species is largely known for its predominance for the *Lactobacillus* in almost the whole of the traditional products. This predominance is with a great number of properties, the acidification and the antimicrobial metabolites production will be quoted. *Lactobacillus delbruekii* also is very represented among the isolated strains. The latter presents three subspecies *lactis* (3 strains), delbruekii with 4 strains and finally *bulgaricus* with 5 strains. *Lactobacillus casei* is represented by 8 strains (*casei* and *rhamnosus*). The presence of the lactobacilles in this traditional product is surely with the methodology used which consists in acidifying milk for 24 hours period before starting the separation of the fat contents. Milk thus acidified contributes to the selection of the acidifying flora. The following stage, separation of the fat contents, are made in an enclosure cook some which supports the conditions of anaerobiose to nevertheless largely decreasing the oxygen contribution facilitates the development of the lactic CO_2 bacteria producing as *Leuconostoc* which are present with under species *Leuconostoc mesenteroides* subsp. *dextranicum*, strains producing dextran and *Leuconostoc mesenteroides* subsp. *mesenteroides*. The lactocoques ones are represented by only one species which is *Lactococcus lactis* subsp. *cremoris*. It will be announced that the phynotypic classification of this species gives ambiguous results. It was confirmed by several research that the strains which were identified like *cremoris*, genotypicly is lactis and screw-poured [28-30]. Generally, the species identified in the present study, were in good agreement with other studies. The lactic acid strains were tested for their capability to inhibit the growth of *Staphylococcus aureus* and *Escherichia coli* showed their antagonistic capacity towards these two strains by producing antimicrobial substance in the culture medium. This production was confirmed by using culture supernatant tested by well method diffusion. The lactic bacteria isolated and identified show a capacity to inhibit *Staphylococcus aureus*, *Escherichia coli*. These two undesirable strains are known like contaminant of the acidified dairy products.

The antibiotic resistance pattern of the lactic acid bacteria shows a resistance to the oxaciline and the vancomycine. Essid *et al*., [31] working on lactobacilli, find that almost the totality of the strains are sensitive to the amikacin (30 µg), the cefuroxime (30 µg), the gentamicin (10 µg), the NOR fl oxacine (10 µg) and the streptomycin (10 µg). These same authors find 88.2% and 82.3% of the strains resistant to the erythromycin and the rifampicin respectively. 70.5% of their strains were resistant to the ampicillin and the penicillin G. Nguyen *et al.*, [32] find that *Lactobacillus plantarum* resist to the tetracycline, gentamicin and the penicillin and sensitive to the erythromicine and ampicillin. Gevers *et al.*, [33] find that 100%, 79% and 64% of the lactic bacteria isolated from fermented sausage and dried are resistant to the tetracycline, the gentamicin and the penicillin respectively. It should be known that before using a lactic starter, it is important to check that the bacterial strains cannot transfer genes from resistance [23]. The proteolytic and lipolytic activity are activities very required in the selection of the lactic starters for the industry of several dairy products and especially that of cheeses as brings back several authors [31,34]. The lactobacilli isolated from traditional butter did not express a lipolytic activity. Two strains of *Leuconostoc* represented by the strain B and M5 expressed a lipolytic activity. With these two strains *Lactococcus lactis* subsp *cremoris* A20 expressed same activity. Studies made by Ammor *et al.*, [23] confirms our results by finding the majority of their strains of *Lactobacillus* unable to express a lipolytic activity. In the same way of another work arrives at the same conclusion [35,36].

CONCLUSION

Traditional butter is a culinary food product of first required the study of the ecology of the lactic bacteria, enabled us to insulate, to identify and characterize 76 isolates of which 52,34% are bacilli and 47,66 are cocci. The most dominant flora is represented by the lactobacilles which finds in the product an acid environment. *Lactobacillus plantarum* is the most dominant species with 28,57% of the isolated lactobacilli. *Lactobacillus casei* subsp. *casei* is represented by 3 strains. This strain was found proteolytic and its presence in butter contributes largely to the development of the organoleptic characteristic of butter like end product. The traditional method is for large-thing in the preselection of these strains. *Leuconostoc* are also present and especially under known species *dextranicum* for the production of the dextran. The lactic bacteria isolated and identified show a capacity to inhibit *Staphylococcus aureus* and *Escherichia coli*. These two undesirable strains are known like contaminant of the acidified dairy products. Two strains of *Leuconostoc* (B and M5 strains) expressed a lipolytic activity. *Lactococcus lactis* subsp. *cremoris* A20 expressed same

activity. the antibiotic resistance pattern of our strains shows us an almost total sensitivity of all the strains tested.

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