

## Characterization of Antibacterial Lactic Acid Bacteria Isolated from Moroccan fermented Olives

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The research for antibacterial activities of lactic acid bacteria isolated from Moroccan table olives, revealed 15 bacterial strains having an inhibitory activity against pathogenic germs: *Listeria monocytogenes*, *Listeria innocua* and *Staphylococcus aureus*; as well as Gram-negative germs, such as *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis* and others. From 127 samples of table olives, 143 bacterial strains with antibacterial effect were isolated by the double layer method, 15 strains were retained. All are Gram positive, catalase negative and non-sporulating. Cocci constitute 66.33% of the total effective. The remaining 33.33% are represented by bacilli/Ovoid. The lactic acid bacteria retained have a greater inhibitory effect against Gram positive and Gram negative bacteria. *Listeria monocytogenes* CECT 4032 and *Staphylococcus aureus* MBLA were inhibited by all the lactic strains studied. The smallest inhibition zones were recorded for the two isolates LB<sub>15</sub> and LB<sub>96</sub> against *E. coli* 87739, with diameter less than 10 mm.

**Keywords:** Lactic acid bacteria, Tables olives, Bacteriocins, antibacterial activity.

Table olive is obtained by lactic fermentation, which consists in promoting the development of lactic acid bacteria (LAB). These bacteria acidify gradually the medium culture by producing lactic acid, which prevents the proliferation of other microorganisms, in particular pathogenic or undesirable microorganisms.

Lactic acid bacteria as probiotics, witch when administered in adequate amounts, confer a health benefit on the host (FAO/WHO., 2006). They are present in many fermented and unfermented foods, and they produce a great diversity of bacteriocins whose nisin is the most widely used as a food preservative.

The bacteriocins produced by LAB have attracted increasing attention because they are active in a nanomolar range and have no toxicity. They are defined as protein-like molecules produced by lactic acid bacteria, which inhibit the growth of other bacteria, especially pathogenic bacteria such as *Listeria monocytogenes* (Klaenhammer, 1988).

In addition to the synthetic pathway and the concentration required for inhibitory activity, bacteriocins differ from antibiotics in that they have a relatively narrow spectrum of action and bactericidal activity is directed against taxonomically close strains of the producing bacterium (Dobson *et al.*, 2012).

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Bacterial fermentation of perishable raw materials has been used for centuries to preserve the nutritional value of foods and beverages, a long period of time (Deegan *et al.*, 2006). In recent years, bio-preservation systems such as bacteriocinogenic cultures and/or their bacteriocins have attracted increasing attention, and new approaches to the control of pathogenic microorganisms have been developed (Ross *et al.*, 1999).

Bacteriocins produced by LAB are of particular interest to the food industry since these bacteria have generally been considered safe. The interest in LAB occurring in foods is primarily due to the biotechnological potential of new bacterial species and strains. In the present study we selected table olives as a potential source of new species or strains of LAB because table olives represent a fermented product that supports the dominance of LAB.

This study aimed to isolate and characterize LAB from fermented olives of Morocco and to evaluate their biotechnological properties.

## MATERIAL AND METHODS

### Isolation of LAB strains for their antibacterial activity

143 bacterial strains with antibacterial effects were isolated from 127 samples of Moroccan table olives by the double layer method.

The isolation was carried out on De Man Rogosa and Sharpe (MRS) agar (Biokar Diagnostics, Beauvais, France) (De Man *et al.*, 1960). Temperature incubation was 30°C for 24h. The well isolated colonies were picked up and transferred to two plates of MRS agar. After incubation for 24h at 30°C, one plate is stored at + 4°C and the other is used to detect the antibacterial activity of the isolates. Thus, the latter was overlaid with 5 ml of soft Brain Heart Infusion (HiMedia Laboratories, Mumbai, India) inoculated with the target strain (*Listeria monocytogenes* CECT 4032). After 18 to 24 hours of incubation at 30°C, the antibacterial activity was detected by the appearance of a clear halo around the colonies.

### Physiological and biochemical characterization

Microscopic examination (form, grouping and mobility of cells), Gram staining, catalase test and sporulation were performed. The metabolism

of gas, acetoin, gelatin and sugars was also carried out (Okada *et al.*, 1992; Abriouel *et al.*, 2012).

Only the Gram positive, catalase negative non-motile and non-sporulating strains were retained.

### Preparation of cell free culture supernatants

LAB isolates were grown in MRS broth at 30°C for 24h. The cultures broth obtained were used for the preparation of culture free supernatants (CFS) for assays. The 24h old cultures were centrifuged at 8 000g for 20 min by using a centrifuge (Model Hettich universal 230) and then the supernatants were adjusted to pH 6.5 with 1N NaOH in order to rule out possible inhibition effects due to organic acids.

### Antibacterial activity spectrum of LAB isolates

Indicator strains used for assessment of antimicrobial activity of the selected LAB isolates were; six Gram positive (*Listeria monocytogenes* CECT 4032, *Staphylococcus aureus* 25983 ATCC, *Staphylococcus aureus* 43300 ATCC, *Staphylococcus aureus* 3920 ATCC, *Staphylococcus aureus* MBLA and *Staphylococcus epidermidis* ATCC 12228), and seven Gram negative (*Escherichia coli* 87739 ATCC, *Escherichia coli* 25922 ATCC, *Escherichia coli* MBLA K12, *Klebsiella pneumonia* ATCC 13883, *Proteus mirabilis* (Institut d'hygiène Rabat, Maroc), *Salmonella typhimurium* ATCC 14028 and *Enterococcus faecium* CECT 410).

Several methods for detecting the antibacterial activity of bacteriocin-producing strains have been described. However, the agar well diffusion assay (AWDA) remains the most common methods. It based on diffusion of the antibacterial substance through solid or semi-solid culture media previously inoculated with an indicator strain.

In the present investigation, the antibacterial activity of LAB isolates was performed by AWDA on Mueller Hinton Agar (MHA, Biokar Diagnostics, Beauvais, France) plates overlaid with soft BHI agar inoculated with overnight cultures of target strains. A blunt end of a sterile Pasteur pipette was used to create 4 wells with diameters of 6 mm into the MHA plate. 3 wells were filled with 100  $\mu$ l of CFS sample adjusted to pH 6.5 with 1N NaOH as mentioned above. The fourth well was filled with 100  $\mu$ l sterile distilled water to serve as a negative control. The plates were pre-incubated at 4°C for

4 h to allow uniform diffusion into the agar and then incubated at 30°C for 24h. The antibacterial activity was expressed in millimeters by measuring the diameter of clear zone around the wells.

#### Confirmation of bacteriocins

Cell free culture supernatant from LAB strains was adjusted to pH 6.5 with 1N NaOH. To eliminate the effect of hydrogen peroxide, catalase enzyme was added to the supernatant at a final concentration of 1 mg/mL. The mixture was filtered through a 0.22 µm pore size filter followed by incubation at 25°C for 2 h and the residual activity was examined by the AWDA as described above.

#### Effect of temperature on activity of antibacterial substances

The CFS samples adjusted at pH 6.5 and treated by catalase as described above were heated at different range of 60, 80 and 100 °C each for 30 min and autoclaved at 121°C for 15 min. The heat treated CFS samples were then assayed for antimicrobial activity by AWDA against *L. monocytogenes*. pH adjusted and H<sub>2</sub>O<sub>2</sub> eliminated CFSs without any heat treatments served as a controls. Residual antimicrobial activity of heat-treated CFS was expressed on percent of inhibition zone size (mm) compared to the control (100% at 30°C for 30 min) using *L. innocua* CECT 4030 as the indicator bacteria.

#### Location of the inhibitory substances

By the AWDA described above, the intra and extracellular fraction represented respectively by the pellet and the culture supernatant of each retained LAB strain were tested against *L. innocua* CECT 4030.

#### Measurement of arbitrary unit of antimicrobial activity

Antimicrobial activity was determined essentially by the AWDA. On the surface of the MHA medium, 5 ml of semi-solid BHI medium inoculated with 100 µl of an overnight culture of *L. monocytogenes* CECT 4032, were poured. After solidification of the surface layer, wells were created into the agar plate as mentioned above. A serial two-fold dilution of neutralized supernatant of selected LAB isolate was carried out. 100µl of each dilution were used to fill the wells. The plate was pre-incubated at 4°C for 2h and then incubated at 37°C for 24h. Antibacterial activity was defined as the reciprocal of the dilution that resulted in an

inhibition zone and expressed as arbitrary units (AU) per milliliter.

#### The growth kinetics of selected LAB strains

Bacterial growth was monitored by optical density (OD) measurements as a function of time. In parallel, the pH of the culture was determined.

Overnight culture of each selected LAB isolates was inoculated (2%, v/v) into 100 ml of MRS broth. Each lactic strain was grown for three days at 25°C, 30°C and 37°C. For each bacterial culture, sterile samples were taken at regular time intervals and the pH was determined by using a pH meter (Model PHSJ-3F). In parallel, 1ml of each culture was centrifuged at 8000 g for 20 min at 4°C, the pellet was washed twice, re-suspended in 1ml of physiological water and the OD of the suspension was measured at 620 nm using a spectrophotometer (Model Rayleigh UV-1800). All samples were in duplicates.

## RESULTS

### Lactic Acid Bacteria Isolation

143 LAB strains were isolated from 127 samples of fermented olives collected from diverse geographic regions in Morocco. LAB isolates were selected based on their ability to produce antibacterial substances, against *L. innocua*, on MRS-agar plates using the double-layer method. Only 54 strains exhibited antibacterial activity against indicator bacteria. However, 15 isolates maintained their inhibitory substance production using agar well diffusion assay. These isolates were retained for further tests.

The morphological, physiological and biochemical identification revealed that all retained strains are Gram positive, catalase negative and not producing gas (**Table 1**). Several remarks can be made as soon as these results are read:

- All LAB isolates are immobile, produced no acetoin except LB<sub>6</sub>, LB<sub>8</sub>, LB<sub>21</sub>, LB<sub>48</sub> and LB<sub>97</sub>. They are all the homo-fermentation type, due to the absence of CO<sub>2</sub> production.
- All the isolates are also able to growing in 6.5% NaCl and are all thermophilic.
- Coccoid-shape lactic acid bacteria able to growing in 6.5% NaCl are identified as Enterococci.
- LB<sub>96</sub> unable to grow at 50°C are identified as *Enterococcus faecalis*.

### Antibacterial activity spectrum of selected LAB

The antibacterial activity exhibited by the majority of strains especially toward Gram-negative bacteria may be due to the organic acid effect. For this purpose, the inhibitory effect was checked after supernatant neutralization. By this way, the selected LAB strains retained their antibacterial effect against different Gram-positive and Gram-negative pathogenic bacteria (Table 2). Some isolated strains inhibited almost all pathogenic bacteria tested; but the most susceptibility of them was against Gram-positive pathogens.

The results of antagonistic activity of LAB isolates showed that the inhibitory effect differs depending on the targeted bacterium. The isolated strains have a greater inhibitory effect against the growth of three Gram positive indicator bacteria, whose inhibition zones is often exceed 20 mm (Table 2). *L. monocytogenes* CECT 4032 and *S. aureus* MBLA were inhibited by all the lactic strains studied. *S. aureus* ATCC 3920, *S. aureus* ATCC 3300, *S. aureus* ATCC 25983 and *S. epidermidis* ATCC 12228 showed significant sensitivity to LB<sub>15</sub>, LB<sub>48</sub>, LB<sub>62</sub>, LB<sub>96</sub> and LB<sub>105</sub>.

*Klebsiella pneumoniae* ATCC 13883 and *Proteus mirabilis* showed a sensitive profile in the presence of the 15 lactic strains. *E. coli* MBLA K12, *Enterobacter cloacae*, *Salmonella typhimurium* ATCC 14028, and *E. coli* 25922 ATCC were all inhibited by LB<sub>15</sub>, LB<sub>62</sub> and LB<sub>96</sub>.

The lowest zones of inhibition were shown against *E. coli* 87739, with a diameter less than 10 mm for both LAB strains LB<sub>15</sub> and LB<sub>96</sub>.

### Effect of incubation period on the production of antibacterial substances

The production of antibacterial substances at different days was determined by AWDA measuring the zone of inhibition against *L. monocytogenes*. Antimicrobial metabolites production by the strains was detected at all days of incubation (Table 3). The highest level was obtained after 24h of incubation and then production was declined very slowly over time.

The results group the LAB isolates into two categories: the first is that of the strains LB<sub>15</sub>, LB<sub>48</sub>, LB<sub>78</sub>, LB<sub>98</sub> and LB<sub>105</sub> having a constant inhibition zones over incubation time. The diameters measured are of the order of 19mm, 21mm, 24mm and 18mm respectively. The second

category consists of LB<sub>6</sub>, LB<sub>8</sub>, LB<sub>21</sub>, LB<sub>62</sub>, LB<sub>91</sub>, LB<sub>93</sub>, LB<sub>96</sub>, LB<sub>97</sub>, LB<sub>104</sub> and LB<sub>105</sub> having downward inhibitory potency over incubation time. The best inhibition zones were recorded for LB<sub>8</sub>, LB<sub>21</sub> and LB<sub>62</sub> with diameters of 26mm, 25mm and 27mm, respectively. After incubation for 96 h, the inhibition zone diameter of LB<sub>8</sub> and LB<sub>62</sub> was reduced by 10 mm, while that of LB<sub>21</sub> was reduced by 4mm (25mm to 21mm).

### Confirmation of Bacteriocins

The inhibiting substances produced by isolated LAB strains have undergone several treatments: neutralization of pH, elimination of the effect of hydrogen peroxide, temperature and pH...

The antimicrobial activity of the CFSs was determined thrice (i.e before and after neutralization to pH 6.5 and treatment by catalase) using AWDA. The measure of inhibition zone diameters showed that all of the isolates have varying antibacterial activity against *L. monocytogenes* (Table 4).

It can be seen that pH neutralization doesn't eliminate the effect of substances produced against *Listeria monocytogenes* CECT 4032. On the contrary, the inhibition diameters are greater after pH neutralization for the majority of the strains. This suggests that the substances produced are highly active at neutral pH.

Treatment of the supernatant by catalase showed that the antibacterial effect is not due to the production of hydrogen peroxide. Indeed, neutralization of supernatant and addition of catalase tend to increase the inhibition degree (Labioui *et al.*, 2005).

### Effect of temperature on activity of antibacterial substances

The residual antibacterial activity of cell free supernatants from LAB isolates after thermal treatments at 60°C, 80°C, 100°C for 30 min and in an autoclave at 121°C for 15 min is presented in Table 5.

Inhibitory substances were found to be thermo-stable and retained their activity up to 121 °C for 15 min while partial loss of activity was noticed with continuous increase in temperature.

The antibacterial activity of the isolated strains is more or less stable at 60°C and 80°C. The residual activity was 100% for certain strains and more than 92% for others. Above 100°C, the antibacterial substances are less thermostable and the residual antimicrobial activity decreases

**Table 1.** Morphological, physiological and biochemical characteristics of isolated genera of LAB

	LAB strain																
	LB <sub>6</sub> +	LB <sub>97</sub> +	LB <sub>98</sub> +	LB <sub>8</sub> +	LB <sub>48</sub> +	LB <sub>62</sub> +	LB <sub>78</sub> +	LB <sub>93</sub> +	LB <sub>21</sub> +	LB <sub>91</sub> +	LB <sub>15</sub> +	LB <sub>96</sub> +	LB <sub>104</sub> +	LB <sub>105</sub> +	LB <sub>114</sub> +		
Gram	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Form	bacilli																
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Production	CO <sub>2</sub>																
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	VP																
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mobility	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatinase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth in 6,5% NaCl	-	+/-	+/-	+/-	+/-	+	+	+	+	+	+	+	+	+	+	+	+
Growth at	45C																
	+	+/-	+/-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	50C																
	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
	7°C																
	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
Fermentation of carbohydrate																	
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+
Dextrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arabinose	+	-	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Saccharose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	<i>Lactobacillus</i>																
	<i>Leuconostoc</i>																
	<i>Enterococcus</i>																

Table 2. Antibacterial activity of CFS derived from LAB isolates using Agar Well Diffusion Assay

Targeted strain	LAB strain															
	LB <sub>6</sub>	LB <sub>8</sub>	LB <sub>15</sub>	LB <sub>21</sub>	LB <sub>48</sub>	LB <sub>62</sub>	LB <sub>78</sub>	LB <sub>91</sub>	LB <sub>93</sub>	LB <sub>96</sub>	LB <sub>97</sub>	LB <sub>98</sub>	LB <sub>104</sub>	LB <sub>105</sub>	LB <sub>114</sub>	
<i>Listeria monocytogenes</i> CECT 4032	+++	+++	+++	++	++	++	+++	+++	+++	+++	+++	+++	+++	++	++	++
<i>Staphylococcus epidermidis</i> ATCC 12228	-	-	++	-	++	++	-	-	-	++	-	+	-	++	++	-
<i>Staphylococcus aureus</i> 25983 ATCC	++	-	++	-	++	++	-	+	-	++	-	-	-	++	++	-
<i>Staphylococcus aureus</i> 43300 ATCC	-	-	++	-	++	++	-	-	-	++	-	-	-	++	++	-
<i>Staphylococcus aureus</i> 3920 ATCC	-	++	++	-	++	++	-	-	-	++	-	-	-	++	++	-
<i>Staphylococcus aureus</i> MBLA	+	+	++	+	+++	++	+	+	+	+	++	+	++	+	+	+
<i>Escherichia coli</i> MBLA K12	-	++	+	-	-	+	++	++	+	+	+	+	+	+	+	+
<i>Proteus mirabilis</i> (Institut d'hygiène Rabat, Maroc)	+	+	+	+	+	+	+	+	++	++	++	++	+	+	+	+
<i>Enterococcus faecium</i> CECT 410	-	+	+	-	++	+	+	++	+	+	+	+	-	+	+	+
<i>Salmonella typhimurium</i> ATCC 14028	-	-	+	-	++	++	-	-	-	+	-	-	-	++	-	-
<i>Escherichia coli</i> 87739 ATCC	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-
<i>Escherichia coli</i> 25922 ATCC	+	++	++	+	++	++	-	+	-	++	+	-	-	++	+	+
<i>Klebsiella pneumoniae</i> ATCC 13883	+	+	+	+	+	+	+	++	++	++	+	++	+	++	+	+

+++; ++; +; -; -;

to 78%. The substances produced by LB<sub>97</sub>, LB<sub>98</sub>, LB<sub>58</sub> and LB<sub>78</sub> are considered to be the most thermostable, they retained more than 95% activity at 121°C for 15 min. However, LB<sub>114</sub> produces a less thermostable substance; its residual activity was 78.94%.

#### Location of the inhibitory activity

The cell fraction (pellet) had no effect on the growth of *L. innocua* CECT 4030 while the extracellular fraction corresponding to the supernatant exhibits a strong antibacterial power (Table 6). The result demonstrates that the inhibitory substance is present in the extracellular fraction for all LAB isolates.

#### Antibacterial compounds production

Table 7 shows the titers of the different substances produced. The most important sub-inhibitory concentration recorded is 640 AU/ml for

LB<sub>8</sub>, LB<sub>15</sub> and LB<sub>104</sub> while LB<sub>91</sub> showed a smaller inhibitory concentration of 80 AU/ml.

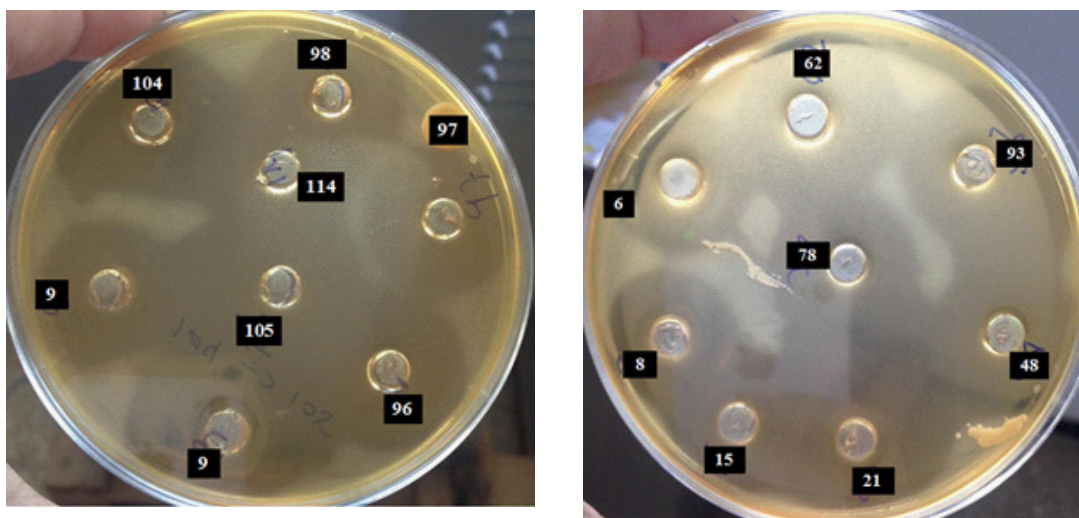
#### Growth kinetics

The behavior of the LAB isolates was determined by the kinetics of cell growth and acidification of medium in MRS broth at 25 °C, 30 °C and 37 °C. The result is shown in Tables 8, 9 and 10.

At 25°C, the initial pH of the LB<sub>6</sub>, LB<sub>62</sub> and LB<sub>21</sub> cultures decreases by approximately 2.4 units after 3 days of incubation (Table 8), followed by LB<sub>98</sub>, LB<sub>15</sub> and LB<sub>93</sub> with 2 pH units. Based on the criteria set by Cogan *et al.* (1997), a fast acidifying strain is able to decrease pH by 1.3 units after the first 6 hours of incubation. At 25°C, LB<sub>6</sub> and LB<sub>21</sub> are considered to be fast acidifying LAB strain.

**Table 3.** Effect of incubation time on the antibacterial substances production expressed by the inhibition diameters (mm)

Incubation time at 30 °C	LAB strain														
	LB <sub>6</sub>	LB <sub>8</sub>	LB <sub>15</sub>	LB <sub>21</sub>	LB <sub>48</sub>	LB <sub>62</sub>	LB <sub>78</sub>	LB <sub>91</sub>	LB <sub>93</sub>	LB <sub>96</sub>	LB <sub>97</sub>	LB <sub>98</sub>	LB <sub>104</sub>	LB <sub>105</sub>	LB <sub>114</sub>
24h	21	26	19	25	21	27	24	22	20	19	24	18	23	18	20
48h	20	21	19	21	21	20	24	21	20	17	23	18	19	18	20
72h	20	19	19	21	21	20	24	18	20	17	22	18	12	18	20
96h	15	17	19	21	21	17	24	12	17	11	22	18	12	18	16



**Fig. 1.** A and B: Agar Well Diffusion Assay showing inhibition zones on Mueller Hinton Agar medium (MHA) by LAB isolates against *L. monocytogenes* CECT 4032 after 24 h of incubation at 30°C after pH neutralization





The optical density recorded during the three days differs from the pH of the medium. When the culture pH decreases, the OD increases. Bacterial biomass often reaches 0.7 to 0.8 OD values. The highest OD 0.9 was obtained for LB<sub>98</sub>. However, the smallest OD values (0.475 and 0.516) were recorded for LB<sub>6</sub> and LB<sub>104</sub>, respectively.

At 30°C, the growth kinetic of LAB strains evaluated by OD and pH measurement is presented in **table 9**. The data show a gradual decrease in pH for all bacterial cultures studied. LB<sub>21</sub> and LB<sub>96</sub> showed a fast acidifying activity (6 to 3.14) after 48 h of incubation. However, LB<sub>78</sub> was as low acidifying strain with an initial and final pH 5.7 and 4.14, respectively. The final OD values ranged from 0.6 to 0.9. LB<sub>96</sub>, LB<sub>105</sub>, LB<sub>8</sub> and LB<sub>62</sub> showed maximum growth of 0.9. However, low optical densities between 0.5 and 0.6 were recorded for LB<sub>6</sub>, LB<sub>48</sub>, LB<sub>98</sub> and LB<sub>104</sub> after 48 h and 72 h of incubation.

At 37°C, the evolution of pH and OD of LAB cultures is presented in **table 10**. It is similar to that observed at 30°C. PH of LB<sub>21</sub> culture decreased by 1.4 units after 6 h, LB<sub>21</sub> is considered as fast acidifying strain. The biomass production is greater at 37°C than 25°C and 30°C. The OD value varies between 0.8 and 0.9 for all strains studied. However, LB<sub>105</sub> reached a maximum biomass of 0.96 after 72 h of incubation.

The growth of isolates at the three temperatures studied 25°C, 30°C and 37°C clearly showed that some isolates grow much better at 30°C and/or 37°C. Other isolates have

almost the similar profile for the three incubation temperatures. Final pH is always between 3 and 4. However, the time required for maximal growth sometimes exceeds 48 hours and may reaches 72 h.

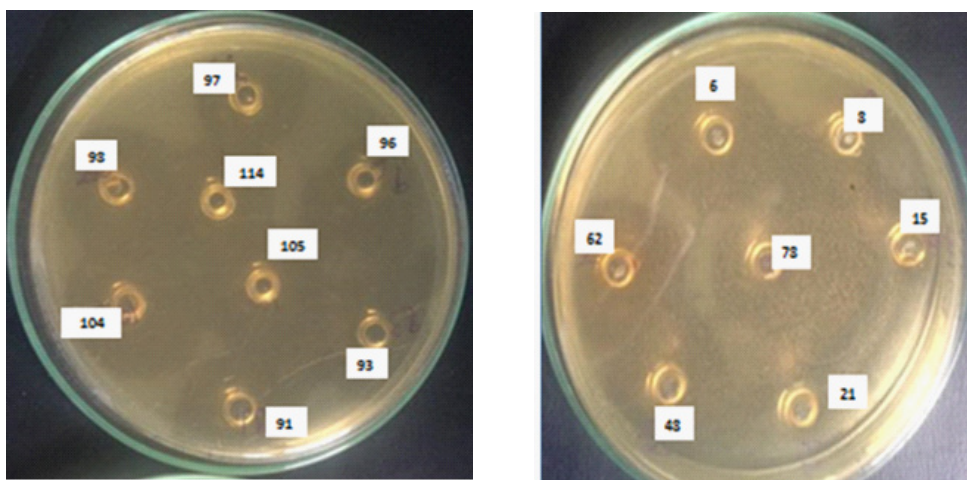
Depending on their behavior, LAB isolates are classified into two categories, the first grouping those with a high acidification rate during the first 6 hours of incubation time, and the second comprising those with low acidification rate.

For the three incubation temperatures 25°C, 30°C and 37°C, the pH of LB<sub>8</sub>, LB<sub>62</sub>, LB<sub>105</sub> and LB<sub>114</sub> decreased by 0.4 pH units after 5 h of incubation. They are therefore considered as strains with a low rate of acidification (Lairini *et al.*, 2014).

The growth of LB<sub>21</sub> as an example of fast acidifying strain and LB<sub>8</sub> as a low acidifying strain was studied in MRS broth at 25°C, 30 °C and 37°C. Growth was followed by optical density (OD<sub>620</sub>) and the production of antibacterial substance was determined against *L. innocua* by the AWDA and expressed in arbitrary units (AU ml<sup>-1</sup>) (Figure 3).

According to the graphs above, the optimal growth temperature varies between 30°C and 37°C. For the two strains with different acidification rates, maximum growth ranged from 48 h to 72 h. The antibacterial activity is the same for the three temperatures studied, and it's reduced when the medium pH is less than 3.5.

The study of different characteristics of LAB isolates, mentioned in Table 11, show that LB<sub>15</sub>, with a high acidifying capacity, and LB<sub>8</sub>, LB<sub>105</sub>, LB<sub>62</sub>, with low acidifying power, produce



**Fig. 2.** A and B: Antibacterial effect of the extracellular fraction of LAB strains against *Listeria innocua*

**Table 7.** Arbitrary unit of antimicrobial activity

	LAB strain														
	LB <sub>6</sub>	LB <sub>8</sub>	LB <sub>15</sub>	LB <sub>21</sub>	LB <sub>48</sub>	LB <sub>62</sub>	LB <sub>78</sub>	LB <sub>91</sub>	LB <sub>93</sub>	LB <sub>96</sub>	LB <sub>97</sub>	LB <sub>98</sub>	LB <sub>104</sub>	LB <sub>114</sub>	
Activity (AU/ml)	320	640	640	640	320	160	160	80	160	320	320	160	640	160	320

**Table 8.** Growth of isolates at 25 °C.

	LAB strain														
	LB <sub>21</sub>	LB <sub>78</sub>	LB <sub>98</sub>	LB <sub>48</sub>	LB <sub>6</sub>	LB <sub>91</sub>	LB <sub>104</sub>	LB <sub>15</sub>	LB <sub>96</sub>	LB <sub>97</sub>	LB <sub>105</sub>	LB <sub>8</sub>	LB <sub>93</sub>	LB <sub>114</sub>	LB <sub>62</sub>
initial pH	5.7	5.7	5.7	5.7	5.8	5.8	5.8	5.9	6	6	6	6.1	6.1	6.1	6.4
final pH	3.4	4.09	3.6	4	3.4	4.12	4	3.79	4.14	4.73	4.3	4.32	4.11	4.4	3.98
O.D	0.713	0.714	0.901	0.736	0.475	0.72	0.516	0.872	0.809	0.88	0.732	0.89	0.79	0.78	0.836
pH	3.4	4.12	3.69	4.07	3.58	4.12	4.1	4.05	4.25	4.29	4.34	4.45	4.24	4.4	4.06
incubation time (h)	72	72	72	48	48	72	48	48	48	72	72	72	48	72	48

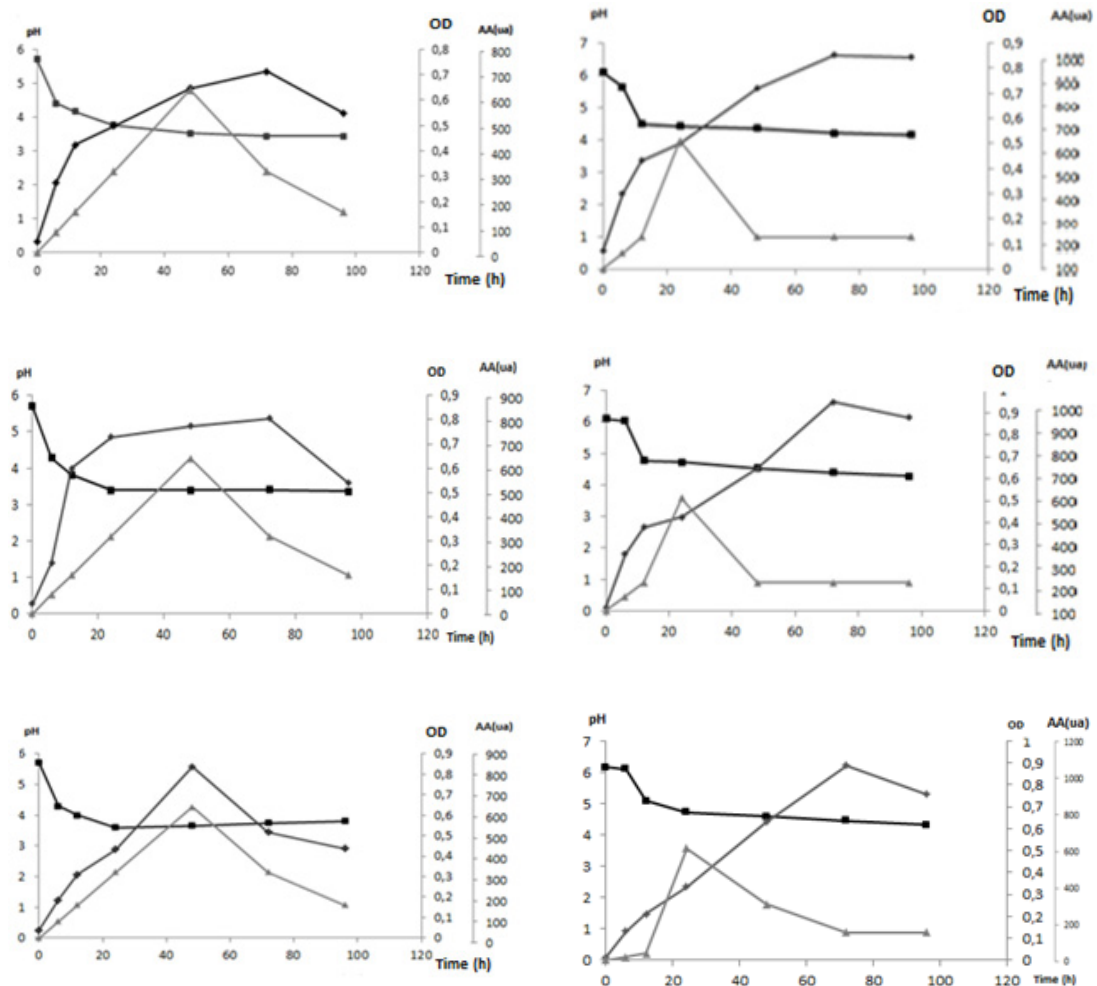
**Table 9.** Growth of LAB isolates at 30°C

	LAB strain														
	LB <sub>21</sub>	LB <sub>78</sub>	LB <sub>98</sub>	LB <sub>48</sub>	LB <sub>6</sub>	LB <sub>91</sub>	LB <sub>104</sub>	LB <sub>15</sub>	LB <sub>96</sub>	LB <sub>97</sub>	LB <sub>105</sub>	LB <sub>8</sub>	LB <sub>93</sub>	LB <sub>114</sub>	LB <sub>62</sub>
initial pH	5.7	5.7	5.7	5.7	5.8	5.8	5.8	5.9	6	6	6	6.1	6.1	6.1	6.4
final pH	3.8	4.14	3.56	4	3.4	4.08	3.9	4.05	3.14	3.82	4.19	4.27	4.19	4.11	4
O.D	0.836	0.782	0.658	0.667	0.683	0.789	0.524	0.851	0.902	0.75	0.941	0.948	0.838	0.77	0.96
pH	3.66	4.13	3.56	4.04	3.58	4.11	4.09	4.05	4.22	4.38	4.29	4.4	4.28	4.5	4.19
incubation time (h)	48	72	72	48	48	48	48	72	48	48	72	72	48	72	48

heat-resistant inhibiting substances. The inhibitory substance titer is important for LB<sub>15</sub>, LB<sub>8</sub>. However, it is less important for LB<sub>105</sub> and LB<sub>62</sub>. The strains LB<sub>104</sub>, LB<sub>6</sub>, LB<sub>21</sub> and LB<sub>96</sub> have a high rate of acidification, heat-resistant inhibiting substances and a high arbitrary unit, with the exception of LB<sub>96</sub> which shows a small inhibitory substance titer and a high bactericidal power. The strains LB<sub>48</sub>, LB<sub>114</sub> and LB<sub>97</sub> with medium acidification rate have a high inhibitory substance titer; Hence, LB<sub>48</sub> and LB<sub>114</sub> are considered as the best strains inhibiting pathogenic bacteria.

**DISCUSSION**

Analysis of 127 samples of Moroccan fermented table olives resulted in the isolation of 143 bacterial strains by the double layer method. 54 strains were gram positive, catalase negative, immobile and non-sporulating. After confirmation of their belonging to lactic acid bacteria, the 54 isolates were purified and then tested for their antibacterial effect against pathogenic bacteria. Only, 15 isolates retained their antibacterial activity using agar well diffusion assay



**Fig. 3.** Time course of growth and inhibitory substances production by LB8 (a) and LB21 (b) at 25°C, 30°C and 37°C

**Table 10.** Growth of LAB isolates at 37°C

	LAB strain															
	LB <sub>21</sub>	LB <sub>78</sub>	LB <sub>98</sub>	LB <sub>48</sub>	LB <sub>6</sub>	LB <sub>91</sub>	LB <sub>104</sub>	LB <sub>15</sub>	LB <sub>96</sub>	LB <sub>97</sub>	LB <sub>105</sub>	LB <sub>8</sub>	LB <sub>93</sub>	LB <sub>114</sub>	LB <sub>62</sub>	
initial pH	5.7	5.7	5.7	5.7	5.7	5.8	5.8	5.8	5.9	6	6	6	6.1	6.1	6.1	6.4
final pH	3.36	4.04	3.6	3.6	4.1	3.39	4.05	4.05	3.6	4.13	3.51	3.89	4.16	4.04	4.14	3.86
O.D	0.805	0.826	0.801	0.801	0.892	0.825	0.869	0.849	0.94	0.867	0.852	0.968	0.852	0.892	0.757	0.891
pH	3.41	4.11	3.6	3.6	4.11	3.52	4.19	4.21	3.62	4.15	4.16	4.12	4.21	4.14	4.25	4.31
incubation time (h)	72	72	72	72	48	48	48	48	72	48	48	72	72	48	72	48

**Table 11.** Important characteristics of LAB isolates

	LAB strain															
	LB <sub>15</sub>	LB <sub>8</sub>	LB <sub>104</sub>	LB <sub>6</sub>	LB <sub>21</sub>	LB <sub>96</sub>	LB <sub>98</sub>	LB <sub>105</sub>	LB <sub>62</sub>	LB <sub>78</sub>	LB <sub>91</sub>	LB <sub>93</sub>	LB <sub>48</sub>	LB <sub>114</sub>	LB <sub>97</sub>	
Acidification rate	++++	-	++++	++++	++++	++++	++++	+	-	++++	+	+	+	+	+	
Thermoresistance	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Antibacterial activity	++++	++++	++++	+	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+	++++
Arbitrary unit of antimicrobial activity	++++	++++	++++	+	++++	+	+	+	+	+	+	+	++++	++++	++++	++++

Based on their biochemical characteristics, the retained strains are homo-fermented because they don't produce CO<sub>2</sub>. The genus-specific identification of strains allowed us to classify strains LB<sub>8</sub>, LB<sub>15</sub>, LB<sub>62</sub>, LB<sub>104</sub>, LB<sub>105</sub>, LB<sub>96</sub> and LB<sub>114</sub> as *Enterococcus* due to their ability to grow in a medium containing 6.5% NaCl. The inability of LB<sub>48</sub> and LB<sub>96</sub> to grow at 50°C, suggests that they are *Enterococcus faecalis*. The antibacterial effect of the LAB strains was tested against a range of 13 Gram positive and Gram negative bacteria. The results revealed an important inhibitory effect of isolated bacteria, especially against Gram-positive bacteria.

Indeed, the study showed a potential antibacterial activity, revealed by large inhibition zones against Gram-positive bacteria, notably *L. monocytogenes* CECT 4032 and *S. aureus* EMBL. On the other hand, *Proteus mirabilis* and *Klebsiella pneumonia* ATCC 13883 were more sensitive than the other Gram-negative bacteria used.

LB<sub>15</sub> and LB<sub>96</sub> are considered to be the most effective, and have been retained for their bactericidal effect against all the target pathogenic bacteria. LB<sub>62</sub>, LB<sub>105</sub> and LB<sub>48</sub> inhibited all indicator strains tested with the exception of *E. coli* 25922 and *E. coli* K12 not inhibited by LB<sub>48</sub>.

Similar results have been reported by Gaamouche *et al.* (2014) who have isolated, from the brine of traditional table olives, 40 lactic strains with antibacterial activity against *L. monocytogenes*, from which 8 strains were also active against *E. coli* O157. Also, Labioui *et al.* (2005) demonstrated that the isolated lactic strains were all active against Gram-positive, but only one strain was effective against *Proteus mirabilis* a Gram-negative bacteria.

The study of antimicrobial activity of thermophilic lactobacilli, carried out by Allouche *et al.* (2010), revealed a narrow spectrum of inhibition, directed in particular towards the target germs: *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. Also, Todorov (2008) found a *Lactobacillus plantarum* strain that inhibits the growth of *Listeria spp.*, *Escherichia coli* and *Klebsiella pneumoniae*.

The antibacterial activity of lactic acid bacteria may be due to various compounds such as hydrogen peroxide, organic acids or bacteriocins (Luo *et al.*, 2011). The results obtained after

addition of catalase, neutralization of the pH and incubation of the CFS at high temperature, showed that the molecules produced are thermostable and exerted an antibacterial activity with respect to pathogenic bacteria. These molecules meet the criteria retained by Klaenhammer (1988) and can therefore be considered as bacteriocins.

The bactericidal activity of the all LAB isolates is detected in extracellular fraction. Similar result was obtained by Labioui *et al.* (2005) reported that the bactericidal activity of bacteriocin BLh5 is found exclusively in the culture medium.

The effect of incubation period on antibacterial activity of LAB isolates at 30°C showed that the inhibition zones of LB<sub>15</sub>, LB<sub>48</sub>, LB<sub>78</sub>, LB<sub>98</sub> and LB<sub>105</sub> incubated for 24h, remained constant after 96h of incubation under the same conditions. However, LB<sub>6</sub>, LB<sub>8</sub>, LB<sub>21</sub>, LB<sub>62</sub>, LB<sub>91</sub>, LB<sub>93</sub>, LB<sub>96</sub>, LB<sub>97</sub>, LB<sub>104</sub> and LB<sub>105</sub> showed a reduction in their antibacterial effect after 24h of incubation at 30°C; as shown in Table 7. The pH measured after 48h of incubation is between 3 and 4. These results are similar to those obtained during the study of pH effect on the production of inhibiting substances, the antimicrobial activity is reduced when the pH is below 4.5 (Kebede *et al.*, 2007; Johanningsmeier and McFeeters, 2011; Medeiros *et al.*, 2015). The work of Corsetti *et al.* (2004) showed that *Lactobacillus* strains were fully active at pH values ranging from 3 to 8, but *Lb. plantarum* 4DE and *Lb. plantarum* 3DM showed a reduction in antibacterial activity also at pH values of 3-4 and 8-9. In the work of Batdorj *et al.* (2006), the two bacteriocins studied were active over a wide pH range (2-10). Other studies realized by Bhattacharya and Das (2010) and Patil (2013) on the stability of bacteriocins at different pH levels, showed that these compounds are more stable in acidic than basic pH. Karaoglu *et al.* (2003) showed that the six bacteriocins tested were stable between pH 4.5 and 7 but sensitive outside this interval.

On the other hand, several studies of the influence of incubation time on the bactericidal activity of bacteriocins have been carried out. Tufail *et al.* (2011) have demonstrated that bacteriocin production by *Lactobacillus bulgaricus* is maximal after 48h of incubation. The work of Noordiana *et al.* (2013) reported that the antibacterial activity of the isolates is optimal between 48 and 72h of incubation, and is reduced after 96h of incubation.

Recently, Kp *et al.* (2016) have found that the best incubation time for maximum bacteriocin activity was 48 h.

Our study also showed that the optimal incubation temperature varies between 25°C and 30°C for some lactic strains, but all isolated strains showed an optimal growth at 30°C and 37°C. Similar results have been reported by Chramostova *et al.* (2014) who showed that 37°C is the optimum growth temperature of *Lactobacillus acidophilus*, *Bifidobacterium sp.* and *Streptococcus thermophilus*. For their part, Panesar *et al.* (2010) showed a maximum production of lactic acid bacteria at 37 °C. Recently, Patel and Parikh (2016) showed that 37 °C is the optimum growth temperature of *Lactobacillus sp.*

### CONCLUSION

In this study, morphological, physiological and biochemical criteria of the identified isolates allowed to link them to three lactic genera, the most dominant being the genus of *Enterococcus* (66.6%) followed by *Leuconostoc* (26.6%) and *Lactobacillus* (6.8%).

In view of the results, the bacterial species isolated from fermented table olives of Morocco, have interesting technological characteristics. Six isolates are considered as good acidifiers, they are the main agents of acidification and preservation of table olives. However, nine isolates are less acidifying, and they can be used for the production of flavors and for post-acidification operations.

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