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Identification and characterization of lactic acid bacteria isolated from cow milk and olives brine

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Abstract
A total of 24 strains of lactic acid bacteria were isolated from cow milk and olives brine, out of which 4 strains, having the best criteria of conservation, were selected for their identification and their characterization. These lactic isolates underwent preliminary testing of morphological and biochemical such as: gram reaction, catalase test, mobility and morphology. Other tests were held for characterization such as: fermentation type, fermentation mode of sugars, tolerance to heat, salinity and pH, test of Voges Proskauer, hydrolysis test of Citrate and Arginine. The lactic isolates were classified into 3 species as follows: the BLN3 and BLN4 strains belong to the species *Lactobacillus plantarum*, the BLN8 strain belongs to *Lactobacillus delbrueckii*, and the BLN17 strain is classified as *Lactobacillus fermentum*. BLN3 has only one particularity; she has high tolerance to grow in salt and acid medium.

Keywords: Lactic acid bacteria, fermentation, conservation, identification, characterization.

1. Introduction
Milk and olives are popular environments for bacteria including lactic acid bacteria. These microbes are used in the production of fermented food products [1] by their contribution to the improvement of the texture and flavor (di-acetyl) [2].; they are also characterized by their acidifying power producing organic acids such as lactic acid and acetic acid; the synthesis of hydrogen peroxide (H_2O_2) and the antagonistic activities against pathogenic micro-organisms via secretion bacteriocin [3] example: Nisin, which has a known effect on *Listeria monocytogenes* [4]. In this study we sought to identify the four selected lactic strains and their individual behavioral against different factors such as pH, salinity and temperature in order to exploit them in the future in the bioconservation.

2. Experimental
2.1. Isolation and purification of lactic acid bacteria
Samples of milk and olives liquid are conducted in our laboratory under sterile conditions. One milliliter of each sample was pour-plated in the selective solid medium MRS (Man Regosa Sharp). The cultures were incubated at 30°C for 24 hours in an anaerobic jar and darkness [3], the suspected colonies of lactic acid bacteria are marked and isolated. The isolates strains were purified by successive streaking onto the same medium.

2.2. *Conservation of lactic acid bacteria*
The working cultures were kept on MRS agar slant at 4°C and streaked every month. For the long time preservation, the purified strains of lactobacillus were stored at –20°C in cryotubes containing MRS broth (80%) supplemented with 20% of glycerol [4].

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2.3. *Preliminary identification of lactic isolates*
Each colony suspected to be a lactic acid bacterium has undergone a preliminary identification; we are based on the following studies:
- Microscopic (cell morphology, mobility, arrangement and gram stain).
- Hydrolytic (catalase test, fermentative type).

2.4. *Gas production from glucose*
In order to define that the lactic isolates are homofermentatives, the CO_2 production was performed. The bacterial strains are inoculated vertically in MRS medium at 8 % of agar. After inoculation, a white layer at 20 % agar is added to each tube. The results are taken after 24hours of incubation at 30°C. Gas accumulation in tubes was taken as the evidence for CO_2 production from glucose.

2.5. Carbohydrate fermentation test
The four isolates were tested for their carbohydrate fermentation. The news tubes from overnights cultures of bacteria lactic are prepared. A colony of the each culture is spread on MRS medium containing Phenol red (0.05g/l) as pH indicator. After inoculation and incubation at 30°C for 24 hours, the acid production was indicated by a change of color from red to yellow. The utilized carbohydrates are: lactose, glucose, sucrose, galactose, dextrose, sorbitol, mannose, arabinose, xylose, fructose, maltose, rhamnose, raffinose, ribose, mannetol [5].

2.6. *Tests of Voges Proskauer, Citrate and Arginine.*
Each lactic colony is inoculated in the MR-VP broth medium and incubated at 30°C for 24 hours. After incubation; 0,5 ml of the alpha-naphthol solution and 0.5 ml of KOH solution are added to the medium. The tubes are shaken for 2 min and they are slope to increase the aeration. After 20 minutes, the positive reaction is indicated by development of pink color. It means VP positive. In a different situation, the test is negative [6]. For the Test of Citrate hydrolysis, the lactic acid strains to test are seeded on the Simmons Citrate agar dispensed into inclined tubes, and then incubated at 30°C for 48 hours. The change of the medium color to blue indicates a positive reaction. In the opposite, the medium remains greenish and the test is denoted negative. For the detection of the Arginine dihydrolase among isolates lactic, 1ml of overnight cultures were inoculated into 5 ml Arginine MRS broth, and were incubated for 24hours at 30ºC. After the incubation, production of ammoniac is demonstrated by the color change from red to yellow.

2.7. *Effect of pH*
The evolution of the growth of lactic acid bacteria according to the pH of the culture medium MRS is assessed by determination of bacterial biomass using a spectro-photometer at 600 nm. The pH is 3.5; 4.5; 6; 7 and 8.5. The results are taken after incubation at 30°C for 24 hours.

2.8. *Effect of temperature*
1ml of overnight cultures was transferred into the tubes which contain 5 ml MRS broth. After inoculation, they were incubated for 24 hours to following temperature: 10ºC, 30ºC and 45ºC. Cells growth at any of these temperatures was detected by measuring the absorbance at 600 nm [7,8].

2.9. *Tolerance of salinity*
The effect of NaCl on the growth of lactic acid bacteria is carried out by inoculation the each bacterial strain in 5 ml MRS broth supplemented with different salt levels (2.5%, 4.5% and 6.5%). They are incubated at 30°C for 24hours. The results are taken by spectrophotometer at 600 nm.

3. Results and discussion

3.1. *Isolation and Purification*

The macroscopic examination of morphological characteristics for the colonies gave the following characteristics: circular shape; pure white, small and milky aspect. After five successive subcultures on the same medium (MRS), the colonies obtained are pure.

3.2. *Identification of lactic acid bacteria at the genus level*

The microscopic observation and biochemical reaction revealed that the four lactic strains are: stick form, gram positive, immobile, catalase negative. The growth of lactic isolates in the MRS medium was clear. Their growth wasn't accompanied by any appearance of gas bubbles or detachment of the agar up. The total lack of gas (CO_2) for the four strains tested is an indicator of the homo-fermentative type. The preliminary tests make it possible to classify the four isolates to Lactobacillus genus on the basis of identification mentioned by Holzapfel [9].

Table 1: Characterization of 4 lactic acid bacteria

Biotope	Isolats	Gram	Catalase	Fermentation Type	Morphology	Genus
Milk cow	BLN3	+	_	Homo*	Rods	lactobacillus
	BLN4	+	_	Homo*	Rods	lactobacillus
	BLN8	+	_	Homo*	Rods	lactobacillus
Olives brine	BLN17	+	_	Homo*	Rods	lactobacillus

* : fermentative

After their microscopic examination, the four lactic strains were tested by sugar fermentation pattern, reaction of Citrate, VP, Arginine, and tolerance towards pH, salinity and temperature.

3.3. *Sugar fermentation tests*

Lactic acid bacteria are known by their ability to ferment carbohydrates. Each lactic strain ferments sugars differently. This variability is an identification tool. The tested strains were differentiated by their capacity to form acid from sugars through the observation of the Petri dish. Incubation of agar plates was performed under anaerobic conditions at 30ºC for 24 hours. The color change from red to yellow indicates that there is use of the sugar [10]. Otherwise, the test is scored negative (picture: 1).

4 positive tests 4 negative tests

Photo 1: Plates showing the fermentation test

All characters to identify the four lactic strains and the corresponding results of fermentation sugars are shown in table 2.

Table 2: Fermentation sugars by the four lactic acid bacteria

Sugars tested	BLN3	BLN4	BLN8	BLN17
Glucose	+	+	+	+
Galactose	+	+	−	−
Lactose	+	+	−	+
Arabinose	v	+	+	+
Raffinose	+	+	−	+
Mannitol	+	+	−	+
Sucrose	+	+	+	+
Xylose	+	+	+	+
Rhamnose	v	−	+	−
Maltose	+	+	+	+
Dextrose	+	+	−	+
Fructose	+	+	+	+
Sorbitol	+	+	−	−
Ribose	+	+	−	+
Mannose	+	+	+	−

+ Positive,− : negative and v: variable

3.4. *Test of Citrate, Arginine and Voges Proskauer*

The change of color to blue in the tubes is a positive indicator of citrate utilization by the four lactic isolates (BLN3, BLN4, BLN8 and BLN17) and therefore these tests indicated citrate utilization. While for the Voges Proskauer test, the absence of color changes was noted in tubes of tree strains (BLN3, BLN4 and BLN8), they can't form ammoniac from Arginine, but the BLN17 was able to do.

3.5. *Identification of lactic acid bacteria to the species level*

The classification for four lactic species is based on the following reference works [11, 12]. Four strains (BLN3, BLN4, BLN8 and BLN17) don't belong to the group Thermobacterium, because no bacteria can grow at 45°C. Since they are homofermentative, they don't classify as Betabacterium gender and therefore, they are part of Streptobacterium group [13, 14]. Two strains (BLN3 and BLN4) out of 4 isolates lactic have arrived to ferment raffinose, mannitol and ribose; these characteristics suggest their classification as *Lactobacillus Plantarum* [15, 16]. The BLN8 strain is characterized by his ability to ferment lactose and raffinose as *Lactobacillus delbrueckii* [17]. The strain BLN17 belonged to the species *Lactobacillus fermentum* by his character fermentative [18, 19].

3.6. *Characterization of lactic acid bacteria*

The study of bacterial growth according to three parameters (pH, temperature and salinity) is evaluated by measuring bacterial biomass in a spectrophotometer at 600 nm. For temperature, 30°C corresponds to the favorable temperature for optimal growth of lactic acid bacteria; so all these strains are mesophilic. For pH, the optical density of the 4 lactic acid bacteria increases progressively when the pH values changed from 3.5 to 6. Beyond pH=6, the biomass of bacteria populations decreases to very low values; the 4 lactic strains are then acidophilic. It is interesting to note that the BLN3 has ability to grow in an acid environment (pH=3.5). But for salinity, the growth of the 4 bacterial strains in different NaCl concentration showed that the best performance of the bacterial growth is obtained with a concentration (4.5%). For BLN3 strain, she can growth to 6.5% of salinity [20].

Table 3: The DO according to Temperature

Temperature	BLN3	BLN4	BLN8	BLN17
10°C	2.08	1.57	1.32	1.15
30°C	2.56	2.52	2.53	2.40
45°C	0.06	0.01	0.02	0.03

Table 4: The DO according to pH

Ph	3.5	4.5	6	7	8.5
BLN3	2.23	2.26	2.35	1.04	0.37
BLN4	2.02	2.21	2.23	1.07	0.82
BLN8	2.06	2.23	2.28	0.99	0.52
BLN17	2.03	2.11	2.10	1.02	0.63

Table 5: The DO according to NaCl

NaCl Concentration (%)	BLN3	BLN4	BLN8	BLN17
2.5	2.45	1.95	2.12	2.15
4.5	2.27	1.73	1.64	2.13
6.5	1.98	0.14	0.15	0.14

Conclusion

The obtained results of identification for four isolates lactic, demonstrated that two strains (BLN3 and BLN4) belong to the species *Lactobacillus Plantarum*, the BLN8 belongs to the species *Lactobacillus Delbrueckii* and the BLN17 belong to *Lactobacillus Fermentum*. The physico-chemical analyzes of these 4 lactic isolates have classified them as mesophilic and acidophilic bacteria. The rate of 4.5% NaCl is the optimal concentration for the good growth to 4 lactic acid bacteria. Exceptionally, the BLN3 hat recorded a significant biomass at pH=3.5 and [NaCl] = 6.5%.

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